(Strategies), 5, 81 (1992)]、C600 [ジェネティックス(Genetics), 39, 440 (1954)]、Y1088、Y1090 [サイエンス(Science), 222, 778 (1983)]、NM522 [ジャーナル・オブ・モレキュラー・バイオロジー(J. Mol. Biol.), 166, 1 (1983)]、K802 [ジャーナル・オブ・モレキュラー・バイオロジー(J. Mol. Biol.), 16, 118 (1966)] 及びJM105 [ジーン(Gene), 38, 275 (1985)] 等が用いられる。

cDNA ライブラリーからのヒト以外の動物の抗体のH鎖及びL鎖 V 領域をコードするcDNA クローンの選択法としては、アイソトープ或いは蛍光標識したプローブを用いたコロニー・ハイブリダイゼーション法或いはプラーク・ハイブリダイゼーション法 [モレキュラー・クローニング：ア・ラボラトリー・マニュアル(Molecular Cloning: A Laboratory Manual), Cold Spring Harbor Lab. Press NewYork, 1989] により選択することができる。また、プライマーを調製し、mRNA から合成した cDNA 或いは cDNA ライブラリーを鋳型として、Polymerase Chain Reaction [以下、PCR 法と表記する；モレキュラー・クローニング：ア・ラボラトリー・マニュアル(Molecular Cloning: A Laboratory Manual), Cold Spring Harbor Lab. Press New York, 1989；カレント・プロトコールズ・イン・モレキュラー・バイオロジー(Current Protocols in Molecular Biology), Supplement 1-34] によりH鎖及びL鎖V領域をコードする cDNA を調製することもできる。

上記方法により選択された cDNA を、適当な制限酵素などで切断後、pBluescript SK(-) (Stratagene 社製) 等のプラスミドにクローニングし、通常用いられる塩基配列解析方法、例えば、サンガー (Sanger) らのジデオキシ法 [プロシーディングス・オブ・ザ・ナショナル・アカデミー・オブ・サイエンス(Proc. Natl. Acad. Sci.,U.S.A.), 74, 5463 (1977)] 等の反応を行い、塩基配列自動分析装置、例えば、A. L. F. DNA シークエンサー (Pharmacia 社製) 等を用いて解析することで該 cDNA の塩基配列を決定することができる。

決定した塩基配列からH鎖及びL鎖V領域の全アミノ酸配列を推定し、既知の抗体のH鎖及びL鎖V領域の全アミノ酸配列 [シーケンシズ・オブ・プロテインズ・オブ・イムノロジカル・インタレスト(Sequences of Proteins of Immunological Interest), US Dept. Health and Human Services, 1991] と比較することにより、取得した cDNA が分泌シグナル配列を含む抗体のH鎖及びL鎖V領域の完全なアミノ酸配列をコードしているかを確認することができる。

（3）ヒト以外の動物の抗体のV領域のアミノ酸配列の解析

分泌シグナル配列を含む抗体のH鎖及びL鎖V領域の完全なアミノ酸配列に関して

は、既知の抗体のH鎖及びL鎖V領域の全アミノ酸配列［シーケンシズ・オブ・プロテインズ・オブ・イムノロジカル・インタレスト(Sequences of Proteins ofImmunological Interest), US Dept. Health and Human Services, 1991］と比較することにより、分泌シグナル配列の長さ及びN末端アミノ酸配列を推定でき、更にはそれらが属するサブグループを知ることができる。また、H鎖及びL鎖V領域の各CDRのアミノ酸配列についても、既知の抗体のH鎖及びL鎖V領域のアミノ酸配列［シーケンシズ・オブ・プロテインズ・オブ・イムノロジカル・インタレスト(Sequences of Proteins of Immunological Interest), US Dept. Health and Human Services, 1991］と比較することによって見出すことができる。

（4）ヒト型キメラ抗体発現ベクターの構築

本項4の（1）に記載のヒト化抗体発現用ベクターのヒト抗体のH鎖及びL鎖C領域をコードする遺伝子の上流に、ヒト以外の動物の抗体のH鎖及びL鎖V領域をコードするcDNAをクローニングし、ヒト型キメラ抗体発現ベクターを構築することができる。例えば、ヒト以外の動物の抗体のH鎖及びL鎖V領域をコードするcDNAを、ヒト以外の動物の抗体H鎖及びL鎖V領域の3'末端側の塩基配列とヒト抗体のH鎖及びL鎖C領域の5'末端側の塩基配列とから成り、かつ適当な制限酵素の認識配列を両端に有する合成DNAとそれぞれ連結し、それぞれを本項4の（1）に記載のヒト化抗体発現用ベクターのヒト抗体のH鎖及びL鎖C領域をコードする遺伝子の上流にそれらが適切な形で発現するようにクローニングし、ヒト型キメラ抗体発現ベクターを構築することができる。

（5）ヒト型CDR移植抗体のV領域をコードするcDNAの構築

ヒト型CDR移植抗体のH鎖及びL鎖V領域をコードするcDNAは、以下のようにして構築することができる。まず、目的のヒト以外の動物の抗体のH鎖及びL鎖V領域のCDRを移植するヒト抗体のH鎖及びL鎖V領域のフレームワーク（以下、FRと表記する）のアミノ酸配列を選択する。ヒト抗体のH鎖及びL鎖V領域のFRのアミノ酸配列としては、ヒト抗体由来のものであれば、いかなるものでも用いることができる。例えば、Protein Data Bank等のデータベースに登録されているヒト抗体のH鎖及びL鎖V領域のFRのアミノ酸配列、ヒト抗体のH鎖及びL鎖のV領域のFRの各サブグループの共通アミノ酸配列［シーケンシズ・オブ・プロテインズ・オブ・イムノロジカル・インタレスト(Sequences of Proteins of Immunological Interest), US Dept. Health and Human Services, 1991］等があげられるが、その中でも、十分な活性を有するヒト型CDR移植抗体を作製するためには、目的のヒト以外の動物の抗体のH鎖

及びL鎖V領域のFRのアミノ酸配列とできるだけ高い相同性（少なくとも60%以上）を有するアミノ酸配列を選択することが望ましい。

次に、選択したヒト抗体のH鎖及びL鎖V領域のFRのアミノ酸配列に目的のヒト以外の動物の抗体のH鎖及びL鎖V領域のCDRのアミノ酸配列を移植し、ヒト型CDR移植抗体のH鎖及びL鎖V領域のアミノ酸配列を設計する。設計したアミノ酸配列を抗体の遺伝子の塩基配列に見られるコドンの使用頻度[シーケンシズ・オブ・プロテインズ・オブ・イムノロジカル・インタレスト(Sequences of Proteins of Immunological Interest), US Dept. Health and Human Services, 1991]を考慮してDNA配列に変換し、ヒト型CDR移植抗体のH鎖及びL鎖V領域のアミノ酸配列をコードするDNA配列を設計する。設計したDNA配列に基づき、100塩基前後の長さから成る数本の合成DNAを合成し、それらを用いてPCR法を行う。この場合、PCRでの反応効率及び合成可能なDNAの長さから、H鎖、L鎖とも6本の合成DNAを設計することが好ましい。

また、両端に位置する合成DNAの5'末端に適当な制限酵素の認識配列を導入することで、本項4の（1）で構築したヒト化抗体発現用ベクターに容易にクローニングすることができる。PCR後、増幅産物をpBluescript SK(-)（Stratagene社製）等のプラスミドにクローニングし、本項4の（2）に記載の方法により、塩基配列を決定し、所望のヒト型CDR移植抗体のH鎖及びL鎖V領域のアミノ酸配列をコードするDNA配列を有するプラスミドを取得する。

（6）ヒト型CDR移植抗体のV領域のアミノ酸配列の改変

ヒト型CDR移植抗体は、目的のヒト以外の動物の抗体のH鎖及びL鎖V領域のCDRのみをヒト抗体のH鎖及びL鎖V領域のFRに移植しただけでは、その抗原結合活性は元のヒト以外の動物の抗体に比べて低下してしまうことが知られている[バイオ/テクノロジー(BIO/TECHNOLOGY), 9, 266 (1991)]。この原因としては、元のヒト以外の動物の抗体のH鎖及びL鎖V領域では、CDRのみならず、FRのいくつかのアミノ酸残基が直接的或いは間接的に抗原結合活性に関与しており、それらアミノ酸残基がCDRの移植に伴い、ヒト抗体のH鎖及びL鎖V領域のFRの異なるアミノ酸残基へと変化してしまうことが考えられている。この問題を解決するため、ヒト型CDR移植抗体では、ヒト抗体のH鎖及びL鎖V領域のFRのアミノ酸配列の中で、直接抗原との結合に関与しているアミノ酸残基やCDRのアミノ酸残基と相互作用したり、抗体の立体構造を維持し、間接的に抗原との結合に関与しているアミノ酸残基を同定し、それらを元のヒト以外の動物の抗体に見出されるアミノ酸残基に改変し、低下した抗原結合活性を上昇させることが行われている[バイオ/テクノロジー(BIO/TECHNOLOGY), 9,

266 (1991)]。

ヒト型 CDR 移植抗体の作製においては、それら抗原結合活性に関わる FR のアミノ酸残基を如何に効率よく同定するかが、最も重要な点であり、そのために X 線結晶解析［ジャーナル・オブ・モレキュラー・バイオロジー(J. Mol. Biol.), 112, 535 (1977)］或いはコンピューターモデリング［プロテイン・エンジニアリング(Protein Engineering), 7, 1501 (1994)］等による抗体の立体構造の構築及び解析が行われている。これら抗体の立体構造の情報は、ヒト型 CDR 移植抗体の作製に多くの有益な情報をもたらして来たが、その一方、あらゆる抗体に適応可能なヒト型 CDR 移植抗体の作製法は未だ確立されておらず、現状ではそれぞれの抗体について数種の改変体を作製し、それぞれの抗原結合活性との相関を検討する等の種々の試行錯誤が必要である。

ヒト抗体の H 鎖及び L 鎖 V 領域の FR のアミノ酸残基の改変は、改変用合成 DNA を用いて本項４の（５）に記載の PCR 法を行うことにより、達成できる。PCR 後の増幅産物について本項４の（２）に記載の方法により、塩基配列を決定し、目的の改変が施されたことを確認する。

（７）ヒト型 CDR 移植抗体発現ベクターの構築

本項４の（１）に記載のヒト化抗体発現用ベクターのヒト抗体の H 鎖及び L 鎖 C 領域をコードする遺伝子の上流に、本項４の（５）及び（６）で構築したヒト型 CDR 移植抗体の H 鎖及び L 鎖 V 領域をコードする cDNA をクローニングし、ヒト型 CDR 移植抗体発現ベクターを構築することができる。例えば、本項４の（５）及び（６）でヒト型 CDR 移植抗体の H 鎖及び L 鎖 V 領域を構築する際に用いる合成 DNA のうち、両端に位置する合成 DNA の 5' 末端に適当な制限酵素の認識配列を導入することで、本項４の（１）に記載のヒト化抗体発現用ベクターのヒト抗体の H 鎖及び L 鎖 C 領域をコードする遺伝子の上流にそれらが適切な形で発現するようにクローニングし、ヒト型 CDR 移植抗体発現ベクターを構築することができる。

（８）ヒト化抗体の安定的生産

本項４の（４）及び（７）に記載のヒト化抗体発現ベクターを適当な動物細胞に導入することによりヒト化抗体を安定に生産する形質転換株を得ることができる。

動物細胞への発現ベクターの導入法としては、エレクトロポレーション法［特開平 2-257891、サイトテクノロジー(Cytotechnology), 3 ,133 (1990)］等があげられる。

ヒト化抗体発現ベクターを導入する動物細胞としては、ヒト化抗体を生産させることができる動物細胞であれば、いかなる細胞でも用いることができるが、好ましくは生産される抗体の Fc 領域に付加する N-アセチルグルコサミンにフコースを付加させ

る酵素活性の低いまたは酵素活性を有しない細胞があげられる。

抗体のFc領域に付加するN-アセチルグルコサミンにフコースを付加させる酵素活性の低いまたは酵素活性を有しない細胞とは、α1,6結合に関与する酵素が少ない、またはない細胞であり、具体的にはフコシルトランスフェラーゼ、好ましくはFUT8活性が少ない、またはない細胞があげられる。

抗体のFc領域に付加するN-アセチルグルコサミンにフコースを付加させる酵素活性の低い、または酵素活性を有しない細胞としては、ラットミエローマ細胞であるYB2/0細胞などがあげられるが、α1,6結合に関与する酵素の遺伝子を欠損させたり、該遺伝子への変異を与えて酵素活性を下げるか欠失させた細胞を抗体生産細胞として用いることもできる。

具体的には、マウスミエローマ細胞であるNSO細胞、SP2/0細胞、チャイニーズハムスター卵巣細胞CHO/dhfr-細胞、CHO/DG44細胞、ラットミエローマYB2/0細胞、IR983F細胞、ヒトミエローマ細胞であるナマルバ細胞などがあげられる。好ましくは、チャイニーズハムスター卵巣細胞であるCHO/DG44細胞等があげられる。

発現ベクターの導入後、ヒト化抗体を安定に生産する形質転換株は、特開平2-257891に開示されている方法に従い、G418 sulfate（以下、G418と表記する；SIGMA社製）等の薬剤を含む動物細胞培養用培地により選択できる。動物細胞培養用培地としては、RPMI1640培地（日水製薬社製）、GIT培地（日本製薬社製）、EX-CELL302培地（JRH社製）、IMDM培地（GIBCO BRL社製）、Hybridoma-SFM培地（GIBCO BRL社製）、またはこれら培地に牛胎児血清（以下、FBSと表記する）等の各種添加物を添加した培地等を用いることができる。得られた形質転換株を培地中で培養することで培養上清中にヒト化抗体を生産蓄積させることができる。培養上清中のヒト化抗体の生産量及び抗原結合活性は酵素免疫抗体法［以下、ELISA法と表記する；アンティボディズ：ア・ラボラトリー・マニュアル(Antibodies: A Laboratory Manual), Cold Spring Harbor Laboratory, Chapter 14, 1998、モノクローナル・アンティボディズ：プリンシプルズ・アンド・プラクティス(Monoclonal Antibodies: Principles and Practice), Academic Press Limited, 1996］等により測定できる。また、形質転換株は、特開平2-257891に開示されている方法に従い、DHFR遺伝子増幅系等を利用してヒト化抗体の生産量を上昇させることができる。

ヒト化抗体は、形質転換株の培養上清よりプロテインAカラムを用いて精製することができる［アンティボディズ：ア・ラボラトリー・マニュアル(Antibodies: A Laboratory Manual), Cold Spring Harbor Laboratory, Chapter 8, 1988、モノクロ

ーナル・アンティボディズ：プリンシプルズ・アンド・プラクティス(Monoclonal Antibodies: Principles and Practice), Academic Press Limited, 1996]。また、その他に通常、蛋白質の精製で用いられる精製方法を使用することができる。例えば、ゲル濾過、イオン交換クロマトグラフィー及び限外濾過等を組み合わせて行い、精製することができる。精製したヒト化抗体のH鎖、L鎖或いは抗体分子全体の分子量は、ポリアクリルアミドゲル電気泳動[以下、SDS-PAGEと表記する；ネイチャー(Nature), 227, 680 (1970)]やウエスタンブロッティング法[アンティボディズ：ア・ラボラトリー・マニュアル(Antibodies: A Laboratory Manual), Cold Spring Harbor Laboratory, Chapter 12, 1988、モノクローナル・アンティボディズ：プリンシプルズ・アンド・プラクティス(Monoclonal Antibodies: Principles and Practice), Academic Press Limited, 1996]等で測定することができる。

以上、動物細胞を宿主とした抗体の製造方法を示したが、上記3にあるように、細菌、酵母、昆虫細胞、植物細胞または動物個体あるいは植物個体においても製造することができる。

（9）ヒト化抗体の活性評価

精製したヒト化抗体の抗原との結合活性、抗原陽性培養細胞株に対する結合活性はELISA法及び蛍光抗体法[キャンサー・イムノロジー・イムノセラピー(Cancer Immunol. Immunother.), 36, 373 (1993)]等により測定できる。抗原陽性培養細胞株に対する細胞障害活性は、CDC活性、ADCC活性等を測定することにより、評価することができる[キャンサー・イムノロジー・イムノセラピー(Cancer Immunol. Immunother.), 36, 373 (1993)]。更にヒト化抗体のヒトでの安全性、治療効果は、カニクイザル等のヒトに比較的近い動物種の適当なモデルを用いて評価することができる。

5．免疫機能分子の使用方法

上記4記載のヒト化抗体の例にあるように、高いADCC活性を有する抗体は、癌、アレルギー、循環器疾患、またはウィルスあるいは細菌感染をはじめとする各種疾患の予防および治療において有用である。

癌、すなわち悪性腫瘍は癌細胞が増殖する。通常の抗癌剤は癌細胞の増殖を抑制することを特徴とする。しかし、高いADCC活性を有する抗体は、殺細胞効果により癌細胞を障害することにより癌を治療することができるので、通常の抗癌剤よりも治療薬として有効である。

アレルギー反応は、免疫細胞によるメディエータ分子の放出により惹起されるため、高いADCC活性を有する抗体を用いて免疫細胞を除去することにより、アレルギー反

応を抑えることができる。

循環器疾患としては、動脈硬化などがあげられる。動脈硬化は、現在バルーンカテーテルによる治療を行うが、治療後の再狭窄での動脈細胞の増殖を高い ADCC 活性を有する抗体を用いて抑えることより、循環器疾患を予防および治療することができる。

ウィルスまたは細菌に感染細胞を、高い ADCC 活性を有する抗体を用いてウィルスまたは細菌に感染細胞の増殖を抑えることにより、ウィルスまたは細菌感染をはじめとする各種疾患の予防および治療することができる。

また、ADCC 活性が抑制された抗体は、自己免疫疾患の予防および治療において有用である。また、ADCC 活性が抑制された抗体は、自己免疫疾患において亢進された免疫反応を押さえるという観点から、自己免疫疾患の予防および治療において有用である。

本発明の抗体を含有する医薬は、治療薬として単独で投与することも可能ではあるが、通常は薬理学的に許容される一つあるいはそれ以上の担体と一緒に混合し、製剤学の技術分野においてよく知られる任意の方法により製造した医薬製剤として提供するのが望ましい。

投与経路は、治療に際して最も効果的なものを使用するのが望ましく、経口投与、または口腔内、気道内、直腸内、皮下、筋肉内および静脈内等の非経口投与をあげることができ、抗体製剤の場合、望ましくは静脈内投与をあげることができる。

投与形態としては、噴霧剤、カプセル剤、錠剤、顆粒剤、シロップ剤、乳剤、座剤、注射剤、軟膏、テープ剤等があげられる。

経口投与に適当な製剤としては、乳剤、シロップ剤、カプセル剤、錠剤、散剤、顆粒剤等があげられる。

乳剤およびシロップ剤のような液体調製物は、水、ショ糖、ソルビトール、果糖等の糖類、ポリエチレングリコール、プロピレングリコール等のグリコール類、ごま油、オリーブ油、大豆油等の油類、ｐ－ヒドロキシ安息香酸エステル類等の防腐剤、ストロベリーフレーバー、ペパーミント等のフレーバー類等を添加剤として用いて製造できる。

カプセル剤、錠剤、散剤、顆粒剤等は、乳糖、ブドウ糖、ショ糖、マンニトール等の賦形剤、デンプン、アルギン酸ナトリウム等の崩壊剤、ステアリン酸マグネシウム、タルク等の滑沢剤、ポリビニルアルコール、ヒドロキシプロピルセルロース、ゼラチン等の結合剤、脂肪酸エステル等の界面活性剤、グリセリン等の可塑剤等を添加剤として用いて製造できる。

非経口投与に適当な製剤としては、注射剤、座剤、噴霧剤等があげられる。

注射剤は、塩溶液、ブドウ糖溶液、あるいは両者の混合物からなる担体等を用いて

調製される。または、ヒト化抗体を常法に従って凍結乾燥し、これに塩化ナトリウムを加えることによって粉末注射剤を調製することもできる。

座剤はカカオ脂、水素化脂肪またはカルボン酸等の担体を用いて調製される。

また、噴霧剤は該化合物そのもの、ないしは受容者の口腔および気道粘膜を刺激せず、かつ該化合物を微細な粒子として分散させ吸収を容易にさせる担体等を用いて調製される。

担体として具体的には乳糖、グリセリン等が例示される。該化合物および用いる担体の性質により、エアロゾル、ドライパウダー等の製剤が可能である。また、これらの非経口剤においても経口剤で添加剤として例示した成分を添加することもできる。

投与量または投与回数は、目的とする治療効果、投与方法、治療期間、年齢、体重等により異なるが、通常成人1日当たり10μg/kg～20mg/kgである。

また、抗体の各種腫瘍細胞に対する抗腫瘍効果を検討する方法は、インビトロ実験としては、CDC活性測定法、ADCC活性測定法等があげられ、インビボ実験としては、マウス等の実験動物での腫瘍系を用いた抗腫瘍実験等があげられる。

CDC活性、ADCC活性、抗腫瘍実験は、文献［キャンサー・イムノロジー・イムノセラピー(Cancer Immunology Immunotherapy), 36, 373 (1993)；キャンサー・リサーチ(Cancer Research), 54, 1511 (1994)］等記載の方法に従って行うことができる。

6．免疫機能分子の活性を促進または抑制させる方法

上述の方法によりフコースが存在しない糖鎖が結合された抗体、ペプチドまたは蛋白質を製造することにより免疫機能分子の活性を促進させることができる。

活性が促進された免疫機能分子を生体内に投与することにより、生体内では、ADCC活性を担うエフェクター細胞であるキラー細胞、ナチュラルキラー細胞、活性化マクロファージ等の細胞をはじめとする各種免疫細胞が活性化され、種々の免疫反応を調節することが可能となる。

また、上述の方法によりフコースが存在する糖鎖を結合された抗体、ペプチドまたは蛋白質を製造することにより免疫機能分子の活性を抑制させることができる。

活性が抑制された免疫機能分子を生体内に投与することにより、生体内では、ADCC活性を担う各種免疫細胞の活性が弱まり、種々の免疫反応を調節することが可能となる。

以下に、本発明の実施例を示すが、これにより本発明の範囲が限定されるものではない。

図面の簡単な説明

第1図　精製した5種類の抗GD3キメラ抗体のSDS-PAGE(4～15%グラジエントゲルを使用）の電気泳動パターンを示した図である。上図が非還元条件、下図が還元条件でそれぞれ電気泳動を行った図である。レーン1が高分子量マーカー、2がYB2/0-GD3キメラ抗体、3がCHO/DG44-GD3キメラ抗体、4がSP2/0-GD3キメラ抗体、5がNSO-GD3キメラ抗体(302)、6がNSO-GD3キメラ抗体(GIT)、7が低分子量マーカーの泳動パターンをそれぞれ示す。

第2図　精製した5種類の抗GD3キメラ抗体のGD3との結合活性を抗体濃度を変化させて測定した図である。縦軸はGD3との結合活性、横軸は抗体濃度をそれぞれ示す。○がYB2/0-GD3キメラ抗体、●がCHO/DG44-GD3キメラ抗体、□がSP2/0-GD3キメラ抗体、■がNSO-GD3キメラ抗体(302)、△がNSO-GD3キメラ抗体(GIT)の活性をそれぞれ示す。

第3図　精製した5種類の抗GD3キメラ抗体のヒトメラノーマ細胞株G-361に対するADCC活性を示した図である。縦軸に細胞障害活性、横軸に抗体濃度をそれぞれ示す。○がYB2/0-GD3キメラ抗体、●がCHO/DG44-GD3キメラ抗体、□がSP2/0-GD3キメラ抗体、■がNSO-GD3キメラ抗体(302)、△がNSO-GD3キメラ抗体(GIT)の活性をそれぞれ示す。

第4図　精製した3種類の抗hIL-5Rα CDR移植抗体のSDS-PAGE（4～15%グラジエントゲルを使用）の電気泳動パターンを示した図である。上図が非還元条件、下図が還元条件でそれぞれ電気泳動を行った図である。レーン1が高分子量マーカー、2がYB2/0-hIL-5RCDR抗体、3がCHO/d-hIL-5RCDR抗体、4がNSO-hIL-5RCDR抗体、5が低分子量マーカーの泳動パターンをそれぞれ示す。

第5図　精製した3種類の抗hIL-5Rα CDR移植抗体のhIL-5Rαとの結合活性を抗体濃度を変化させて測定した図である。縦軸はhIL-5Rαとの結合活性、横軸は抗体濃度をそれぞれ示す。○がYB2/0-hIL-5RCDR抗体、●がCHO/d-hIL-5RCDR抗体、□がNSO-hIL-5RCDR抗体の活性をそれぞれ示す。

第6図　精製した3種類の抗hIL-5Rα CDR移植抗体のhIL-5R発現マウスT細胞株CTLL-2(h5R)に対するADCC活性を示した図である。縦軸に細胞障害活性、横軸に抗体濃度をそれぞれ示す。○がYB2/0-hIL-5RCDR抗体、●がCHO/d-hIL-5RCDR抗体、□がNSO-hIL-5RCDR抗体の活性をそれぞれ示す。

第7図　精製した3種類の抗hIL-5Rα CDR移植抗体のカニクイザルのhIL-5誘発好酸球増加モデルに対する抑制作用を示した図である。縦軸に末梢血中好酸球数、横軸

に日数（抗体及びhIL-5の投与開始日を0日とした）をそれぞれ示す。101、102が抗体非投与群、301、302、303がYB2/0-hIL-5RCDR抗体投与群、401、402、403がCHO/d-hIL-5RCDR抗体投与群、501、502、503がNSO-hIL-5RCDR抗体投与群の結果をそれぞれ示す。

第8図　YB2/0が生産した精製抗hIL-5Rα CDR移植抗体（上側）およびNSOが生産した精製抗hIL-5Rα CDR移植抗体（下側）のPA化糖鎖の逆相HPLC溶離の溶離図（左図）とそのPA化糖鎖をα-L-フコシダーゼ処理した後に逆相HPLCで分析して得た溶離図（右図）を示したものである。縦軸に相対蛍光強度、横軸に溶出時間をそれぞれ示す。

第9図　CHO/d細胞が生産した精製抗hIL-5Rα CDR移植抗体からPA化糖鎖を調製し、逆相HPLCで分析して得た溶離図を示したものである。縦軸に相対蛍光強度、横軸に溶出時間をそれぞれ示す。

第10図　非吸着画分、吸着画分の一部のGD3との結合活性を、抗体濃度を変化させて測定した図である。縦軸はGD3との結合活性、横軸は抗体濃度をそれぞれ示す。●が非吸着画分、○が吸着画分の一部をそれぞれ示す。下図は非吸着画分、吸着画分の一部のヒトメラノーマ細胞株G-361に対するADCC活性を示した図である。縦軸に細胞障害活性、横軸に抗体濃度をそれぞれ示す。●が非吸着画分、○が吸着画分の一部をそれぞれ示す。

第11図　非吸着画分、吸着画分の一部から調製したPA化糖鎖を逆相HPLCで分析して得た溶離図を示したものである。左図に非吸着画分の溶離図、右図に吸着画分の一部の溶離図をそれぞれ示す。縦軸に相対蛍光強度、横軸に溶出時間をそれぞれ示す。

第12図　ラットFUT8配列をスタンダード、内部コントロールに用いた場合の各宿主細胞株におけるFUT8転写産物の量を示す。■がCHO細胞株、□がYB2/0細胞株を宿主細胞として用いた場合をそれぞれ示す。

発明を実施するための最良の形態

実施例1．抗ガングリオシドGD3ヒト型キメラ抗体の作製

1．抗ガングリオシドGD3ヒト型キメラ抗体のタンデム型発現ベクターpChiLHGM4の構築

抗ガングリオシドGD3ヒト型キメラ抗体（以下、抗GD3キメラ抗体と表記する）のL鎖の発現ベクターpChi641LGM4［ジャーナル・オブ・イムノロジカル・メソッズ(J. Immunol. Methods), 167, 271 (1994)］を制限酵素MluI（宝酒造社製）とSalI（宝酒造社製）で切断して得られるL鎖cDNAを含む約4.03kbの断片と動物細胞用発現ベ

クターpAGE107［サイトテクノロジー(Cytotechnology), 3, 133 (1990)］を制限酵素MluI（宝酒造社製）とSalI（宝酒造社製）で切断して得られるG418耐性遺伝子及びスプライシングシグナルを含む約3.40kbの断片をDNA Ligation Kit（宝酒造社製）を用いて連結、大腸菌HB101株［モレキュラー・クローニング：ア・ラボラトリー・マニュアル(Molecular Cloning: A Laboratory Manual),Cold Spring Harbor Lab. Press New York, 1989］を形質転換してプラスミドpChi641LGM40を構築した。

次に、上記で構築したプラスミドpChi641LGM40を制限酵素ClaI（宝酒造社製）で切断後、DNA Blunting Kit（宝酒造社製）を用いて平滑末端化し、更にMluI（宝酒造社製）で切断して得られるL鎖cDNAを含む約5.68kbの断片と抗GD3キメラ抗体のH鎖の発現ベクターpChi641HGM4［ジャーナル・オブ・イムノロジカル・メソッズ(J. Immunol. Methods), 167, 271 (1994)］を制限酵素XhoI（宝酒造社製）で切断後、DNA Blunting Kit（宝酒造社製）を用いて平滑末端化し、更にMluI（宝酒造社製）で切断して得られるH鎖cDNAを含む約8.40kbの断片をDNA Ligation Kit（宝酒造社製）を用いて連結、大腸菌HB101株［モレキュラー・クローニング：ア・ラボラトリー・マニュアル(Molecular Cloning: A Laboratory Manual), Cold Spring Harbor Lab. Press New York, 1989］を形質転換して抗 GD3 キメラ抗体のタンデム型発現ベクターpChi641LHGM4を構築した。

2．抗GD3キメラ抗体の安定生産細胞の作製

上記実施例1の1項で構築した抗 GD3 キメラ抗体のタンデム型発現ベクターpChi641LHGM4 を用いて抗 GD3 キメラ抗体の安定生産細胞を以下のようにして作製した。

（1）ラットミエローマYB2/0細胞を用いた生産細胞の作製

抗GD3キメラ抗体発現ベクターpChi641LHGM4の5μgを4×10^6細胞のラットミエローマ YB2/0 細胞へエレクトロポレーション法［サイトテクノロジー(Cytotechnology), 3, 133 (1990)］により導入後、40ml の RPMI1640-FBS(10)［FBS(GIBCO BRL社製)を10%含むRPMI1640培地］に懸濁し、96ウェル培養用プレート（住友ベークライト社製）に200μl/ウェルずつ分注した。5%CO_2インキュベーター内で37℃、24時間培養した後、G418を0.5mg/mlになるように添加して1～2週間培養した。G418耐性を示す形質転換株のコロニーが出現し、増殖の認められたウェルより培養上清を回収し、上清中の抗GD3キメラ抗体の抗原結合活性を実施例1の3項に示すELISA法により測定した。

培養上清中に抗GD3キメラ抗体の生産が認められたウェルの形質転換株については、

DHFR 遺伝子増幅系を利用して抗体生産量を増加させる目的で、G418 を 0.5mg/ml、DHFR の阻害剤であるメソトレキセート（以下、MTX と表記する；SIGMA 社製）を 50nM 含む RPMI1640-FBS(10)培地に 1～2×10^5 細胞/ml になるように懸濁し、24 ウェルプレート（Greiner 社製）に 2ml ずつ分注した。5%CO_2 インキュベーター内で 37℃で 1～2 週間培養して、50nM MTX 耐性を示す形質転換株を誘導した。形質転換株の増殖が認められたウェルの培養上清中の抗 GD3 キメラ抗体の抗原結合活性を実施例 1 の 3 項に示す ELISA 法により測定した。培養上清中に抗 GD3 キメラ抗体の生産が認められたウェルの形質転換株については、上記と同様の方法により、MTX 濃度を 100nM、200nM と順次上昇させ、最終的に G418 を 0.5mg/ml、MTX を 200nM の濃度で含む RPMI1640-FBS(10)培地で増殖可能かつ、抗 GD3 キメラ抗体を高生産する形質転換株を得た。得られた形質転換株については、2 回の限界希釈法による単一細胞化（クローン化）を行った。

このようにして得られた抗 GD3 キメラ抗体を生産する形質転換細胞クローン 7-9-51 は平成１１年４月５日付で工業技術院生命工学工業技術研究所(日本国茨城県つくば市東１丁目１番３号）に FERM BP-6691 として寄託されている。

（２）CHO/DG44 細胞を用いた生産細胞の作製

抗 GD3 キメラ抗体発現ベクターpChi641LHGM4 の 4 μg を 1.6×10^6 細胞の CHO/DG44 細胞へエレクトロポレーション法［サイトテクノロジー(Cytotechnology), 3, 133 (1990)］により導入後、10ml の IMDM-FBS(10)［FBS を 10%、HT supplement(GIBCO BRL 社製)を 1 倍濃度で含む IMDM 培地］に懸濁し、96 ウェル培養用プレート（岩城硝子社製）に 200 μl/ウェルずつ分注した。5%CO_2 インキュベーター内で 37℃、24 時間培養した後、G418 を 0.5mg/ml になるように添加して 1～2 週間培養した。G418 耐性を示す形質転換株のコロニーが出現し、増殖の認められたウェルより培養上清を回収し、上清中の抗 GD3 キメラ抗体の抗原結合活性を実施例 1 の 3 項に示す ELISA 法により測定した。

培養上清中に抗 GD3 キメラ抗体の生産が認められたウェルの形質転換株については、DHFR 遺伝子増幅系を利用して抗体生産量を増加させる目的で、G418 を 0.5mg/ml、MTX を 10nM 含む IMDM-dFBS(10)培地［透析牛胎児血清(以下、dFBS と表記する；GIBCO BRL 社製)を 10%含む IMDM 培地］に 1～2×10^5 細胞/ml になるように懸濁し、24 ウェルプレート（岩城硝子社製）に 0.5ml ずつ分注した。5%CO_2 インキュベーター内で 37℃で 1～2 週間培養して、10nM MTX 耐性を示す形質転換株を誘導した。増殖が認められたウェルの形質転換株については、上記と同様の方法により、MTX 濃度を 100nM に上昇させ、最終的に G418 を 0.5mg/ml、MTX を 100nM の濃度で含む IMDM-dFBS(10)培地で増殖

可能かつ、抗GD3キメラ抗体を高生産する形質転換株を得た。得られた形質転換株については、2回の限界希釈法による単一細胞化（クローン化）を行った。

（3）マウスミエローマNSO細胞を用いた生産細胞の作製

抗GD3キメラ抗体発現ベクターpChi641LHGM4の5μgを4×10^6細胞のマウスミエローマNSO細胞へエレクトロポレーション法[サイトテクノロジー(Cytotechnology), 3, 133, 1990]により導入後、40mlのEX-CELL302-FBS(10)[FBSを10%、L-グルタミン(以下、L-Glnと表記する；GIBCO BRL社製)を2mM含むEX-CELL302培地]に懸濁し、96ウェル培養用プレート（住友ベークライト社製）に200μl/ウェルずつ分注した。5%CO_2インキュベーター内で37℃、24時間培養した後、G418を0.5mg/mlになるように添加して1～2週間培養した。G418耐性を示す形質転換株のコロニーが出現し、増殖の認められたウェルより培養上清を回収し、上清中の抗GD3キメラ抗体の抗原結合活性を実施例1の3項に示すELISA法により測定した。

培養上清中に抗GD3キメラ抗体の生産が認められたウェルの形質転換株については、DHFR遺伝子増幅系を利用して抗体生産量を増加させる目的で、G418を0.5mg/ml、MTXを50nM含むEX-CELL302-dFBS(10)培地（dFBSを10%、L-Glnを2mM含むEX-CELL302培地）に1～2×10^5細胞/mlになるように懸濁し、24ウェルプレート（Greiner社製）に2mlずつ分注した。5%CO_2インキュベーター内で37℃で1～2週間培養して、50nM MTX耐性を示す形質転換株を誘導した。形質転換株の増殖が認められたウェルの培養上清中の抗GD3キメラ抗体の抗原結合活性を実施例1の3項に示すELISA法により測定した。培養上清中に抗GD3キメラ抗体の生産が認められたウェルの形質転換株については、上記と同様の方法により、MTX濃度を100nM、200nMと順次上昇させ、最終的にG418を0.5mg/ml、MTXを200nMの濃度で含むEX-CELL302-dFBS(10)培地で増殖可能かつ、抗GD3キメラ抗体を高生産する形質転換株を得た。得られた形質転換株については、2回の限界希釈法による単一細胞化（クローン化）を行った。

3．抗体のGD3に対する結合活性の測定（ELISA法）

抗体のGD3に対する結合活性は以下のようにして測定した。

4nmolのGD3を10μgのジパルミトイルフォスファチジルコリン（SIGMA社製）と5μgのコレステロール（SIGMA社製）とを含む2mlのエタノール溶液に溶解した。該溶液の20μl(40pmol/ウェルとなる)を96ウェルのELISA用のプレート(Greiner社製）の各ウェルにそれぞれ分注し、風乾後、1%牛血清アルブミン（以下、BSAと表記する；SIGMA社製）を含むPBS（以下、1%BSA-PBSと表記する）を100μl/ウェルで加え、室温で1時間反応させて残存する活性基をブロックした。1%BSA-PBSを捨て、

形質転換株の培養上清或いは精製したヒト型キメラ抗体の各種希釈溶液を50μl/ウェルで加え、室温で1時間反応させた。反応後、各ウェルを0.05%Tween20（和光純薬社製）を含むPBS（以下、Tween-PBSと表記する）で洗浄後、1%BSA-PBSで3000倍に希釈したペルオキシダーゼ標識ヤギ抗ヒトIgG(H&L)抗体溶液(American Qualex社製)を二次抗体溶液として、50μl/ウェルで加え、室温で1時間反応させた。反応後、Tween-PBSで洗浄後、ABTS基質液［2,2'-アジノ-ビス(3-エチルベンゾチアゾリン-6-スルホン酸)アンモニウムの0.55gを1Lの0.1Mクエン酸緩衝液(pH4.2)に溶解し、使用直前に過酸化水素を1μl/mlで添加した溶液)を50μl/ウェルで加えて発色させ、415nmの吸光度（以下、OD415と表記する）を測定した。

4．抗GD3キメラ抗体の精製

（1）YB2/0細胞由来の生産細胞の培養及び抗体の精製

上記実施例1の2項（1）で得られた抗GD3キメラ抗体を生産する形質転換細胞クローン　をBSAを0.2%、MTXを200nM、トリヨードチロニン（以下、T3と表記する；SIGMA社製）を100nMの濃度で含むHybridoma-SFM培地に3×10^5細胞/mlとなるように懸濁し、2.0Lスピナーボトル（岩城硝子社製）を用いて50rpmの速度で撹拌培養した。37℃の恒温室内で10日間培養後、培養上清を回収した。培養上清よりProsep-A（Bioprocessing社製）カラムを用いて、添付の説明書に従い、抗GD3キメラ抗体を精製した。精製した抗GD3キメラ抗体は、YB2/0-GD3キメラ抗体と名付けた。

（2）CHO/DG44細胞由来の生産細胞の培養及び抗体の精製

上記実施例1の2項（2）で得られた抗GD3キメラ抗体を生産する形質転換細胞クローンをL-Glnを3mM、脂肪酸濃縮液（以下、CDLCと表記する；GIBCO BRL社製）を0.5%、プルロニックF68（以下、PF68と表記する；GIBCO BRL社製）を0.3%の濃度で含むEX-CELL302培地に1×10^6細胞/mlとなるように懸濁し、175mm^2フラスコ(Greiner社製）に50mlずつ分注した。5%CO2インキュベーター内で37℃で4日間培養後、培養上清を回収した。培養上清よりProsep-A（Bioprocessing社製）カラムを用いて、添付の説明書に従い、抗GD3キメラ抗体を精製した。精製した抗GD3キメラ抗体は、CHO/DG44-GD3キメラ抗体と名付けた。

（3）NS0細胞由来の生産細胞の培養及び抗体の精製

上記実施例1の2項（3）で得られた抗GD3キメラ抗体を生産する形質転換細胞クローンをL-Glnを2mM、G418を0.5mg/ml、MTXを200nM、FBSを1%の濃度で含むEX-CELL302培地に1×10^6細胞/mlとなるように懸濁し、175mm^2フラスコ（Greiner社製）に200mlずつ分注した。5%CO_2インキュベーター内で37℃で4日間培養後、培養上清

を回収した。培養上清よりProsep-A (Bioprocessing社製) カラムを用いて、添付の説明書に従い、抗GD3キメラ抗体を精製した。精製した抗GD3キメラ抗体は、NSO-GD3キメラ抗体 (302) と名付けた。また、該形質転換細胞クローンをG418を0.5mg/ml、MTXを200nMの濃度で含むGIT培地に3×10^5細胞/mlとなるように懸濁し、175mm²フラスコ (Greiner社製) に200mlずつ分注した。5%CO_2インキュベーター内で37℃で10日間培養後、培養上清を回収した。培養上清よりProsep-A (Bioprocessing社製) カラムを用いて、添付の説明書に従い、抗GD3キメラ抗体を精製した。精製した抗GD3キメラ抗体は、NSO-GD3キメラ抗体 (GIT) と名付けた。

(4) SP2/0細胞由来の生産細胞の培養及び抗体の精製

特開平 5-304989 に記載の抗 GD3 キメラ抗体を生産する形質転換細胞クローンをG418を0.5mg/ml、MTXを200nMの濃度で含むGIT培地に3×10^5細胞/mlとなるように懸濁し、175mm²フラスコ (Greiner社製) に200mlずつ分注した。5%CO_2インキュベーター内で 37℃で 8 日間培養後、培養上清を回収した。培養上清より Prosep-A (Bioprocessing社製) カラムを用いて、添付の説明書に従い、抗GD3キメラ抗体を精製した。精製した抗GD3キメラ抗体は、SP2/0-GD3キメラ抗体と名付けた。

5．精製した抗GD3キメラ抗体の解析

上記実施例1の4項で得られた各種動物細胞で生産、精製した5種類の抗GD3キメラ抗体の各 4 μg を公知の方法 [ネイチャー(Nature), 227,680, 1970] に従ってSDS-PAGEし、分子量及び製精度を解析した。その結果を第1図に示した。第1図に示したように、精製した各抗GD3キメラ抗体は、いずれも非還元条件下では分子量が約150キロダルトン (以下、Kdと表記する) の単一のバンドが、還元条件下では約50Kdと約25Kdの2本のバンドが認められた。これらの分子量は、抗体のH鎖及びL鎖のcDNAの塩基配列から推定される分子量 (H鎖：約49Kd、L鎖：約23Kd、分子全体：約144Kd) とほぼ一致し、更に、IgG 型の抗体は、非還元条件下では分子量は約 150Kdであり、還元条件下では分子内のジスルフィド結合 (以下、S-S結合と表記する) が切断され、約50Kdの分子量を持つH鎖と約25Kdの分子量を持つL鎖に分解されるという報告 [アンティボディズ：ア・ラボラトリー・マニュアル(Antibodies: A Laboratory Manual), Cold Spring Harbor Laboratory, Chapter 14, 1988、モノクローナル・アンティボディズ：プリンシプルズ・アンド・プラクティス(Monoclonal Antibodies: Principles and Practice), Academic Press Limited, 1996] と一致し、各抗GD3キメラ抗体が正しい構造の抗体分子として発現され、かつ精製されたことが確認された。

実施例２．抗 GD3 キメラ抗体の活性評価

１．抗 GD3 キメラ抗体の GD3 に対する結合活性（ELISA 法）

上記実施例１の４項で得られた５種類の精製抗 GD3 キメラ抗体の GD3（雪印乳業社製）に対する結合活性を実施例１の３項に示す ELISA 法により測定した。第２図は、添加する抗 GD3 キメラ抗体の濃度を変化させて結合活性を検討した結果である。第２図に示したように、５種類の抗 GD3 キメラ抗体は、ほぼ同等の GD3 に対する結合活性を示した。この結果は抗体の抗原結合活性は、抗体を生産する動物細胞やその培養方法に関わらず、一定であることを示している。また、NSO-GD3 キメラ抗体（302）と NSO-GD3 キメラ抗体（GIT）の比較から抗原結合活性は、培養に用いる培地にも依らず、一定であることが示唆された。

２．抗 GD3 キメラ抗体の in vitro 細胞障害活性（ADCC 活性）

上記実施例１の４項で得られた５種類の精製抗 GD3 キメラ抗体の in vitro 細胞障害活性を評価するため、以下に示す方法に従い、ADCC 活性を測定した。

（１）標的細胞溶液の調製

RPMI1640-FBS(10)培地で培養したヒトメラノーマ培養細胞株 G-361（ATCC CRL1424）の 1×10^6 細胞を調製し、放射性物質である $Na_2{}^{51}CrO_4$ を 3.7MBq 当量加えて 37℃で１時間反応させ、細胞を放射標識した。反応後、RPMI1640-FBS(10)培地で懸濁及び遠心分離操作により３回洗浄し、培地に再懸濁し、4℃で 30 分間氷中に放置して放射性物質を自然解離させた。遠心分離後、RPMI1640-FBS(10)培地を 5ml 加え、2×10^5 細胞/ml に調製し、標的細胞溶液とした。

（２）エフェクター細胞溶液の調製

健常人静脈血 50ml を採取し、ヘパリンナトリウム（武田薬品社製）0.5ml を加え穏やかに混ぜた。これを Lymphoprep（Nycomed Pharma AS 社製）を用いて使用説明書に従い、遠心分離して単核球層を分離した。RPMI1640-FBS(10)培地で３回遠心分離して洗浄後、培地を用いて 2×10^6 細胞/ml の濃度で再懸濁し、エフェクター細胞溶液とした。

（３）ADCC 活性の測定

96 ウェルU字底プレート（Falcon 社製）の各ウェルに上記（１）で調製した標的細胞溶液の 50 μl（1×10^4 細胞/ウェル）を分注した。次いで（２）で調製したエフェクター細胞溶液を 100 μl（2×10^5 細胞/ウェル、エフェクター細胞と標的細胞の比は 20:1 となる）添加した。更に、各種抗 GD3 キメラ抗体を各最終濃度 0.0025～2.5 μg/ml となるように加え、37℃で４時間反応させた。反応後、プレートを遠心分離

し、上清の ^{51}Cr 量をγ-カウンターにて測定した。自然解離 ^{51}Cr 量は、エフェクター細胞溶液、抗体溶液の代わりに培地のみを用いて上記と同様の操作を行い、上清の ^{51}Cr 量を測定することにより求めた。全解離 ^{51}Cr 量は、抗体溶液の代わりに培地のみを、エフェクター細胞溶液の代わりに1規定塩酸を添加し、上記と同様の操作を行い、上清の ^{51}Cr 量を測定することにより求めた。ADCC活性は下式により求めた。

$$\text{ADDC 活性(\%)} = \frac{\text{検体上清中の } ^{51}Cr \text{ 量} - \text{自然解離 } ^{51}Cr \text{ 量}}{\text{全解離 } ^{51}Cr \text{ 量} - \text{自然解離 } ^{51}Cr \text{ 量}} \times 100$$

その結果を第3図に示した。第3図に示したように、5種類の抗GD3キメラ抗体のうち、YB2/0-GD3キメラ抗体が最も高いADCC活性を示し、次いでSP2/0-GD3キメラ抗体、NSO-GD3キメラ抗体、CHO-GD3キメラ抗体の順に高いADCC活性を示した。培養に用いた培地の異なるNSO-GD3キメラ抗体（302）とNSO-GD3キメラ抗体（GIT）では、それらのADCC活性に差は認められなかった。以上の結果は、抗体のADCC活性は、生産に用いる動物細胞によって大きく異なることを示している。その機構としては、抗原結合活性が同等であったことから、抗体のFc領域の構造の差に起因していることが推定された。

実施例3．抗ヒトインターロイキン5レセプターα鎖ヒト型CDR移植抗体の作製

1．抗ヒトインターロイキン5レセプターα鎖ヒト型CDR移植抗体の安定生産細胞の作製

（1）ラットミエローマYB2/0細胞を用いた生産細胞の作製

WO97/10354に記載の抗ヒトインターロイキン5レセプターα鎖ヒト型CDR移植抗体（以下、抗hIL-5R α CDR移植抗体と表記する）の発現ベクターpKANTEX1259HV3LV0を用いて抗hIL-5R α CDR移植抗体の安定生産細胞を以下のようにして作製した。

抗hIL-5R α CDR移植抗体発現ベクターpKANTEX1259HV3LV0の5 μgを 4×10^6 細胞のラットミエローマYB2/0細胞へエレクトロポレーション法［サイトテクノロジー(Cytotechnology), 3, 133, 1990］により導入後、40mlのRPMI1640-FBS(10)に懸濁し、96ウェル培養用プレート（住友ベークライト社製）に200 μl/ウェルずつ分注した。5%CO2インキュベーター内で37℃、24時間培養した後、G418を0.5mg/mlになるように添加して1～2週間培養した。G418耐性を示す形質転換株のコロニーが出現し、増殖の認められたウェルより培養上清を回収し、上清中の抗hIL-5R α CDR移植

抗体の抗原結合活性を実施例３の２項に示すELISA法により測定した。

培養上清中に抗hIL-5Rα CDR移植抗体の生産が認められたウェルの形質転換株については、DHFR 遺伝子増幅系を利用して抗体生産量を増加させる目的で、G418 を0.5mg/ml、MTXを50nM含むRPMI1640-FBS(10)培地に1～2×10^5細胞/mlになるように懸濁し、24ウェルプレート（Greiner社製）に2mlずつ分注した。5%CO_2インキュベーター内で37℃で1～2週間培養して、50nM MTX耐性を示す形質転換株を誘導した。形質転換株の増殖が認められたウェルの培養上清中の抗hIL-5Rα CDR移植抗体の抗原結合活性を実施例３の２項に示すELISA法により測定した。培養上清中に抗hIL-5Rα CDR移植抗体の生産が認められたウェルの形質転換株については、上記と同様の方法により、MTX濃度を100nM、200nMと順次上昇させ、最終的にG418を0.5mg/ml、MTXを200nMの濃度で含むRPMI1640-FBS(10)培地で増殖可能かつ、抗hIL-5Rα CDR移植抗体を高生産する形質転換株を得た。得られた形質転換株については、２回の限界希釈法による単一細胞化（クローン化）を行った。このようにして得られた抗hIL-5Rα CDR移植抗体を生産する形質転換細胞クローンNo.3は平成１１年４月５日付で工業技術院生命工学工業技術研究所（日本国茨城県つくば市東１丁目１番３号）にFERM BP-6690として寄託されている。

（２）CHO/dhfr-細胞を用いた生産細胞の作製

WO97/10354に記載の抗hIL-5Rα CDR移植抗体発現ベクターpKANTEX1259HV3LV0の４μgを1.6×10^6細胞のCHO/dhfr-細胞へエレクトロポレーション法［サイトテクノロジー(Cytotechnology), 3, 133 (1990)］により導入後、10mlのIMDM-FBS(10)に懸濁し、96ウェル培養用プレート（岩城硝子社製）に200μl/ウェルずつ分注した。5%CO_2インキュベーター内で37℃、24時間培養した後、G418を0.5mg/mlになるように添加して1～2週間培養した。G418耐性を示す形質転換株のコロニーが出現し、増殖の認められたウェルより培養上清を回収し、上清中の抗hIL-5Rα CDR移植抗体の抗原結合活性を実施例３の２項に示すELISA法により測定した。

培養上清中に抗hIL-5Rα CDR移植抗体の生産が認められたウェルの形質転換株については、DHFR 遺伝子増幅系を利用して抗体生産量を増加させる目的で、G418 を0.5mg/ml、MTXを10nM含むIMDM-dFBS(10)培地に1～2×10^5細胞/mlになるように懸濁し、24ウェルプレート（岩城硝子社製）に0.5mlずつ分注した。5%CO_2インキュベーター内で37℃で1～2週間培養して、10nM MTX耐性を示す形質転換株を誘導した。増殖が認められたウェルの形質転換株については、上記と同様の方法により、MTX濃度を100nM、500nMに上昇させ、最終的にG418を0.5mg/ml、MTXを500nMの濃度で含

む IMDM-dFBS(10)培地で増殖可能かつ、抗 hIL-5R α CDR 移植抗体を高生産する形質転換株を得た。得られた形質転換株については、2 回の限界希釈法による単一細胞化（クローン化）を行った。

（3）マウスミエローマ NSO 細胞を用いた生産細胞の作製

ヤラントン(Yarranton)らの方法[バイオ/テクノロジー(BIO/TECHNOLOGY), 10, 169 (1992)] に従い、WO97/10354 に記載の抗 hIL-5R α CDR 移植抗体発現ベクター pKANTEX1259HV3LV0 上の抗体 H 鎖及び L 鎖 cDNA を用いて抗 hIL-5R α CDR 移植抗体発現ベクターを作製し、NSO 細胞を形質転換し、抗 hIL-5R α CDR 移植抗体を高生産する形質転換株を得た。得られた形質転換株については、2 回の限界希釈法による単一細胞化（クローン化）を行った。

2．抗体の hIL-5R α に対する結合活性の測定（ELISA 法）

抗体の hIL-5R α に対する結合活性は以下のようにして測定した。

WO97/10354 に記載の抗 hIL-5R α マウス抗体 KM1257 を PBS で 10 μg/ml の濃度に希釈した溶液の 50 μl を 96 ウェルの ELISA 用のプレート（Greiner 社製）の各ウェルにそれぞれ分注し、4℃で 20 時間反応させた。反応後、1%BSA-PBS を 100 μl/ウェルで加え、室温で 1 時間反応させて残存する活性基をブロックした。1%BSA-PBS を捨て、WO97/10354 に記載の可溶性 hIL-5R α を 1%BSA-PBS で 0.5 μg/ml の濃度に希釈した溶液を 50 μl/ウェルで加え、4℃で 20 時間反応させた。反応後、各ウェルを Tween-PBS で洗浄後、形質転換株の培養上清或いは精製したヒト型 CDR 移植抗体の各種希釈溶液を 50 μl/ウェルで加え、室温で 2 時間反応させた。反応後、各ウェルを Tween-PBS で洗浄後、1%BSA-PBS で 3000 倍に希釈したペルオキシダーゼ標識ヤギ抗ヒト IgG(H&L) 抗体溶液（American Qualex 社製）を二次抗体溶液として、50 μl/ウェルで加え、室温で 1 時間反応させた。反応後、Tween-PBS で洗浄後、ABTS 基質液 [2,2'-アジノ-ビス(3-エチルベンゾチアゾリン-6-スルホン酸)アンモニウムの 0.55g を 1L の 0.1M クエン酸緩衝液(pH4.2)に溶解し、使用直前に過酸化水素を 1 μl/ml で添加した溶液] を 50 μl/ウェルで加えて発色させ、OD415 を測定した。

3．抗 hIL-5R α CDR 移植抗体の精製

（1）YB2/0 細胞由来の生産細胞の培養及び抗体の精製

上記実施例 3 の 1 項（1）で得られた抗 hIL-5R α CDR 移植抗体を生産する形質転換細胞クローンを G418 を 0.5mg/ml、MTX を 200nM の濃度で含む GIT 培地に 3×10^5 細胞/ml となるように懸濁し、175mm_2 フラスコ（Greiner 社製）に 200ml ずつ分注した。5%CO_2 インキュベーター内で 37℃で 8 日間培養後、培養上清を回収した。培養上清よ

りイオン交換クロマトグラフィー及びゲル濾過法を用いて抗 hIL-5R α CDR 移植抗体を精製した。精製した抗 hIL-5R α CDR 移植抗体は、YB2/0-hIL-5RCDR 抗体と名付けた。

（２）CHO/dhfr-細胞由来の生産細胞の培養及び抗体の精製

上記実施例３の１項（２）で得られた抗 hIL-5R α CDR 移植抗体を生産する形質転換細胞クローンを L-Gln を 3mM、CDLC を 0.5%、PF68 を 0.3%の濃度で含む EX-CELL302 培地に 3×10^5 細胞/ml となるように懸濁し、4.0L スピナーボトル（岩城硝子社製）を用いて 100rpm の速度で撹拌培養した。37℃の恒温室内で 10 日間培養後、培養上清を回収した。培養上清よりイオン交換クロマトグラフィー及びゲル濾過法を用いて抗 hIL-5R α CDR 移植抗体を精製した。精製した抗 hIL-5R α CDR 移植抗体は、CHO/d-hIL-5RCDR 抗体と名付けた。

（３）NS0 細胞由来の生産細胞の培養及び抗体の精製

上記実施例３の１項（３）で得られた抗 hIL-5R α CDR 移植抗体を生産する形質転換細胞クローンをヤラントン（Yarranton）らの方法［バイオ/テクノロジー(BIO/TECHNOLOGY), 10, 169 (1992)］に従い、培養後、培養上清を回収した。培養上清よりイオン交換クロマトグラフィー及びゲル濾過法を用いて抗 hIL-5R α CDR 移植抗体を精製した。精製した抗 hIL-5R α CDR 移植抗体は、NS0-hIL-5RCDR 抗体と名付けた。

４．精製した抗 hIL-5R α CDR 移植抗体の解析

上記実施例３の３項で得られた各種動物細胞で生産、精製した 3 種類の抗 hIL-5R α CDR 移植抗体の各 4 μg を公知の方法［ネイチャー(Nature), 227, 680 (1970)］に従って SDS-PAGE し、分子量及び製精度を解析した。その結果を第 4 図に示した。第 4 図に示したように、精製した各抗 hIL-5R α CDR 移植抗体は、いずれも非還元条件下では分子量が約 150Kd の単一のバンドが、還元条件下では約 50Kd と約 25Kd の 2 本のバンドが認められた。これらの分子量は、抗体の H 鎖及び L 鎖の cDNA の塩基配列から推定される分子量（H 鎖：約 49Kd、L 鎖：約 23Kd、分子全体：約 144Kd）とほぼ一致し、更に、IgG 型の抗体は、非還元条件下では分子量は約 150Kd であり、還元条件下では分子内のジスルフィド結合（以下、S-S 結合と表記する）が切断され、約 50Kd の分子量を持つ H 鎖と約 25Kd の分子量を持つ L 鎖に分解されるという報告［アンティボディズ：ア・ラボラトリー・マニュアル(Antibodies: A Laboratory Manual), Cold Spring Harbor Laboratory, Chapter 14, 1988、モノクローナル・アンティボディズ:プリンシプルズ・アンド・プラクティス(Monoclonal Antibodies: Principles and Practice), Academic Press Limited, 1996］と一致し、各抗 hIL-5R α CDR 移植抗体

が正しい構造の抗体分子として発現され、かつ、精製されたことが確認された。

実施例４．抗hIL-5R α CDR移植抗体の活性評価

１．抗hIL-5R α CDR移植抗体のhIL-5R αに対する結合活性（ELISA法）

上記実施例３の３項で得られた３種類の精製抗hIL-5R α CDR移植抗体のhIL-5R αに対する結合活性を実施例３の２項に示すELISA法により測定した。第５図は、添加する抗hIL-5R α CDR移植抗体の濃度を変化させて結合活性を検討した結果である。第５図に示したように、３種類の抗hIL-5R α CDR移植抗体は、ほぼ同等のhIL-5R αに対する結合活性を示した。この結果は実施例２の１項の結果と同様に、抗体の抗原結合活性は、抗体を生産する動物細胞やその培養方法に関わらず、一定であることを示している。

２．抗hIL-5R α CDR移植抗体のin vitro細胞障害活性（ADCC活性）

上記実施例３の３項で得られた３種類の精製抗hIL-5R α CDR移植抗体のin vitro細胞障害活性を評価するため、以下に示す方法に従い、ADCC活性を測定した。

（１）標的細胞溶液の調製

WO97/10354に記載のhIL-5R α鎖及びβ鎖を発現しているマウスT細胞株CTLL-2(h5R)をRPMI1640-FBS(10)培地で培養し、1×10^6細胞/0.5mlとなるように調製し、放射性物質である$Na_2{}^{51}CrO_4$を3.7MBq当量加えて37℃で１.５時間反応させ、細胞を放射標識した。反応後、RPMI1640-FBS(10)培地で懸濁及び遠心分離操作により３回洗浄し、培地に再懸濁し、4℃で30分間氷中に放置して放射性物質を自然解離させた。遠心分離後、RPMI1640-FBS(10)培地を5ml加え、2×10^5細胞/mlに調製し、標的細胞溶液とした。

（２）エフェクター細胞溶液の調製

健常人静脈血50mlを採取し、ヘパリンナトリウム（武田薬品社製）0.5mlを加え穏やかに混ぜた。これをPolymorphprep（Nycomed Pharma AS社製）を用いて使用説明書に従い、遠心分離して単核球層を分離した。RPMI1640-FBS(10)培地で３回遠心分離して洗浄後、培地を用いて9×10^6細胞/mlの濃度で再懸濁し、エフェクター細胞溶液とした。

（３）ADCC活性の測定

96ウェルU字底プレート（Falcon社製）の各ウェルに上記（１）で調製した標的細胞溶液の50μl（1×10^4細胞/ウェル）を分注した。次いで（２）で調製したエフェクター細胞溶液を100μl（9×10^5細胞/ウェル、エフェクター細胞と標的細胞の比は90:1となる）添加した。更に、各種抗hIL-5R α CDR移植抗体を各最終濃度0.001

～0.1 μg/ml となるように加え、37℃で 4 時間反応させた。反応後、プレートを遠心分離し、上清の ^{51}Cr 量をγ-カウンターにて測定した。自然解離 ^{51}Cr 量は、エフェクター細胞溶液、抗体溶液の代わりに培地のみを用いて上記と同様の操作を行い、上清の ^{51}Cr 量を測定することにより求めた。全解離 ^{51}Cr 量は、抗体溶液の代わりに培地のみを、エフェクター細胞溶液の代わりに 1 規定塩酸を添加し、上記と同様の操作を行い、上清の ^{51}Cr 量を測定することにより求めた。

ADCC 活性は下式により求めた。

$$\text{ADDC 活性(\%)} = \frac{\text{検体上清中の } ^{51}Cr \text{ 量} - \text{自然解離 } ^{51}Cr \text{ 量}}{\text{全解離 } ^{51}Cr \text{ 量} - \text{自然解離 } ^{51}Cr \text{ 量}} \times 100$$

その結果を第 6 図に示した。第 6 図に示したように、3 種類の抗 hIL-5R α CDR 移植抗体のうち、YB2/0-hIL-5RCDR 抗体が最も高い ADCC 活性を示し、次いで CHO/d-hIL-5RCDR 抗体、NSO-hIL-5RCDR 抗体の順に高い ADCC 活性を示した。以上の結果は実施例 2 の 2 項の結果と同ように、抗体の ADCC 活性は、生産に用いる動物細胞によって大きく異なることを示している。更に、2 種類のヒト化抗体のいずれの場合も YB2/0 細胞で生産した抗体が最も高い ADCC 活性を示したことから、YB2/0 細胞を用いることにより、ADCC 活性の高い抗体を製造できることが明らかとなった。

3．抗 hIL-5R α CDR 移植抗体の in vivo における活性評価

上記実施例 3 の 3 項で得られた 3 種類の精製抗 hIL-5R α CDR 移植抗体の in vivo における活性を評価するため、以下に示す方法に従い、カニクイザルの hIL-5 誘発好酸球増加モデルに対する抑制作用を検討した。

カニクイザルに初日より hIL-5（調製方法は WO97/10354 に記載）を 1 μg/kg で 1 日 1 回、計 14 回背部皮下より投与した。各種抗 hIL-5R α CDR 移植抗体を 0 日の hIL-5 の投与 1 時間前に 0.3mg/kg で静脈内に単回投与した。抗体非投与群をコントロールとして用いた。抗体投与群は各群 3 頭（No.301、No.302、No.303、No.401、No.402、No.403、No.501、No.502、No.503）、抗体非投与群は 2 頭（No.101、No.102）のカニクイザルを用いた。投与開始の 7 日前より投与後 42 日目まで経時的に約 1ml の血液を伏在静脈または大腿静脈より採取し、1 μl の末梢血中の好酸球数を測定した。その結果を第 7 図に示した。第 7 図に示したように、YB2/0-hIL-5RCDR 抗体を投与した群では、血中好酸球の増加が完全に抑制された。一方、CHO/d-hIL-5RCDR 抗体の投与群では、1 頭で完全な抑制作用が認められたものの、2 頭ではその抑制作用は不充分

であった。NSO-hIL-5RCDR 抗体の投与群では、完全な抑制作用は認められず、その効果は不充分であった。以上の結果は、抗体の in vivo 活性は、生産に用いる動物細胞によって大きく異なることを示している。更に、抗 hIL-5R α CDR 移植抗体ではその in vivo 活性の高さは、実施例４の２項で述べた ADCC 活性の高さと正の相関が認められたことから、その活性発現には、ADCC 活性の高さが極めて重要であることが示唆された。

以上の結果から、ADCC 活性の高い抗体は、ヒトの各種疾患の臨床においても有用であることが期待される。

実施例５．ADCC 活性を高める糖鎖の解析

1．2-アミノピリジン標識糖鎖（PA 化糖鎖）の調製

本発明のヒト化抗体を塩酸による酸加水分解にてシアル酸を除去した。塩酸を完全に除去した後、ヒドラジン分解により糖鎖を蛋白質から切断した［メソッド・オブ・エンザイモロジー（Method of Enzymology），83,263,1982］。ヒドラジンを除去した後、酢酸アンモニウム水溶液と無水酢酸加えて N-アセチル化を行った。凍結乾燥後、2-アミノピリジンによる蛍光標識を行った［ジャーナル・オブ・バイオケミストリー（J.Biochem.），95,197（1984）］。蛍光標識した糖鎖（PA 化糖鎖）を、Surperdex Peptide HR 10/30 カラム（Pharmacia 社製）を用いて不純物と分離した。糖鎖画分を遠心濃縮機にて乾固させ、精製 PA 化糖鎖とした。

２．精製抗 hIL-5R α CDR 移植抗体の PA 化糖鎖の逆相 HPLC 分析

上記実施例５の１項で調製した各種抗 hIL-5RCDR 抗体の PA 化糖鎖を用いて、CLC-ODS カラム（Shimadzu 社製）による逆相 HPLC 分析を行った。過剰量のα-L-フコシダーゼ（ウシ腎由来、SIGMA 社製）を PA 化糖鎖に添加して消化を行い（37℃、15 時間）、逆相 HPLC で分析した（第８図）。アスパラギン結合糖鎖は 30 分間から 80 分間の範囲に溶出することを TaKaRa 社製 PA 化糖鎖スタンダードを用いて確認した。α-L-フコシダーゼ消化によって、逆相 HPLC の溶出位置が移動する糖鎖（48 分間から 78 分間に溶出される糖鎖）の全体に占める割合を計算した。結果を第１表に示す。

第　1　表

抗体の生産細胞	α 1-6 フコース結合糖鎖（%）
YB2/0	47
NSO	73

YB2/0 細胞で生産させた抗 hIL-5RCDR 移植抗体は約 47%、NS0 細胞で生産させた抗 hIL-5RCDR 移植抗体は約 73%がα 1-6 フコースをもつ糖鎖であった。よって、YB 2/0 細胞で生産した抗体は、NS0 細胞で生産した抗体と比較してα 1-6 フコースを持たない糖鎖が多かった。

3. 精製抗 hIL-5R α CDR 移植抗体の単糖組成分析

トリフルオロ酢酸による酸加水分解により、YB2/0 細胞、NS0 細胞および CHO/d 細胞で生産した抗 hIL-5R α CDR 移植抗体の糖鎖を単糖に分解し、BioLC (Dionex 社製) を用いて単糖組成分析を行った。

N-グリコシド結合糖鎖のうち、コンプレックス型では、1 本の糖鎖におけるマンノース数は 3 であるため、マンノースを 3 として計算した場合の各単糖の相対比を第 2 表に示す。

第 2 表

抗体の生産細胞	Fuc	GlcNAc	Gal	Man	ADCC 活性(%) *
YB2/0	0.60	4.98	0.30	3.00	42.27
NS0	1.06	3.94	0.66	3.00	16.22
CHO/dhFr-	0.85	3.59	0.49	3.00	25.73
	0.91	3.80	0.27	3.00	

＊抗体濃度 0.01 μ g/ml

フコースの相対比は、 YB2/0<CHO/d<NS0 であり、本結果でも YB2/0 細胞で生産した抗体の糖鎖はフコース含量が最も低かった。

実施例 6.CHO/dhfr-細胞生産抗体の糖鎖解析

CHO/dhfr-細胞で生産した精製抗 hIl-5R α CDR 移植抗体から PA 化糖鎖を調製し、CLC-ODS カラム (島津社製) を用いて逆相 HPLC 分析を行った(第 9 図)。第 9 図において、溶出時間 35～45 分間がフコースを持たない糖鎖、45～60 分間がフコースを持つ糖鎖であった。CHO/dhfr-細胞で生産した抗 hIl-5R α CDR 移植抗体は、マウスミエローマ NS0 細胞で生産させた抗体と同様に、ラットミエローマ YB2/0 細胞で生産させた抗体よりもフコースを持たない糖鎖の含量が少なかった。

実施例 7.高 ADCC 活性抗体の分離

フコースを持つ糖鎖に結合するレクチンカラムを用いて、ラットミエローマ YB2/0細胞で生産させた抗 hIl-5R α CDR 移植抗体の分離を行った。HPLC は島津社製 LC-6A を用い、流速は 1ml/分、カラム温度は室温で行った。50mM トリス-硫酸緩衝液(pH7.3)で平衡化し、精製された抗 hIL-5R α CDR 移植抗体を注入後、0.2M α-メチルマンノシド(ナカライテスク社製)の直線濃度勾配(60 分間)にて溶出した。抗 hIl-5R α CDR 移植抗体を非吸着画分と吸着画分とに分離した。非吸着画分、吸着画分の一部をとり、hIL-5R α に対する結合活性を測定すると、同様の結合活性を示した(第 10 図 上図)。ADCC 活性を測定すると、非吸着画分の方が吸着画分の一部よりも高い ADCC 活性を示した(第 10 図 下図)。さらに、非吸着画分、吸着画分の一部から PA 化糖鎖を調製し、CLC-ODS カラム（島津社製）を用いて逆相 HPLC 分析を行った(第 11 図)。非吸着画分は主としてフコースのない糖鎖をもつ抗体であり、吸着画分の一部は主としてフコースがある糖鎖もつ抗体であった。

実施例８．宿主細胞株におけるα1,6-フコシルトランスフェラーゼ（FUT8）遺伝子の転写物の定量

（１）各種細胞株由来一本鎖cDNAの調製

チャイニーズハムスター卵巣由来CHO/DG44細胞を、10% FBS (Life Technologies社) および１倍濃度のHT supplement (Life Technologies社) を添加したIMDM培地(Life Technologies社)に懸濁し、2×10^5cells/mlの密度で接着細胞培養用T75フラスコ（Greiner社）に播種した。またラットミエローマ由来YB2/0細胞を、10% FBS (Life Technologies社)、４mM グルタミン (Life Technologies社) を添加したRPMI1640培地(Life Technologies社)に懸濁し、2×10^5cells/mlの密度で浮遊細胞培養用T75フラスコ (Greiner社) に播種した。これらを37℃の5% CO_2インキュベーター内で培養し、培養１日目、２日目、３日目、４日目および５日目に各宿主細胞1×10^7cellsを回収し、RNAeasy (QIAGEN社製) により全RNAを抽出した。

全RNAを45μlの滅菌水に溶解し、RQ1 Rnase-Free DNase (Promega社) 0.5U/μl、付属の10×DNase buffer 5μl、RNasin Ribonuclease inhibitor (Promega社) 0.5μlをそれぞれに添加して、37℃で30分間反応させた。反応後、RNAeasy (QIAGEN社) により全RNAを再精製し、50μlの滅菌水に溶解した。

得られた各々の全RNA3μgについて、オリゴ(dT)をプライマーとしてSUPERSCRIPT™ Preamplification System for First Strand cDNA Synthesis (Life Technologies社) により、20μlの系で逆転写反応を行い、cDNAを合成した。各宿主細胞由来FUT8およびβ-アクチンのクローニングには逆転写反応後の溶液の１倍濃度液を、競合的PCRに

よる各遺伝子転写量の定量には逆転写反応後の溶液を水で50倍希釈したものを用い、各々使用するまで-80℃で保管した。

（2）チャイニーズハムスターFUT8およびラットFUT8の各cDNA部分断片の取得

チャイニーズハムスターFUT8およびラットFUT8の各cDNA部分断片の取得は、以下のように行った。まず、ヒトFUT8のcDNA[Journal of Biochemistry, 121, 626 (1997)]およびブタFUT8のcDNA[Journal of Biological Chemistry, 271, 27810 (1996)]に共通の塩基配列に対して特異的なプライマー（配列番号1および配列番号2に示す）を設計した。

次にDNAポリメラーゼExTaq(宝酒造社)を用いて、（1）で調製した培養2日目のCHO細胞由来cDNAおよびYB2/0細胞由来cDNAを各々1μlを含む25μlの反応液[ExTaq buffer(宝酒造社)、0.2mM dNTPs、0.5μM 上記特異的プライマー(配列番号1および配列番号2)]を調製し、ポリメラーゼ連鎖反応（PCR）を行った。PCRは、94℃で1分間の加熱の後、94℃で30秒間、55℃で30秒間、72℃で2分間からなる反応を1サイクルとして30サイクルの後、さらに72℃で10分間加熱する条件で行った。PCRにより得た各特異的増幅断片979bpをTOPO TA cloning Kit（Invitrogen社）を用いてプラスミドpCR2.1へ連結し、チャイニーズハムスターFUT8およびラットFUT8の各cDNA部分断片を含むプラスミド（CHFT8-pCR2.1およびYBFT8-pCR2.1）を取得した。

取得した各cDNAの塩基配列について、DNAシークエンサー377（Parkin Elmer社）およびBigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Parkin Elmer社)を用いて決定し、取得したcDNAがチャイニーズハムスターFUT8およびラットFUT8（配列番号3および4に示す）のオープンリーディングフレーム（ORF）部分配列をコードすることを確認した。

（3）チャイニーズハムスターβ-アクチンおよびラットβ-アクチンcDNAの取得

β-アクチン遺伝子は各細胞において恒常的に転写されており、その転写量は細胞間で同程度と考えられているため、各細胞由来cDNA合成反応の効率の目安としては、β-アクチン遺伝子の転写量を定量する。

チャイニーズハムスターβ-アクチンおよびラットβ-アクチンの取得は、以下の方法で行った。まず、チャイニーズハムスターβ-アクチンゲノム配列[GenBank,U20114]およびラットβ-アクチンゲノム配列[Nucleic Acid Research, 11, 1759 (1983)]より、翻訳開始コドンを含む共通配列に特異的なフォワードプライマー（配列番号5に示す）および翻訳終止コドンを含む各配列特異的なリバースプライマー(配列番号6および配列番号7に示す)を設計した。

次にDNAポリメラーゼKOD(東洋紡社)を用いて、（1）で調製した培養2日目のCHO細胞由来cDNAおよびYB2/0細胞由来cDNA 1μlを含む25μlの反応液[KOD buffer #1(東洋紡社)、0.2mM dNTPs、1mM $MgCl_2$、0.4μM 上記遺伝子特異的プライマー（配列番号5および配列番号6、または配列番号5および配列番号7）、5% DMSO]を調製し、ポリメラーゼ連鎖反応（PCR）を行った。PCRは、94℃で4分間の加熱の後、98℃で15秒間、65℃で2秒間、74℃で30秒間からなる反応を1サイクルとして、25サイクル行った。PCRにより得た各特異的増幅断片1128bpの5'末端をMEGALABEL(宝酒造社)によりリン酸化した後、pBluescriptII KS(+)（Strategene社）に、制限酵素EcoRVにより切断して得られる断片（2.9Kb）をLigation High(東洋紡社)で連結し、チャイニーズハムスターβ-アクチンおよびラットβ-アクチンの各cDNAのORF全長を含むプラスミド（CHAc-pBS、YBAc-pBS）を得た。

取得した各cDNAの塩基配列を、DNAシークエンサー377（Parkin Elmer社製）およびBigDye Terminator Cycle Sequencing FS Ready Reaction Kit（Parkin Elmer社製）を用いて決定し、各々チャイニーズハムスターβ-アクチンおよびラットβ-アクチンの各cDNAのORF全長配列をコードすることを確認した。

（4）スタンダードおよび内部配列のコントロールの調製

生産細胞内のFUT8遺伝子からのmRNA転写量を測定するために、まず検量線を作成した。

検量線に用いるFUT8のスタンダードとしては、（2）で得たチャイニーズハムスターFUT8およびラットFUT8の各cDNA部分断片をpCR2.1に組み込んだプラスミドであるCHFT8-pCR2.1およびYBFT8-pCR2.1を制限酵素EcoRIで切断して得られたDNAを直鎖化して用いた。

FUT8定量の内部コントロールとしては、CHFT8-pCR2.1およびYBFT8-pCR2.1のうち、チャイニーズハムスターFUT8およびラットFUT8の内部塩基配列のScaI-HindIII間203bpを欠失させることにより得られたCHFT8d-pCR2.1およびYBFT8d-pCR2.1を、制限酵素EcoRIで切断して得られたDNAを直鎖化して用いた。

生産細胞内のβ-アクチン遺伝子からのmRNA転写量のスタンダードとしては、（3）で得たチャイニーズハムスターβ-アクチンおよびラットβ-アクチンのそれぞれのcDNAのORF全長をpBluescriptII KS(+)に組み込んだプラスミドであるCHAc-pBSおよびYBAc-pBSを、前者はHindIIIおよびPstIで、後者はHindIIIおよびKpnIで、各々切断して得られたDNAを直鎖化して用いた。

β-アクチン定量の内部コントロールとしては、CHAc-pBSおよびYBAc-pBSのうち、テ

ャイニーズハムスターβ-アクチンおよびラットβ-アクチンの内部塩基配列のDraIII-DraIII間180bpを欠失させることにより得られたCHAcd-pBSおよびYBAcd-pBSを、前者はHindIIIおよびPstIで、後者はHindIIIおよびKpnIで、各々切断して得られたDNAを直鎖化して用いた。

（５）競合的RT-PCRによる転写量の定量

FUT8の転写産物の定量は以下のように行った。まず（２）で得たチャイニーズハムスターFUT8およびラットFUT8のORF部分配列の内部配列に対し、共通配列特異的なプライマーセット（配列番号８および９に示す）を設計した。

次に、（１）で得られた各宿主細胞株由来のcDNA溶液の50倍希釈液　５μlおよび内部コントロール用プラスミド５μl（10fg）を含む総体積20μlの反応液[ExTaq buffer(宝酒造社)、0.2mM dNTPs、0.5μM　上記遺伝子特異的プライマー（配列番号８および配列番号９）、５% DMSO]で、DNAポリメラーゼExTaq(宝酒造社)を用いてPCRを行った。PCRは、94℃で3分間の加熱の後、94℃で1分間、60℃で1分間、72℃で1分間からなる反応を１サイクルとして32サイクル行った。

β-アクチンの転写産物の定量は、以下のように行った。（３）で得たチャイニーズハムスターβ-アクチンおよびラットβ-アクチンORF全長の内部配列に対し、各遺伝子特異的なプライマーセット（前者を配列番号１０および配列番号１１に、後者を配列番号１２および配列番号１３に示す）をそれぞれ設計した。

次に、（１）で得られた各宿主細胞株由来のcDNA溶液の50倍希釈液　５μlおよび内部コントロール用プラスミド５μl（１pg）を含む総体積20μlの反応液[ExTaq buffer(宝酒造社)、0.2mM dNTPs、0.5μM　上記遺伝子特異的プライマー（配列番号１０および配列番号１１、または配列番号１２および配列番号１３）、５% DMSO]で、DNAポリメラーゼExTaq(宝酒造社)を用いてPCRを行った。PCRは、94℃で3分間の加熱の後、94℃で30秒間、65℃で1分間、72℃で2分間からなる反応を１サイクルとして17サイクル行った。

第 3 表

ターゲット遺伝子	*プライマーセット	PCR増幅産物のサイズ(bp) ターゲット	コンペティター
FUT8	F：5'-GTCCATGGTGATCCTGCAGTGTGG-3' R：5'-CACCAATGATATCTCCAGGTTCC-3'	638	431
β-actin (チャイニーズハムスター)	F：5'-GATATCGCTGCGCTCGTTGTCGAC-3' R：5'-CAGGAAGGAAGGCTGGAAAAGAGC-3'	789	609
β-actin (ラット)	F：5'-GATATCGCTGCGCTCGTCGTCGAC-3' R：5'-CAGGAAGGAAGGCTGGAAGAGAGC-3'	789	609.

*F:フォワードプライマー、R:リバースプライマー

第３表に記載のプライマーセットを用いて定量的PCRを行った。その結果、各遺伝子転写産物および各スタンダードからは、第３表のターゲット欄に示したサイズのDNA断片を、各内部コントロールからは、第３表のコンペティター欄に示したサイズのDNA断片を増幅させることができた。

PCR後の溶液のうち、７μlを1.75%アガロースゲル電気泳動に供した後、ゲルをSYBR Green I Nucleic Acid Gel Stain (Molecular Probes社) で染色した。増幅された各DNA断片の発光強度をフルオロイメージャー (FluorImager SI; Molecular Dynamics社) で算出することにより、増幅されたDNA断片の量を測定した。

一方、細胞由来cDNAに代えて（４）で調製したスタンダードプラスミド量を0.1fg、１fg、５fg、10fg、50fg、100fgおよび500fgとしてPCRをそれぞれ行い、増幅産物量を測定した。そして、その測定値とスタンダードプラスミド量をプロットして検量線を作成した。

上述の検量線を用いて、各細胞由来全cDNAを鋳型とした場合の増幅産物の量より各細胞中の目的遺伝子cDNA量を算出し、これを各細胞におけるmRNA転写量とした。

ラットFUT8配列をスタンダード、内部コントロールに用いた場合の各宿主細胞株におけるFUT8転写産物の量を第１２図に示した。培養期間を通じてCHO細胞株はYB2/0細胞株の10倍以上の転写量を示した。この傾向は、チャイニーズハムスターFUT8配列をスタンダード、内部コントロールに用いた場合にも認められた。

また、第４表にβ-actin転写産物の量との相対値としてFUT8転写量を示した。

第 4 表

	培養日数				
細胞株	Day1	Day2	Day3	Day4	Day5
CHO	2.0	0.90	0.57	0.52	0.54
YB2/0	0.07	0.13	0.13	0.05	0.02

YB2/0細胞株のFUT8転写量がβ-actinの0.1%前後であるのに対し、CHO細胞株は0.5～2%であった。

以上の結果より、YB2/0細胞株のFUT8転写産物量はCHO細胞株のそれよりも有意に少ないことが示された。

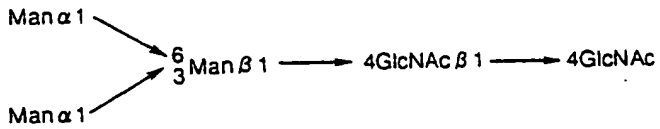
産業上の利用可能性

本発明は、抗体、蛋白質またはペプチドなどの免疫機能分子の有する活性を調節する糖鎖、および糖鎖を有する抗体、蛋白質またはペプチドに関する。本発明は更に、該糖鎖および糖鎖を有する抗体、蛋白質またはペプチドの製造法、ならびにそれらを有効成分として含有する診断薬、予防薬および治療薬に関する。

請求の範囲

1． 免疫機能分子に結合するN－グリコシド結合糖鎖の還元末端であるN-アセチルグルコサミンへのフコースの結合の有無による、免疫機能分子の活性を調節する方法。

2． 免疫機能分子に結合するN-グリコシド結合糖鎖が、



を含む糖鎖であることを特徴とする、請求の範囲1記載の方法。

3． N－グリコシド結合糖鎖の還元末端のN-アセチルグルコサミンにフコースが存在しない糖鎖を免疫機能分子に結合させることを特徴とする、免疫機能分子の活性を促進させる方法。

4． 糖鎖が、

を含む糖鎖であることを特徴とする、請求の範囲3記載の方法。

5． 糖鎖が、還元末端のN-アセチルグルコサミンにフコースを付加する酵素活性の低いか、または当該酵素活性を有しない細胞が合成する糖鎖であることを特徴とする、請求の範囲3記載の方法。

6． 還元末端のN-アセチルグルコサミンにフコースを付加する酵素がフコシルトランスフェラーゼである、請求の範囲5記載の方法。

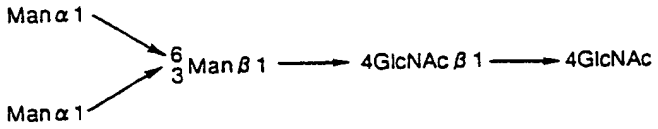
7． フコシルトランスフェラーゼがα1,6-フコシルトランスフェラーゼである、請求の範囲6記載の方法。

8． 糖鎖が、ラットミエローマ細胞が合成する糖鎖である、請求の範囲3記載の方法。

9． ラットミエローマ細胞が、ラットミエローマ細胞 YB2/3HL.P2.G11.16Ag.20細胞（ATCC CRL1662）である、請求の範囲8記載の方法。

１０．　N－グリコシド結合糖鎖の還元末端のN-アセチルグルコサミンにフコースが存在する糖鎖を免疫機能分子に結合させることを特徴とする、免疫機能分子の活性を抑制させる方法。

１１．　糖鎖が、



を含む糖鎖であることを特徴とする、請求の範囲１０記載の方法。

１２．　糖鎖が、還元末端のN-アセチルグルコサミンにフコースを付加する酵素活性が高い細胞が合成する糖鎖であることを特徴とする、請求の範囲１０記載の方法。

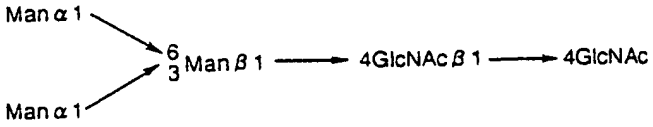
１３．　還元末端のN-アセチルグルコサミンにフコースを付加する酵素がフコシルトランスフェラーゼである、請求の範囲１２記載の方法。

１４．　フコシルトランスフェラーゼがα1,6-フコシルトランスフェラーゼである、請求の範囲１３記載の方法。

１５．　免疫機能分子が抗体、蛋白質、ペプチドである請求の範囲１～１４記載の方法。

１６．　N－グリコシド結合糖鎖の還元末端のN-アセチルグルコサミンにフコースが存在しない糖鎖を含有する免疫機能分子活性促進剤。

１７．　糖鎖が、



を含む糖鎖を含有する請求の範囲１６記載の免疫機能分子活性促進剤。

１８．　糖鎖が、還元末端のN-アセチルグルコサミンにフコースを付加する酵素活性の低いか、または当該酵素活性を有しない細胞が合成する糖鎖を含有する請求の範囲１６記載の免疫機能分子活性促進剤。

１９．　還元末端のN-アセチルグルコサミンにフコースを付加する酵素がフコシルトランスフェラーゼである、請求の範囲１８記載の免疫機能分子活性促進剤。

２０．フコシルトランスフェラーゼがα1,6-フコシルトランスフェラーゼである、請求の範囲１９記載の免疫機能分子活性促進剤。

２１．糖鎖が、ラットミエローマ細胞が合成する糖鎖である、請求の範囲１６記載の免疫機能分子活性促進剤。

２２．ラットミエローマ細胞が、ラットミエローマ細胞YB2/3HL.P2.G11.16Ag.20細胞（ATCC CRL1662）である、請求の範囲２１記載の免疫機能分子活性促進剤。

２３．免疫機能分子が抗体、蛋白質、ペプチドである請求の範囲１６～２２記載の免疫機能分子活性促進剤。

２４．Ｎ－グリコシド結合糖鎖の、還元末端のN-アセチルグルコサミンにフコースが存在しない糖鎖が結合することにより免疫機能活性が促進された免疫機能分子。

２５．Ｎ－グリコシド結合糖鎖の、還元末端のN-アセチルグルコサミンにフコースが存在する糖鎖が結合することにより免疫機能活性が抑制された免疫機能分子。

２６．免疫機能分子が抗体、蛋白質、ペプチドである請求の範囲２４記載の免疫機能分子。

２７．免疫機能分子が抗体、蛋白質、ペプチドである請求の範囲２５記載の免疫機能分子。

２８．還元末端のN-アセチルグルコサミンにフコースを付加する酵素活性の低い、または当該酵素活性を有しない細胞を用いることを特徴とする、請求の範囲２４記載の免疫機能分子の製造方法。

２９．還元末端のN-アセチルグルコサミンにフコースを付加する酵素がフコシルトランスフェラーゼである、請求の範囲２８記載の製造方法。

３０．フコシルトランスフェラーゼが、α1,6-フコシルトランスフェラーゼである、請求の範囲２９記載の製造方法。

３１．免疫機能活性が抑制された免疫機能分子の製造法が、ラットミエローマ細胞を用いた製造法であることを特徴とする、請求の範囲２４記載の免疫機能分子の製造方法。

３２．ラットミエローマ細胞が、YB2/3HL.P2.G11.16Ag.20細胞である、請求の範囲３１記載の製造方法。

３３．還元末端のN-アセチルグルコサミンにフコースを付加する酵素活性が高い細胞を用いることを特徴とする、請求の範囲２５記載の免疫機能分子の製造方法。

３４．還元末端のN-アセチルグルコサミンにフコースを付加する酵素がフコシルトランスフェラーゼである、請求の範囲３３記載の製造方法。

３５．　フコシルトランスフェラーゼが、α1,6-フコシルトランスフェラーゼである、請求の範囲３４記載の製造方法。

３６．　抗体が、腫瘍関連抗原を認識する抗体である請求の範囲２６記載の免疫機能分子。

３７．　腫瘍関連抗原がガングリオシドGD3である、請求の範囲３６記載の免疫機能分子。

３８．　抗体が、7-9-51（FERM BP-6691）より生産される抗体である請求の範囲３６記載の免疫機能分子。

３９．　抗体が、アレルギーまたは炎症に関連する抗原を認識する抗体である請求の範囲２６記載の免疫機能分子。

４０．　アレルギーまたは炎症に関連する抗原が、ヒトインターロイキン５レセプターα鎖である請求の範囲３９記載の免疫機能分子。

４１．　抗体が、No.3（FERM BP-6690）より生産される抗体である請求の範囲３９記載の免疫機能分子。

４２．　抗体が、循環器疾患に関連する抗原を認識する抗体である請求の範囲２６記載の免疫機能分子。

４３．　抗体が、自己免疫疾患に関連する抗原を認識する抗体である請求の範囲２７記載の免疫機能分子。

４４．　抗体が、ウィルスまたは細菌感染に関連する抗原を認識する抗体である請求の範囲２６記載の免疫機能分子。

４５．　請求の範囲３６記載の免疫機能分子を有効成分として含有する、癌の診断薬。

４６．　請求の範囲３６記載の免疫機能分子を有効成分として含有する、癌の治療薬。

４７．　請求の範囲３６記載の免疫機能分子を有効成分として含有する、癌の予防薬。

４８．　請求の範囲３９記載の抗体を有効成分として含有する、アレルギーまたは炎症の診断薬。

４９．　請求の範囲３９記載の抗体を有効成分として含有する、アレルギーまたは炎症の治療薬。

５０．　請求の範囲３９記載の抗体を有効成分として含有する、アレルギーまたは炎症の予防薬。

５１．　請求の範囲４２記載の抗体を有効成分として含有する、循環器疾患の診断薬。

５２．　請求の範囲４２記載の抗体を有効成分として含有する、循環器疾患の治療薬。

５３．　請求の範囲４２記載の抗体を有効成分として含有する、循環器疾患の予防薬。

５４．　請求の範囲４３記載の抗体を有効成分として含有する、自己免疫疾患の診断薬。

５５．　請求の範囲４３記載の抗体を有効成分として含有する、自己免疫疾患の治療薬。

５６．　請求の範囲４３記載の抗体を有効成分として含有する、自己免疫疾患の予防薬。

５７．　請求の範囲４４記載の抗体を有効成分として含有する、ウィルスまたは細菌感染症の診断薬。

５８．　請求の範囲４４記載の抗体を有効成分として含有する、ウィルスまたは細菌感染症の治療薬。

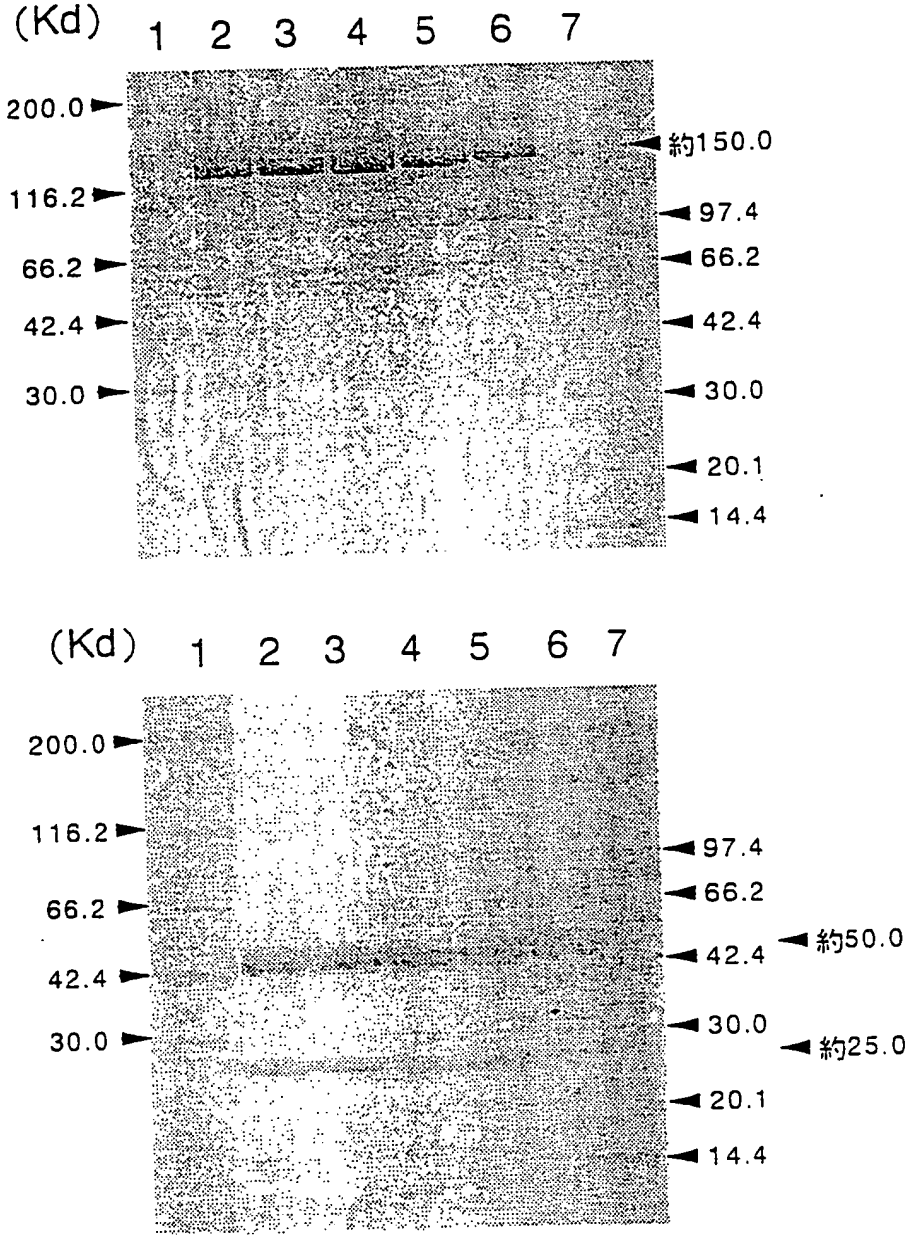
５９．　請求の範囲４４記載の抗体を有効成分として含有する、ウィルスまたは細菌感染症の予防薬。

６０．　請求の範囲２６または２７記載のペプチドまたは蛋白質を有効成分として含有する、各種疾患の診断薬。

６１．　請求の範囲６０記載のペプチドまたは蛋白質を有効成分として含有する、各種疾患の治療薬。

６２．　請求の範囲６０記載のペプチドまたは蛋白質を有効成分として含有する、各種疾患の予防薬。

第 1 図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116.2
66.2
42.4
30.0
約150.0
97.4
66.2
42.4
30.0
20.1
14.4
(Kd)
1 2 3 4 5 6 7
200.0
116.2
66.2
42.4
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30.0
20.1
14.4
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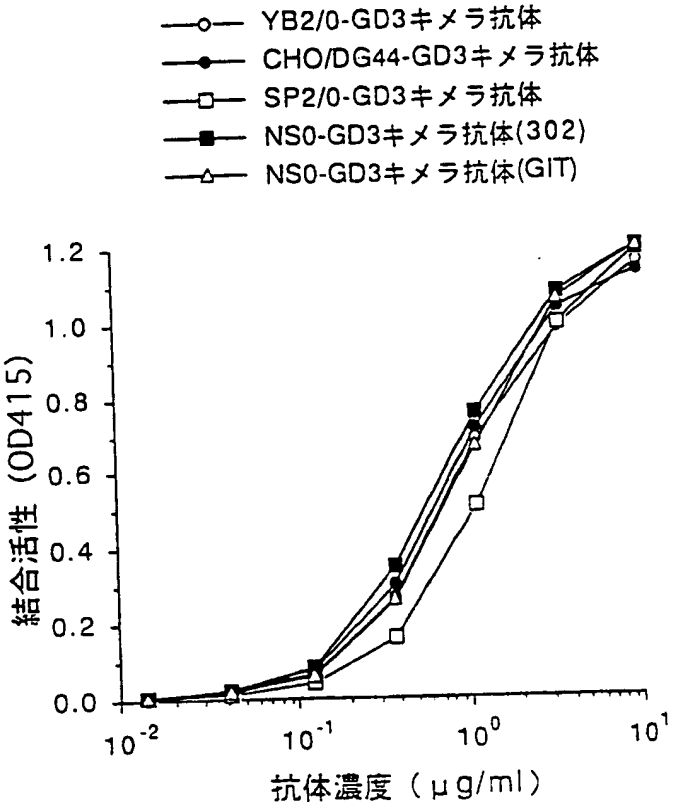

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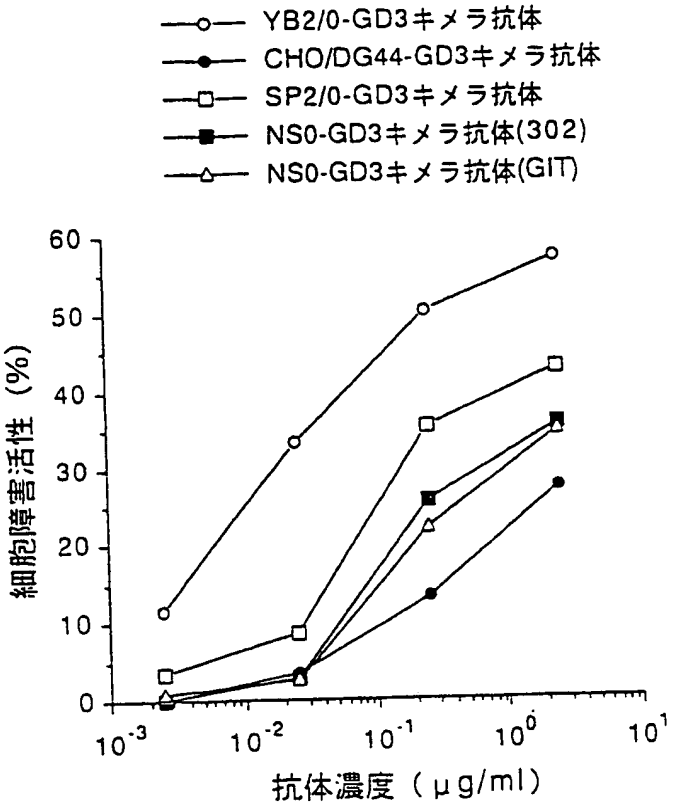
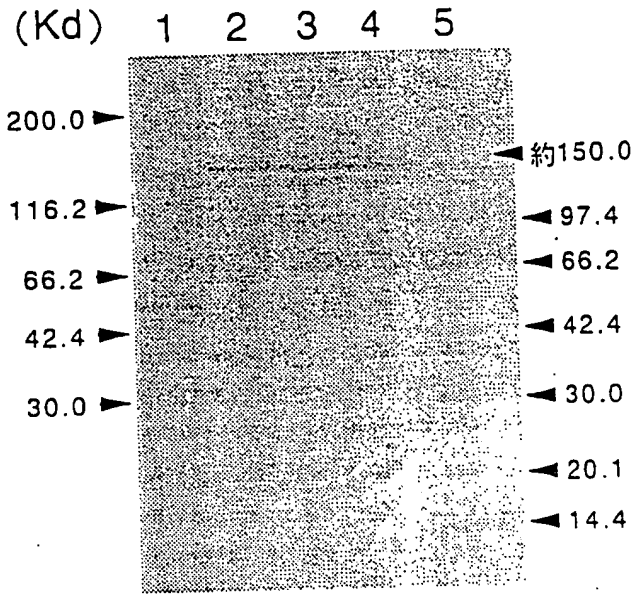
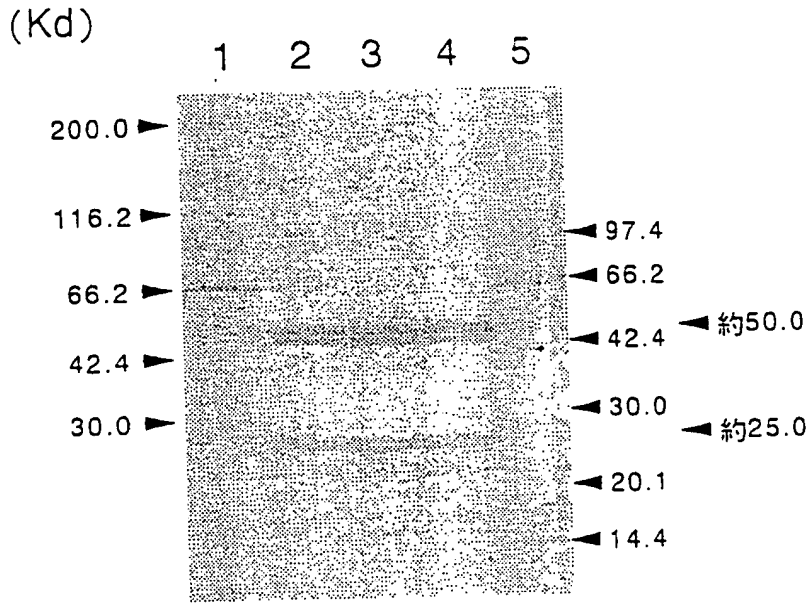

YB2/0-GD3キメラ抗体
CHO/DG44-GD3キメラ抗体
SP2/0-GD3キメラ抗体
NS0-GD3キメラ抗体(302)
NS0-GD3キメラ抗体(GIT)
細胞障害活性（%）
60
50
40
30
20
10
0
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
抗体濃度（μg/ml）

第 4 図





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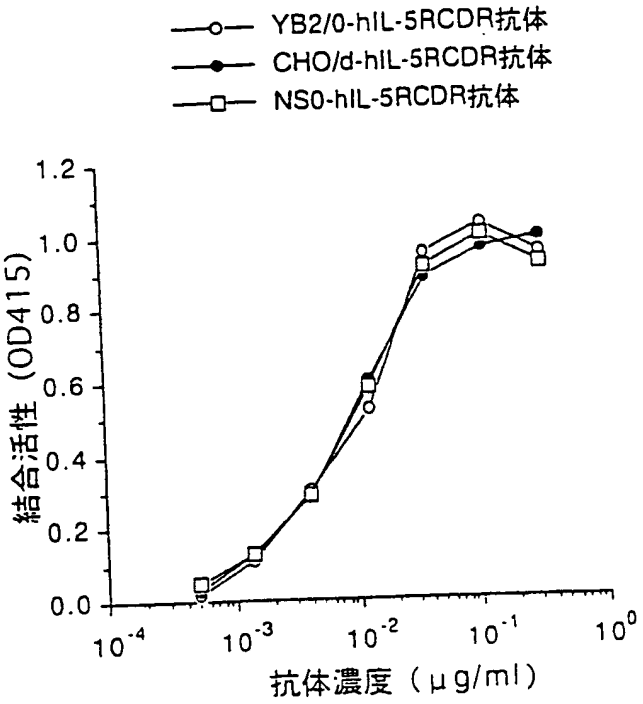
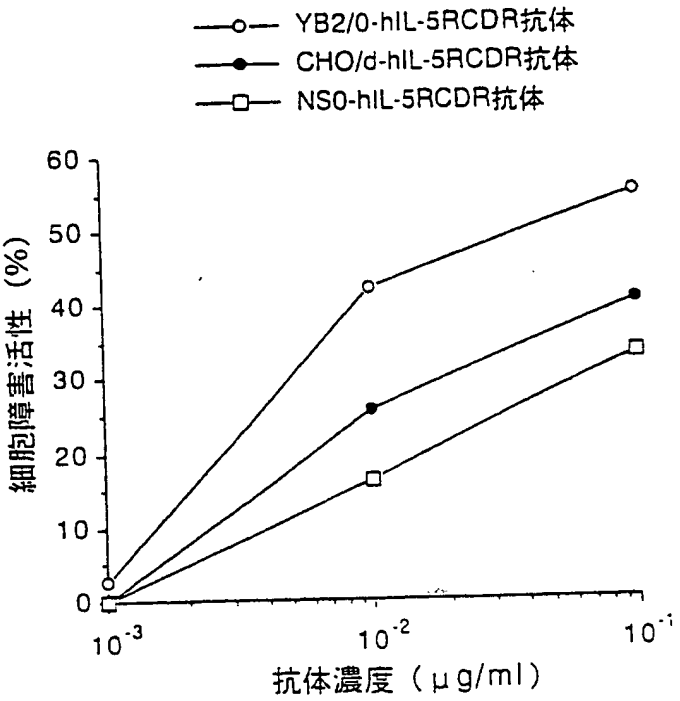
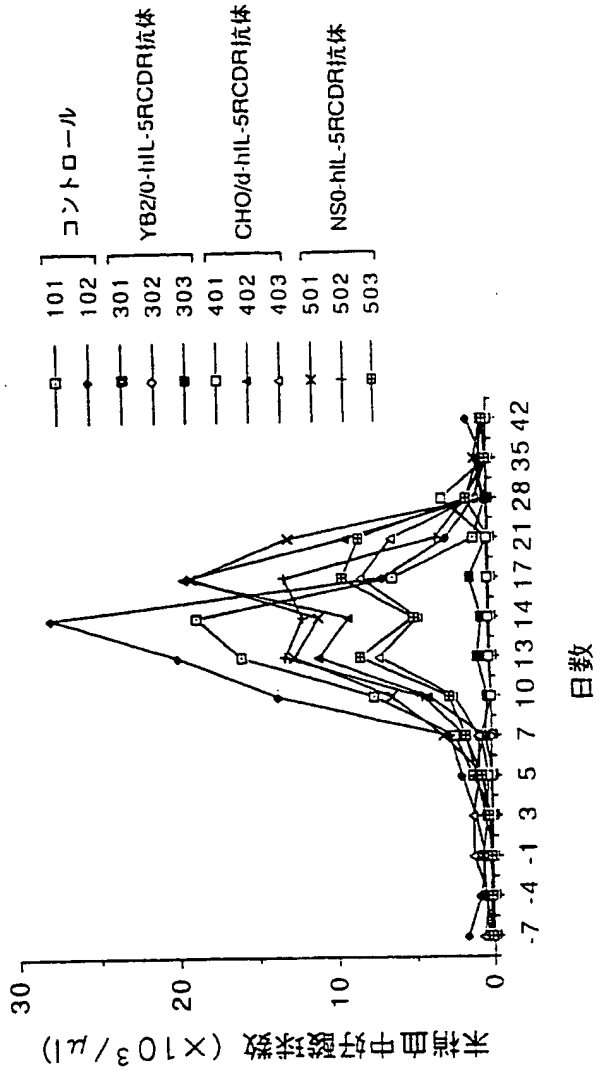

YB2/0-hIL-5RCDR抗体
CHO/d-hIL-5RCDR抗体
NS0-hIL-5RCDR抗体
結合活性 (OD415)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10^{-4}
10^{-3}
10^{-2}
10^{-1}
10^{0}
抗体濃度（μg/ml）

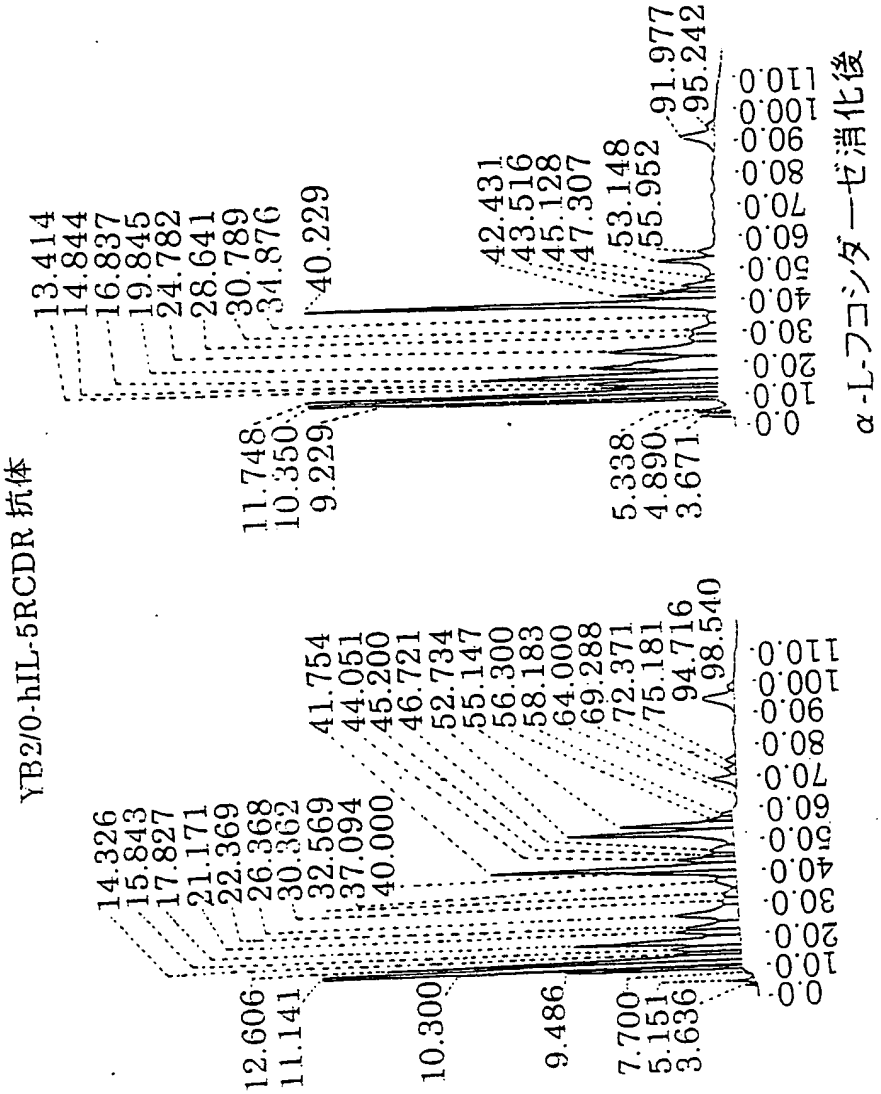
第 6 図


YB2/0-hIL-5RCDR抗体
CHO/d-hIL-5RCDR抗体
NS0-hIL-5RCDR抗体
細胞障害活性（%）
60
50
40
30
20
10
0
10^{-3}
10^{-2}
10^{-1}
抗体濃度（μg/ml）

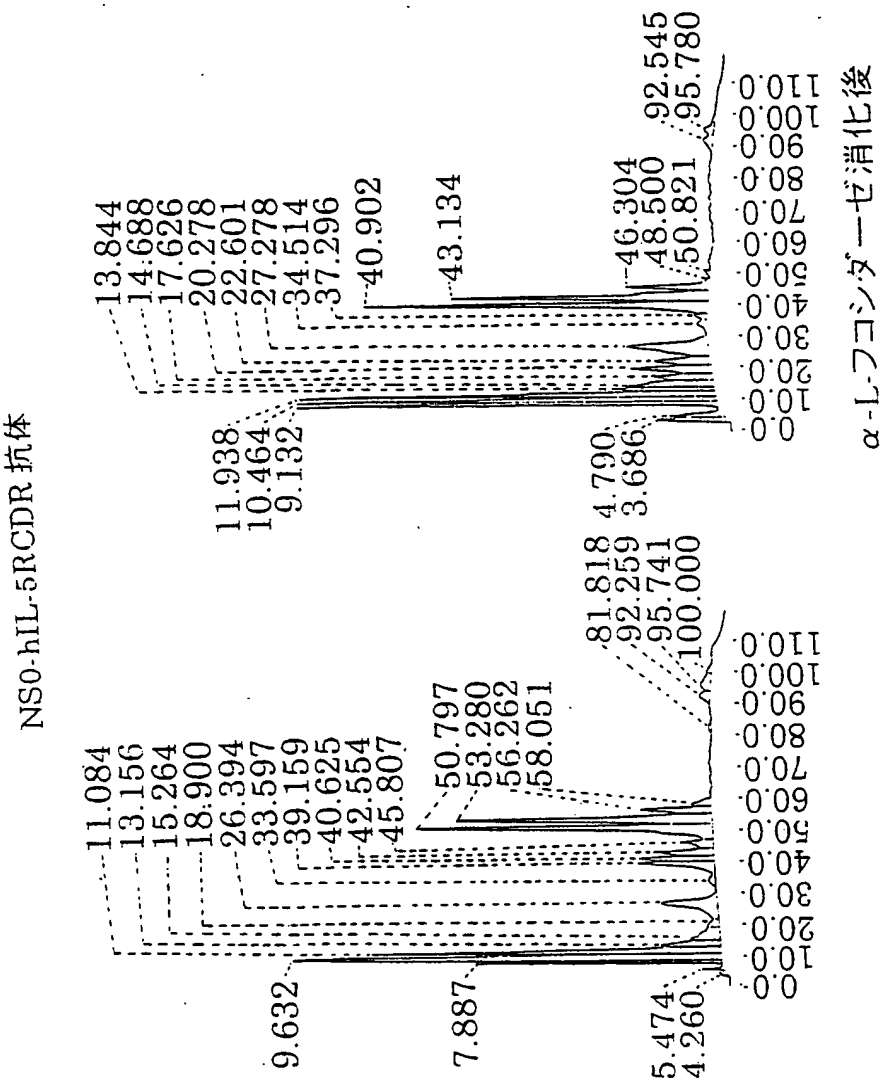
第 7 図



第 8 図



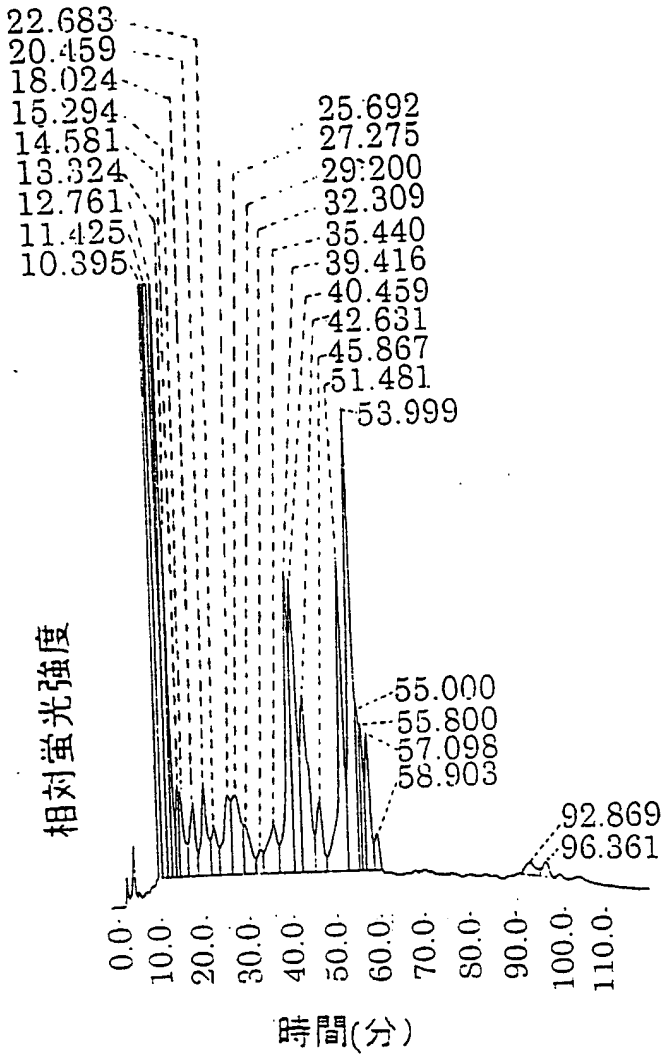
差替え用紙（規則26）



8/11

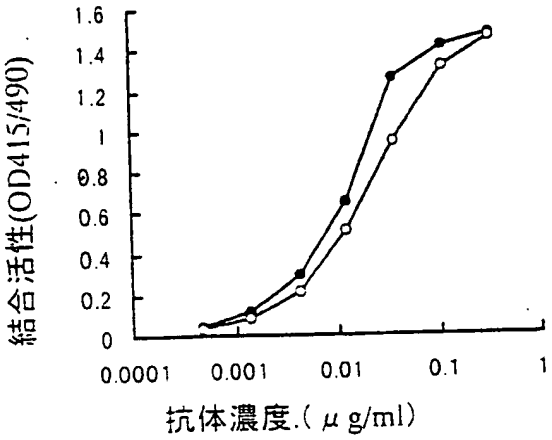
差替え用紙（規則26）

第 9 図



差替え用紙（規則26）

第 10 図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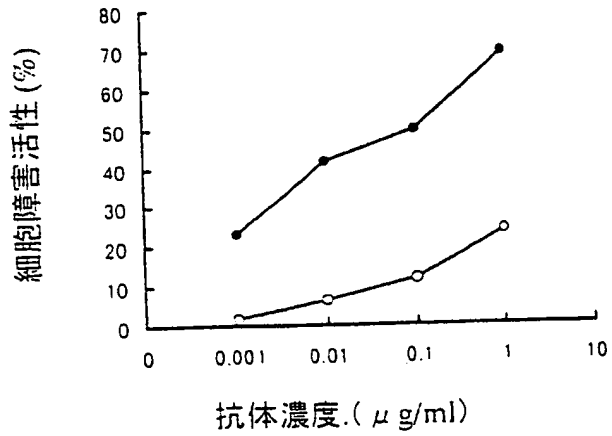
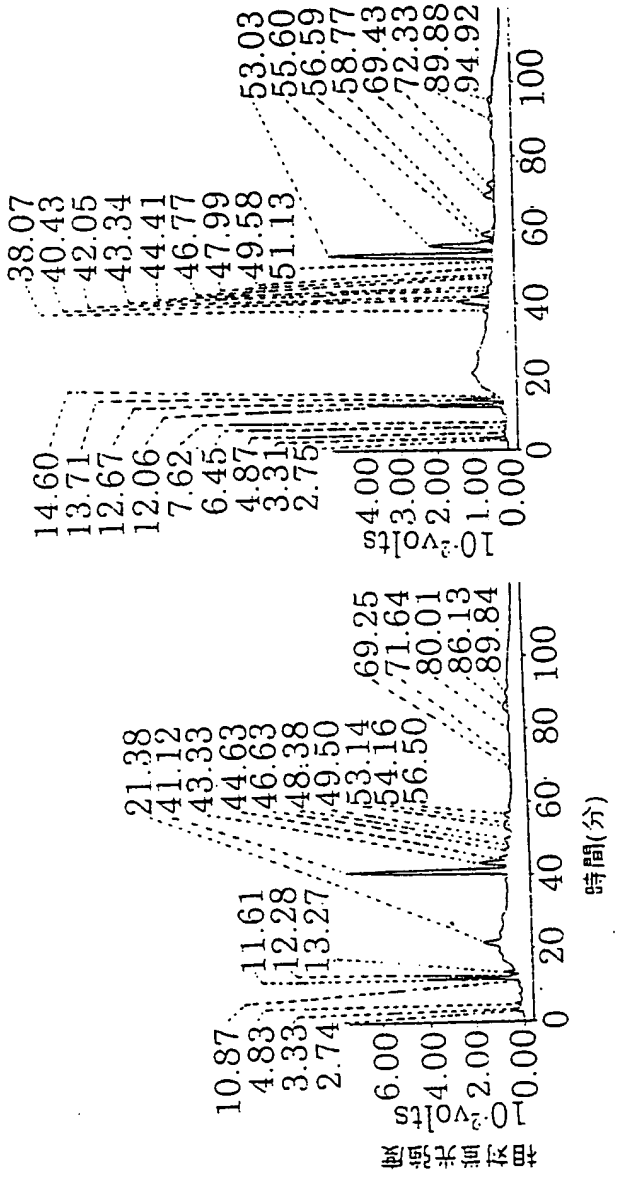
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.0001
0.001
0.01
0.1
1
抗体濃度.(μ g/ml)

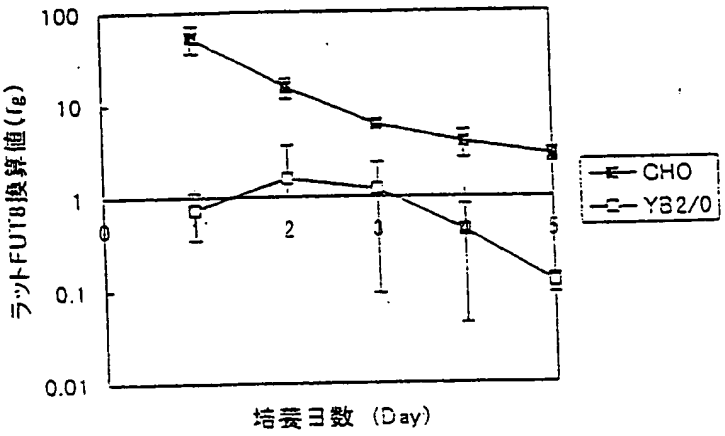

細胞障害活性 (%)
80
70
60
50
40
30
20
10
0
0
0.001
0.01
0.1
1
10
抗体濃度.(μ g/ml)

第 11 図



差替え用紙（規則26）

第 12 図



11/1/11

差替え用紙（規則26）

配 列 表

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end.

<400> 13
caggaaggaa ggctggaaga gagc 24

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP00/02260

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl[7] C12N 15/09, C12P 21/00, C12P 21/08, A61K 39/00, G01N 33/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl[7] C12N 15/09, C12P 21/00, C12P 21/08, A61K 39/00, G01N 33/53

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MDLINE (STN), WPI (DIALOG), BIOSIS(DIALOG)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Kenya Shitara et al., "A new vector for the high level expression of chimeric antibodies in myeloma cells", Journal of Immunological Methods (1994), Vol. 167, No. 1-2 pp.271-278	24-45,48, 51,54,57,60
X	EP, 882794, A2 (KYOWA HAKKO KOGYO KK), 09 December, 1998 (09.12.98) & JP, 10-257893, A & AU, 9859420, A	24-45,48, 51,54,57,60
X	WO, 94/16094, A2 (BIOGEN INC), 21 July, 1994 (21.07.94) & EP, 678122, A1 & JP, 8-507680, A & AU, 9459936, A	24-45,48, 51,54,57,60
A	Iain B. H. Wilson et al., "Sructural analysis of N-glycans from allergenic grass, ragweed and tree pollens: Core α1, 3-linked fucose and xylose present in all pollens examined", Glycoconjugate Journal (1998), Vol.15, No.11, pp.1055-1070	1-62
A	EP, 623352, A2 (BEHRINGWERKE AKTINEN GESELLSCHAFT), 09 November, 1994 (09.11.94)	1-62

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 July, 2000 (12.07.00)	25 July, 2000 (25.07.00)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP00/02260

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	& JP, 6-319554, A & DE, 4314556, A1 & AU, 9461829, A & CA, 2122745, A	

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

国際調査報告

国際出願番号 PCT／JP00／02260

A. 発明の属する分野の分類（国際特許分類（IPC））

Int.Cl' C12N 15／09, C12P 21／00, C12P 21／08, A61K 39／00, G01N 33／53

B. 調査を行った分野

調査を行った最小限資料（国際特許分類（IPC））

Int.Cl' C12N 15／09, C12P 21／00, C12P 21／08, A61K 39／00, G01N 33／53

最小限資料以外の資料で調査を行った分野に含まれるもの

国際調査で使用した電子データベース（データベースの名称、調査に使用した用語）

MDLINE(STN), WPI(DIALOG), BIOSIS(DIALOG)

C. 関連すると認められる文献

引用文献のカテゴリー*	引用文献名 及び一部の箇所が関連するときは、その関連する箇所の表示	関連する請求の範囲の番号
X	Kenya Shitara et al., "A new vector for the high level expression of chimeric antibodies in myeloma cells", Journal of Immunological Methods (1994), Vol.167, No.1-2 p.271-278	24-45, 48, 51, 54, 57, 60
X	EP, 882794, A2 (KYOWA HAKKO KOGYO KK) 9.12月.1998 (09.12.98) & JP, 10-257893, A & AU, 9859420, A	24-45, 48, 51, 54, 57, 60

☒ C欄の続きにも文献が列挙されている。　☐ パテントファミリーに関する別紙を参照。

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「&」同一パテントファミリー文献

国際調査を完了した日 12.07.00	国際調査報告の発送日 25.07.00
国際調査機関の名称及びあて先 日本国特許庁（ISA／JP） 郵便番号100－8915 東京都千代田区霞が関三丁目4番3号	特許庁審査官（権限のある職員） 小暮 道明　4B 9358 電話番号 03－3581－1101 内線 3448

様式PCT／ISA／210（第2ページ）（1998年7月）

C（続き）．　関連すると認められる文献

引用文献のカテゴリー＊	引用文献名　及び一部の箇所が関連するときは、その関連する箇所の表示	関連する請求の範囲の番号
X	WO, 94/16094, A2 (BIOGEN INC)　21.7月.1994 (21.07.94) & EP, 678122, A1 & JP, 8-507680, A & AU, 9459936, A	24-45, 48, 51, 54, 57, 60
A	Iain B.H.Wilson et al., "Sructural analysis of N-glycans from allergenic grass, ragweed and tree pollens: Core α1, 3-linked fucose and xylose present in all pollens examined", Glycoconjugate Journal (1998), Vol.15, No.11, p.1055-1070	1－62
A	EP, 623352, A2 (BEHRINGWERKE AKTINEN GESELLSCHAFT) 9.11月.1994 (09.11.94) JP, 6-319554, A & DE, 4314556, A1 & AU, 9461829, A & CA, 2122745, A	1－62

様式PCT／ISA／210（第2ページの続き）（1998年7月）

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Remarks:
The applicant has subsequently filed a sequence listing and declared, that it includes no new matter.

(54) Human complementarity determining region (CDR)-grafted antibody to ganglioside gm2

(57) A human CDR-grafted antibody which specifically reacts with ganglioside GM_2, wherein said antibody comprises CDR 1, CDR 2 and CDR 3 of heavy chain (H chain) variable region (V region) comprising amino acid sequences described in SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3, and CDR 1, CDR 2 and CDR 3 of light chain (L chain) V region comprising amino acid sequences described in SEQ ID NO:4, SEQ ID NO: 5 and SEQ ID NO:6, and wherein at least one of the frameworks (FR) of said H chain and L chain V regions comprises an amino acid sequence selected from common sequences (HMHCS; human most homologous consensus sequence) derived from human antibody subgroups.

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Description

FIELD OF THE INVENTION

This invention relates to a human complementarity determining region (referred to as "CDR" hereinafter) grafted antibody to ganglioside GM_2 (referred to as "GM_2" hereinafter). This invention also relates to a DNA fragment encoding the above-described antibody, particularly its variable region (referred to as "V region" hereinafter). This invention relates to an expression vector which contains the DNA fragment and to a host transformed with the expression vector. This invention further relates to a method for the production of the human CDR-grafted antibody specific for GM_2 and to its therapeutic and diagnostic use.

BACKGROUND OF THE INVENTION

It is known in general that, when a mouse antibody is administered to human, the mouse antibody is recognized as foreign matter in the human body and thus induces a human antibody to a mouse antibody (human anti-mouse antibody, referred to as "HAMA" hereinafter) which reacts with the administered mouse antibody to produce adverse effects (Dillman, R.O. *et al., J. Clin. Oncol.*, 2, 881 (1984); Meeker, T.C. *et al., Blood*, 65, 1349 (1985); LoBuglio, A.F. *et al., J. Natl. Cancer Inst.*, 80, 932 (1988); Houghton, A.N. *et al., Proc. Natl. Acad. Sci. U.S.A.*, 82, 1242 (1985)), and the administered mouse antibody is quickly cleared (Pimm, M.V. *et al., J. Nucl. Med.*, 26, 1011 (1985); Meeker, T.C. *et al., Blood*, 65, 1349 (1985); Khazaeli, M.B. *et al., J. Natl. Cancer Inst.*, 80, 937 (1988)) to reduce effects of the antibody (Shawler, D.L. *et al., J. Immunol.*, 135, 1530 (1985); Courtenay-Luck, N.S. *et al., Cancer Res.*, 46, 6489 (1986)).

In order to solve these problems, attempts have been made to convert a mouse antibody into a humanized antibody such as a human chimeric antibody or a human CDR-grafted antibody. The human chimeric antibody is an antibody in which its V region is derived from an antibody of nonhuman animal and its constant region (referred to as "C region" hereinafter) is derived from a human antibody (Morrison, S.L. *et al., Proc. Natl. Acad. Sci. U.S.A.*, 81, 6851 (1984)). Furthermore, it is reported that, when this type of antibody is administered to human, HAMA is hardly induced and its half-life in blood increases six times (LoBuglio, A.F. *et al., Proc. Natl. Acad. Sci. U.S.A.*, 86, 4220 (1989)). The human CDR-grafted antibody is an antibody in which the CDR of human antibody is replaced by other CDR derived from nonhuman animal (Jones, P.T. *et al., Nature*, 321, 522 (1986)), which is also called a reshaped human antibody. It is reported that, in a test of a human CDR-grafted antibody in monkeys, its immunogenicity is reduced and its half-life in blood is increased four to five times, in comparison with a mouse antibody (Hakimi, J. *et al., J. Immunol.*, 147, 1352 (1991)).

Also, with regard to the cytotoxicity of antibodies, it is reported that the Fc region of a human antibody activates human complement and human effector cells more effectively than the Fc region of mouse antibody. For example, it is reported that human effector cell-mediated anti-tumor effects of a mouse antibody to GD_2 is increased when the antibody is converted into a human chimeric antibody having human antibody Fc region (Mueller, B.M. *et al., J. Immunol.*, 144, 1382 (1990)), and similar results are reported on a human CDR-grafted antibody to CAMPATH-1 antigen (Reichmann, L. *et al., Nature*, 332, 323 (1988)). These results indicate that humanized antibodies are more desirable than mouse antibodies as antibodies to be clinically used in human.

Ganglioside as a glycolipid having sialic acid is a molecule which constitutes an animal cell membrane, and comprises a carbonhydrate chain as a hydrophilic side chain and sphingosine and fatty acid as hydrophobic side chains. It is known that types and expression quantities of ganglioside vary depending on the cell species, organ species, animal species and the like. It is known also that the expression of ganglioside changes quantitatively and qualitatively in the process of cancer development of cells (Hakomori, S. *et al., Cancer Res.*, 45, 2405 (1985)). For example, it is reported that gangliosides GD_2, GD_3, GM_2 and the like which are hardly observed in normal cells are expressed in nerve ectoderm system tumors considered to have high malignancy, such as neuroblastoma, pulmonary small cell carcinoma and melanoma (Pukel, C.S. *et al., J. Exp. Med.*, 155, 1133 (1982); Nudelman, E. *et al., J. Biol. Chem.*, 257, 12752 (1982); Werkmeister, J.A. *et al., Cancer Res.*, 47, 225 (1987); Mujoo, K. *et al., Cancer Res.*, 47, 1098 (1987); Cheung, N.V. *et al., Cancer Res.*, 45, 2642 (1985); Tai, T. et *al., Proc. Natl. Acad. Sci.* U.S.A., 80, 5392 (1983)), and antibodies to these gangliosides are considered to be useful for diagnosis and treatment of various cancers in human.

It is indicated that human antibodies to GM_2 are useful for treatment of human melanoma (Irie, R.F. *et al., Lancet*, I, 786 (1989)). However, the antibodies to GM_2 so far reported are either those which are derived from nonhuman animal or a human antibody belonging to the IgM class (Natoli, E.J. *et al., Cancer Res.*, 46, 4116 (1986); Miyake, M. *et al., Cancer Res.*, 48, 6154 (1988); Cahan, L.D. *et al., Proc. Natl. Acad. Sci. U.S.A.*, 79, 7629 (1982); Fredman, P. *et al., J. Biol. Chem.*, 264, 12122 (1989)). The antibody of the IgM class, however, is unsuitable for applying to human, because it has a pentameric structure having a large molecular weight (about 900,000) in comparison with the antibody of IgG class which has a molecular weight of about 150,000, thus posing a problem in carrying out its purification, in addition to other problems such as its short half-life in blood and weak anti-tumor effect (Bernstein, I.D. *et al., Monoclonal Antibodies, Plenum Press*, p.275 (1980)).

Because of the above, it is desirable to develop a humanized antibody to GM_2 of the IgG class which, when applied to human, does not induce HAMA in the human body, causes less adverse effects, shows prolonged half-life in blood and has improved anti-tumor effect, so that its high diagnostic and therapeutic effects on human cancers can be expected.

The inventors of the present invention disclose in JP-A-6-205694 (the term "JP-A" as used herein means an "unexamined published Japanese patent application") (corresponding to EP-A-0 598 998) a method for producing an IgG class human chimeric antibody and a human CDR-grafted antibody, which can specifically reacts with GM_2 and are useful for diagnosis and treatment of human cancers. However, there are no reports on a human CDR-grafted antibody which, when compared with a human chimeric antibody, has similar levels of binding activity and binding specificity for GM_2 and anti-tumor effects upon GM_2-positive cells.

SUMMARY OF THE INVENTION

As described in the foregoing, it is considered that human CDR-grafted antibodies are useful for diagnosis and treatment of human cancers and the like. However, the antibody activity is reduced when the CDRs of the heavy chain (referred to as "H chain" hereinafter) V region and light chain (referred to as "L chain" hereinafter) V region of an antibody of nonhuman animal are replaced only with the CDRs of the H chain V region and L chain V region of a human antibody, so that great concern has been directed toward the establishment of a method for the production of a human CDR-grafted antibody to GM_2 belonging to the IgG class (referred to as "human CDR-grafted anti-GM_2 antibody" hereinafter) which, when compared with a human chimeric antibody, has similar levels of binding activity and binding specificity for GM_2 and anti-tumor effects upon GM_2-positive cells, as well as a method for producing a human CDR-grafted antibody, which can be applied to all antibodies.

This invention relates to a human CDR-grafted antibody which specifically reacts with ganglioside GM_2, wherein said antibody comprises CDR 1, CDR 2 and CDR 3 of H chain V region comprising amino acid sequences of SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3 or functional equivalents thereof, and CDR 1, CDR 2 and CDR 3 of L chain V region comprising amino acid sequences of SEQ ID NO:4, SEQ ID NO:5 and SEQ ID NO:6 or functional equivalents thereof, and wherein at least one of the frameworks (referred to as "FR" hereinafter) of said H chain and L chain V regions comprises an amino acid sequence selected from common sequences (human most homologous consensus sequence (referred to as "HMHCS" hereinafter) derived from human antibody subgroups.

Furthermore, the present invention relates to the above human CDR-grafted antibody, wherein said FR of H chain or L chain V region of the human CDR-grafted antibody comprises an amino acid sequence in which at least one amino acid is replaced by an other amino acid, and wherein said antibody has antigen-binding activity, binding specificity, antibody dependent cell mediated cytotoxicity (ADCC), and complement dependent cytotoxicity (CDC) comparable to those of a human chimeric antibody having a V region of a monoclonal antibody derived from nonhuman animal which specifically reacts with ganglioside GM_2.

Moreover, the present invention relates to the above human CDR-grafted antibody, wherein said H chain C region of the antibody is derived from an antibody belonging to the human antibody IgG class.

BRIEF EXPLANATION OF THE DRAWINGS

Fig. 1 shows a construction scheme for a plasmid named pBSA.
Fig. 2 shows a construction scheme for a plasmid named pBSAE.
Fig. 3 shows a construction scheme for a plasmid named pBSH-S.
Fig. 4 shows a construction scheme for a plasmid named pBSK-H.
Fig. 5 shows a construction scheme for plasmids named pBSH-SA and pBSK-HA.
Fig. 6 shows a construction scheme for plasmids named pBSH-SAE and pBSK-HAE.
Fig. 7 shows a construction scheme for plasmids named pBSH-SAEE and pBSK-HAEE.
Fig. 8 shows a construction scheme for a plasmid named pBSK-HAEESal.
Fig. 9 shows a construction scheme for a plasmid named pBSX-S.
Fig. 10 shows a construction scheme for a plasmid named pBSX-SA.
Fig. 11 shows a construction scheme for a plasmid named pBSSC.
Fig. 12 shows a construction scheme for a plasmid named pBSMo.
Fig. 13 shows a construction scheme for a plasmid named pBSMoS.
Fig. 14 shows a construction scheme for a plasmid named pChilgLA1S.
Fig. 15 shows a construction scheme for a plasmid named pMohCκ.
Fig. 16 shows a construction scheme for a plasmid named pBSMoSal.
Fig. 17 shows a construction scheme for a plasmid named pBSMoSalS.
Fig. 18 shows a construction scheme for a plasmid named pBShCγ1.

Fig. 19 shows a construction scheme for a plasmid named pMohCγ1.

Fig. 20 shows a construction scheme for a plasmid named pMoγ1SP.

Fig. 21 shows a construction scheme for a plasmid named pMoκγ1SP.

Fig. 22 shows a construction scheme for a plasmid named pKANTEX93.

Fig. 23 shows a construction scheme for a plasmid named pBSNA.

Fig. 24 shows a construction scheme for a plasmid named pBSH3.

Fig. 25 shows a construction scheme for a plasmid named pBSES.

Fig. 26 shows a construction scheme for a plasmid named pBSL3.

Fig. 27 shows a construction scheme for a plasmid named pKANTEX796H.

Fig. 28 shows a construction scheme for a plasmid named pKANTEX796.

Fig. 29 shows a construction scheme for a plasmid named pT796.

Fig. 30 is a graphic representation of transient mouse-human chimeric anti-GM_2 antibody expression by the plasmids pKANTEX796 and pT796. The ordinate denotes the antibody concentration that showed GM_2-binding activity, and the abscissa denotes the time after introduction of the plasmid.

Fig. 31 shows a construction scheme for a plasmid named pBSH10.

Fig. 32 shows a construction scheme for a plasmid named pBSL16.

Fig. 33 illustrates a process for mutagenesis by PCR and a process for cloning DNA fragments mutated.

Fig. 34 shows a construction scheme for a plasmid named pBSLV1+2.

Fig. 35 shows a construction scheme for a plasmid named pBSLm-28.

Fig. 36 shows a construction scheme for a plasmid named pBSHSGL.

Fig. 37 shows a construction scheme for a plasmid named pT796LCDR.

Fig. 38 shows a construction scheme for plasmids named pT796HLCDR, pT796HLCDRHV2 and pT796HLCDRHV4.

Fig. 39 shows a construction scheme for a plasmid named pT796HLCDRH10.

Fig. 40 shows construction scheme for plasmids named pT796HCDR, pT796HCDRHV2, pT796HCDRHV4 and pT796HCDRH10.

Fig. 41 is a graphic representation of the results of human CDR-grafted anti-GM_2 antibody activity evaluation in terms of transient expression as obtained using the plasmids pT796, pT796HCDR, pT796HCDRHV2, pT796HCDRHV4 and pT796HCDRH10. The ordinate denotes the plasmid used, and the abscissa denotes the relative activity value with the activity obtained with the chimera antibody being taken as 100%.

Fig. 42 shows a construction scheme for plasmids named pT796HLCDRLV1, pT796HLCDRLV2, pT796HLCDRLV3, pT796HLCDRLV4, pT796HLCDRLV8, pT796HLCDRLm-2, pT796HLCDRLm-8, pT796HLCDRLm-28 and pT796HLCDRHSGL.

Fig. 43 is a graphic representation of the results of human CDR-grafted anti-GM_2 antibody activity evaluation in terms of transient expression as obtained using the plasmids pT796, pT796HLCDR, pT796HLCDRLV1, pT796HLCDRLV2, pT796HLCDRLV3, pT796HLCDRLV4, pT796HLCDRLV8, pT796HLCDRLm-2, pT796HLCDRLm-8, pT796HLCDRLm-28 and pT796HLCDRHSGL. The ordinate denotes the plasmid used, and the abscissa denotes the relative activity value with the activity obtained with the chimera antibody being taken as 100%.

Fig. 44 shows a construction scheme for plasmids named pKANTEX796HLCDRLm-28 and pKANTEX796HLCDRHSGL.

Fig. 45 shows electrophoretic patterns obtained for mouse-human chimeric anti-GM_2 antibody KM966 and purified human CDR-grafted anti-GM_2 antibodies KM8966 and KM8967 by SDS-PAGE (4 to 15% gradient gels used). The patterns shown on the left side are those obtained under reducing conditions, and those on the right under nonreducing conditions. From the left of each lane, the electrophoretic patterns for high-molecular-weight marker, KM966, KM8966, KM8967, low-molecular-weight marker, KM966, KM8966 and KM8967 are shown in that order.

Fig. 46 is a graphic representation of the GM_2-binding activities of mouse-human chimeric anti-GM_2 antibody KM966 and purified human CDR-grafted anti-GM_2 antibodies KM8966 and KM8967. The ordinate denotes the GM_2-binding activity, and the abscissa the antibody concentration.

Fig. 47 is a graphic representation of the reactivities of mouse-human chimeric anti-GM_2 antibody KM966 and purified human CDR-grafted anti-GM_2 antibodies KM8966 and KM8967 against various gangliosides. The ordinate denotes the ganglioside species, and the abscissa the binding activity. $AcGM_2$ stands for N-acetyl-GM_2, $GcGM_2$ for N-glycolyl-GM_2, AcGM3 for N-acetyl-GM_3 and GcGM3 for N-glycolyl-GM_3.

Fig. 48 is a graphic representation of the reactivities of mouse-human chimeric anti-GM_2 antibody KM966 and purified human CDR-grafted anti-GM_2 antibodies KM8966 and KM8967 against the human lung small cell carcinoma cell line SBC-3. The ordinate denotes the number of cells, and the abscissa the fluorescence intensity. From the lowermost graph, the reactivities of control, KM8967, KM8966 and KM966 are shown in that order.

Fig. 49 graphically shows the CDC activities of mouse-human chimeric anti-GM_2 antibody KM966 and purified human CDR-grafted anti-GM_2 antibodies KM8966 and KM8967 against the human lung small cell carcinoma cell line

SBC-3. The ordinate indicates the cytotoxic activity and the abscissa the concentration of the antibody.

Fig. 50 graphically shows the ADCC activities of mouse-human chimeric anti-GM_2 antibody KM966 and purified human CDR-grafted anti-GM_2 antibodies KM8966 and KM8967 against the human lung small cell carcinoma cell line SBC-3. The ordinate indicates the cytotoxicity and the abscissa the concentration of the antibody.

Fig. 51 shows a construction scheme for plasmids, pKANTEX796HM1Lm-28, pKANTEX796HM2Lm-28, pKANTEX796HM3Lm-28, pKANTEX796HM31Lm-28 and pKANTEX796HM32Lm-28.

Fig. 52 shows the electrophoretic patterns in SDS-PAGE (using 4-15% gradient gels) of mouse-human chimeric anti-GM_2 antibody KM966, human CDR-grafted anti-GM_2 antibody KM8966 and human CDR-grafted anti-GM_2 antibodies each having various types of substitution. The pattern obtained under nonreducing conditions is shown on the left side and that obtained under reducing conditions on the right side. M stands for molecular weight markers (from the top, the arrows indicate the molecular weight of 205 Kd, 140 Kd, 83 Kd, 45 Kd, 32.6 Kd, 18 Kd and 7.5 Kd in that order) and 1, 2, 3, 4, 5, 6 and 7 stand for the electrophoretic patterns of KM966, KM8966, M1-28, M2-28, M3-28, M31-28 and M32-28, respectively.

Fig. 53 graphically shows the CDC activities of mouse-human chimeric anti-GM_2 antibody KM966, human CDR-grafted anti-GM_2 antibody KM8966 and human CDR-grafted anti-GM_2 antibodies each having various types of substitution against the human lung small cell carcinoma cell line SBC-3. The ordinate indicates the cytotoxic activity and the abscissa the concentration of the antibody.

Fig. 54 shows a construction scheme for plasmids, pKANTEX796HLm-28 No.1, pKANTEX796HM1Lm-28 No.1, pKANTEX796HM2Lm-28 No.1 and pKANTEX796HM3Lm-28 No.1.

Fig. 55 shows the electrophoretic patterns in SDS-PAGE (using 4-15% gradient gels) of mouse-human chimeric anti-GM_2 antibody KM966 and human CDR-grafted anti-GM_2 antibodies each having various types of substitution. The pattern obtained under nonreducing conditions is shown on the left side and that obtained under reducing conditions on the right side. M stands for molecular weight markers (from the top, the arrows indicate the molecular weight of 205 Kd, 140 Kd, 83 Kd, 45 Kd, 32.6 Kd, 18 Kd and 7.5 Kd in that order) and 1, 2, 3, 4 and 5 stand for the electrophoretic patterns of KM966, h796H-No.1, M1-No.1, M2-No.1 and M3-No.1, respectively.

Fig. 56 graphically shows the CDC activities of mouse-human chimeric anti-GM_2 antibody KM966, human CDR-grafted anti-GM_2 antibodies KM8966 and KM8970 and human CDR-grafted anti-GM_2 antibodies each having various types of substitution against the human lung small cell carcinoma cell line SBC-3. The ordinate indicates the cytotoxic activity and the abscissa the concentration of the antibody.

Fig. 57 graphically shows the GM_2-binding activities of mouse-human chimeric anti-GM_2 antibody KM966 and human CDR-grafted anti-GM_2 antibodies KM8969 and KM8970. The ordinate indicates the GM_2-binding activity and the abscissa the concentration of the antibody.

Fig. 58 graphically shows the reactivities of mouse-human chimeric anti-GM_2 antibody KM966 and human CDR-grafted anti-GM_2 antibodies KM8969 and KM8970 against various gangliosides. The ordinate indicates the ganglioside species and the abscissa the binding activity. $AcGM_2$ stands for N-acetyl-GM_2, $GcGM_2$ for N-glycolyl-GM_2, $AcGM_3$ for N-acetyl-GM_3 and $GcGM_3$ for N-glycolyl-GM_3.

Fig. 59 graphically shows the reactivities of mouse-human chimeric anti-GM_2 antibody KM966 and human CDR-grafted anti-GM_2 antibodies KM8969 and KM8970 against the human lung small cell carcinoma cell line SBC-3. The ordinate indicates the number of cells and the abscissa the fluorescence intensity. From the lowermost graph, the reactivities of control, KM966, KM8970 and KM8969 are shown in that order.

Fig. 60 graphically shows the ADCC activities of mouse-human chimeric anti-GM_2 antibody KM966 and human CDR-grafted anti-GM_2 antibodies KM8966, KM8969 and KM8970 against the human lung small cell carcinoma cell line SBC-3. The ordinate indicates the cytotoxicity and the abscissa the concentration of the antibody.

Fig. 61 graphically shows the CDC activities of mouse-human chimeric anti-GM_2 antibody KM966 and human CDR-grafted anti-GM_2 antibodies KM8966, KM8969 and KM8970 against the human lung small cell carcinoma cell line SBC-3 obtained when the reaction was carried out for 1 hour and 4 hours after the addition of the human complement. The ordinate indicates the cytotoxicity and the abscissa the concentration of the antibody.

DETAILED DESCRIPTION OF THE INVENTION

In the human CDR-grafted antibody, only CDRs of the H chain and L chain V regions comprise amino acid sequences of an antibody derived from nonhuman animal, and FRs of the H and L chain V regions and the C region comprise of amino acid sequences of a human antibody. Examples of the nonhuman animal include mouse, rat, hamster, rabbit and the like, as long as a hybridoma can be prepared therefrom.

With regard to the FR of the V regions of H chain and L chain, any amino acid sequence of known human antibodies can be used, such as an amino acid sequence selected from human antibody amino acid sequences, HMHCS, registered at the Protein Data Bank. Preferably, an amino acid sequence of the FR of HMHCS, which has a high homology with the FR of a monoclonal antibody of nonhuman animal, may be used.

As described in the foregoing, the antibody activity is reduced when the CDRs of the H chain V region and L chain V region of an antibody of nonhuman animal are replaced only with the CDRs of the H chain V region and L chain V region of a human antibody. In consequence, the present invention relates to a human CDR-grafted antibody wherein at least one amino acid in the FR of H chain and L chain V regions of the human CDR-grafted antibody is replaced by an other amino acid, so that it can show certain levels of antigen-binding activity, binding specificity and antibody dependent cell mediated cytotoxicity (ADCC), as well as complement dependent cytotoxicity (CDC), which are comparable to those of a human chimeric antibody having the V region of a monoclonal antibody derived from nonhuman animal which specifically reacts with ganglioside GM_2, and to a method for producing the same.

The replacement of at least one amino acid in the FR of H chain and L chain V regions of the human CDR-grafted antibody of the present invention means that amino acid residues desired to be replaced in the FR of H chain and L chain V regions of the human CDR-grafted antibody having a human antibody amino acid sequence are replaced by an other amino acid residues at corresponding positions in the FR of H chain and L chain V regions of a monoclonal antibody derived from nonhuman animal which specifically reacts with ganglioside GM_2. For example, at least one amino acid of positions 38, 40, 67, 72, 84 and 98 in the FR of H chain V region and positions 4, 11, 15, 35, 42, 46, 59, 69, 70, 71, 72, 76, 77 and 103 in the FR of L chain V region is replaced by an other amino acid.

Mouse anti-GM_2 monoclonal antibody KM796 (FERM BP-3340, JP-A-4-311385) can be cited as an example of the monoclonal antibody derived from nonhuman animal which specifically reacts with ganglioside GM_2. A chimeric anti-GM_2 antibody KM966 (FERM BP-3931, JP-A- 6-205694) can be cited as an example of the human chimeric antibody having the V region of a monoclonal antibody which is derived from nonhuman animal which specifically reacts with ganglioside GM_2.

Examples of the antibody having certain levels of antigen-binding activity, binding specificity and antibody dependent cell mediated cytotoxicity (ADCC), which are comparable to those of a human chimeric antibody having a V region of a monoclonal antibody derived from nonhuman animal which specifically reacts with ganglioside GM_2 include KM8966 produced by a transformant cell line KM8966 (FERM BP-5105), KM8967 produced by a transformant cell line KM8967 (FERM BP-5106) and KM8970 produced by a transformant cell line KM8970 (FERM BP-5528).

KM8969 produced by a transformant cell line KM8969 (FERM BP-5527) can be cited as an example of the antibody having certain levels of antigen-binding activity, binding specificity, antibody dependent cell mediated cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC), which are comparable to those of a human chimeric antibody having a V region of a monoclonal antibody derived from nonhuman animal which specifically reacts with ganglioside GM_2.

A method for producing the human CDR-grafted anti-GM_2 antibody is discussed below.

1. Construction of humanized antibody expression vector

The humanized antibody expression vector is an expression vector for use in animal cells, in which cDNA molecules encoding the C regions of H chain and L chain of a human antibody are integrated, and can be constructed by inserting the cDNA molecules encoding the C regions of H chain and L chain of a human antibody into respective expression vectors for animal cell use or by inserting the cDNA molecules which encode the C regions of H chain and L chain of a human antibody into a single expression vector for animal cell use (such a vector is called a tandem cassette vector). The C regions of human antibody can be any of C regions of human antibody H chain and L chain, and examples thereof include γ1 type C region (referred to as "Cγ1" hereinafter) and γ4 type C region (referred to as "Cγ4" hereinafter) of the human antibody H chain and κ type C region (referred to as "Cκ" hereinafter) of the human antibody L chain. Any expression vector for animal cell use can be used, as long as the cDNA encoding the human antibody C region can be integrated and expressed. Examples thereof include pAGE107 (Miyaji, H. *et al., Cytotechnology*, 3, 133 (1990)), pAGE103 (Mizukami, T. *et al., J. Biochem.*, 101, 1307 (1987)), pHSG274 (Brady, G. *et al., Gene*, 27, 223 (1984)), pKCR (O'Hare, K. *et al, Proc. Natl. Acad. Sci. U.S.A.*, 78, 1527 (1981)), and pSG1βd2-4 (Miyaji, H. *et al., Cytotechnology*, 4, 173 (1990)). Examples of the promoter and enhancer to be used in the expression vector for animal cell use include early promoter and enhancer of SV40 (Mizukami, T. *et al., J. Biochem.*, 101, 1397 (1987)), LTR promoter and enhancer of Moloney mouse leukemia virus (Kuwana, Y. *et al., Biochem. Biophys. Res. Comm.*, 149, 960 (1987)) and promoter (Mason, J.O. *et al., Cell*, 41, 479 (1985)) and enhancer (Gillies, S.D. *et al., Cell*, 33, 717 (1983)) of immunoglobulin H chain. The thus constructed humanized antibody expression vector can be used for expressing the human chimeric antibody and human CDR-grafted antibody in animal cells.

2. Preparation of cDNA encoding the V region of antibody of nonhuman animal

The cDNA encoding the H chain V region and L chain V region of the antibody of nonhuman animal to GM_2 is obtained in the following manner.

cDNA molecules are synthesized by extracting mRNA from cells of a hybridoma which produces the anti-GM_2 mon-

oclonal antibody. A library is prepared from the thus synthesized cDNA using a phage or a plasmid. Using cDNA corresponding to the C region moiety or cDNA corresponding to the V region moiety of each chain of a mouse antibody as a probe, a recombinant phage or recombinant plasmid having a cDNA which encodes the V region of H chain or a recombinant phage or recombinant plasmid having a cDNA encoding the V region of L chain is isolated from the library, and complete nucleotide sequences of the intended H chain V region and L chain V region of the antibody on the recombinant phage or recombinant plasmid are determined. Complete amino acid sequences of the H chain V region and L chain V region are deduced from the thus determined nucleotide sequences.

KM796 (FERM BP-3340, JP-A-4-311385) can be cited as an example of the hybridoma cells which produce the anti-GM_2 monoclonal antibody.

The guanidine thiocyanate-cesium trifluoroacetate method [*Methods in Enzymol.*, 154, 3 (1987)] can be exemplified as a method for prepering total RNA from hybridoma cells KM796, and the oligo (dT) immobilized cellulose column method [*Molecular Cloning*; A Laboratory Manual (2nd ed.)] can be exemplified as a method for preparing poly(A)$^+$ RNA from the total RNA. As a kit for use in the preparation of mRNA from the hybridoma KM796 cells, Fast Track mRNA Isolation Kit; manufactured by Invitrogen), Quick Prep mRNA Purification Kit; manufactured by Pharmacia) or the like can be exemplified.

With regard to the method for synthesizing cDNA and preparing cDNA library, the methods described in Molecular Cloning; A Laboratory Manual (2nd ed.) and Current Protocols in Molecular Biology, supplements 1 - 34 and the like, or a method which uses a commercially available kit such as Super Script™ Plasmid System for cDNA Synthesis and Plasmid Cloning (manufactured by Life Technologies) or Zap-cDNA Synthesis Kit (manufactured by Stratagene) can be exemplified. In preparing a cDNA library, any vector can be used as the vector into which the cDNA synthesized using the mRNA extracted from the hybridoma cells KM796 is to be integrated, as long as the cDNA can be integrated therein. Examples of such vectors include ZAP Express [*Strategies*, 5, 58 (1992)], pBluescript II SK(+) [*Nucleic Acids Research*, 17, 9494 (1989)], λzap II (manufactured by Stratagene), λgt10, λgt11 [*DNA Cloning*, A Practical Approach, Vol.1, 49 (1985)], Lambda BlueMid (manufactured by Clontech), λExCell, pT7T3 18U (manufactured by Pharmacia), pcD2 [*Mol. Cell. Biol.*, 3, 280 (1983)] and pUC18 [*Gene*, 33, 103 (1985).

As *Escherichia coli* into which a cDNA library constructed by the vector is to be introduced, any strain can be used, as long as the cDNA library can be introduced, expressed and maintained. Examples of such strains include XL1-Blue NRF' [*Strategies*, 5, 81 (1992)], C600 [*Genetics*, 39, 440 (1954)], Y1088, Y1090 [*Science*, 222, 778 (1983)], NM522 [*J. Mol. Biol.*, 166, 1 (1983)], K802 [*J. Mol. Biol.*, 16, 118 (1966)] and JM105 [*Gene*, 38, 275 (1985)]. Selection of cDNA clones encoding the V regions of H chain and L chain of the antibody of nonhuman animal from the cDNA library can be carried out by a colony hybridization or place hybridization method in which a probe labeled with an isotope or a fluorescence is used [*Molecular Cloning*; A Laboratory Manual (2nd ed.)]. Also, a DNA fragment encoding the V regions of H chain and L chain can be prepared by preparing primers and carrying out the polymerase chain reaction (referred to as "PCR" hereinafter) method [*Molecular Cloning*; A Laboratory Manual (2nd ed.), Current Protocols in Molecular Biology, supplements 1 - 34] using cDNA or cDNA library synthesized from poly(A)$^+$ RNA or mRNA as the template.

Nucleotide sequence of the DNA can be determined by digesting the cDNA clone selected by the aforementioned method with appropriate restriction enzymes, cloning the digests into a plasmid such as pBluescript SK(-) (manufactured by Stratagene) and then analyzing the resulting clones by a generally used nucleotide sequence analyzing method such as the dideoxy method of Sanger *et al.* [*Proc. Natl. Acad. Sci., U.S.A.*, 74, 5463 (1977)]. Analysis of the nucleotide sequence can be carried out using an automatic nucleotide sequence analyzer such as 373A DNA Sequencer (manufactured by Applied Biosystems).

3. Identification of CDR of the antibody of nonhuman animal

Each V region of H chain and L chain of the antibody forms an antigen binding site. Each of the V regions of H chain and L chain comprises four FRs whose sequences are relatively stable and three CDRs which connect them and are rich in sequence changes (Kabat, E.A. *et al.*, "*Sequences of Proteins of Immunological Interest*", US Dept. Health and Human Services, 1991). Each CDR can be found by comparing it with the V region amino acid sequences of known antibodies (Kabat, E.A. *et al.*, *Sequences of Proteins of Immunological Interest*, US Dept. Health and Human Services, 1991).

4. Construction of CDR of the antibody of nonhuman animal

The DNA sequences encoding the H chain V region and L chain V region of the human CDR-grafted anti-GM_2 antibody are obtained in the following manner.

First, an amino acid sequence of the V region of each of the H chain and L chain of the human antibody is selected for grafting the CDR of the V region of the anti-GM_2 antibody of nonhuman animal. As the amino acid sequence of the human antibody V region, any of the known V region amino acid sequences derived from human antibodies can be

used. For example, an amino acid sequence selected from human antibody V region amino acid sequences, HMHCS, registered at the Protein Data Bank may be used. However, in order to create a human CDR-grafted antibody having activities of interest such as binding activity and binding specificity for GM_2 or anti-tumor effect on GM_2-positive cells, it is desirable that the sequence has a high homology with the amino acid sequence of the V region of monoclonal antibody derived from nonhuman animal. Next, the DNA sequence encoding the FR in the selected V region amino acid sequence of human antibody is connected with the DNA sequence which encodes the amino acid sequence of the CDR, that becomes the source of the creation, of the V region of monoclonal antibody originated from nonhuman animal, thereby designing a DNA sequence which encodes the amino acid sequence of the V region of each of the H chain and L chain. A total of 6 synthetic DNA fragments are designed for each chain in such a manner that they can cover the thus designed DNA sequence, and PCR is carried out using them. Alternatively, 6 or 7 of each of anti-sense and sense DNA sequences, each comprising 35 to 84 bases, are synthesized in such a manner that they can cover the thus designed DNA sequence, and they are annealed to form double-stranded DNA fragments which are then subjected to the linking reaction. Thereafter, the amplification reaction product or the linking reaction product is subcloned into an appropriate vector and then its nucleotide sequence is determined, thereby obtaining a plasmid which contains the DNA sequence that encodes the amino acid sequence of the V region of each chain of the human CDR-grafted antibody of interest.

5. Modification of amino acid sequence of the V region of human CDR-grafted antibody.

Modification of amino acid sequence of the V region of human CDR-grafted antibody is carried out by a mutation introducing method using PCR. Illustratively, a sense mutation primer and an anti-sense mutation primer, comprising 20 to 40 bases and containing a DNA sequence which encodes amino acid residues after the modification, are synthesized and PCR is carried out using, as the template, a plasmid containing a DNA sequence which encodes the amino acid sequence of the V region to be modified. The amplified fragments are subcloned into an appropriate vector and then their nucleotide sequences are determined to obtain a plasmid which contains a DNA sequence in which the mutation of interest is introduced.

6. Construction of human CDR-grafted antibody expression vector

The human CDR-grafted antibody expression vector can be constructed by inserting the DNA sequences obtained in the above paragraphs 4 and 5, encoding V regions of H chain and L chain of the human CDR-grafted antibody, into upstream of the cDNA, corresponding to the C regions of H chain and L chain of human antibody, of the humanized antibody expression vector prepared in the above paragraph 1. For example, they are inserted into upstream of the cDNA of desired human antibody C regions so that they are properly expressed, by introducing appropriate restriction enzyme recognition sequences into the 5'- and 3'-termini of a synthetic DNA when PCR is carried out in order to construct a DNA sequence which encodes amino acid sequences of the V regions of H chain and L chain of the human CDR-grafted antibody.

7. Expression of the human CDR-grafted antibody and its activity evaluation

A transformant cell line capable of producing the human CDR-grafted antibody can be obtained by introducing the human CDR-grafted antibody expression vector prepared in the above paragraph 6.

Electroporation (JP-A-2-257891; Miyaji, H. *et al.*, *Cytotechnology*, 3, 133 (1990)) or the like can be used as the introduction method of the expression vector into host cells.

With regard to the host cells into which the human CDR-grafted antibody expression vector is introduced, any type of host cells can be used with the proviso that the human CDR-grafted antibody can be expressed therein. Examples of such cells include mouse SP2/0-Ag14 cells (ATCC CRL1581, referred to as "SP2/0 cells" hereinafter), mouse P3X63-Ag8.653 cells (ATCC CRL1580), dihydrofolate reductase gene (referred to as "DHFR gene" hereinafter)-deficient CHO cells (Urlaub, G. *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 77, 4216 (1980)), rat YB2/3HL.P2.G11.16Ag.20 cells (ATCC CRL1662, referred to as "YB2/0 cells" hereinafter) and the like.

After introduction of the vector, a transformant cell line capable of producing the human CDR-grafted antibody is selected in accordance with the method disclosed in JP-A-2-257891, using the RPMI 1640 medium containing geneticin (manufactured by Gibco, referred to as "G418" hereinafter) and fetal calf serum (referred to as "FCS" hereinafter). By culturing the thus obtained transformant cell line in a medium, the human CDR-grafted antibody can be produced and accumulated in the culture supernatant. Activity of the human CDR-grafted antibody in the culture supernatant is measured, for example, by the enzyme-linked immunosorbent assay (referred to as "ELISA method" hereinafter; Harlow, E. *et al.*, *Antibodies, A laboratory Manual*, Cold Spring Harbor Laboratory, Chapter 14 (1988)). In addition, production of the human CDR-grafted antibody by the transforaant cell line can be improved in accordance

with the method disclosed in JP-A-2-257891 making use of a DHFR gene amplifying system and the like.

The human CDR-grafted antibody can be purified from the aforementioned culture supernatant using a protein A column (Harlow, E. *et al.*, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Chapter 8 (1988)). Alternatively, other purification methods usually used for proteins can be employed. For example, it can be purified by carrying out gel filtration, ion exchange chromatography, ultrafiltration and the like techniques in an appropriate combination. Molecular weight of the H chain, L chain or entire antibody molecule of the thus purified human CDR-grafted antibody is measured for example by polyacrylamide gel electrophoresis (referred to as "SDS-PAGE" hereinafter; Laemmli, U.K. *et al.*, *Nature*, 227, 680 (1970) or western blot technique (Harlow, E. *et al.*, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Chapter 12 (1988).

Reactivity of the purified human CDR-grafted antibody with antigens and its binding activity to cultured cancer cell lines are measured by ELISA method, fluorescent antibody technique and the like means. Its complement dependent cytotoxicity (referred to as "CDC" hereinafter) activity and antibody dependent cell mediated cytotoxicity (referred to as "ADCC" hereinafter) activity upon cultured cancer cell lines are measured by the method of Shitara, K. *et al.* (*Cancer Immunol. Immunother.*, 36, 373 (1993)).

Since the human CDR-grafted antibody of the present invention binds to cultured cancer cell lines of human origin in a specific fashion and shows cytotoxic activities such as CDC activity and ADCC activity, it is useful in the diagnosis and treatment of human cancers and the like. In addition, since most portions of said antibody are originated from the amino acid sequence of a human antibody, when compared with monoclonal antibodies of animal origins excluding human, it is expected that it will exert strong anti-tumor effect without showing immunogenicity and that the effect will be maintained for a prolonged period of time.

The human CDR-grafted antibody of the present invention can be used as an anti-tumor composition, alone or together with at least one pharmaceutically acceptable auxiliary (carrier). For example, the human CDR-grafted antibody is made into an appropriate pharmaceutical composition by dissolving it in physiological saline or an aqueous solution of glucose, lactose, mannitol or the like. Alternatively, the human CDR-grafted antibody is freeze-dried in the usual way and then mixed with sodium chloride to prepare powder injections. As occasion demands, the pharmaceutical composition may contain pharmaceutically acceptable salts and the like additives commonly known in the field of pharmaceutical preparations.

Though the dosage of the pharmaceutical preparation varies depending on the age, symptoms and the like of each patient, the human CDR-grafted antibody is administered to animals including human at a dose of from 0.2 to 20 mg/kg/day. The administration is carried out once a day (single administration or every day administration) or 1 to 3 times a week or once in 2 to 3 weeks, by intravenous injection.

The present invention will be illustrated by the following Examples; however, the present invention is not limited thereto.

EXAMPLE 1

Construction of tandem cassette humanized antibody expression vector, pKANTEX93:

A tandem cassette humanized antibody expression vector, pKANTEX93, for the expression of a human CDR-grafted antibody in animal cells was constructed based on the plasmid pSE1UK1SEd1-3 described in JP-A-2-257891 by inserting a cDNA fragment coding for a human CDR-grafted antibody H chain V region and a cDNA fragment coding for a human CDR-grafted antibody L chain V region into said plasmid upstream of the human antibody Cγ1 cDNA and human antibody Cκ cDNA, respectively, in the following manner. The humanized antibody expression vector thus constructed can be also used for expressing a mouse-human chimeric antibody.

1. Modification of *Apa*I and *Eco*RI restriction enzyme sites occurring in rabbit β-globin gene splicing and poly A signals

For making it possible to construct a human CDR-grafted antibody expression vector by inserting human CDR-grafted antibody V regions cassette-wise in the form of *Not*I-*Apa*I (H chain) and EcoRI-*Spl*I (L chain) restriction fragments into a vector for humanized antibody expression, the *Apa*I and *Eco*RI restriction sites occurring in the rabbit β-globin gene splicing and poly A signals of the plasmid pSE1UK1SEd1-3 were modified in the following manner.

Three μg of the plasmid pBluescript SK(-) (Stratagene) was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Apa*I (Takara Shuzo) was further added, and the digestion reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, and the 3' cohesive ends resulting from *Apa*I digestion were rendered blunt using DNA Blunting Kit (Takara Shuzo), followed by ligation using DNA Ligation Kit (Takara Shuzo). The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101. Thus was obtained a plasmid, pBSA, shown in Fig. 1.

Furthermore, 3 μg of the plasmid pBSA thus obtained was added to 10 μl of 50 mM Tris-hydrochloride buffer (pH

7.5) containing 10 mM magnesium chloride, 100 mM sodium chloride and 1 mM DTT, 10 units of the restriction enzyme *Eco*RI (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, and the 5' cohesive ends resulting from *Eco*RI digestion were rendered blunt using DNA Blunting Kit (Takara Shuzo), followed by ligation using DNA Ligation Kit (Takara Shuzo). The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101. Thus was obtained the plasmid pBSAE shown in Fig. 2.

Then, 3 μg of the thus-obtained plasmid pBSAE was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride, 50 mM sodium chloride and 1 mM DTT, 10 units of the restriction enzyme *Hind*III (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was dissolved in 20 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride and 1 mM DTT, and the solution was divided into two 10-μl portions. To one portion, 10 units of the restriction enzyme *Sac*II (Toyobo) was further added and, to the other, 10 units of the restriction enzyme *Kpn*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. Both the reaction mixtures were fractionated by agarose gel electrophoresis, whereby about 0.3 μg each of a *Hind*III-*Sac*II fragment (about 2.96 kb) and a *Kpn*I-*Hind*III fragment (about 2.96 kb) were recovered.

Then, 3 μg of the plasmid pSE1UK1SEd1-3 was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Sac*II (Toyobo) and 10 units of the restriction enzyme *Kpn*I (Takara Shuzo) were further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was dissolved in 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride, 50 mM sodium chloride and 1 mM DTT, 10 units of the restriction enzyme *Hind*III (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, whereby about 0.2 μg each of a *Hind*III-*Sac*II fragment (about 2.42 kb) and a *Kpn*I-*Hind*III fragment (about 1.98 kb) were recovered.

Then, 0.1 μg of the thus-obtained *Hind*III-*Sac*II fragment of pSE1UK1SEd1-3 and 0.1 μg of the above *Hind*III-*Sac*II fragment of pBSAE were dissolved in a total of 20 μl of sterilized water and ligated to each other using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech). The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101 and, as a result, a plasmid, pBSH-S, shown in Fig. 3 was obtained. Furthermore, 0.1 μg of the above-mentioned *Kpn*I-*Hind*III fragment of pSE1UK1SEd1-3 and 0.1 μg of the above-mentioned *Kpn*I-*Hind*III fragment of pBSAE were dissolved in a total of 20 μl of sterilized water and ligated to each other using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech). The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101, and the plasmid pBSK-H shown in Fig. 4 was obtained.

Then, 3 μg each of the thus-obtained plasmids pBSH-S and pBSK-H were respectively added to 10-μl portions of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Apa*I (Takara Shuzo) was further added to each mixture, and the reaction was allowed to proceed at 37°C for 1 hour. Both the reaction mixtures were subjected to ethanol precipitation. With each precipitate, the 3' cohesive ends resulting from *Apa*I digestion were rendered blunt using DNA Blunting Kit (Takara Shuzo), followed by ligation using DNA Ligation Kit (Takara Shuzo). The thus-obtained recombinant DNA solution were used to transform *Escherichia coli* HB101, and the plasmids pBSH-SA and pBSK-HA shown in Fig. 5 were obtained.

Then, 5 μg each of the thus-obtained plasmids pBSH-SA and pBSK-HA were respectively added to 10-μl portions of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride, 100 mM sodium chloride and 1 mM DTT, 1 unit of the restriction enzyme *Eco*RI (Takara Shuzo) was further added to each mixture, and the reaction was allowed to proceed at 37°C for 10 minutes for partial digestion. Both the reaction mixtures were subjected to ethanol precipitation. With each precipitate, the 5' cohesive ends resulting from *Eco*RI digestion were rendered blunt using DNA Blunting Kit (Takara Shuzo), followed by fractionation by agarose gel electrophoresis, whereby about 0.5 μg each of a fragment about 5.38 kb in length and a fragment about 4.94 kb in length were recovered. The thus-recovered fragments (0.1 μg each) were each dissolved in a total of 20 μl of sterilized water and subjected to ligation treatment using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech). The thus-obtained recombinant DNA solutions were respectively used to transform *Escherichia coli* HB101, and the plasmids pBSH-SAE and pBSK-HAE shown in Fig. 6 were obtained.

Then, 3 μg each of the thus-obtained plasmids pBSH-SAE and pBSK-HAE were respectively added to 10-μl portions of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride, 100 mM sodium chloride and 1 mM DTT, 10 units of the restriction enzyme *Eco*RI (Takara Shuzo) was further added to each mixture, and the reaction was allowed to proceed at 37°C for 1 hour. Both the reaction mixtures were subjected to ethanol precipitation. With each precipitate, the 5' cohesive ends resulting from *Eco*RI digestion were rendered blunt using DNA Blunting Kit (Takara Shuzo), followed by ligation using DNA Ligation Kit (Takara Shuzo). The thus-obtained recombinant plasmid DNA solutions were each used to transform *Escherichia coli* HB101, and two plasmids, pBSH-SAEE and pBSK-HAEE, shown in Fig. 7 were obtained. Ten μg each of the thus-obtained plasmids were subjected to sequencing reaction according to the instructions attached to AutoRead Sequencing Kit (Pharmacia Biotech), followed by base sequence determination by electrophoresis on A.L.F. DNA Sequencer (Pharmacia Biotech), whereby it was confirmed that both

the *Apa*I and *Eco*RI sites had disappeared as a result of the above modification.

(2) *Sal*I restriction site introduction downstream from rabbit β-globin gene splicing and poly A signals and SV40 early gene poly A signal

For making it possible to exchange the antibody H chain and L chain expression promoters of the humanized antibody expression vector each for an arbitrary promoter, a *Sal*I restriction site was introduced into the plasmid pSE1UK1SEd1-3 downstream from the rabbit β-globin gene splicing and poly A signals and from the SV40 early gene poly A signal in the following manner.

Three μg of the plasmid pBSK-HAEE obtained in Paragraph 1 of Example 1 was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Nae*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was dissolved in 20 μl of 50 mM Tris-hydrochloride buffer (pH 9.0) containing 1 mM magnesium chloride, 1 unit of alkaline phosphatase (*E. coli* C75, Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour for dephosphorylation at the 5' termini. The reaction mixture was further subjected to phenol-chloroform extraction and then to ethanol precipitation, and the precipitate was dissolved in 20 μl of 10 mM Tris-hydrochloride buffer (pH 8.0) containing 1 mM disodium ethylenediaminetotraacetate (hereinafter briefly referred to as "TE buffer"). One μl of said reaction solution and 0.1 μg of a phosphorylated *Sal*I linker (Takara Shuzo) were added to sterilized water to make a total volume of 20 μl, followed by ligation treatment using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech). The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101, and a plasmid, pBSK-HAEESal, shown in Fig. 8 was obtained. Ten μg of the plasmid thus obtained was subjected to sequencing reaction according to the instructions attached to AutoRead Sequencing Kit (Pharmacia Biotech), followed by electrophoresis on A.L.F. DNA Sequencer (Pharmacia Biotech) for base sequence determination, whereby it was confirmed that one *Sal*I restriction site had been introduced downstream from the rabbit β-globin gene splicing and poly A signals and from the SV40 early gene poly A signal.

3. Modification of *Apa*I restriction site occurring in poly A signal of Herpes simplex virus thymidine kinase (hereinafter referred to as "HSVtk") gene

The *Apa*I restriction site occurring in the HSVtk gene poly A signal downstream from the Tn5 kanamycin phosphotransferase gene of the plasmid pSE1UK1SEd1-3 was modified in the following manner.

Three μg of the plasmid pBSA obtained in Paragraph 1 of Example 1 was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Sac*II (Toyobo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was added to 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Xho*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, whereby about 1 μg of a *Sac*II-*Xho*I fragment (about 2.96 kb) was recovered.

Then, 5 μg of the plasmid pSE1UK1SEd1-3 was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Sac*II (Toyobo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was added to 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Xho*I (Takara Shuzo) was further added, and the reaction was fractionated by agarose gel electrophoresis, whereby about 1 μg of a *Sac*II-*Xho*I fragment (about 4.25 kb) was recovered.

Then, 0.1 μg of the above *Sac*II-*Xho*I fragment of pBSA and the above *Sac*II-*Xho*I fragment of pSE1UK1SEd1-3 were added to a total of 20 μl of sterilized water, followed by ligation using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech). The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101, and the plasmid pBSX-S shown in Fig. 9 was obtained.

Then, 3 μg of the thus-obtained plasmid pBSX-S was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Apa*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the 3' cohesive ends resulting from *Apa*I digestion were rendered blunt using DNA Blunting Kit (Takara Shuzo) and then ligation was carried out using DNA Ligation Kit (Takara Shuzo). The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101, and a plasmid, pBSX-SA, shown in Fig. 10 was obtained. Ten μg of the thus-obtained plasmid was subjected to sequencing reaction according to the instructions attached to AutoRead Sequencing Kit (Pharmacia Biotech), followed by electrophoresis on A.L.F. DNA Sequencer

(Pharmacia Biotech) for base sequence determination, whereby it was confirmed that the *Apa*I restriction site in the HSVtk gene poly A signal had disappeared.

4 . Construction of humanized antibody L chain expression unit

A plasmid, pMohCκ, containing a human antibody Cκ cDNA downstream from the promoter/enhancer of the Moloney mouse leukemia virus long terminal repeat and having a humanized antibody L chain expression unit allowing cassette-wise insertion thereinto of a humanized antibody L chain V region was constructed in the following manner.

Three μg of the plasmid pBluescript SK(-) (Stratagene) was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Sac*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 50 mM sodium chloride, 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Cla*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, and the cohesive ends resulting from *Sac*I and *Cla*I digestion were rendered blunt using DNA Blunting Kit (Takara Shuzo), followed by fractionation by agarose gel electrophoresis, whereby about 1 μg of a DNA fragment about 2.96 kb in length was recovered. A 0.1-μg portion of the DNA fragment recovered was added to a total of 20 μl of sterilized water and subjected to ligation reaction using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech). The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101, and the plasmid pBSSC shown in Fig. 11 was obtained.

Then, 3 μg of the thus-obtained plasmid pBSSC was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Kpn*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was dissolved in 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Xho*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, whereby about 1 μg of a *Kpn*I-*Xho*I fragment (about 2.96 kb) was recovered.

Then, 5 μg of the plasmid pAGE147 described in JP-A-6-205694 was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH7.5) containing 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Kpn*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was dissolved in 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Xho*I (Takara Shuzo) was further added, and the reaction was fractionated by agarose gel electrophoresis, whereby about 0.3 μg of a *Kpn*I-*Xho*I fragment (about 0.66 kb) containing the Moloney mouse leukemia virus long terminal repeat promoter/enhancer was recovered.

Then, 0.1 μg of the *Kpn*I-*Xho*I fragment of pBSSC and 0.1 μg of the *Kpn*I-*Xho*I fragment of pAGE147 each obtained as mentioned above were dissolved in a total of 20 μl of sterilized water and subjected to ligation using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech). The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101, and the plasmid pBSMo shown in Fig. 12 was obtained.

Then, 3 μg of the above plasmid pBSMo was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Kpn*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was dissolved in 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 50 mM sodium chloride, 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Hind*III (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, whereby about 1 μg of a *Kpn*I-*Hind*III fragment (about 3.62 kb) was recovered.

Then, synthetic DNAs respectively having the base sequences shown in SEQ ID NO:12 and SEQ ID NO:13 were synthesized using an automatic DNA synthesizer (Applied Biosystems model 380A). To 15 μl of sterilized water were added 0.3 μg each of the thus-obtained synthetic DNAs, and the mixture was heated at 65°C for 5 minutes. The reaction mixture was allowed to stand at room temperature for 30 minutes and then 2 μl of 10-fold concentrated buffer [500 mM Tris-hydrochloride (pH 7.6), 100 mM magnesium chloride, 50 mM DTT] and 2 μl of 10 mM ATP were added, 10 units of T4 polynucleotide kinase was further added, and the reaction was allowed to proceed at 37°C for 30 minutes for phosphorylation of the 5' termini. To a total of 20 μl of sterilized water were added 0.1 μg of the above *Kpn*I-*Hind*III fragment (3.66 kb) derived from the plasmid pBSMo and 0.05 μg of the phsophorylated synthetic DNA pair, and ligation was effected using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech). The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101, and the plasmid pBSMoS shown in Fig. 13 was obtained. Ten μg of the plasmid thus obtained was subjected to sequencing reaction according to the instructions attached to AutoRead Sequencing Kit (Pharmacia Biotech), followed by electrophoresis on A.L.F. DNA Sequencer (Pharmacia Bio-

tech) for base sequence determination, whereby it was confirmed that the synthetic DNA pair had been introduced as desired.

Then, 3 μg of the plasmid pChilgLA1 described in JP-A-5-304989 was dissolved in 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride and 1 mM DTT, 10 units each of the restriction enzymes *Eco*RI (Takara Shuzo) and *Eco*RV (Takara Shuzo) were further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, whereby about 1 μg of an *Eco*RI-*Eco*RV fragment (about 9.70 kb) was recovered.

Then, synthetic DNAs respectively having the base sequences shown in SEQ ID NO:14 and SEQ ID NO:15 were synthesized using an automatic DNA synthesizer (Applied Biosystems model 380A). To 15 μl of sterilized water were added 0.3 μg each of the thus-obtained synthetic DNAs, and the mixture was heated at 65°C for 5 minutes. The reaction mixture was allowed to stand at room temperature for 30 minutes. Then, 2 μl of 10-fold concentrated buffer [500 mM Tris-hydrochloride (pH 7.6), 100 mM magnesium chloride, 50 mM DTT] and 2 μl of 10 mM ATP were added, 10 units of T4 polynucleotide kinase was further added, and the reaction was allowed to proceed at 37°C for 30 minutes for phosphorylation of the 5' termini. To a total of 20 μl of sterilized water were added 0.1 μg of the above *Eco*RI-*Eco*RV fragment (9.70 kb) derived from the plasmid pChilgLA1 and 0.05 μg of the phsophorylated synthetic DNA, and ligation was effected using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech). The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101, and the plasmid pChilgLA1S shown in Fig. 14 was obtained.

Then, 3 μg of the plasmid pBSMoS obtained in the above manner was dissolved in 10 μl of 20 mM Tris-hydrochloride buffer (pH 8.5) containing 100 mM potassium chloride, 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Hpa*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was dissolved in 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Eco*RI (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, whereby about 1 μg of an *Hpa*I-*Eco*RI fragment (about 3.66 kb) was recovered.

Then, 10 μg of the plasmid pChilgLA1S obtained as mentioned above was dissolved in 10 μl of 20 mM Tris-acetate buffer (pH 7.9) containing 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM DTT and 100 μg/ml BSA, 10 units of the restriction enzyme *Nla*IV (New England BioLabs) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was dissolved in 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Eco*RI (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, whereby about 0.3 μg of an *Nla*IV-*Eco*RI fragment (about 0.41 kb) was recovered.

Then, 0.1 μg of the above *Hpa*I-*Eco*RI fragment of pBSMoS and 0.1 μg of the above *Nla*IV-*Eco*RI fragment of pChilgLA1S were added to a total of 20 μl of sterilized water, and ligation was effected using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech). The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101, and the plasmid pMohCκ shown in Fig. 15 was obtained.

5. Construction of humanized antibody H chain expression unit

A plasmid, pMohCγ1, containing a human antibody Cγ1 cDNA downstream from the promoter/enhancer of the Moloney mouse leukemia virus long terminal repeat and having a humanized antibody H chain expression unit allowing cassette-wise insertion thereinto of a humanized antibody H chain V region was constructed in the following manner.

Three μg of the plasmid pBSMo obtained in Paragraph 4 of Example 1 was added to 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Xho*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was dissolved in 10 μl of 30 mM sodium acetate buffer (pH 5.0) containing 100 mM sodium chloride, 1 mM zinc acetate and 10% glycerol, 10 units of Mung bean nuclease (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 10 minutes. The reaction mixture was subjected to phenol-chloroform extraction and then to ethanol precipitation, the cohesive ends of the precipitate were rendered blunt using DNA Blunting Kit (Takara Shuzo) and ligation was effected using DNA Ligation Kit (Takara Shuzo). The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101, and the plasmid pBSMoSal shown in Fig. 16 was obtained. A 10-μg portion of the plasmid obtained was subjected to sequencing reaction according to the instructions attached to AutoRead Sequencing Kit (Pharmacia Biotech), followed by electrophoresis on A.L.F. DNA Sequencer (Pharmacia Biotech) for base sequence determination, whereby it was confirmed that the *Xho*I restriction site upstream of the Moloney mouse leukemia virus long terminal repeat promoter/enhancer had disappeared.

Then, 3 μg of the plasmid pBSMoSal obtained as mentioned above was added to 10 μl of 10 mM Tris-hydrochloride

buffer (pH 7.5) containing 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Kpn*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was dissolved in 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 50 mM sodium chloride, 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Hind*III (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, whereby about 1 μg of a *Kpn*I-*Hind*III fragment (about 3.66 kb) was recovered.

Then, synthetic DNAs respectively having the base sequences shown in SEQ ID NO:16 and SEQ ID NO:17 were synthesized using an automatic DNA synthesizer (Applied Biosystems model 380A). To 15 μl of sterilized water were added 0.3 μg each of the thus-obtained synthetic DNAs, and the mixture was heated at 65°C for 5 minutes. The reaction mixture was allowed to stand at room temperature for 30 minutes. Then, 2 μl of 10-fold concentrated buffer [500 mM Tris-hydrochloride (pH 7.6), 100 mM magnesium chloride, 50 mM DTT] and 2 μl of 10 mM ATP were added, 10 units of T4 polynucleotide kinase was further added, and the reaction was allowed to proceed at 37°C for 30 minutes for phosphorylation of the 5' termini. To a total of 20 μl of sterilized water were added 0.1 μg of the above *Kpn*I-*Hind*III fragment (3.66 kb) derived from the plasmid pBSMoSa1 and 0.05 μg of the phosphorylated synthetic DNA, and ligation was effected using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech). The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101, and the plasmid pBSMoSa1S shown in Fig. 17 was obtained. A 10-μg portion of the thus-obtained plasmid was subjected to sequencing reaction according to the instructions attached to AutoRead Sequencing Kit (Pharmacia Biotech), followed by electrophoresis on A.L.F. DNA Sequencer (Pharmacia Biotech), for base sequence determination whereby it was confirmed that the synthetic DNA had been introduced as desired.

Then, 10 μg of the plasmid pChilgHB2 described in JP-A-5-304989 was dissolved in 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme Eco52I (Toyobo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was dissolved in 10 μl of 30 mM sodium acetate buffer (pH 5.0) containing 100 mM sodium chloride, 1 mM zinc acetate and 10% glycerol, 10 units of Mung bean nuclease (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 10 minutes. The reaction mixture was subjected to phenol-chloroform extraction and then to ethanol precipitation, and the cohesive ends were rendered blunt using DNA Blunting Kit (Takara Shuzo). After ethanol precipitation, the precipitate was dissolved in 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Apa*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, whereby about 0.7 μg of *Apa*I-blunt end fragment (about 0.99 kb) was recovered.

Then, 3 μg of the plasmid pBluescript SK(-) (Stratagene) was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesia chloride and 1 mM DTT, 10 units of the restriction enzyme *Apa*I (2UTakara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was dissolved in 10 μl of 33 mM Tris-acetate buffer (pH 7.9) containing 10 mM magnesium acetate, 66 mM potassium acetate, 0.5 mM DTT and 100 μg/ml BSA, 10 units of the restriction enzyme *Sma*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, whereby about 1 μg of an *Apa*I-*Sma*I fragment (about 3.0 kb) was recovered.

Then, 0.1 μg of the *Apa*I-blunt end fragment of pChilgHB2 and 0.1 μg of the *Apa*I-*Sma*I fragment of pBluescript SK(-), each obtained as mentioned above, were added to a total of 20 μl of sterilized water and ligated to each other using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech). The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101, and the plasmid pBShCγ1 shown in Fig. 18 was obtained.

Then, 5 μg of the above plasmid pBShCγ1 was dissolved in 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Apa*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was dissolved in 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 50 mM sodium chloride, 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Spe*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, whereby about 1 μg of an *Apa*I-*Spe*I fragment (about 1.0 kb) was recovered.

Then, 3 μg of the plasmid pBSMoSa1S obtained as mentioned above was dissolved in 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Apa*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was dissolved in 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 50 mM sodium chloride, 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Spe*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture

was fractionated by agarose gel electrophoresis, whereby about 1 μg of an *Apa*I-*Spe*I fragment (about 3.66 kb) was recovered.

Then, 0.1 μg of the *Apa*I-*Spe*I fragment of pBShCγ1 and 0.1 μg of the *Apa*I-*Spe*I fragment of pBSMoSa1S, each obtained as mentioned above, were added to a total of 20 μl of sterilized water and ligated to each other using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech). The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101, and the plasmid pMohCγ1 shown in Fig. 19 was obtained.

6. Construction of tandem cassette humanized antibody expression vector, pKANTEX93

A tandem cassette humanized antibody expression vector, pKANTEX93, was constructed using the various plasmids obtained in Paragraphs 1 through 5 of Example 1 in the following manner.

Three μg of the plasmid pBSH-SAEE obtained in Paragraph 1 of Example 1 was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 50 mM sodium chloride, 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Hin*dIII (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was dissolved in 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Sal*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, whereby about 1 μg of a *Hin*dIII-*Sal*I fragment (about 5.42 kb) was recovered.

Then, 5 μg of the plasmid pBSK-HAEE obtained in Paragraph 1 of Example 1 was added to 10 μl of 10 mM Tris-hydrochloride buffer (ph 7.5) containing 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Kpn*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was dissolved in 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 50 mM sodium chloride, 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Hin*dIII (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, whereby about 0.8 μg of a *Kpn*I-*Hin*dIII fragment (about 1.98 kb) containing the rabbit β-globin gene splicing and poly A signals, the SV40 early gene poly A signal and the SV40 early gene promoter was recovered.

Then, 5 μg of the plasmid pMohCγ1 obtained in Paragraph 5 of Example 1 was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Kpn*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was dissolved in 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Sal*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, whereby about 0.8 μg of a human CDR-grafted antibody H chain expression unit-containing *Kpn*I-*Sal*I fragment (about 1.66 kb) was recovered.

Then, 0.1 μg of the *Hin*dIII-*Sal*I fragment of pBSH-SAEE, 0.1 μg of the *Kpn*I-*Hin*dIII fragment of pBSK-HAEE and 0.1 μg of the *Kpn*I-*Sal*I fragment of pMohCγ1, each obtained as mentioned above, were added to a total of 20 μl of sterilized water and ligated together using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech). The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101, and the plasmid pMoγ1SP shown in Fig. 20 was obtained.

Then, 3 μg of the above plasmid pMoγ1SP was added to 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Sal*I (Takara Shuzo) and 10 units of the restriction enzyme *Xho*I were further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, whereby about 1 μg of a *Sal*I-*Xho*I fragment (about 9.06 kb) was recovered.

Then, 5 μg of the plasmid pBSK-HAEESal obtained in Paragraph 2 of Example 1 was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Kpn*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was dissolved in 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Sal*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, whereby about 0.7 μg of a *Kpn*I-*Sal*I fragment (about 1.37 kb) containing the rabbit β-globin gene splicing and poly A signals and the SV40 early gene poly A signal was recovered.

Then, 5 μg of the plasmid pMohCκ obtained in Paragraph 4 of Example 1 was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride and 1mM DTT, 10 units of the restriction enzyme *Kpn*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was dissolved in 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5)

containing 100 mM sodium chloride, 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Xho*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, whereby about 0.7 μg of a human CDR-grafted antibody L chain expression unit-containing *Kpn*I-*Xho*I fragment (about 1.06 kb) was recovered.

Then, 0.1 μg of the *Sal*I-*Xho*I fragment of pMoγ1SP, 0.1 μg of the *Kpn*I-*Sal*I fragment of pBSK-HAEESal and 0.1 μg of the *Kpn*I-*Xho*I fragment of pMohCκ, each obtained as mentioned above, were added to a total of 20 μl of sterilized water and ligated together using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech). The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101, and the plaid pMoκγ1SP shown in Fig. 21 was obtained.

Then, 3 μg of the above plasmid pMoκγ1SP was added to 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Xho*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride and 1 mM DTT, 1 units of the restriction enzyme *Sac*II (Toyobo) was further added, and the reaction was allowed to proceed at 37°C for 10 minutes for partial digestion. The reaction mixture was fractionated by agarose gel electrophoresis, and about 0.2 μg of a *Sac*II-*Xho*I fragment (about 8.49 kb) was recovered.

Then, 3 μg of the plaid pBSX-SA obtained in Paragraph 3 of Example 1 was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Sac*II (Toyobo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was dissolved in 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Xho*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, and about 1 μg of a *Sac*II-*Xho*I fragment (about 4.25 kb) was recovered.

Then, 0.1 μg of the *Sac*II-*Xho*I fragment of pMoκγ1SP and 0.1 μg of the *Sac*II-*Xho*I fragment of pBSX-SA, each obtained as mentioned above, were added to a total of 20 μl of sterilized water and ligated to each other using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech). The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101, and the plasmid pKANTEX93 shown in Fig. 22 was obtained.

EXAMPLE 2

1. Expression of mouse-human chimeric anti-GM_2 antibody

Mouse-human chimeric anti-GM_2 antibody expression was effected using the humanized antibody expression vector pKANTEX93 mentioned above in Example 1 in the following manner.

(1) Construction of plasmid pBSH3 containing mouse anti-GM_2 antibody KM796 H chain V region cDNA

Three μg of the plasmid pBluescript SK(-) (Stratagene) was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride and 1 mM DTT, 10 units each of the restriction enzymes *Sac*II (Toyobo) and *Kpn*I (Takara Shuzo) were further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, and the precipitate was subjected to blunting treatment for rendering blunt the 3' cohesive ends resulting from the restriction enzyme digestion using DNA Blunting Kit (Takara Shuzo). The resulting reaction was precipitated with ethanol, the precipitate thus obtained was dissolved in 20 μl of a buffer containing 50 mM Tris-hydrochloride buffer (pH 9.0) and 1 mM magnesium chloride, and the mixture thus obtained was allowed to react by adding one unit of alkali phosphatase (*E. coli* C75, Takara Shuzo) at 37°C for 1 hour for dephosphorylation of the 5' termini. Then, fractionation by agarose gel electrophoresis was carried out, and about 1 μg of a DNA fragment about 2.95 kb in size was recovered.

Then, synthetic DNAs respectively having the base sequences shown in SEQ ID NO:18 and SEQ ID NO:19 were synthesized using an automatic DNA synthesizer (Applied Biosystems model 380A). To 15 μl of sterilized water were added 0.3 μg each of the synthetic DNAs obtained, and the mixture was heated at 65°C for 5 minutes. The reaction mixture was allowed to stand at room temperature for 30 minutes and then 2 μl of 10-fold concentrated buffer [500 mM Tris-hydrochloride (pH 7.6), 100 mM magnesium chloride, 50 mM DTT] and 2 μl of 10 mM ATP were added, 10 units of T4 polynucleotide kinase was further added, and the reaction was allowed to proceed at 37°C for 30 minutes for phosphorylating the 5' termini. To a total of 20 μl of sterilized water were added 0.1 μg of the DNA fragment (2.95 kb) derived from the plasmid pBluescript SK(-) and 0.05 μg of the phosphorylated synthetic DNA, each obtained as mentioned above, followed by ligation to each other using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech): The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101, and the plaid pBSNA shown in Fig.

23 was obtained. Ten μg of the plasmid obtained was subjected to sequencing reaction treatment according to the instructions attached to AutoRead Sequencing Kit (Pharmacia Biotech), followed by electrophoresis on A.L.F. DNA Sequencer (Pharmacia Biotech) for base sequence determination, whereby it was confirmed that the synthetic DNA had been introduced as desired.

Then, 3 μg of the plasmid pBSNA obtained as mentioned above was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Apa*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was added to 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride, 1 mM DTT, 100 μg/ml BSA and 0.01% Triton X-100, 10 units of the restriction enzyme *Not*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, and about 1 μg of a DNA fragment about 2.95 kb in size was recovered.

Then, 10 μg of the plaid pChi796HM1 described in JP-A-6-205964 was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Apa*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was added to 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride, 1 mM DTT, 100 μg/ml BSA and 0.01% Triton X-100, 10 units of the restriction enzyme *Not*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, and about 0.3 μg of a DNA fragment about 0.45 kb in size was recovered.

Then, 0.1 μg of the *Apa*I-*Not*I fragment of pBSNA and 0.1 μg of the *Apa*I-*Not*I fragment of pChi796HM1, each obtained as mentioned above, were added to a total of 20 μl of sterilized water and ligated to each other using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech). The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101, and the plasmid pBSH3 shown in Fig. 24 was obtained.

(2) Construction of plasmid pBSL3 containing mouse anti-GM_2 antibody KM796 L chain V region cDNA

Three μg of the plasmid pBluescript SK(-) (Stratagene) was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Kpn*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, and the precipitate was subjected to blunting treatment for rendering blunt the 3' cohesive ends resulting from *Kpn*I digestion using DNA Blunting Kit (Takara Shuzo) and then to ethanol precipitation, the precipitate was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Sac*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, whereby about 1 μg of a DNA fragment about 2.95 kb in size was recovered.

Then, synthetic DNAs respectively having the base sequences shown in SEQ ID NO:20 and SEQ ID NO:21 were synthesized using an automatic DNA synthesizer (Applied Biosystems model 380A). To 15 μl of sterilized water were added 0.3 μg each of the synthetic DNAs obtained, and the mixture was heated at 65°C for 5 minutes. The reaction mixture was allowed to stand at room temperature for 30 minutes. Then, 2 μl of 10-fold concentrated buffer [500 mM Tris-hydrochloride (pH 7.5), 100 mM magnesium chloride, 50 mM DTT] and 2 μl of 10 mM ATP were added, 10 units of T4 polynucleotide kinase was further added, and the reaction was allowed to proceed at 37°C for 30 minutes for phosphorylating the 5' termini. The, 0.1 μg of the DNA fragment (2.95 kb) derived from the plasmid pBluescript SK(-) and 0.05 μg of the phosphorylated synthetic DNA, each obtained as mentioned above, were added to a total of 20 μl of sterilized water and ligated to each other using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech). The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101, and the plasmid pBSES shown in Fig. 25 was obtained. Ten μg of the plasmid obtained was subjected to sequencing reaction treatment according to the instructions attached to AutoRead Sequencing Kit (Pharmacia Biotech), followed by electrophoresis on A.L.F. DNA Sequencer (Pharmacia Biotech) for base sequence determination, whereby it was confirmed that the synthetic DNA had been introduced as desired.

Then, 3 μg of the plasmid pBSES obtained as mentioned above was added to 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride, 1 mM DTT and 100 μg/ml BSA, 10 units each of the restriction enzymes *Eco*RI (Takara Shuzo) and *Spl*I (Takara Shuzo) were further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, and about 1 μg of a DNA fragment about 2.95 kb in size was recovered.

Then, 5 μg of the plasmid pKM796L1 described in JP-A-6-205694 was added to 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride and 1 mM DTT, 10 units each of the restriction enzymes *Eco*RI (Takara Shuzo) and *Afl*III (Takara Shuzo) were further added, and the reaction was allowed to proceed at 37°C for 1

hour. The reaction mixture was fractionated by agarose gel electrophoresis, and about 0.3 μg of an *Eco*RI-*Af*lII fragment about 0.39 kb in size was recovered. Then, synthetic DNAs respectively having the base sequences shown in SEQ ID NO:22 and SEQ ID NO:23 were synthesized using an automatic DNA synthesizer (Applied Biosystems model 380A). To 15 μl of sterilized water were added 0.3 μg each of the synthetic DNAs obtained, and the mixture was heated at 65°C for 5 minutes. The reaction mixture was allowed to stand at room temperature for 30 minutes. Then, 2 μl of 10-fold concentrated buffer [500 mM Tris-hydrochloride (pH 7.6), 100 mM magnesium chloride, 50 mM DTT] and 2 μl of 10 mM ATP were added, 10 units of T4 polynucleotide kinase was further added, and the reaction was allowed to proceed at 37°C for 30 minutes for phosphorylating the 5' termini.

Then, 0.1 μg of the pBSES-derived *Eco*RI-*Spl*I fragment (2.95 kb), 0.1 μg of the pKM796LI-derived *Eco*RI-*Af*lII fragment and 0.05 μg of the phosphorylated synthetic DNA, each obtained as mentioned above, were added to a total of 20 μl of sterilized water and ligated together using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech). The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101, and the plasmid pBSL3 shown in Fig. 26 was obtained. Ten μg of the plasmid obtained was subjected to sequencing reaction treatment according to the instructions attached to AutoRead Sequencing Kit (Pharmacia Biotech), followed by electrophoresis on A.L.F. DNA Sequencer (Pharmacia Biotech) for base sequence determination, whereby it was confirmed that the synthetic DNA had been introduced as desired.

3. Construction of mouse-human chimeric anti-GM_2 antibody expression vector, pKANTEX796

A mouse-human chimeric anti-GM_2 antibody expression vector, pKANTEX796, was constructed using the plasmid pKANTEX93 obtained in Example 1 and the plasmids pBSH3 and pBSL3 respectively obtained in Paragraph 1 (1) and (2) of Example 2, in the following manner.

Three μg of the plasmid pBSH3 was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Apa*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was dissolved in 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride, 1 mM DTT, 100 μg/ml BSA and 0.01% Triton X-100, 10 units of the restriction enzyme *Not*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, and about 0.3 μg of an *Apa*I-*Not*I fragment about 0.46 kb in size was recovered.

Then, 3 μg of the plasmid pKANTEX93 was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Apa*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was dissolved in 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride, 1 mM DTT, 100 μg/ml BSA and 0.01% Triton X-100, 10 units of the restriction enzyme *Not*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, whereby about 1 μg of an *Apa*I-*Not*I fragment about 12.75 kb in size was recovered.

Then, 0.1 μg of the pBSH3-derived *Apa*I-*Not*I fragment and 0.1 μg of the pKANTEX93-derived *Apa*I-*Not*I fragment, each obtained as mentioned above, were added to a total of 20 μl of sterilized water and ligated to each other using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech). The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101, and the plasmid pKANTEX796H shown in Fig. 27 was obtained.

Then, 3 μg of the plasmid pBSL3 was added to 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesia chloride, 1 mM DTT and 100 μg/ml BSA, 10 units each of the restriction enzymes *Eco*RI (Takara Shuzo) and *Spl*I (Takara Shuzo) were further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, and about 0.3 μg of an *Eco*RI-*Spl*I fragment about 0.4 kb in size was recovered.

Then, 3 μg of the plasmid pKANTEX796H was added to 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride, 1 mM DTT and 100 μg/ml BSA, 10 units each of the restriction enzymes *Eco*RI (Takara Shuzo) and *Spl*I (Takara Shuzo) were further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, and about 1 μg of an *Eco*RI-*Spl*I fragment about 13.20 kb in size was recovered.

Then, 0.1 μg of the pBSL3-derived *Eco*RI-*Spl*I fragment and 0.1 μg of the pKANTEX796H-derived *Eco*RI-*Spl*I fragment, each obtained as mentioned above, were added to a total of 20 μl of sterilized water and ligated to each other using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech). The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101, and the plasmid pKANTEX796 shown in Fig. 28 was obtained.

(4) Expression of mouse-human chimeric anti-GM_2 antibody in YB2/0 cells using pKANTEX796

Introduction of the plasmid into YB2/0 cells (ATCC CRL1662) was carried out by the electroporation method (Miyaji, H. *et al.*, *Cytotechnology*, 3, 133 (1990)). A 4 μg portion of pKANTEX796 obtained in Paragraph 1 (3) of Example 2 was introduced into 4×10^6 cells of YB2/0 cells, and the resulting cells were suspended in 40 ml of RPMI1640-FCS (10) medium [RPMI1640 medium (manufactured by Nissui Pharmaceutical) supplemented with 10% of FCS, an appropriate amount of 7.5% sodium bicarbonate solution, 3% of 200 mM L-glutamine solution (manufactured by Gibco) and 0.5% of penicillin-streptomycin solution (manufactured by Gibco, contains 5,000 U/ml of penicillin and 5 mg/ml of streptomycin)] and dispensed in 200 μl portions into wells of a 96 well microplate. After 24 hours of culturing at 37°C in a 5% CO_2 incubator, G418 was added to each well to a final concentration of 0.5 mg/ml, and the cells were cultured for 1 to 2 weeks. Culture supernatants were recovered from wells in which colonies of transformant cell lines have been formed, and the activity of the mouse-human chimeric anti-GM_2 antibody in the culture supernatants was measured by the ELISA method described in the following paragraph (5). Cells in wells in which the activity was found were subjected to gene amplification in the following manner with an attempt to increase expression quantity of the chimera antibody. Firstly, the cells were suspended in the RPMI1640-FCS (10) medium supplemented with 0.5 mg/ml of G418 and 50 nM of methotrexate (manufactured by Sigma, to be referred to as "MTX" hereinafter), to a density of $1\text{-}2 \times 10^5$ cells/ml, and the suspension was dispensed in 2 ml portions in wells of a 24 well plate. The cells were cultured at 37°C for 1 to 2 was in a 5% CO_2 incubator to induce resistant cells to 50 nM MTX. In wells in which the cells resistant to 50 nM MTX have been formed, the final concentration of MTX was increased to 100 nM and then to 200 nM and the expression quantity was evaluated by the ELISA method to select cells having the highest expression quantity. The thus selected cells were subjected twice to cloning by the limiting dilution analysis and then established as the final chimera antibody stable expression cells. The thus established mouse-human chimeric anti-GM_2 antibody stable expression cells showed an expression quantity of about 1 to 2 μg/ml, so that it was confirmed that efficient and stable expression of the humanized antibody can be effected by the use of pKANTEX93.

(5) ELISA method

A 2 ng portion of ganglioside was dissolved in 2 ml of ethanol solution containing 5 ng of phosphatidylcholine (manufactured by Sigma) and 2.5 ng of cholesterol (manufactured by Sigma). This solution or a diluted solution thereof was dispensed in 20 μl portions in wells of a 96 well microplate (manufactured by Greiner), air-dried and then subjected to blowing with a phosphate buffer containing 1% BSA (to be referred to as "PBS" hereinafter). To the resulting plate was added culture supernatant of a transformant cell line, a purified mouse monoclonal antibody a purified mouse-human chimeric antibody or a purified humanized antibody in an amount of from 50 to 100 μl, subsequently carrying out 1 to 2 hours of reaction at room temperature. After the reaction and subsequent washing of each well with PBS, 50 to 100 μl of a peroxidase-labeled rabbit anti-mouse IgG antibody (manufactured by Dako, used by 400 times dilution) or a peroxidase-labeled goat anti-human γ chain antibody (manufactured by Kiyukegard & Perry Laboratory, used by 1,000 times dilution) was added thereto, and 1 to 2 hours of reaction was carried out at room temperature. After washing with PBS, 50 to 100 μl of an ABTS substrate solution [a solution prepared by dissolving 550 mg of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) in 1 liter of 0.1 M citrate buffer (pH 4.2) and adding 1 μl/ml of hydrogen peroxide to the solution just before its use] was added to each well to effect development of color which was then measured at OD_{415}.

2. Transient mouse-human chimeric antibody expression in COS-7 (ATCC CRL 1651) cells

For enabling more rapid activity evaluation of various versions of human CDR-grafted anti-GM_2 antibody, transient expression of mouse-human chimeric anti-GM_2 antibody expression was caused in COS-7 cells by the Lipofectamine method using pKANTEX796 and a variant thereof in the following manner.

(1) Construction of variant of pKANTEX796

Since transient antibody expression in animal cells is dependent on the copy number of an expression vector introduced, it was supposed that an expression vector smaller in size would show a higher expression efficiency. Therefore, a smaller humanized antibody expression vector, pT796, was constructed by deleting a region supposedly having no effect on humanized antibody expression from pKANTEX796 in the following manner.

Thus, 3 μg of the plasmid pKANTEX796 was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 50 mM sodium chloride, 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Hin*dIII (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was dissolved in 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Mlu*I

(Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, and the 5' cohesive ends resulting from the restriction enzyme digestion were rendered blunt using DNA Blunting Kit (Takara Shuzo). The reaction mixture was fractionated by agarose gel electrophoresis and about 1 μg of a DNA fragment about 9.60 kb in size was recovered. A 0.1-μg portion of the thus-recovered DNA fragment was added to a total of 20 μl of sterilized water and subjected to ligation treatment using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech). The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101, and the plasmid pT796 shown in Fig. 29 was obtained.

(2) Transient expression of mouse-human chimeric anti-GM_2 antibody using pKANTEX796 and pT796

A 1×10^5 cells/ml suspension of COS-7 cells was distributed in 2-ml portions into wells of a 6-well plate (Falcon) and cultured overnight at 37°C. Two μg of pKANTEX796 or pT796 was added to 100 μl of OPTI-MEM medium (Gibco), a solution prepared by adding 10 μl of LIPOFECTAMINE reagent (Gibco) to 100 μl of OPTI-MEM medium (Gibco) was further added, and the reaction was allowed to proceed at room temperature for 40 minutes to cause DNA-liposome complex formation. The COS-7 cells cultured overnight were washed twice with 2 ml of OPTI-MEM medium (Gibco), the complex-containing solution was added, and the cells were cultured at 37°C for 7 hours. Then, the solution was removed, 2 ml of DMEM medium (Gibco) containing 10% FCS was added to each well, and the cells were cultured at 37°C. After 24 hours, 48 hours, 72 hours, 96 hours and 120 hours of cultivation, the culture supernatant was recovered and, after concentration procedure as necessary, evaluated for mouse-human chimeric anti-GM_2 antibody activity in the culture supernatant by the ELISA method described in Paragraph 1 (5) of Example 2. The results are shown in Fig. 30. As shown in Fig. 30, higher levels of transient mouse-human chimeric anti-GM_2 antibody expression was observed with pT796 as compared with pKANTEX796. For pT796, the level of expression was highest at 72 to 96 hours, the concentration being about 30 ng/ml (in terms of GM_2 binding activity). The above results indicate that construction of a pKANTEX93-derived vector having a reduced size and introduction thereof into COS-7 cells make it possible to make activity evaluation of expression vector-derived humanized antibodies in a transient expression system. Furthermore, for close activity comparison of various versions of human CDR-grafted anti-GM_2 antibody as mentioned hereinafter, the ELISA method described below under (3) was used to determine antibody concentrations in transient expression culture supernatants.

(3) Determination by sandwich ELISA of humanized antibody concentrations in various culture supernatants

A solution prepared by 400-fold dilution of goat anti-human γ chain antibody (Igaku Seibutugaku Kenkyusho) with PBS was distributed in 50-μl portions into wells of a 96-well microtiter plate and allowed to stand overnight at 4°C for binding to the wells. After removing the antibody solution, blowing was effected with 100 μl of PBS containing 1% BSA at 37°C for 1 hour. Fifty μl of a transient expression culture supernatant or purified mouse-human chimeric anti-GM_2 antibody was added thereto and allowed to react at room temperature for 1 hour. Thereafter, the solution was removed, the wells were washed with PBS, and 50 μl of a solution prepared by 500-fold dilution of peroxidase-labeled mouse anti-human κ chain antibody (Zymet) with PBS was added and allowed to react at room temperature for 1 hour. After washing with PBS, 50 μl of an ABTS substrate solution was added for causing color development, and the OD_{415} was measured.

EXAMPLE 3

Production of human CDR-grafted anti-GM_2 antibody I

A human CDR-grafted anti-GM_2 antibody higher in GM_2-binding activity than the human CDR-grafted anti-GM_2 antibody described in Example 2 of JP-A-6-105694 was produced in the following manner.

(1) Modification of human CDR-grafted anti-GM_2 antibody H chain V region described in Paragraph 1 (1) of Example 2 of JP-A-6-205694

DNAs coding for some versions of the human CDR-grafted anti-GM_2 antibody H chain V region described in Example 2 as derived by replacing several amino acids in the FR with original mouse antibody amino acids were constructed in the following manner. Based on a computer model for the V region of mouse antibody KM796, those amino acid residues that were expected to contribute to restoration of antigen-binding activity as a result of replacement were selected as the amino acid residues to be replacement. First, DNAs respectively having the base sequences of SEQ ID NO:24 and SEQ ID NO:25 were synthesized using an automatic DNA synthesize (Applied Biosystems model 380A).

Then, a version (version 2) of human CDR-grafted antibody H chain V region shown in SEQ ID NO:26 and having

replacement in positions 78 (threonine in lieu of glutamine), 79 (alanine in lieu of phenylalanine) and 80 (tyrosine in lieu of serine) was constructed in the same manner as in Paragraph 1 (1) of Example 2 of JP-A-6-205964 using a synthetic DNA of SEQ ID NO:24 in lieu of the synthetic DNA of SEQ ID NO:27 of JP-A-6-205964.

Then, another version (version 4) of human CDR-grafted antibody H chain V region shown in SEQ ID NO:27 and having replacements in positions 27 (tyrosine in lieu of phenylalanine), 30 (threonine in lieu of serine), 40 (serine in lieu of proline) and 41 (histidine in lieu of proline) was constructed in the same manner as in Paragraph 1 (1) of Example 2 of JP-A-6-205694 using a synthetic DNA of SEQ ID NO:25 in lieu of the synthetic DNA of SEQ ID NO:25 of JP-A-6-205694.

(2) Construction of human CDR-grafted anti-GM_2 antibody H chain V region using known HMHCS of human antibody H chain V region

According to Kabat *et al.* (Kabat E. A. *et al.*, *Sequences of Proteins of Immunological Interest*, US Dept. of Health and Human Services, 1991), known human antiobdy H chain V regions are classifiable into subgroups I to III (Human Sub Groups (HSG) I to III) based on the homology of their FR regions, and coon sequences have been identified for respective subgroups. The present inventors identified HMHCS as one meaning from the common sequences, a human CDR-grafted anti-GM_2 antibody H chain V region was constructed based on the HMHCS. First, for selecting HMHCS to serve as the base, the homology was examined between the FR, of the mouse antibody KM796 H chain V region and the FR of the HMHCS of the human antibody H chain V region of each subgroup (Table 1).

TABLE 1

Homology (%) between mouse antibody KM796 H chain V region FR and human antibody H chain V region common sequence FR		
HSG I	HSG II	HSG III
72.1	52.9	58.6

As a result, it was confirmed that subgroup I shows the greatest similarity. Thus, based on the HMHCS of subgroup I, a human CDR-grafted anti-GM_2 antibody H chain V region was constructed by the PCR method in the following manner.

Synthetic DNAs respectively having the base sequences of SEQ ID NO:28 through SEQ ID NO:33 were synthesized using an automatic DNA synthesizer (Applied Systems model 380A). The DNAs synthesized were added, each to a final concentration of 0.1 μM, to 50 μl of 10 mM Tris-hydrochloride buffer (pH 8.3) containing 50 mM potassium chloride, 1.5 mM magnesium chloride, 0.001% gelatin, 200 μM dNTP, 0.5 μM M13 primer RV (Takara Shuzo), 0.5 μM M13 primer M4 (Takara Shuzo) and 2 units of TaKaRa Taq DNA polymerase, the mixture was covered with 50 μl of mineral oil, a DNA thermal cycler (Perkin Elmer model PJ480) was loaded with the mixture, and 30 PCR cycles (2 minutes at 94°C, 2 minutes at 55°C and 2 minutes at 72°C per cycle) were conducted. The reaction mixture was purified using QIAquick PCR Purification Kit (Qiagen) and then made into a solution in 30 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Apa*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was added to 10 μl of 50 mM Tris-hydrochloride (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride, 1 mM DTT, 100 μg/ml BSA and 0.01% Triton X-100, 10 units of the restriction enzyme *Not*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, and about 0.2 μg of an *Apa*I-*Not*I fragment about 0.44 kb in size was recovered.

Then, 3 μg of the plasmid pBSH3 obtained in Paragraph 1 (1) of Example 2 was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Apa*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was added to 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride, 1 mM DTT, 100 μg/ml BSA and 0.01% Triton X-100, 10 units of the restriction enzyme *Not*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agraose gel electrophoresis, and about 1 μg of an *Apa*I-*Not*I fragment about 2.95 kb in size was recovered.

Then, 0.1 μg of the *Apa*I-*Not*I fragment of the human CDR-grafted anti-GM_2 antibody H chain V region and 0.1 μg of the *Apa*I-*Not*I fragment of pBSH3, each obtained as mentioned above, were added to a total of 20 μl of sterilized

water and ligated to each other using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech). The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101. Plasmid DNAs were prepared from 10 transformant clones and their base sequences were determined. As a result, a plasmid, pBSB10, shown in Fig. 31 and having the desired base sequence was obtained. The amino acid sequence and base sequence of the human CDR-grafted anti-GM_2 antibody H chain V region contained in pBSH10 are shown in SEQ ID NO:7. In the amino acid sequence of the thus-constructed human CDR-grafted anti-GM_2 antibody H chain V region, arginine in position 67, alanine in position 72, serine in position 84 and arginine in position 98 in the FR as selected based on a computer model for the V region are replaced by lysine, valine, histidine and threonine, respectively, that are found in the mouse antibody KM796 H chain V region. This is for the purpose of retaining the antigen-binding capacity of mouse antibody KM796.

(3) Modification of human CDR-grafted anti-GM_2 antibody L chain V region described in Paragraph 1 (2) of Example 2 of JP-A-6-205694

First, a DNA having the base sequence of SEQ ID NO:34 was synthesized using an automatic DNA synthesizer (Applied Biosystems model 380A), and a human CDR-grafted anti-GM_2 antibody L chain V region cDNA with a 3' terminus capable of pairing with the restriction enzyme *Spl*I was constructed by following the same reaction procedure as in Paragraph 1 (2) of Example 2 of JP-A-6-205694 using the synthetic DNA in lieu of the synthetic DNA of SEQ ID NO:35 of JP-A-6-205964.

Then, 3 μg of the plasmid pBSL3 obtained in Paragraph 1 (2) of Example 2 was added to 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride, 1 mM DTT and 100 μg/ml BSA, 10 units each of the restriction enzymes *Eco*RI (Takara Shuzo) and *Spl*I (Takara Shuzo) were further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, and about 1 μg of an *Eco*RI-*Spl*I fragment about 2.95 kb in size was recovered.

Then, 0.1 μg of the *Eco*RI-*Spl*I fragment of the human CDR-grafted anti-GM_2 antibody L chain V region obtained as mentioned above and 0.1 μg of the above *Eco*RI-*Spl*I fragment of pBSL3 were added to a total of 20 μl of sterilized water and ligated to each other using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech). The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101, and the plasmid pBSL16 shown in Fig. 32 was obtained.

Then, DNAs coding for certain versions of the human CDR-grafted anti-GM_2 antibody L chain V region contained in the above plasmid pBSL16 were constructed by replacing a certain number of amino acids in the FR with original mouse antibody amino acids by mutagenesis by means of PCR in the following manner (Fig. 33). Based on a computer model for the V region of mouse antibody KM796, those amino acid residues that were expected to contribute to restoration of antigen-binding activity as a result of replacement were selected as the amino acid residues to be replaced.

Antisense and sense DNA primers for introducing mutations were synthesized using an automatic DNA synthesizer (Applied Biosystems model 380A). A first PCR reaction was conducted in the same manner as in Paragraph 1 (2) of Example 3 using a final concentration each of 0.5 μM of M13 primer RV (Takara Shuzo) and the antisense DNA primer and of M13 primer M4 (Takara Shuzo) and the sense DNA primer, with 1 ng of pBSL16 as the template. Each reaction mixture was purified using QIAquick PCR Purification Kit (Qiagen) with elution with 20 μl of 10 mM Tris-hydrochloride (pH 8.0). Using 5 μl of each elute, a second PCR reaction was conducted in the same manner as in Paragraph 1 (2) of Example 3. The reaction mixture was purified using QIAaquick PCR Purification Kit (Qiagen) and then made into a solution in 30 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride, 1 mM DTT and 100 μg/ml BSA, 10 units each of the restriction enzymes *Eco*RI (Takara Shuzo) and *Spl*I (Takara Shuzo) were further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, and about 0.2 μg of an *Eco*RI-*Spl*I fragment (about 0.39 kb) of each replacement version of the human CDR-grafted anti-GM_2 antibody L chain V region was recovered.

Then, 0.1 μμg of the above *Eco*RI-*Spl*I fragment of each replacement version of the human CDR-grafted anti-GM_2 antibody L chain V region and 0.1 μg of the *Eco*RI-*Spl*I fragment of pBSL3 were added to a total of 20 μl of sterilized water and ligated to each other using Ready-To-Go T4 DNA ligase (Pharmacia Biotech). The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101, and a plasmid DNA was prepared from a transformant clone, and the base sequence of said plasmid was determined. In this way, plasmids respectively containing a base sequence having a desired mutation or mutations were obtained.

Thus, a plasmid, pBSLV1, containing version 1, shown in SEQ ID NO:37, of the human CDR-grafted anti-GM_2 antibody L chain V region was obtained following the above procedure using the synthetic DNA of SEQ ID NO:35 as the mutant antisense primer and the synthetic DNA of SEQ ID NO:36 as the mutant sense primer. In the amino acid sequence of the version 1 human CDR-grafted anti-GM_2 antibody L chain V region, the amino acid valine in position 15 in the FR is replaced by proline that is found in the mouse antibody KM796 L chain V region. This is for the purpose of retaining the antigen-binding capacity of mouse antibody KM796.

A plasmid, pBSLV2, containing version 2, shown in SEQ ID NO:40, of the human CDR-grafted anti-GM_2 antibody

L chain V region was obtained following the above procedure using the synthetic DNA of SEQ ID NO:38 as the mutant antisense primer and the synthetic DNA of SEQ ID NO:39 as the mutant sense primer. In the amino acid sequence of the version 2 human CDR-grafted anti-GM_2 antibody L chain V region, the amino acid leucine in positions 46 in the FR is replaced by tryptophan that is found in the mouse antibody KM796 L chain V region. This is for the purpose of retaining the antigen-binding capacity of mouse antibody KM796.

A plasmid, pBSLV3, containing version 3, shown in SEQ ID NO:43, of the human CDR-grafted anti-GM_2 antibody L chain V region was obtained following the above procedure using the synthetic DNA of SEQ ID NO:41 as the mutant antisense primer and the synthetic DNA of SEQ ID NO:42 as the mutant sense primer. In the amino acid sequence of the version 3 human CDR-grafted anti-GM_2 antibody L chain V region, proline in position 79 and isoleucine in position 82 in the FR are both replaced by alanine that is found in the mouse antibody KM796 L chain V region. This is for the purpose of retaining the antigen-binding capacity of mouse antibody KM796.

Then, a plasmid, pBSLV1+2, containing a human CDR-grafted anti-GM_2 antibody L chain V region having both the version 1 and version 2 replacements was constructed in the following manner.

Three μg of the plasmid pBSLV1 obtained as mentioned above was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 50 mM sodium chloride, 10 mM magnesium chloride and 1 mM DTT, 10 units each of the restriction enzymes *Eco*RI (Takara Shuzo) and *Hin*dIII (Takara Shuzo) were further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, and about 0.2 μg of an *Eco*RI-*Hin*dIII fragment about 0.20 kb in size was recovered.

Then, 3 μg of the plasmid pBSLV2 obtained as mentioned above was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 50 mM sodium chloride, 10 mM magnesium chloride and 1 mM DTT, 10 units each of the restriction enzymes *Eco*RI (Takara Shuzo) and *Hin*dIII (Takara Shuzo) were further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, and about 1 μg of an *Eco*RI-*Hin*dIII fragment about 3.2 kb in size was recovered.

Then, 0.1 μg of the *Eco*RI-*Hin*dIII fragment of pBSLV1 and 0.1 μg of the *Eco*RI-*Hin*dIII fragment of pBSLV2, each obtained as mentioned above, were added to a total of 20 μl of sterilized water and ligated to each other using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech). The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101, and the plasmid pBSLV1+2 shown in Fig. 34 was obtained.

Then, the PCR reaction procedure mentioned above was followed using 1 ng of the plasmid pBSLV1+2 obtained as mentioned above as the template, a synthetic DNA having the base sequence of SEQ ID NO:44 as the mutant antisense primer and a synthetic DNA having the base sequence of SEQ ID NO:45 as the mutant sense primer, whereby a plasmid, pBSLV4, containing a version 4 human CDR-grafted anti-GM_2 antibody L chain V region set forth in SEQ ID NO:46 was obtained. In the amino acid sequence of the version 4 human CDR-grafted anti-GM_2 antibody L chain V region, valine in position 15, leucine in position 46, aspartic acid in position 69, phenylalanine in position 70 and threonine in position 71 in the FR are replaced by proline, tryptophan, serine, tyrosine and serine, respectively, that are found in the mouse antibody KM796 L chain V region. This is for the purpose of retaining the antigen-binding capacity of mouse antibody KM796.

Then, the PCR reaction procedure mentioned above was followed using 1 ng of the plasmid pBSLV1+2 obtained as mentioned above as the template, a synthetic DNA having the base sequence of SEQ ID NO:47 as the mutant antisense primer and a synthetic DNA having the base sequence of SEQ ID NO:48 as the mutant sense primer, whereby a plasmid, pBSLV8, containing a version 8 human CDR-grafted anti-GM_2 antibody L chain V region set forth in SEQ ID NO:49 was obtained. In the amino acid sequence of the version 8 human CDR-grafted anti-GM_2 antibody L chain V region, valine in position 15, leucine in position 46, aspartic acid in position 69, phenylalanine in position 70, threonine in position 71, serine in position 76, leucine in position 77 and glutamine in position 78 in the FR are replaced by proline, tryptophan, serine, tyrosine, serine, arginine, methionine and glutamic acid, respectively, that are found in the mouse antibody KM796 L chain V region. This is for the purpose of retaining the antigen-binding capacity of mouse antibody KM796.

Then, the PCR reaction procedure mentioned above was followed using 1 ng of the plasmid pBSLV4 obtained as mentioned above as the template, a synthetic DNA having the base sequence of SEQ ID NO:50 as the mutant antisense primer and a synthetic DNA having the base sequence of SEQ ID NO:51 as the mutant sense primer, whereby a plasmid, pBSLm-2, containing a version Lm-2 human CDR-grafted anti-GM_2 antibody L chain V region set forth in SEQ ID NO:52 was obtained. In the amino acid sequence of the version Lm-2 human CDR-grafted anti-GM_2 antibody L chain V region, valine in position 15, tyrosine in position 35, leucine in position 46, aspartic acid in position 69, phenylalanine in position 70 and threonine in position 71 in the FR are replaced by proline, phenylalanine, tryptophan, serine, tyrosine and serine, respectively, that are found in the mouse antibody KM796 L chain V region. This is for the purpose of retaining the antigen-binding capacity of mouse antibody KM796.

Then, the PCR reaction procedure mentioned above was followed using 1 ng of the plasmid pBSLV4 obtained as mentioned above as the template, a synthetic DNA having the base sequence of SEQ ID NO:53 as the mutant antisense primer and a synthetic DNA having the base sequence of SEQ ID NO:54 as the mutant sense primer, whereby

a plasmid, pBSLm-8, containing a version Lm-8 human CDR-grafted anti-GM_2 antibody L chain V region set forth in SEQ ID NO:55 was obtained. In the amino acid sequence of the version Lm-8 human CDR-grafted anti-GM_2 antibody L chain V region, valine in position 15, leucine in position 46, aspartic acid in position 69, phenylalanine in position 70, threonine in position 71, phenylalanine in position 72 and serine in position 76 in the FR are replaced by proline, tryptophan, serine, tyrosine, serine, leucine and arginine, respectively, that are found in the mouse antibody KM796 L chain V region. This is for the purpose of retaining the antigen-binding capacity of mouse antibody KM796.

Then, a plasmid, pBSLm-28, containing a human CDR-grafted anti-GM_2 antibody L chain V region having both the version Lm-2 and version Lm-8 replacements was constructed in the following manner.

Three μg of the plasmid pBSLm-2 obtained as mentioned above was added to 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Eco*RI (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 50 mM sodium chloride, 10 mM magnesium chloride, 1 mM DTT and 100 μg/ml BSA, 10 units of the restriction enzyme *Xba*I (Takara Shuzo) was further added, and the reaction as allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, and about 0.2 μg of an *Eco*RI-*Xba*I fragment about 0.24 kb in size was recovered.

Then, 3 μg of the plasmid pBSLm-8 obtained as mentioned above was added to 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Eco*RI (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 50 mM sodium chloride, 10 mM magnesium chloride, 1 mM DTT and 100 μg/ml BSA, 10 units of the restriction enzyme *Xba*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, and about 1 μg of an *Eco*RI-*Xba*I fragment about 3.16 kb in size was recovered.

Then, 0.1 μg of the *Eco*RI-*Xba*I fragment of pBSLm-2 and 0.1 μg of the *Eco*RI-*Xba*I fragment of pBSLm-8, each obtained as mentioned above, were added to a total of 20 μl of sterilized water and ligated to each other using Ready-To-go T4 DNA Ligase (Pharmacia Biotech). The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101, and the plasmid pBSLm-28 shown in Fig. 35 was obtained. The version Lm-28 human CDR-grafted anti-GM_2 antibody L chain V region contained in the plasmid pBSLm-28 is shown in SEQ ID NO:8. In the amino acid sequence of the version Lm-28 human CDR-grafted anti-GM_2 antibody L chain V region thus constructed, valine in position 15, tyrosine in position 35, leucine in position 46, aspartic acid in position 69, phenylalanine in position 70, threonine in position 71, phenylalanine in position 72 and serine in position 76 are replaced by proline, phenylalanine, tryptophan, serine, tyrosine, serine, leucine and arginine, respectively, that are found in the mouse antibody KM796 L chain V region. This is for the intended purpose of retaining the antigen-binding capacity of mouse antibody KM796.

(4) Construction of human CDR-grafted anti-GM_2 antibody L chain V region using known HMHCS of human antibody L chain V region

According to Kabat *et al.* (Kabat E. A. *et al.*, "*Sequences of Proteins of Immunological Interest*", US Dept. of Health and Human Services, 1991), known human antibody L chain V regions are classifiable into subgroups I to IV based on the homology of their FR regions, and common sequences have been identified for respective subgroups. The present inventors identified HMHCS as one meaning from the common sequences, a human CDR-grafted anti-GM_2 antibody L chain V region was constructed based on the HMHCs. First, for selecting common sequences to serve as the base, the homology was exmined between the FR of the mouse antibody KM796 L chain V region and the FR of the HMHCS of the human antibody L chain V region of each subgroup (Table 2).

TABLE 2

Homology (%) between mouse antibody KM796 L chain V region FR and human antibody L chain V region common sequence FR			
HSG I	HSG II	HSG III	HSG IV
70.0	65.0	68.8	67.5

As a result, it was confirmed that subgroup I shows the greatest similarity. Thus based on the common sequence of subgroup I, a human CDR-grafted anti-GM_2 antibody L chain V region was constructed by the PCR method in the

following manner.

Synthetic DNAs respectively having the base sequences of SEQ ID NO:56 through SEQ ID NO:61 were synthesized using an automatic DNA synthesizer (Applied Systems model 380A). The DNAs synthesized were added, each to a final concentration of 0.1 μM, to 50 μl of 10 mM Tris-hydrochloride buffer (pH 8.3) containing 50 mM potassium chloride, 1.5 mM magnesium chloride, 0.001% gelatin, 200 μM dNTP, 0.5 μM M13 primer RV (Takara Shuzo), 0.5 μM M13 primer M4 (Takara Shuzo) and 2 units of TaKaRa Taq DNA polymerase. The mixture was covered with 50 μl of mineral oil, a DNA thermal cycler (Perkin Elmer model PJ480) was loaded with the mixture, and 30 PCR cycles (2 minutes at 94°C, 2 minutes at 55°C and 2 minutes at 72°C per cycle) were conducted. The reaction mixture was purified using QIAquick PCR Purification Kit (Qiagen) and then made into a solution in 30 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride, 1 mM DTT and 100 μg/ml BSA, 10 units each of the restriction enzymes *Eco*RI (Takara Shuzo) and *Spl*I (Takara Shuzo) were further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, and about 0.2 μg of an *Eco*RI-*Spl*I fragment about 0.39 kb in size was recovered.

Then, 0.1 μg of the above *Eco*RI-*Spl*I fragment of the human CDR-grafted anti-GM_2 antibody L chain V region and 0.1 μg of the *Eco*RI-*Spl*I fragment of pBSL3 were added to a total of 20 μl of sterilized water and ligated to each other using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech). The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101. Plasmid DNAs were prepared from 10 transformant clones and their base sequences were determined. As a result, a plasmid, pBSHSGL, shown in Fig. 36 and having the desired base sequence was obtained. The amino acid sequence and base sequence of the human CDR-grafted anti-GM_2 antibody L chain V region contained in pBSHSGL are shown in SEQ ID NO:9. In the amino acid sequence of the thus-constructed human CDR-grafted anti-GM_2 antibody L chain V region, methionine in position 4, leucine in position 11, valine in position 15, tyrosine in position 35, alanine in position 42, leucine in position 46, aspartic acid in position 69, phenylalanine in position 70, threonine in position 71, leucine in position 77 and valine in position 103 in the FR as selected based on a computer model for the V region are replaced by leucine, methionine, proline, phenylalanine, serine, tryptophan, serine, tyrosine, serine, methionine and leucine, respectively, that are found in the mouse antibody KM796 L chain V region. This is for the intended purpose of retaining the antigen-binding capacity of mouse antibody KM796.

2. Activity evaluation of replacement versions of human CDR-grafted anti-GM_2 antibody in terms of transient expression

Various replacement version human CDR-grafted anti-GM_2 antibodies composed of the human CDR-grafted anti-GM_2 antibody H chain and L chain V regions constructed in Paragraphs 3 (1) through (4) of Example 3 and having various replacements were evaluated for activity in terms of transient expression in the following manner.

First, for evaluating the human CDR-grafted anti-GM_2 antibody H chain V regions having various replacements, expression vectors, pT796HCDRHV2, pT796HCDRHV4 and pT796HCDRH10, were constructed by replacing the mouse H chain V region of the mouse-human chimeric anti-GM_2 antibody transient expression vector pT796 obtained in Paragraph 1 (1) of Example 2 of JP-A-6-205694 with the human CDR-grafted anti-GM_2 antibody H chain V regions having various replacements, in the following manner. For comparison, an expression vector, pT796HCDR was constructed by replacing the mouse H chain V region of pT796 with the human CDR-grafted anti-GM_2 antibody H chain V region obtained in Paragraph 1 (1) of Example 2.

Three μg of the plasmid pT796 was added to 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride, 1mM DTT and 100 μg/ml BSA, 10 units each of the restriction enzymes *Eco*RI (Takara Shuzo) and *Spl*I (Takara Shuzo) were further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, and about 1 μg of an *Eco*RI-*Spl*I fragment about 9.20 kb in size was recovered. Then, 3 μg of the plasmid pBSL16 obtained in Paragraph 1 (3) of Example 3 was added to 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride, 1 mM DTT and 100 μg/ml BSA, 10 units each of the restriction enzymes *Eco*RI (Takara Shuzo) and *Spl*I (Takara Shuzo) were further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, and about 0.3 μg of an *Eco*RI-*Spl*I fragment about 0.39 kb in size was recovered.

Then, 0.1 μg of the *Eco*RI-*Spl*I fragment of pT796 and 0.1 μg of the *Eco*RI-*Spl*I fragment of pBSL16, each obtained as mentioned above, were added to a total of 20 μl of sterilized water and ligated to each other using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech). The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101, and the plasmid pT796LCDR shown in Fig. 37 was obtained.

Then, 3 μg of the above plasmid pT796LCDR was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Apa*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was added to 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride, 1 mM DTT, 100 μg/ml BSA and 0.01% Triton X-100, 10 units of the restriction

enzyme *Not*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, and about 1 μg of an *Apa*I-*Not*I fragment about 9.11 kb in size was recovered.

Then, 0.1 μg of the human CDR-grafted anti-GM_2 antibody H chain V region obtained in Paragraph 1 (1) of Example 2 of JP-A-6-205694 or the replacement version 2 or 4 human CDR-grafted anti-GM_2 antibody H chain V region obtained in Paragraph 1 (1) of Example 3 and 0.1 μg of the *Apa*I-*Not*I fragment of pT796LCDR were added to a total of 20 μl of sterilized water and ligated to each other using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech). Each recombinant plasmid DNA solution thus obtained was used to transform *Escherichia coli* HB101. The plasmids pT796HLCDR, pT796HLCDRHV2 and pT796HLCDRHV4 shown in Fig. 38 were obtained.

Then, 3 μg of the plasmid pBSH10 obtained in Paragraph 1 (2) of Example 3 was added to 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Apa*I (Takara Shuzo) was further added, and the restriction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was added to 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride, 1 mM DTT, 100 μg/ml BSA and 0.01% Triton X-100, 10 units of the restriction enzyme *Not*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, and about 0.3 μg of an *Apa*I-*Not*I fragment about 0.44 kb in size was recovered.

Then, 0.1 μg of the *Apa*I-*Not*I fragment of pBSM10 and 0.1 μg of the *Apa*I-*Not*I fragment of pT796LCDR were added to a total of 20 μl of sterilized water and ligated to each other using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech). The thus-obtained recombinant plasmid DNA solution was used to transform *Escherichia coli* HB101, and the plasmid pT796HLCDRH10 shown in Fig. 39 was obtained.

Then, 3 μg each of the plasmids pT796HLCDR, pT796HLCDRHV2, pT796HLCDRHV4 and pT796HLCDRH10 were respectively added to 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride, 1 mM DTT and 100 μg/ml BSA, 10 units each of the restriction enzymes *Eco*RI (Takara Shuzo) and *Sph*I (Takara Shuzo) were further added, and the reaction was allowed to proceed at 37°C for 1 hour. Each reaction mixture was fractionated by agarose gel electrophoresis, and about 1 μg of an *Eco*RI-*Sph*I fragment about 9.15 kb in size was recovered.

Then, 5 μg of the plasmid pBSL3 obtained in Paragraph 1 (2) of Example 2 was added to 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride, 1 mM DTT and 100 μg/ml BSA, 10 units each of the restriction enzymes *Eco*RI (Takara Shuzo) and *Sph*I (Takara Shuzo) were further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, and about 0.4 μg of an *Eco*RI-*Sph*I fragment about 0.39 kb in size was recovered.

Then, 0.1 μg of the *Eco*RI-*Sph*I fragment of each of pT796HLCDR, pT796HLCDRHV2, pT796HLCDRHV4 and pT796HLCDRH10 and 0.1 μg of the *Eco*RI-*Sph*I fragment of pBSL3 were added to a total of 20 μl of sterilized water and ligated to each other using Ready-To-Go DNA Ligase (Pharmacia Biotech). Each recombinant plasmid DNA solution thus obtained was used to transform *Escherichia coli* HB101. In this way, the plasmids pT796HCDR, pT796HCDRHV2, pT796HCDRHV4 and pT796HCDRH10 shown in Fig. 40 were obtained.

Then, 2 μg each of the plasmids pT796HCDR, pT796HCDRHV2, pT796HCDRHV4 and pT796HCDRH10 thus obtained were used for transient human CDR-grafted anti-GM_2 antibody expression and for culture supernatant human CDR-grafted anti-GM_2 antibody activity evaluation by the procedures described in Paragraphs 1 (5), 2 (2) and (3) of Example 2. After introduction of each plasmid, the culture supernatant was recovered at 72 hours, and the GM_2-binding activity and antibody concentration in the culture supernatant were determined by ELISA and the relative activity was calculated with the activity of the positive control chimera antibody taken as 100%. The results are shown in Fig. 41.

The results revealed that the amino acid residue replacements alone in replacement versions 2 and 4 have little influence on the restoration of the antigen-binding activity of the human CDR-grafted anti-GM_2 antibody but that the use of the pBSH10-derived human CDR-grafted antibody H chain V region constructed based on the known HMHCS of the human antiobdy H chain V region, contributes to the restoration of the antigen-binding activity.

In view of the above results , the human CDR-grafted anti-GM_2 antibody H chain V region constructed based on the known HMHCS of the human antibody H chain V region as shown in SEQ ID NO:7 was selected as a novel human CDR-grafted anti-GM_2 antibody H chain V region.

Then, for evaluating the human CDR-grafted anti-GM_2 antibody L chain V regions having various replacements, expression vectors, pT796HLCDRLV1, pT796HLCDRLV2, pT796HLCDRLV3, pT796HLCDRLV4, pT796HLCDRLV8, pT796HLCDRLm-2, pT796HLCDRLm-8, pT796HLCDRLm-28, and pT796HLCDRHSGL, were constructed in the following manner by replacing the mouse L chain V region of the vector pT796HCDRH10 for transient human CDR-grafted anti-GM_2 antibody expression obtained as mentioned above with the human CDR-grafted anti-GM_2 antibody L chain V regions having various replacements.

Thus, 3 μg of the plasmid pT796HCDRH10 was added to 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride, 1 mM DTT and 100 μg/ml BSA, 10 units each of the restric-

tion enzymes *Eco*RI (Takara Shuzo) and *Spl*I (Takara Shuzo) were further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, and about 1 μg of an *Eco*RI-*Spl*I fragment about 9.15 kb in size was recovered.

Then, 3 μg of the plasmid pBSLV1, pBSLV2, pBSLV3, pBSLV4, pBSLV8, pBSLm-2, pBSLm-8, pBSLm-28 or pBSHSGL obtained in Paragraph 1 (3) or (4) of Example 3 was added to 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride, 1 mM DTT and 100 μg/ml BSA, 10 units each of the restriction enzymes *Eco*RI (Takara Shuzo) and *Spl*I (Takara Shuzo) were further added, and the reaction was allowed to proceed at 37°C for 1 hour. Each reaction mixture was fractionated by agarose gel electrophoresis, and about 0.3 μg of an *Eco*RI-*Spl*I fragment about 0.39 kb in size was recovered.

Then, 0.1 μg of the *Eco*RI-*Spl*I fragment of the pT796HCDRH10, and 0.1 μg of the *Eco*RI-*Spl*I fragment of each replacement version human CDR-grafted anti-GM_2 antibody L chain V region were added to a total of 20 μl of sterilized water and ligated to each other using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech). Each recombinant plasmid DNA solution thus obtained was used to transform *Escherichia coli* HB101. In this way, the plasmids pT796HLCDRLV1, pT796HLCDRLV2, pT796HLCDRLV3, pT796HLCDRLV4, pT796HLCDRLV8, pT796HLCDRLm-2, pT796HLCDRLm-8, pT796HLCDRLm-28 and pT796HLCDRHSGL were obtained as shown in Fig. 42.

Then, 2 μg each of the thus-obtained plasmids pT796HLCDRLV1, pT796HLCDRLV2, pT796HLCDRLV3, pT796HLCDRLV4, pT796HLCDRLV8, pT796HLCDRLm-2, pT796HLCDRLm-8, pT796HLCDRLm-28 and pT796HLCDRHSGL and of the plasmid pT796HLCDR described in Example 2 of JP-A-6-205694 and capable of expressing human CDR-grafted anti-GM_2 antibody were used for transient human CDR-grafted anti-GM_2 antibody expression and for culture supernatant human CDR-grafted anti-GM_2 antibody activity evaluation by the procedures described in Paragraphs 1 (5) and 2 (2) and (3) of Example 2. After introduction of each plasmid, the culture supernatant was recovered at 72 hours, and the GM_2-binding activity and antibody concentration in the culture supernatant were determined by ELISA and the relative activity was calculated with the activity of the positive control chimera antibody taken as 100%. The results are shown in Fig. 43.

The results revealed that the amino acid residue replacements alone in replacement versions 1, 2, 3, 4 and 8 have little influence on the restoration of the antigen-binding activity of the human CDR-grafted anti-GM_2 antibody but that the amino acid residue replacements in replacement versions Lm-2 and Lm-8 contributes to the restoration of the antigen-binding activity. Furthermore, version Lm-28 having both the amino acid residue replacements of Lm-2 and Lm-8 showed a high level of antigen-biding activity almost comparable to that of the chimera antibody, revealing that those amino acid residues replaced in producing Lm-28 were very important from the antigen-binding activity viewpoint.

In view of the above results, the version Lm-28 human CDR-grafted anti-GM_2 antibody L chain V region shown in SEQ ID NO:8 was selected as a first novel human CDR-grafted anti-GM_2 antibody L chain V region.

It was further revealed that the antigen-binding activity can be restored when the pBSHSGL-derived human CDR-grafted anti-GM_2 antibody L chain V region, namely the human CDR-grafted anti-GM_2 antibody L chain V region constructed based on the known HMHCS of the human antibody L chain V region, is used.

In view of the above result, the human CDR-grafted anti-GM_2 antibody L chain V region constructed based on the known HMHCS of the human antibody L chain V region as set forth in SEQ ID NO:9 was selected as a second novel human CDR-grafted anti-GM_2 antibody an L chain V region.

It is to be noted that in those human CDR-grafted anti-GM_2 antibody L chain V regions that showed high binding activity against GM_2, certain amino acid residues which cannot be specified by deduction fro known human CDR-grafted antibody production examples have been replaced by amino acid residues found in the mouse L chain V region. Thus, obviously, it was very important, in human CDR-grafted anti-GM_2 antibody production, to identify these amino acid residues.

Furthermore, the fact that the human CDR-grafted anti-GM_2 antibodies having those human CDR-grafted anti-GM_2 antibody H chain and L chain V regions based on the known HMHCS of the human antibody V region showed high antigen binding activity is proof of the usefulness of the present process in human CDR-grafted antibody production.

3. Acquisition of cell lines for stable production of human CDR-grafted anti-GM_2 antibodies

Based on the results of Paragraph 2 (5) of Example 3, two cell lines, KM8966 and KM8967, capable of stably expressing KM8966, which has the amino acid sequence set forth in SEQ ID NO:7 as the H chain V region and the amino acid sequence set forth in SEQ ID NO:8 as the L chain V region, and KM8967, which has the amino acid sequence set forth in SEQ ID NO:7 as the H chain V region and the amino acid sequence set forth in SEQ ID NO:9 as the L chain V region, respectively as human CDR-grafted anti-GM_2 antibodies having higher antigen-binding activity than the human CDR-grafted anti-GM_2 antibody described in Example 2 of JP-A-6-205694 were obtained in the following manner.

Three μg each of the plasmids pT796HLCDRLm-28 and pT796HLCDRHSGL obtained in Paragraph 2 (5) of Example 3 were respectively added to 10 μl of 20 mM Tris-hydrochloride buffer (pH 8.5) containing 100 mM potassium chlo-

ride, 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Bam*HI (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. Each reaction mixture was subjected to ethanol precipitation, the precipitate was added to 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Xho*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. Each reaction mixture was fractionated by agarose gel electrophoresis, and about 1 μg of a *Bam*HI-*Xho*I fragment about 4.93 kb in size was recovered.

Then, 3 μg of the plasmid pKANTEX93 obtained in Example 1 was added to 10 μl of 20 mM Tris-hydrochloride buffer (pH 8.5) containing 100 mM potassium chloride, 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Bam*HI (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was subjected to ethanol precipitation, the precipitate was added to 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride and 1 mM DTT, 10 units of the restriction enzyme *Xho*I (Takara Shuzo) was further added, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis, and about 1 μg of a *Bam*HI-*Xho*I fragment about 8.68 kb in size was recovered.

Then, 0.1 μg of the *Bam*HI-*Xho*I fragment of pT796HLCDRLm-28 or pT796HLCDRHSGL and 0.1 μg of the *Bam*HI-*Xho*I fragment of pKANTEX93, each obtained as mentioned above, were added to a total of 20 μl of sterilized water and ligated to each other using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech). Each recombinant plasmid DNA solution thus obtained was used to transform *Escherichia coli* HB101. In this way, the plasmids pKANTEX796HLCDRLm-28 and pKANTEX796HLCDRHSGL shown in Fig. 44 were obtained.

Then, 4 μg each of the above plasmids pKANTEX796HLCDRLm-28 and pKANTEX796HLCDRHSGL were respectively used to transform YB2/0 (ATCC CRL 1581) cells according to the procedure described in Paragraph 1 (4) of Example 2 and, after final selection using G418 (0.5 mg/ml) and MTX (200 nM), a transformant cell line, KM8966, capable of producing about 40 μg/ml of KM8966, i.e. the pKANTEX796HLCDRLm-28-derived human CDR-grafted anti-GM_2 antibody, and a transformant cell line, KM8967, capable of producing about 30 μg/ml of KM8967, i.e. the pKANTEX796HLCDRHSGL-derived human CDR-grafted anti-GM_2 antibody, were obtained.

The transformants KM8966 and KM8967 have been deposited with National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (Higashi 1-1-3, Tsukuba, Ibaraki, Japan; hereinafter the address is the same as this) on May 23, 1995 under the deposit numbers FERM BP-5105, and FERM BP-5106, respectively.

4. Purification of human CDR-grafted anti-GM_2 antibodies KM8966 and KM8967

The transformant cell lines KM8966 and 8967 obtained in Paragraph 3 of Example 3 were respectively suspended in GIT medium (Nippon Pharmaceutical) containing 0.5 mg/ml G418 and 200 nM MTX and, according to the procedure of Paragraph 11 of Example 1 of JP-A-6-205694, 18 mg of purified human CDR-grafted anti-GM_2 antibody KM8966 and 12 mg of purified KM8967 were obtained each from about 0.5 liter of culture fluid. Three μg each of the purified human CDR-grafted anti-GM_2 antibodies obtained and the mouse-human chimeric anti-GM_2 antibody KM966 were subjected to electrophoresis by the known method [Laemli, U.K., *Nature*, 227, 680 (1979)] for molecular weight determination. The results are shown in Fig. 45. As shown in Fig. 45, under reducing conditions, both antibody H chains showed a molecular weight of about 50 kilodaltons and both antibody L chains showed a molecular weight of about 25 kilodaltons. Expression of H and L chains of correct molecular weights was thus confirmed. Under nonreducing conditions, both human CDR-grafted anti-GM_2 antibodies showed a molecular weight of about 150 kilodaltons and it was thus confirmed that antibodies each composed of two H chains and two L chains and having a correct size had been expressed. Furthermore, the H and L chains of each human CDR-grafted anti-GM_2 antibody were analyzed for N-terminal amino acid sequence by automatic Edman degradation using a protein sequencer (Applied Biosystems model 470A), whereby an amino acid sequence deducible from the base sequence of the V region DNA constructed was revealed.

5. *In vitro* reactivity of human CDR-grafted anti-GM_2 antibodies KM8966 and KM8967 against GM_2

The mouse-human chimeric anti-GM_2 antibody KM966 and the purified human CDR-grafted anti-GM_2 antibodies KM8966 and KM8967 were tested for reactivity against GM_2 by ELISA as described in Paragraph 1 (5) of Example 2. The results are shown in Fig. 46. GM_2 (N-acetyl-GM_2) used was purified from cultured cell line HPB-ALL [Oboshi *et al.*, *Tanpakushitsu, Kakusan & Koso* (*Protein, Nucleic Acid & Enzyme*), 23, 697 (1978)] in accordance with the known method [*J. Biol. Chem.*, 263, 10915 (1988)]. As shown, it was found that the purified human CDR-grafted anti-GM_2 antibody KM8966 exerted the binding activity comparable to that of the mouse-human chimeric anti-GM_2 antibody KM966. On the other hand, the binding activity of purified human CDR-grafted anti-GM_2 antibody KM8967 was about 1/4 to 1/5 of that of the mouse-human chimeric anti-GM_2 antibody KM966.

6. Reaction specificity of human CDR-grafted anti-GM_2 antibodies KM8966 and KM8967

The mouse-human chimeric anti-GM_2 antibody KM966 and the human CDR-grafted anti-GM_2 antibodies KM8966 and KM8967 were tested for reactivity against the gangliosides GM_1, N-acetyl-GM_2, N-glycolyl-GM_2, N-acetyl-GM_3, N-glycolyl-GM_3, GD_{1a}, GD_{1b} (Iatron), GD_2, GD_3 (Iatron) and GQ_{1b} (Iatron) by ELISA as described in Paragraph 1 (5) of Example 2. The results are shown in Fig. 47. GM_1 and GD_{1a} were purified from bovine brain, N-acetyl-GM_2 from cultured cell line HPB-ALL [Oboshi *et al.*, *Tanpakushitsu, Kakusan & Koso (Protein, Nucleic acid & Enzyme)*, 23, 697 (1978)], N-glycolyl-GM_2 and N-glycolyl-GM_3 from mouse liver, N-acetyl-GM_3 canine erythrocytes, and GD_2 from cultured cell line IMR32 (ATCC CCL127), respectively by the *per se* known method [*J. Biol. Chem.*, 263, 10915 (1988)]. Each antibody was used in a concentration of 10 μg/ml.

As shown in Fig. 47, it was confirmed that the human CDR-grafted anti-GM_2 antibodies KM8966 and KM8967 react specifically with GM_2 (N-acetyl-GM_2 and N-glycolyl-GM_2) like the mouse-human chimeric anti-GM_2 antibody KM966.

7. Reactivity of human CDR-grafted anti-GM_2 antibodies KM8966 and KM8967 against cancer cells

The human lung small cell carcinoma culture cell line SBC-3 (JCRB 0818) (1×10^6 cells) was suspended in PBS, the suspension was placed in a microtube (TREF) and centrifuged (1200 rpm, 2 minutes). To the thus-washed cells was added 50 μl (50 μg/ml) of the mouse-human chimeric anti-GM_2 antibody KM966 or the purified human CDR-grafted anti-GM_2 antibody KM8966 or KM8967, followed by stirring and 1 hour of standing at 4°C. After the above reaction step, the cells were washed three times with PBS, each time followed by centrifugation. Then, 20 μl of fluorescein isocyanate-labeled protein A (30-fold dilution, Boehringer Mannheim) was added and, after stirring, the reaction was allowed to proceed at 4°C for 1 hour. Thereafter, the cells were washed three times with PBS, each time followed by centrifugation, then further suspended in PBS and subjected to analysis using a flow cytometer, EPICS Elite (Coulter). In a control run, the above procedure was followed without addition of the human CDR-grafted anti-GM_2 antibody and analyzed. The results are shown in Fig. 48. It was found that the purified human CDR-grafted anti-GM_2 antibodies KM8966 and KM8967 strongly reacted with the human lung small cell carcinoma culture cell line SBC-3 like the mouse-human chimeric anti-GM_2 antibody KM966.

8. *In vitro* antitumor activity of human CDR-grafted anti-GM_2 antibodies KM8966 and KM8967: CDC activity

(1) Preparation of target cells

The target cells SBC-3, cultured in RPMI1640-FCS (10) medium supplemented with 10% FCS, here adjusted to a cell concentration of 5×10^5 cells/500 μl, 3.7 MBq of $Na_2{}^{51}CrO_4$ (Daiichi Pure Chemicals Co., Ltd.) was added thereto. Then, the reaction was allowed to proceed at 37°C for 1 hour, and the cells were washed three times with the medium. The cells were then allowed to stand in the medium at 4°C for 30 minutes and, after centrifugation, the medium was added to adjust the cell concentration to 1×10^6 cells/ml.

(2) Preparation of the complement

Sera from healthy subjects were combined and used as a complement source.

(3) CDC activity measurement

The mouse-human chimeric anti-GM_2 antibody KM966 or purified human CDR-grafted anti-GM_2 antibody KM8966 or KM8967 was added to wells of 96-well U-bottom plates within the final concentration range of 0.05 to 50 μg/ml and then 50 μl (5×10^4 cells/well) of the target cells prepared in (1) were added to each well. The reaction was allowed to proceed at room temperature for 1 hour. After centrifugation, the supernatants were discarded, the human complement obtained in (2) was added to each well to give a final concentration of 15% v/v, and the reaction was allowed to proceed at 37°C for 1 hour. After centrifugation, the amount of ^{51}Cr in each supernatant was determined using a gamma counter. The amount of spontaneously dissociated ^{51}Cr was determined by adding to the target cells the medium alone in stead of the antibody and complement solutions and measuring the amount of ^{51}Cr in the supernatant in the same manner as mentioned above. The total amount of dissociated ^{51}Cr was determined by adding to the target cells 1 N hydrochloric acid in stead of the antibody and complement solutions and measuring the amount of ^{51}Cr in the supernatant in the same manner as mentioned above. The CDC activity was calculated as follows:

$$\text{CDC activity (\%)} = \frac{\text{Amount of } {}^{51}\text{Cr in sample supernatant - Amount of } {}^{51}\text{Cr spontaneously dissociated}}{\text{Total amount of } {}^{51}\text{Cr dissociated - Amount of } {}^{51}\text{Cr spontaneously dissociated}} \times 100$$

The results thus obtained are shown in Fig. 49. It was shown that CDC activity of the human CDR-grafted anti-GM_2 antibodies KM8966 and KM8967 was lower than that of the mouse-human chimeric anti-GM_2 antibody KM966.

9. *In vitro* antitumor activity of human CDR-grafted anti-GM_2 antibodies KM8966 and KM8967: ADCC activity

(1) Preparation of target cells

The target cells SBC-3 cultured in RPMI1640-FCS (10) medium supplemented with 10% FCS were adjusted to a cell concentration of 1×10^6 cells/500 µl, 3.7 MBq of $Na_2{}^{51}CrO_4$ (Daiichi Pure Chemicals Co., Ltd.) was added thereto. Then, the reaction was allowed to proceed at 37°C for 1 hour and the cells were washed three times with the medium. The cells were then allowed to stand in the medium at 4°C for 30 minutes and then, after centrifugation, the medium was added to adjust the cell concentration to 2×10^5 cells/ml.

(2) Preparation of effector cells

Human venous blood (50 ml) was collected, 0.5 ml of heparin sodium (Takeda Chemical Industries; 1,000 units/ml) was added, and the mixture was gently stirred. This mixture was overlaid on Polymorphprep (Nycomed) and centrifuged to separate the lymphocyte layer (PBMC). The resulting lymphocytes were washed three times by centrifugation with RPMI1640 medium supplemented with 10% FCS, and the cells were suspended in the medium (5×10^5 cells/ml) for use as effector cells.

(3) ADCC activity measurement

The mouse-human chimeric anti-GM_2 antibody KM966 or purified human CDR-grafted anti-GM_2 antibodies KM8966 or KM8967 was added to wells of 96-well U-bottom plates within the final concentration range of 0.05 to 50 µg/ml and then 50 µl (1×10^4 cells/well) of the target cell suspension prepared in (1) and 100 µl (5×10^5 cells/well) of the effector cell suspension prepared in (2) were added to each well. The reaction was allows to proceed at 37°C for 4 hours and, after centrifugation, the amount of ^{51}Cr in each supernatant was measured using a gamma counter. The amount of spontaneously dissociated ^{51}Cr was determined by adding to the target cells the medium alone in lieu of the antibody and effector cells and measuring the amount of ^{51}Cr in the supernatant in the same manner as mentioned above. The total amount of dissociated ^{51}Cr was determined by adding to the target cells 1 N hydrochloric acid in lieu of the antibody and effector cells and measuring the amount of ^{51}Cr in the supernatant in the same manner as mentioned above. The ADCC activity was calculated as follows:

$$\text{ADCC activity (\%)} = \frac{\text{Amount of } {}^{51}\text{Cr in sample supernatant - Amount of } {}^{51}\text{Cr spontaneously dissociated}}{\text{Total amount of } {}^{51}\text{Cr dissociated - Amount of } {}^{51}\text{Cr spontaneously dissociated}} \times 100$$

The results thus obtained are shown in Fig. 50. The human CDR-grafted anti-GM_2 antibody KM8966 showed ADCC activity comparable to that of the mouse-human chimeric anti-GM_2 antibody KM966, whereas the human CDR-grafted anti-GM_2 antibody KM8967 showed ADCC activity slightly lower than that of the mouse-human chimeric anti-GM_2 antibody KM966.

EXAMPLE 4

Production of human CDR-grafted anti-GM_2 antibodies II

The human CDR-grafted anti-GM_2 antibodies KM8966 and KM8967 showed antigen binding activity (ELISA), binding specificity and ADCC activity comparable to those of the mouse-human chimeric anti-GM_2 antibody KM966, while its CDC activity was lower than that of the chimeric antibody. In order to improve the CDC activity, human CDR-grafted anti-GM_2 antibodies were produced in the following manner.

1. Modification of human CDR-grafted anti-GM_2 antibody KM8966 H chain V region

Among the human CDR-grafted anti-GM_2 antibodies prepared in Example 3, the antibody KM8966 showing higher CDC activity was subjected to amino acid residue replacements at the H chain V region (SEQ ID NO:7) in order to improve CDC activity. The amino acid residues to be replaced were selected at random with reference to the results of various replacement obtained in Example 3 and a computer model for the V region of mouse antibody KM796. Replacements were introduced by PCR method using as a template 1 ng of the plasmid pBSH10 containing the human CDR-grafted anti-GM_2 antibody H chain V region obtained in Paragraph 1 (2) of Example 3 and using as a primer antisense and sense synthetic DNA containing mutations described in Paragraph 1 (3) of Example 3.

The reaction was carried out in the same manner as described in Paragraph 1 (3) of Example 3 using the synthetic DNA of SEQ ID NO:62 as the mutant antisense primer and the synthetic DNA of SEQ ID NO:63 as the mutant sense primer to obtain the plasmid pBSHM1 containing version HM1, shown in SEQ ID NO:64, of the human CDR-grafted anti-GM_2 antibody H chain V region. In the amino acid sequence of the version HM1, arginine in position 38, alanine in position 40, glutamine in position 43 and glycine in position 44 in the FR shown in SEQ ID NO:7 were replaced by lysine, serine, lysine and serine, respectively, that are found in the mouse antibody KM796 H chain V region.

The plasmid pBSHM2 containing version HM2, shown in SEQ ID NO:10, of the human CDR-grafted anti-GM_2 antibody H chain V region was obtained following the reaction described in Paragraph 1 (3) of Example 3 using the synthetic DNA of SEQ ID NO:65 as the mutant antisense primer and the synthetic DNA of SEQ ID NO:66 as the mutant sense primer. In the amino acid sequence of the version HM2, arginine in position 38 and alanine in position 40 in the FR shown in SEQ ID NO:7 were replaced by lysine and serine, respectively, that are found in the mouse antibody KM796 H chain V region.

The plasmid pBSHM3 containing version BM3, shown in SEQ ID NO:69, of the human CDR-grafted anti-GM_2 antibody H chain V region was obtained following the reaction described in Paragraph 1 (3) of Example 3 using the synthetic DNA of SEQ ID NO:67 as the mutant antisense primer and the synthetic DNA of SEQ ID NO:68 as the mutant sense primer. In the amino acid sequence of the version HM3, valine in position 68 and isoleucine in position 70 in the FR shown in SEQ ID NO:7 were replaced by alanine and leucine, respectively, that are found in the mouse antibody KM796 H chain V region.

The plasmid pBSHM31 containing version HM31, shown in SEQ ID NO:70, of the human CDR-grafted anti-GM_2 antibody H chain V region was obtained following the reaction described in Paragraph 1 (3) of Example 3 using 1 ng of the plasmid pBSHM3 as the template, the synthetic DNA of SEQ ID NO:62 as the mutant antisense primer and the synthetic DNA of SEQ ID NO:63 as the mutant sense primer. In the amino acid sequence of the version HM31, arginine in position 38, alanine in position 40, glutamine in position 43 and glycine in position 44 in the FR of the version HM3 were replaced by lysine, serine, lysine and serine, respectively, that are found in the mouse antibody KM796 H chain V region.

Further, the plasmid pBSHM32 containing version HM32, shown in SEQ ID NO:71, of the human CDR-grafted anti-GM_2 antibody H chain V region was obtained following the reaction described in Paragraph 1 (3) of Example 3 using 1 ng of the plasmid pBSHM3 as the template, the synthetic DNA of SEQ ID NO:65 as the mutant antisense primer and the synthetic DNA of SEQ ID NO:66 as the mutant sense primer. In the amino acid sequence of the version HM32, arginine in position 38 and alanine in position 40 in the FR of the version HM3 were replaced by lysine and serine, respectively, that are found in the mouse antibody KM796 H chain V region.

2. Evaluation of CDC activity of human CDR-grafted anti-GM_2 antibodies having various replacements in the human CDR-grafted anti-GM_2 antibody H chain V region

(1) Construction of expression vectors

Expression vectors for various human CDR-grafted anti-GM_2 antibodies containing the H chain V region of human CDR-grafted anti-GM_2 antibodies having various replacements obtained in Paragraph 1 of Example 4 and the L chain V region of KM8966 (SEQ ID NO:8) were prepared in the following manner.

Three μg each of the plasmids pBSHM1, pBSHM2, pBSHM3, pBSHM31 and pBSHM32 obtained in Paragraph 1 of Example 4 were dissolved in 10 μl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride and 1 mM DTT, 10 units of *Apa*I (Takara Shuzo) were added thereto and the mixture was allowed to react at 37°C for 1 hour. The resulting mixture was subjected to ethanol precipitation and the thus-obtained precipitate was dissolved in 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride, 1 mM DTT, 100 μg/ml BSA and 0.01% of Triton X-100. Ten units of *Not*I (Takara Shuzo) were further added thereto to allow the mixture to react at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis to recover about 0.2 μg of the ApaI-NotI fragment of about 0.44 kb.

Then, 3 μg of the plasmid pKANTEX796HLCDRLm-28 obtained in Paragraph 3 (3) of Example 3 was dissolved in

10 µl of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM magnesium chloride and 1 mM DTT, 10 units of *Apa*I (Takara Shuzo) were added thereto and the mixture was allowed to react at 37°C for 1 hour. The resulting mixture was subjected to ethanol precipitation and the thus-obtained precipitate was dissolved in 10 µl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride, 1 mM DTT, 100 µg/ml BSA and 0.01% of Triton X-100. 10 units of *Not*I (Takara Shuzo) were added thereto to allow the mixture to react at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis to recover about 1 µg of the *Apa*I-*Not*I fragment of about 13.14 kb.

About 0.1 µg each of the thus-obtained *Apa*I-*Not*I fragment of pBSHM1, pBSHM2, pBSHM3, pBSHM31 and pBSHM32 and 0.1 µg of the *Apa*I-*Not*I fragment of pKANTEX796HLCDRLm-28 were added in a total of 20 µl of sterilized water and ligated to each other using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech). Each of the resulting recombinant plasmid DNA solutions was used to transform *Escherichia coli* HB101 and plasmids, pKANTEX796HM1Lm-28, pKANTEX796HM2Lm-28, pKANTEX796HM3Lm-28, pKANTEX796HM31Lm-28 and pKANTEX796HM32Lm-28 shown in Fig. 51 were obtained.

(2) Expression of replacement versions of human CDR-grafted anti-GM_2 antibodies

Four µg each of the plasmids pKANTEX796HM1Lm-28, pKANTEX796HM2Lm-28, pKANTEX796HM3Lm-28, pKANTEX796HM31Lm-28 and pKANTEX796HM32Lm-28 obtained in Paragraph 2 (1) of Example 4 were used to transform YB2/0 cells (ATCC CRL 1581) in accordance with the method as described in Paragraph 1 (4) of Example 2. The cells were ultimately selected using G418 (0.5 mg/ml) and MTX (200 nM) to obtain about 2 to 5 µg/ml of transformants capable of producing human CDR-grafted anti-GM_2 antibodies derived from the corresponding expression vectors.

(3) Purification of replacement versions of human CDR-grafted anti-GM_2 antibodies

Cells of each transformant obtained in Paragraph 2 (2) of Example 4 were suspended in GIT medium (Nihon Pharmaceutical) containing 0.5 mg/ml G418 and 200 nM MTX and about 1 to 3 mg of purified human CDR-grafted anti-GM_2 antibodies were obtained from about 0.6 liter of the culture broth in accordance with the method described in Paragraph 11 of Example 1 of JP-A-6-205694. The human CDR-grafted anti-GM_2 antibodies derived from the plasmids pKANTEX796HM1Lm-28, pKANTEX796HM2Lm-28, pKANTEX796HM3Lm-28, pKANTEX796HM31Lm-28 and pKANTEX796HM32Lm-28 are hereinafter referred to as "M1-28", "M2-28", "M3-28", "M31-28" and "M32-28", respectively. 4 µg each of the purified human CDR-grafted anti-GM_2 antibodies, the human CDR-grafted anti-GM_2 antibody KM8966 and the mouse-human chimeric anti-GM_2 antibody KM966 were electrophoresed by the conventional method [Laemmli: *Nature*, 227, 680 (1970)] for molecular weight checking. The results are shown in Fig. 52. As shown in Fig. 52, under reducing conditions, the molecular weight of the antibody H chain was about 50 KDa and the molecular weight of the antibody L chain was about 25 KDa, thus confirming the expression of the H chain and L chain having the correct molecular weight. Under nonreducing conditions, the molecular weight of the human CDR-grafted anti-GM_2 antibodies was about 150 KDa, confirming that the antibody expressed was composed of two H chains and two L chains and was correct in size. The N-terminal amino acid sequence of the H and L chains of each purified human CDR-grafted anti-GM_2 antibodies was examined by automatic Edman degradation using a protein sequencer (Applied Biosystems model 470A). As a result, it was confirmed that the amino acid sequence was consistent with that deduced from the synthesized V region DNA sequence.

(4) CDC activity of replacement versions of human CDR-grafted anti-GM_2 antibodies

CDC activity of the replacement versions of the human CDR-grafted anti-GM_2 antibodies obtained in Paragraph 2 (3) of Example 4, the human CDR-grafted anti-GM_2 antibody KM8966 and the mouse-human chimeric anti-GM_2 antibody KM966 was measured in accordance with the method described in Paragraph 8 of Example 3. The results are shown in Fig. 53. As shown in Fig. 53, it was found that, among the replacement versions of the human CDR-grafted anti-GM_2 antibodies, the human CDR-grafted anti-GM_2 antibody M2-28 derived from the plasmid pKANTEX796HM2Lm-28 showed the highest CDC activity which was higher than that of the human CDR-grafted anti-GM_2 antibody KM8966 prepared in Example 3. This result indicates that the replaced amino acid residues of the version HM2 among the various replacement versions prepared in Paragraph 1 of Example 4 play an important role for improving CDC activity. It was assumed from the computer model for the V region of mouse antibody KM796 that the replacement of the amino acid residues of the version HM2 would influence on the entire structure of the V region since these amino acid residues are located at the site which interacts with the L chain V region. Recent study of the production of human CDR-grafted antibody reveals that the amino acid residues which affect the structure of the antibody varies in each antibody. No method for precisely predicting such amino acid residues has been established and the above results provide a significant finding for the production of the human CDR-grafted antibody.

The human CDR-grafted anti-GM_2 antibody M2-28 derived from the plasmid pKANTEX796HM2Lm-28 was designated as KM8970 and the antibody KM8970-producing trasformant KM8970 has been deposited with National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology as of May 9, 1996 under the deposit number FERM BP-5528.

3. Modification of human CDR-grafted anti-GM_2 antibody KM8966 L chain V region

The human CDR-grafted anti-GM_2 antibody KM8966 prepared in Example 3 was subjected to amino acid residue replacements in the L chain V region (SEQ ID NO:8) to improve CDC activity. As an amino acid residue to be replaced, serine residue in position 59 was selected based on the results of various replacements obtained in Paragraph 1 (3) of Example 3 which suggested that it was important to support the structure of CDR2 for the human CDR-grafted anti-GM_2 antibody activity. Replacements were introduced by PCR method using as a template 1 ng of the plasmid pBSLm-28 containing the human CDR-grafted anti-GM_2 antibody L chain V region obtained in Paragraph 1 (3) of Example 3 and using as a primer antisense and sense synthetic DNA containing mutations described in Paragraph 1 (3) of Example 3.

The reaction was carried out in the same manner as described in Paragraph 1 (3) of Example 3 using the synthetic DNA of SEQ ID NO:72 as the mutant antisense primer and the synthetic DNA of SEQ ID NO:73 as the mutant sense primer to obtain the plasmid pBSLm-28 No.1, containing version Lm-28 No.1, shown in SEQ ID NO:11, of the human CDR-grafted anti-GM_2 antibody L chain V region. In the amino acid sequence of the version Lm-28 No.1, serine in position 59 in the FR shown in SEQ ID NO:83 was replaced by alanine that is found in the mouse antibody KM796 L chain V region.

4. Evaluation of CDC activity of human CDR-grafted anti-GM_2 antibody having new replacement in human CDR-grafted anti-GM_2 antibody L chain V region

(1) Construction of expression vectors

Expression vectors for the human CDR-grafted anti-GM_2 antibody containing the human CDR-grafted anti-GM_2 antibody L chain V region having the replacement obtained in Paragraph 3 of Example 4 and the human CDR-grafted anti-GM_2 antibody H chain V region were obtained in the following manner.

Six μg of the plasmid pBSLm-28 No.1 obtained in Paragraph 3 of Example 4 was dissolved in 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride, 1 mM DTT and 100 μg/ml BSA. 10 units each of *Eco*RI (Takara Shuzo) and *Spl*I (Takara Shuzo) were added thereto to allow the mixture to react at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis to recover about 0.4 μg of the *Eco*RI-*Spl*I fragment of about 0.39 kb.

Then, 3 μg each of the plasmid pKANTEX796HLCDRLm-28 obtained in Paragraph 3 of Example 3 and the plasmids pKANTEX796HM1Lm-28, pKANTEX796HM2Lm-28 and pKANTEX796HM3Lm-28 obtained in Paragraph 2 (1) of Example 4 were dissolved in 10 μl of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 100 mM sodium chloride, 10 mM magnesium chloride and 1 mM DTT and 100 μg/ml BSA, 10 units each of *Eco*RI (Takara Shuzo) and *Spl*I were added thereto and the mixture was allowed to react at 37°C for 1 hour. The reaction mixture was fractionated by agarose gel electrophoresis to recover about 1 μg of the *Eco*RI-*Spl*I fragment of about 13.19 kb.

A 0.1 μg portion each of the thus-obtained *Eco*RI-*Spl*I fragment of pBSLm-28 No.1 and 0.1 μg of the *Eco*RI-*Spl*I of pKANTEX796HLCDRLm-28, pKANTEX796HM1Lm-28, pKANTEX796HM2Lm-28 and pKANTEX796HM3Lm-28 were added in a total of 20 μl of sterilized water and ligated to each other using Ready-To-Go T4 DNA Ligase (Pharmacia Biotech). Each of the resulting recombinant plasmid DNA solutions was used to transform *Escherichia coli* HB101 and the plasmids pKANTEX796HLm-28 No.1, pKANTEX796HM1Lm-28 No.1, pKANTEX796HM2Lm-28 No.1 and pKANTEX796HM3Lm-28 No.1 shown in Fig. 54 were obtained.

(2) Expression of human CDR-grafted anti-GM_2 antibodies having replacements in the L chain V region

Four μg each of the plasmids pKANTEX796HLm-28 No.1, pKANTEX796HM1Lm-28 No.1, pKANTEX796HM2Lm-28 No.1 and pKANTEX796HM3Lm-28 No.1 obtained in Paragraph 4 (1) of Example 4 was used to transform YB2/0 cells (ATCC CRL 1581) in accordance with the method as described in Paragraph 11 of Example 1. The cells were ultimately selected using G418 (0.5 mg/ml) and MTX (200 nM) to obtain about 2 to 5 μg/ml of transformants capable of producing human CDR-grafted anti-GM_2 antibodies derived from the corresponding expression vectors.

(3) Purification of human CDR-grafted anti-GM_2 antibodies having replacements in the L chain V region

Cells of each transformant obtained in Paragraph 4 (2) of Example 4 were suspended in GIT medium (Nihon Pharmaceutical) containing 0.5 mg/ml G418 and 200 nM MTX and about 1 to 3 mg of purified human CDR-grafted anti-GM_2 antibodies were obtained from about 0.6 liter of the culture broth in accordance with the method described in Paragraph 11 of Example 1 of JP-A-6-205694. The human CDR-grafted anti-GM_2 antibodies derived from the plasmids pKANTEX796HLm-28 No.1, pKANTEX796HM1Lm-28 No.1, pKANTEX796HM2Lm-28 No.1 and pKANTEX796HM3Lm-28 No.1 are hereinafter referred to as "h796H-No.1", "M1-No.1", "M2-No.1" and "M3-No.1", respectively. Four μg each of the purified human CDR-grafted anti-GM_2 antibodies and the mouse-human chimeric anti-GM_2 antibody KM966 was electrophoresed by the conventional method [Laemmli: *Nature*, 227, 680 (1970)] for molecular weight checking. The results are shown in Fig. 55. As shown in Fig. 55, under reducing conditions, the molecular weight of the antibody H chain was about 50 KDa and the molecular weight of the antibody L chain was about 25 KDa, thus confirming the expression of the H chain and L chain having the correct molecular weight. Under nonreducing conditions, the molecular weight of the human CDR-grafted anti-GM_2 antibodies was about 150 KDa, confirming that the antibody expressed was composed of two H chains and two L chains and was correct in size. The N-terminal amino acid sequence of the H and L chains of each purified human CDR-grafted anti-GM_2 antibodies was examined by automatic Edman degradation using a protein sequencer (Applied Biosystems model 470A). As a result, it was confirmed that the amino acid sequence was consistent with that deduced from the synthesized V region DNA sequence.

(4) CDC activity of human CDR-grafted anti-GM_2 antibodies having replacements in the L chain V region

CDC activity of the human CDR-grafted anti-GM_2 antibodies having replacements in the L chain V region obtained in Paragraph 4 (3) of Example 4, the human CDR-grafted anti-GM_2 antibody KM8970, the human CDR-grafted anti-GM_2 antibody KM8966 and the mouse-human chimeric anti-GM_2 antibody KM966 was measured in accordance with the method described in Paragraph 8 of Example 3. The results are shown in Fig. 56. Comparing CDC activity of KM8966 with that of h796H-No.1, it was found that the replacement introduced into only the L chain V region showed improved CDC activity. Among the replaced antibodies having replacements in both of the L chain V region and the H chain V region, M2-No.1 having replacement in the human CDR-grafted anti-GM_2 antibody KM8970 H and L chain V region obtained in Paragraph 2 of Example 4 showed the highest CDC activity, which was comparable to or higher than that of KM8970. These results indicates that the replaced amino acid residue in position 59 in the FR of the L chain V region prepared in Paragraph 3 of Example 4 played an important role for improving its CDC activity and it interacted with the replaced amino acid residue in the H chain V region of KM8970 for improving its CDC activity cooperatively. It was not assumed from the computer model for the V region of mouse antibody KM796 that the replacement of the amino acid residue in position 59 in the FR of the version Lm-28 No.1 would be involved in direct action with antigen GM_2 and interaction with each CDR residue. However, the above results suggested that they were quite important for maintaining the entire structure of the whole V region. This knowledge cannot be predicted from the known production method of a humanized antibody, and the above findings will provide an important indication for the production of human CDR-grafted antibody.

The human CDR-grafted anti-GM_2 antibody M2-No.1 derived from the plasmid pKANTEX796HM2Lm-28 No. 1 was designated as KM8969 and the antibody KM8969-producing trasformant KM8969 has been deposited with National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology as of May 9, 1996 under the deposit number FERM BP-5527.

5. *In vitro* reactivity of human CDR-grafted anti-GM_2 antibodies KM8969 and KM8970 with GM_2

Reactivities of the mouse-human chimeric anti-GM_2 antibody KM966 and the human CDR-grafted anti-GM_2 antibodies KM8969 and KM8970 with GM_2 were measured in accordance with the method described in Paragraph 1 (5) of Example 2. The results are shown in Fig. 57. As shown in Fig. 57, the human CDR-grafted anti-GM_2 antibodies KM8969 and KM8970 showed binding activity comparable to that of the mouse-human chimeric anti-GM_2 antibody KM966.

6. Reaction specificity of human CDR-grafted anti-GM_2 antibodies KM8969 and KM8970

The mouse-human chimeric anti-GM_2 antibody KM966 and the human CDR-grafted anti-GM_2 antibodies KM8969 and KM8970 were examined for reactivity with various gangliosides in accordance with the method described in Paragraph 6 of Example 3. The results are shown in Fig. 58. As shown in Fig. 58, it was found that the human CDR-grafted anti-GM_2 antibodies KM8969 and KM8970 specifically reacted with GM_2 (N-acetyl GM_2 and N-glycolyl GM_2) like the mouse-human chimeric anti-GM_2 antibody KM966.

7. Reactivity of human CDR-grafted anti-GM_2 antibodies KM8969 and KM8970 with cancer cells

The mouse-human chimeric anti-GM_2 antibody KM966 and the human CDR-grafted anti-GM_2 antibodies KM8969 and KM8970 were examined for reactivity with the human lung small cell carcinoma cell line SBC-3 (JCRB 0818) using fluorescein isocyanate-labeled rabbit anti-human IgG antibody (Dako) as a second antibody in accordance with the method described in Paragraph 7 of Example 3. The results are shown in Fig. 59. As shown in Fig. 59, the human CDR-grafted anti-GM_2 antibodies KM8969 and KM8970 strongly reacted with the human lung small cell carcinoma cell line SBC-3 like the mouse-human chimeric anti-GM_2 antibody KM966.

8. In vitro antitumor effect of human CDR-grafted anti-GM_2 antibodies KM8969 and KM8970: antibody dependent cell mediated cytotoxicity (ADCC)

The mouse-human chimeric anti-GM_2 antibody KM966 and the human CDR-grafted anti-GM_2 antibodies KM8966, KM8969 and KM8970 were examined for ADCC activity against the human lung small cell carcinoma cell line SBC-3 (JCRB 0818) in accordance with the method described in Paragraph 9 of Example 3. The results are shown in Fig. 123. As shown in Fig. 123, the human CDR-grafted anti-GM_2 antibodies KM8969 and KM8970 showed ADCC activity comparable to that of the mouse-human chimeric anti-GM_2 antibody KM966.

9. Comparison of *in vitro* anti-tumor activities of humanized anti-GM_2 antibodies: comparison of CDC activity

CDC activities of various humanized anti-GM_2 antibodies (KM966, KM8966, KM8969 and KM8970) established in the aforementioned Inventive Examples 3 and 4 were compared by prolonging the reaction time. Illustratively, the reaction time of the method described in the item 8 of Inventive Example 3 after addition of the human complement was set to 4 hours. The results are shown in Fig. 61. As shown in Fig. 61, it was revealed that the CDC activity of each of these humanized antibodies increases by the 4 hours of reaction and, at an antibody concentration of 5 μg/ml or more, the mouse-human chimeric anti-GM_2 antibody KM966 and the human CDR-grafted anti-GM_2 antibodies KM8966, KM8969 and KM8970 show almost the same level of CDC activity. Particularly, KM8969 showed the highest CDC activity which was about 1/2 of that of the mouse-human chimeric anti-GM_2 antibody KM966, so that it was revealed that a human CDR-grafted anti-GM_2 antibody having further high CDC activity was able to be produced by the examination of Inventive Example 4.

Thus, production method of human CDR-grafted anti-GM_2 antibodies and evaluation of their various activities have been described, and these results show that the established human CDR-grafted anti-GM_2 antibodies are useful for the treatment of human cancers.

By the present invention, human CDR-grafted antibodies to ganglioside GM_2, whose binding activity and binding specificity for GM_2 and anti-tumor effect upon ganglioside GM_2-positive cells are comparable to the levels of chimeric human antibodies, and the production method thereof are provided.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Kyowa Hakko Kogyo Co., Ltd

(ii) TITLE OF INVENTION: Human complementarity determining Region (CDR)-grafted antibody to ganglioside GM2

(iii) NUMBER OF SEQUENCES: 73

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Kyowa Hakko Kogyo Co., Ltd.
(B) STREET: 6-1, Ohtemachi 1-chome, Chiyoda-ku
(C) CITY: Tokyo
(E) COUNTRY: Japan

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: 98105047.9
(B) FILING DATE: 10.03.1998

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Kinzebach, Werner, Dr.
(C) REFERENCE/DOCKET NUMBER: M/39063

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (089) 998397-0
(B) TELEFAX: (089) 987304

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Asp Tyr Asn Met Asp
1 5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Tyr Ile Tyr Pro Asn Asn Gly Gly Thr Gly Tyr Asn Gln Lys Phe Lys
1 5 10 15

Ser
17

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Tyr Gly His Tyr Tyr Gly Tyr Met Phe Ala Tyr
1 5 10 11

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Ser Ala Ser Ser Ser Val Ser Tyr Met His
1 5 10

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Ser Thr Ser Asn Leu Ala Ser
1 5 7

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Gln Gln Arg Ser Ser Tyr Pro Tyr Thr
 1               5               9
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 433 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(ix) FEATURES:
(A) NAME/KEY: sig peptide
(B) LOCATION: -19..-1
(C) IDENTIFICATION METHOD: S
(A) NAME/KEY: domain
(B) LOCATION: 31..35
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 1
(A) NAME/KEY: domain
(B) LOCATION: 50..66
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 2
(A) NAME/KEY: domain
(B) LOCATION: 99..109
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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ATG GGA TGG AGC TGG ATC TTT CTC TTC CTC CTG TCA GGA ACT GCA GGT   48
Met Gly Trp Ser Trp Ile Phe Leu Phe Leu Leu Ser Gly Thr Ala Gly
 1               5                  10                  15

GTC CTC TCT GAG GTG CAG CTG GTG CAG TCT GGA GCA GAG GTG AAG AAG   96
Val Leu Ser Glu Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys
            20                  25                  30

CCT GGG GCC TCA GTG AAG GTC TCC TGC AAG GCT TCC GGA TAC ACC TTC  144
Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe
        35                  40                  45

ACT GAC TAC AAC ATG GAC TGG GTG CGA CAG GCC CCT GGA CAA GGG CTC  192
Thr Asp Tyr Asn Met Asp Trp Val Arg Gln Ala Pro Gly Gln Gly Leu
    50                  55                  60

GAG TGG ATG GGA TAT ATT TAT CCT AAC AAT GGT GGT ACT GGC TAC AAC  240
Glu Trp Met Gly Tyr Ile Tyr Pro Asn Asn Gly Gly Thr Gly Tyr Asn
65                  70                  75                  80

CAG AAG TTC AAG AGC AAG GTC ACC ATT ACC GTA GAC ACA TCC ACG AGC  288
Gln Lys Phe Lys Ser Lys Val Thr Ile Thr Val Asp Thr Ser Thr Ser
                85                  90                  95

ACA GCC TAC ATG GAG CTG CAC AGC CTG AGA TCT GAG GAC ACG GCC GTG  336
Thr Ala Tyr Met Glu Leu His Ser Leu Arg Ser Glu Asp Thr Ala Val
            100                 105                 110
```

```
TAT TAC TGT GCG ACC TAC GGT CAT TAC TAC GGC TAC ATG TTT GCT TAC  384
Tyr Tyr Cys Ala Thr Tyr Gly His Tyr Tyr Gly Tyr Met Phe Ala Tyr
        115                 120                 125

TGG GGC CAG GGA ACC CTG GTC ACC GTC TCC TCA GCC TCC ACC AAG GGC  442
Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly
    130                 135                 140             144

C                                                               443
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(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 390 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(ix) FEATURES:
(A) NAME/KEY: sig peptide
(B) LOCATION: -22..-1
(C) IDENTIFICATION METHOD: S
(A) NAME/KEY: domain
(B) LOCATION: 24..33
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 1
(A) NAME/KEY: domain
(B) LOCATION: 49..55
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 2
(A) NAME/KEY: domain
(B) LOCATION: 86..96
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

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ATG CAT TTT CAA GTG CAG ATT TTC AGC TTC CTG CTA ATC AGT GCC TCA   48
Met His Phe Gln Val Gln Ile Phe Ser Phe Leu Leu Ile Ser Ala Ser
 1               5                  10                  15

GTC ATA ATG TCC AGA GGA GAT ATC CAG CTG ACC CAG AGC CCA AGC AGC   96
Val Ile Met Ser Arg Gly Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser
            20                  25                  30

CTG AGC GCT AGC CCA GGT GAC AGA GTG ACC ATC ACG TGC AGT GCC AGC  144
Leu Ser Ala Ser Pro Gly Asp Arg Val Thr Ile Thr Cys Ser Ala Ser
        35                  40                  45

TCA AGT GTA AGT TAC ATG CAC TGG TTC CAG CAG AAA CCA GGT AAG GCT  192
Ser Ser Val Ser Tyr Met His Trp Phe Gln Gln Lys Pro Gly Lys Ala
    50                  55                  60

CCA AAG CTT TGG ATC TAC AGC ACA TCC AAC CTG GCT TCT GGT GTG CCA  240
Pro Lys Leu Trp Ile Tyr Ser Thr Ser Asn Leu Ala Ser Gly Val Pro
65                  70                  75                  80

TCT AGA TTC AGC GGT AGC GGT AGC GGT ACA TCT TAC TCT CTC ACC ATC  288
Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile
                85                  90                  95
```

```
AGC CGA CTC CAG CCA GAG GAC ATC GCT ACA TAC TAC TGC CAG CAA AGG  336
Ser Arg Leu Gln Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Arg
            100                 105                 110

AGT AGT TAC CCG TAC ACG TTC GGC GGG GGG ACC AAG GTG GAA ATC AAA  384
Ser Ser Tyr Pro Tyr Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
        115                 120                 125

CGT ACG                                                          390
Arg Thr
    130
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 390 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(ix) FEATURES:
(A) NAME/KEY: sig peptide
(B) LOCATION: -22..-1
(C) IDENTIFICATION METHOD: S
(A) NAME/KEY: domain
(B) LOCATION: 24..33
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 1
(A) NAME/KEY: domain
(B) LOCATION: 49..55
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 2
(A) NAME/KEY: domain
(B) LOCATION: 86..96
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

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ATG CAT TTT CAA GTG CAG ATT TTC AGC TTC CTG CTA ATC AGT GCC TCA   48
Met His Phe Gln Val Gln Ile Phe Ser Phe Leu Leu Ile Ser Ala Ser
 1               5                  10                  15

GTC ATA ATG TCC AGA GGA GAC ATC CAG CTG ACC CAG TCT CCA TCC TCC   96
Val Ile Met Ser Arg Gly Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser
            20                  25                  30

ATG TCT GCA TCT CCA GGA GAC AGA GTC ACC ATC ACT TGT AGT GCA AGT  144
Met Ser Ala Ser Pro Gly Asp Arg Val Thr Ile Thr Cys Ser Ala Ser
        35                  40                  45

TCA AGT GTA AGT TAC ATG CAC TGG TTT CAG CAG AAA CCA GGG AAA TCA  192
Ser Ser Val Ser Tyr Met His Trp Phe Gln Gln Lys Pro Gly Lys Ser
    50                  55                  60

CCT AAG CTC TGG ATC TAC TCA ACT TCA AAT TTA GCT TCT GGT GTG CCA  240
Pro Lys Leu Trp Ile Tyr Ser Thr Ser Asn Leu Ala Ser Gly Val Pro
65                  70                  75                  80
```

```
TCT AGA TTC AGC GGT AGC GGT AGC GGT ACA TCT TAC TCT CTC ACC ATC  288
Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile
                85                  90                  95

AGC AGC ATG CAG CCT GAA GAT TTT GCA ACT TAT TAC TGT CAG CAA AGG  336
Ser Ser Met Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Arg
            100                 105                 110

AGT AGT TAC CCG TAC ACG TTC GGC CAG GGG ACC AAG CTG GAA ATC AAA  384
Ser Ser Tyr Pro Tyr Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile Lys
        115                 120                 125

CGT ACG                                                         390
Arg Thr
    130
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 433 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(ix) FEATURES:
(A) NAME/KEY: sig peptide
(B) LOCATION: -19..-1
(C) IDENTIFICATION METHOD: S
(A) NAME/KEY: domain
(B) LOCATION: 31..35
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 1
(A) NAME/KEY: domain
(B) LOCATION: 50..66
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 2
(A) NAME/KEY: domain
(B) LOCATION: 99..109
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

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 1               5                  10                  15

GTC CTC TCT GAG GTG CAG CTG GTG CAG TCT GGA GCA GAG GTG AAG AAG   96
Val Leu Ser Glu Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys
            20                  25                  30

CCT GGG GCC TCA GTG AAG GTC TCC TGC AAG GCT TCC GGA TAC ACC TTC  144
Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe
        35                  40                  45

ACT GAC TAC AAC ATG GAC TGG GTG AAG CAG AGC CCT GGA CAA GGG CTC  192
Thr Asp Tyr Asn Met Asp Trp Val Lys Gln Ser Pro Gly Gln Gly Leu
    50                  55                  60
```

```
GAG TGG ATG GGA TAT ATT TAT CCT AAC AAT GGT GGT ACT GGC TAC AAC  240
Glu Trp Met Gly Tyr Ile Tyr Pro Asn Asn Gly Gly Thr Gly Tyr Asn
65                  70                  75                  80

CAG AAG TTC AAG AGC AAG GTC ACC ATT ACC GTA GAC ACA TCC ACG AGC  288
Gln Lys Phe Lys Ser Lys Val Thr Ile Thr Val Asp Thr Ser Thr Ser
                85                  90                  95

ACA GCC TAC ATG GAG CTG CAC AGC CTG AGA TCT GAG GAC ACG GCC GTG  336
Thr Ala Tyr Met Glu Leu His Ser Leu Arg Ser Glu Asp Thr Ala Val
            100                 105                 110

TAT TAC TGT GCG ACC TAC GGT CAT TAC TAC GGC TAC ATG TTT GCT TAC  384
Tyr Tyr Cys Ala Thr Tyr Gly His Tyr Tyr Gly Tyr Met Phe Ala Tyr
        115                 120                 125

TGG GGC CAG GGA ACC CTG GTC ACC GTC TCC TCA GCC TCC ACC AAG GGC  432
Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly
    130                 135                 140                 144

C                                                               433
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 390 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(ix) FEATURES:
(A) NAME/KEY: sig peptide
(B) LOCATION: -22..-1
(C) IDENTIFICATION METHOD: S
(A) NAME/KEY: domain
(B) LOCATION: 24..33
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 1
(A) NAME/KEY: domain
(B) LOCATION: 49..55
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 2
(A) NAME/KEY: domain
(B) LOCATION: 86..96
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
ATG CAT TTT CAA GTG CAG ATT TTC AGC TTC CTG CTA ATC AGT GCC TCA   48
Met His Phe Gln Val Gln Ile Phe Ser Phe Leu Leu Ile Ser Ala Ser
 1               5                  10                  15

GTC ATA ATG TCC AGA GGA GAT ATC CAG CTG ACC CAG AGC CCA AGC AGC   96
Val Ile Met Ser Arg Gly Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser
            20                  25                  30

CTG AGC GCT AGC CCA GGT GAC AGA GTG ACC ATC ACG TGC AGT GCC AGC  144
Leu Ser Ala Ser Pro Gly Asp Arg Val Thr Ile Thr Cys Ser Ala Ser
        35                  40                  45
```

```
TCA AGT GTA AGT TAC ATG CAC TGG TTC CAG CAG AAA CCA GGT AAG GCT  192
Ser Ser Val Ser Tyr Met His Trp Phe Gln Gln Lys Pro Gly Lys Ala
    50                  55                  60

CCA AAG CTT TGG ATC TAC AGC ACA TCC AAC CTG GCT TCT GGT GTG CCA  240
Pro Lys Leu Trp Ile Tyr Ser Thr Ser Asn Leu Ala Ser Gly Val Pro
65                  70                  75                  80

GCT AGA TTC AGC GGT AGC GGT AGC GGT ACA TCT TAC TCT CTC ACC ATC  288
Ala Arg Phe Ser Gly Ser Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile
                85                  90                  95

AGC CGA CTC CAG CCA GAG GAC ATC GCT ACA TAC TAC TGC CAG CAA AGG  336
Ser Arg Leu Gln Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Arg
            100                 105                 110

AGT AGT TAC CCG TAC ACG TTC GGC GGG GGG ACC AAG GTG GAA ATC AAA  384
Ser Ser Tyr Pro Tyr Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
        115                 120                 125

CGT ACG                                                         390
Arg Thr
    130
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CACTCAGTGT TAACTGAGGA GCAGGTGAAT TC 32

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AGCTGAATTC ACCTGCTCCT CAGTTAACAC TGAGTGGTAC 40

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AATTCGTACG GTGGCTGCAC C 21

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GGTGCAGCCA CCGTACG 17

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CTCGCGACTA GTGGGCCCGC GGCCGC 26

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AGCTGCGGCC GCGGGCCCAC TAGTCGCGAG GTAC 34

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GTGGCGGCCG CTTGGGCCCG 20

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CGGGCCCAAG CGGCCGCCAC 20

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CATGAATTCT TCGTACGGTT CGATAAATCG ATACCG 36

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CGGTATCGAT TTATCGAACC GTACGAAGAA TTCATGAGCT 40

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CACGTTCGGA GGGGGGACCA AGCTGGAAAT AAAAC 35

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GTACGTTTTA TTTCCAGCTT GGTCCCCCCT CCGAA 35

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 61 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TCGACACCAG CAAGAACACA GCCTACCTGA GACTCAGCAG CGTGACAGCC GCCGACACCG 60

C 61

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CCGGATACAC ATTCACTGAC TACAACATGG ACTGGGTGAG ACAGAGCCAT GGACGAGGTC 60

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 442 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(ix) FEATURES:
(A) NAME/KEY: sig peptide
(B) LOCATION: -19..-1
(C) IDENTIFICATION METHOD: S
(A) NAME/KEY: domain
(B) LOCATION: 31..35
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 1
(A) NAME/KEY: domain
(B) LOCATION: 50..66
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 2
(A) NAME/KEY: domain
(B) LOCATION: 99..109
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

```
GGCCGCACC ATG GGA TGG AGC TGG ATC TTT CTC TTC CTC CTG TCA GGA ACT  51
          Met Gly Trp Ser Trp Ile Phe Leu Phe Leu Leu Ser Gly Thr
           1               5                  10

GCT GGT GTC CTC TCT CAG GTC CAA CTG CAG GAG AGC GGT CCA GGT CTT   99
Ala Gly Val Leu Ser Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu
15                  20                  25                  30

GTG AGG CCT AGC CAG ACC CTG AGC CTG ACC TGC ACC GTG TCC GGA TTC  147
Val Arg Pro Ser Gln Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Phe
                35                  40                  45

ACC TTC AGC GAC TAC AAC ATG GAC TGG GTG AGA CAG CCA CCT GGA CGA  195
Thr Phe Ser Asp Tyr Asn Met Asp Trp Val Arg Gln Pro Pro Gly Arg
            50                  55                  60

GGT CTC GAG TGG ATT GGA TAT ATT TAT CCT AAC AAT GGT GGT ACT GGC  243
Gly Leu Glu Trp Ile Gly Tyr Ile Tyr Pro Asn Asn Gly Gly Thr Gly
        65                  70                  75

TAC AAC CAG AAG TTC AAG AGC AGA GTG ACA ATG CTG GTC GAC ACC AGC  291
Tyr Asn Gln Lys Phe Lys Ser Arg Val Thr Met Leu Val Asp Thr Ser
    80                  85                  90

AAG AAC ACA GCC TAC CTG AGA CTC AGC AGC GTG ACA GCC GCC GAC ACC  339
Lys Asn Thr Ala Tyr Leu Arg Leu Ser Ser Val Thr Ala Ala Asp Thr
95                  100                 105                 110

GCG GTC TAT TAT TGT GCA ACC TAC GGT CAT TAC TAC GGC TAC ATG TTT  387
Ala Val Tyr Tyr Cys Ala Thr Tyr Gly His Tyr Tyr Gly Tyr Met Phe
                115                 120                 125

GCT TAC TGG GGT CAA GGT ACC ACC GTC ACA GTC TCC TCA GCC TCC ACC  435
Ala Tyr Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Ala Ser Thr
            130                 135                 140

AAG GGC C                                                       442
Lys Gly
    144
```

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 442 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(ix) FEATURES:
(A) NAME/KEY: sig peptide
(B) LOCATION: -19..-1
(C) IDENTIFICATION METHOD: S
(A) NAME/KEY: domain
(B) LOCATION: 31..35
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 1
(A) NAME/KEY: domain
(B) LOCATION: 50..66
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 2
(A) NAME/KEY: domain
(B) LOCATION: 99..109
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

```
GGCCGCACC ATG GGA TGG AGC TGG ATC TTT CTC TTC CTC CTG TCA GGA ACT 51
          Met Gly Trp Ser Trp Ile Phe Leu Phe Leu Leu Ser Gly Thr
           1               5                  10

GCT GGT GTC CTC TCT CAG GTC CAA CTG CAG GAG AGC GGT CCA GGT CTT   99
Ala Gly Val Leu Ser Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu
15                  20                  25                  30

GTG AGG CCT AGC CAG ACC CTG AGC CTG ACC TGC ACC GTG TCC GGA TAC  147
Val Arg Pro Ser Gln Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Tyr
                35                  40                  45

ACC TTC ACT GAC TAC AAC ATG GAC TGG GTG AGA CAG AGC CAT GGA CGA  195
Thr Phe Thr Asp Tyr Asn Met Asp Trp Val Arg Gln Ser His Gly Arg
            50                  55                  60

GGT CTC GAG TGG ATT GGA TAT ATT TAT CCT AAC AAT GGT GGT ACT GGC  243
Gly Leu Glu Trp Ile Gly Tyr Ile Tyr Pro Asn Asn Gly Gly Thr Gly
        65                  70                  75

TAC AAC CAG AAG TTC AAG AGC AGA GTG ACA ATG CTG GTC GAC ACC AGC  291
Tyr Asn Gln Lys Phe Lys Ser Arg Val Thr Met Leu Val Asp Thr Ser
    80                  85                  90

AAG AAC CAG TTC AGC CTG AGA CTC AGC AGC GTG ACA GCC GCC GAC ACC  339
Lys Asn Gln Phe Ser Leu Arg Leu Ser Ser Val Thr Ala Ala Asp Thr
95                  100                 105                 110

GCG GTC TAT TAT TGT GCA ACC TAC GGT CAT TAC TAC GGC TAC ATG TTT  387
Ala Val Tyr Tyr Cys Ala Thr Tyr Gly His Tyr Tyr Gly Tyr Met Phe
                115                 120                 125

GCT TAC TGG GGT CAA GGT ACC ACC GTC ACA GTC TCC TCA GCC TCC ACC  435
Ala Tyr Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Ala Ser Thr
            130                 135                 140
```

AAG GGC C 442
Lys Gly
144

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 100 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CAGGAAACAG CTATGACGCG GCCGCCACCA TGGGATGGAG CTGGATCTTT CTCTTCCTCC 60

TGTCAGGAAC TGCAGGTGTC CTCTCTGAGG TGCAGCTGGT 100

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 100 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

AGTCAGTGAA GGTGTATCCG GAAGCCTTGC AGGAGACCTT CACTGAGGCC CCAGGCTTCT 60

TCACCTCTGC TCCAGACTGC ACCAGCTGCA CCTCAGAGAG 100

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 100 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CGGATACACC TTCACTGACT ACAACATGGA CTGGGTGCGA CAGGCCCCTG GACAAGGGCT 60

CGAGTGGATG GGATATATTT ATCCTAACAA TGGTGGTACT 100

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 94 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

AGCTCCATGT AGGCTGTGCT CGTGGATGTG TCTACGGTAA TGGTGACCTT GCTCTTGAAC 60

TTCTGGTTGT AGCCAGTACC ACCATTGTTA GGAT 94

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 96 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

AGCACAGCCT ACATGGAGCT GCACAGCCTG AGATCTGAGG ACACGGCCGT GTATTACTGT 60

GCGACCTACG GTCATTACTA CGGCTACATG TTTGCT 96

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 90 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

GTTTTCCCAG TCACGACGGG CCCTTGGTGG AGGCTGAGGA GACGGTGACC AGGGTTCCCT 60

GGCCCCAGTA AGCAAACATG TAGCCGTAGT 90

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 68 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GTACTACTGC CAGCAAAGGA GTAGTTACCC GTACACGTTC GGCGGGGGGA CCAAGGTGGA 60

AATCAAAC 68

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

ACTCTGTCAC CTGGGCTAGC GCTCA 25

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

TGAGCGCTAG CCCAGGTGAC AGAGT 25

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 390 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(ix) FEATURES:
(A) NAME/KEY: sig peptide
(B) LOCATION: -22..-1
(C) IDENTIFICATION METHOD: S
(A) NAME/KEY: domain
(B) LOCATION: 24..33
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 1
(A) NAME/KEY: domain
(B) LOCATION: 49..55
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 2
(A) NAME/KEY: domain
(B) LOCATION: 86..96
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

```
ATG CAT TTT CAA GTG CAG ATT TTC AGC TTC CTG CTA ATC AGT GCC TCA   48
Met His Phe Gln Val Gln Ile Phe Ser Phe Leu Leu Ile Ser Ala Ser
 1               5                  10                  15

GTC ATA ATG TCC AGA GGA GAT ATC CAG CTG ACC CAG AGC CCA AGC AGC   96
Val Ile Met Ser Arg Gly Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser
            20                  25                  30
```

```
CTG AGC GCT AGC CCA GGT GAC AGA GTG ACC ATC ACG TGC AGT GCC AGC  144
Leu Ser Ala Ser Pro Gly Asp Arg Val Thr Ile Thr Cys Ser Ala Ser
        35                  40                  45

TCA AGT GTA AGT TAC ATG CAC TGG TAT CAG CAG AAA CCA GGT AAG GCT  192
Ser Ser Val Ser Tyr Met His Trp Tyr Gln Gln Lys Pro Gly Lys Ala
    50                  55                  60

CCA AAG CTT CTG ATC TAC AGC ACA TCC AAC CTG GCT TCT GGT GTG CCA  240
Pro Lys Leu Leu Ile Tyr Ser Thr Ser Asn Leu Ala Ser Gly Val Pro
65                  70                  75                  80

TCT AGA TTC AGC GGT AGC GGT AGC GGT ACA GAC TTC ACC TTC ACC ATC  288
Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile
                85                  90                  95

AGC AGC CTC CAG CCA GAG GAC ATC GCT ACA TAC TAC TGC CAG CAA AGG  336
Ser Ser Leu Gln Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Arg
            100                 105                 110

AGT AGT TAC CCG TAC ACG TTC GGC GGG GGG ACC AAG GTG GAA ATC AAA  384
Ser Ser Tyr Pro Tyr Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
        115                 120                 125

CGT ACG                                                         390
Arg Thr
    130
```

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

GTGCTGTAGA TCCAAAGCTT TGGAG 25

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

CTCCAAAGCT TTGGATCTAC AGCAC 25

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 390 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(ix) FEATURES:
(A) NAME/KEY: sig peptide
(B) LOCATION: -22..-1
(C) IDENTIFICATION METHOD: S
(A) NAME/KEY: domain
(B) LOCATION: 24..33
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 1
(A) NAME/KEY: domain
(B) LOCATION: 49..55
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 2
(A) NAME/KEY: domain
(B) LOCATION: 86..96
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

```
ATG CAT TTT CAA GTG CAG ATT TTC AGC TTC CTG CTA ATC AGT GCC TCA   48
Met His Phe Gln Val Gln Ile Phe Ser Phe Leu Leu Ile Ser Ala Ser
 1               5                  10                  15

GTC ATA ATG TCC AGA GGA GAT ATC CAG CTG ACC CAG AGC CCA AGC AGC   96
Val Ile Met Ser Arg Gly Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser
            20                  25                  30

CTG AGC GCT AGC GTG GGT GAC AGA GTG ACC ATC ACG TGC AGT GCC AGC  144
Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Ser Ala Ser
        35                  40                  45

TCA AGT GTA AGT TAC ATG CAC TGG TAT CAG CAG AAA CCA GGT AAG GCT  192
Ser Ser Val Ser Tyr Met His Trp Tyr Gln Gln Lys Pro Gly Lys Ala
    50                  55                  60

CCA AAG CTT TGG ATC TAC AGC ACA TCC AAC CTG GCT TCT GGT GTG CCA  240
Pro Lys Leu Trp Ile Tyr Ser Thr Ser Asn Leu Ala Ser Gly Val Pro
65                  70                  75                  80

TCT AGA TTC AGC GGT AGC GGT AGC GGT ACA GAC TTC ACC TTC ACC ATC  288
Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile
                85                  90                  95

AGC AGC CTC CAG CCA GAG GAC ATC GCT ACA TAC TAC TGC CAG CAA AGG  336
Ser Ser Leu Gln Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Arg
            100                 105                 110

AGT AGT TAC CCG TAC ACG TTC GGC GGG GGG ACC AAG GTG GAA ATC AAA  384
Ser Ser Tyr Pro Tyr Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
        115                 120                 125

CGT ACG                                                         390
Arg Thr
    130
```

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

ACGTAGCAGC ATCTTCAGCC TGGAG 25

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

CTCCAGGCTG AAGATGCTGC TACGT 25

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 390 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(ix) FEATURES:
(A) NAME/KEY: sig peptide
(B) LOCATION: -22..-1
(C) IDENTIFICATION METHOD: S
(A) NAME/KEY: domain
(B) LOCATION: 24..33
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 1
(A) NAME/KEY: domain
(B) LOCATION: 49..55
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 2
(A) NAME/KEY: domain
(B) LOCATION: 86..96
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

```
ATG CAT TTT CAA GTG CAG ATT TTC AGC TTC CTG CTA ATC AGT GCC TCA   48
Met His Phe Gln Val Gln Ile Phe Ser Phe Leu Leu Ile Ser Ala Ser
 1               5                  10                  15
```

```
GTC ATA ATG TCC AGA GGA GAT ATC CAG CTG ACC CAG AGC CCA AGC AGC   96
Val Ile Met Ser Arg Gly Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser
            20                  25                  30

CTG AGC GCT AGC GTG GGT GAC AGA GTG ACC ATC ACG TGC AGT GCC AGC  144
Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Ser Ala Ser
        35                  40                  45

TCA AGT GTA AGT TAC ATG CAC TGG TAT CAG CAG AAA CCA GGT AAG GCT  192
Ser Ser Val Ser Tyr Met His Trp Tyr Gln Gln Lys Pro Gly Lys Ala
    50                  55                  60

CCA AAG CTT CTG ATC TAC AGC ACA TCC AAC CTG GCT TCT GGT GTG CCA  240
Pro Lys Leu Leu Ile Tyr Ser Thr Ser Asn Leu Ala Ser Gly Val Pro
65                  70                  75                  80

TCT AGA TTC AGC GGT AGC GGT AGC GGT ACA GAC TTC ACC TTC ACC ATC  288
Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile
                85                  90                  95

AGC AGC CTC CAG GCT GAA GAT GCT GCT ACA TAC TAC TGC CAG CAA AGG  336
Ser Ser Leu Gln Ala Glu Asp Ala Ala Thr Tyr Tyr Cys Gln Gln Arg
            100                 105                 110

AGT AGT TAC CCG TAC ACG TTC GGC GGG GGG ACC AAG GTG GAA ATC AAA  384
Ser Ser Tyr Pro Tyr Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
        115                 120                 125

CGT ACG                                                          390
Arg Thr
    130
```

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

ATGGTGAAAG AGTAAGATGT ACCGC 25

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

GCGGTACATC TTACTCTTTC ACCAT 25

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 390 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(ix) FEATURES:
(A) NAME/KEY: sig peptide
(B) LOCATION: -22..-1
(C) IDENTIFICATION METHOD: S
(A) NAME/KEY: domain
(B) LOCATION: 24..33
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 1
(A) NAME/KEY: domain
(B) LOCATION: 49..55
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 2
(A) NAME/KEY: domain
(B) LOCATION: 86..96
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

```
ATG CAT TTT CAA GTG CAG ATT TTC AGC TTC CTG CTA ATC AGT GCC TCA   48
Met His Phe Gln Val Gln Ile Phe Ser Phe Leu Leu Ile Ser Ala Ser
 1               5                  10                  15

GTC ATA ATG TCC AGA GGA GAT ATC CAG CTG ACC CAG AGC CCA AGC AGC   96
Val Ile Met Ser Arg Gly Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser
            20                  25                  30

CTG AGC GCT AGC CCA GGT GAC AGA GTG ACC ATC ACG TGC AGT GCC AGC  144
Leu Ser Ala Ser Pro Gly Asp Arg Val Thr Ile Thr Cys Ser Ala Ser
        35                  40                  45

TCA AGT GTA AGT TAC ATG CAC TGG TAT CAG CAG AAA CCA GGT AAG GCT  192
Ser Ser Val Ser Tyr Met His Trp Tyr Gln Gln Lys Pro Gly Lys Ala
    50                  55                  60

CCA AAG CTT TGG ATC TAC AGC ACA TCC AAC CTG GCT TCT GGT GTG CCA  240
Pro Lys Leu Trp Ile Tyr Ser Thr Ser Asn Leu Ala Ser Gly Val Pro
65                  70                  75                  80

TCT AGA TTC AGC GGT AGC GGT AGC GGT ACA TCT TAC TCT TTC ACC ATC  288
Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Ser Tyr Ser Phe Thr Ile
                85                  90                  95

AGC AGC CTC CAG CCA GAG GAC ATC GCT ACA TAC TAC TGC CAG CAA AGG  336
Ser Ser Leu Gln Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Arg
            100                 105                 110

AGT AGT TAC CCG TAC ACG TTC GGC GGG GGG ACC AAG GTG GAA ATC AAA  384
Ser Ser Tyr Pro Tyr Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
        115                 120                 125

CGT ACG                                                         390
Arg Thr
    130
```

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

TCTGGCTCCA TTCGGCTGAT GGTGAAAGAG TAAGATGTAC 40

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

GTACATCTTA CTCTTTCACC ATCAGCCGAA TGGAGCCAGA 40

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 390 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(ix) FEATURES:
(A) NAME/KEY: sig peptide
(B) LOCATION: -22..-1
(C) IDENTIFICATION METHOD: S
(A) NAME/KEY: domain
(B) LOCATION: 24..33
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 1
(A) NAME/KEY: domain
(B) LOCATION: 49..55
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 2
(A) NAME/KEY: domain
(B) LOCATION: 86..96
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

ATG CAT TTT CAA GTG CAG ATT TTC AGC TTC CTG CTA ATC AGT GCC TCA 48
Met His Phe Gln Val Gln Ile Phe Ser Phe Leu Leu Ile Ser Ala Ser
1 5 10 15

```
GTC ATA ATG TCC AGA GGA GAT ATC CAG CTG ACC CAG AGC CCA AGC AGC   96
Val Ile Met Ser Arg Gly Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser
            20                  25                  30

CTG AGC GCT AGC CCA GGT GAC AGA GTG ACC ATC ACG TGC AGT GCC AGC  144
Leu Ser Ala Ser Pro Gly Asp Arg Val Thr Ile Thr Cys Ser Ala Ser
        35                  40                  45

TCA AGT GTA AGT TAC ATG CAC TGG TAT CAG CAG AAA CCA GGT AAG GCT  192
Ser Ser Val Ser Tyr Met His Trp Tyr Gln Gln Lys Pro Gly Lys Ala
    50                  55                  60

CCA AAG CTT TGG ATC TAC AGC ACA TCC AAC CTG GCT TCT GGT GTG CCA  240
Pro Lys Leu Trp Ile Tyr Ser Thr Ser Asn Leu Ala Ser Gly Val Pro
65                  70                  75                  80

TCT AGA TTC AGC GGT AGC GGT AGC GGT ACA TCT TAC TCT TTC ACC ATC  288
Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Ser Tyr Ser Phe Thr Ile
                85                  90                  95

AGC CGA ATG GAG CCA GAG GAC ATC GCT ACA TAC TAC TGC CAG CAA AGG  336
Ser Arg Met Glu Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Arg
            100                 105                 110

AGT AGT TAC CCG TAC ACG TTC GGC GGG GGG ACC AAG GTG GAA ATC AAA  384
Ser Ser Tyr Pro Tyr Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
        115                 120                 125

CGT ACG                                                          390
Arg Thr
    130
```

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

TTCTGCTGGA ACCAGTGCAT 20

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

ATGCACTGGT TCCAGCAGAA 20

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 390 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(ix) FEATURES:
(A) NAME/KEY: sig peptide
(B) LOCATION: -22..-1
(C) IDENTIFICATION METHOD: S
(A) NAME/KEY: domain
(B) LOCATION: 24..33
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 1
(A) NAME/KEY: domain
(B) LOCATION: 49..55
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 2
(A) NAME/KEY: domain
(B) LOCATION: 86..96
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

```
ATG CAT TTT CAA GTG CAG ATT TTC AGC TTC CTG CTA ATC AGT GCC TCA   48
Met His Phe Gln Val Gln Ile Phe Ser Phe Leu Leu Ile Ser Ala Ser
 1               5                  10                  15

GTC ATA ATG TCC AGA GGA GAT ATC CAG CTG ACC CAG AGC CCA AGC AGC   96
Val Ile Met Ser Arg Gly Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser
            20                  25                  30

CTG AGC GCT AGC CCA GGT GAC AGA GTG ACC ATC ACG TGC AGT GCC AGC  144
Leu Ser Ala Ser Pro Gly Asp Arg Val Thr Ile Thr Cys Ser Ala Ser
        35                  40                  45

TCA AGT GTA AGT TAC ATG CAC TGG TTC CAG CAG AAA CCA GGT AAG GCT  192
Ser Ser Val Ser Tyr Met His Trp Phe Gln Gln Lys Pro Gly Lys Ala
    50                  55                  60

CCA AAG CTT TGG ATC TAC AGC ACA TCC AAC CTG GCT TCT GGT GTG CCA  240
Pro Lys Leu Trp Ile Tyr Ser Thr Ser Asn Leu Ala Ser Gly Val Pro
65                  70                  75                  80

TCT AGA TTC AGC GGT AGC GGT AGC GGT ACA TCT TAC TCT TTC ACC ATC  288
Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Ser Tyr Ser Phe Thr Ile
                85                  90                  95

AGC AGC CTC CAG CCA GAG GAC ATC GCT ACA TAC TAC TGC CAG CAA AGG  336
Ser Ser Leu Gln Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Arg
            100                 105                 110

AGT AGT TAC CCG TAC ACG TTC GGC GGG GGG ACC AAG GTG GAA ATC AAA  384
Ser Ser Tyr Pro Tyr Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
        115                 120                 125

CGT ACG                                                         390
Arg Thr
    130
```

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

TGGAGTCGGC TGATGGTGAG AGAGT 25

(2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

ACTCTCTCAC CATCAGCCGA CTCCA 25

(2) INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 390 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(ix) FEATURES:
(A) NAME/KEY: sig peptide
(B) LOCATION: -22..-1
(C) IDENTIFICATION METHOD: S
(A) NAME/KEY: domain
(B) LOCATION: 24..33
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 1
(A) NAME/KEY: domain
(B) LOCATION: 49..55
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 2
(A) NAME/KEY: domain
(B) LOCATION: 86..96
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

```
ATG CAT TTT CAA GTG CAG ATT TTC AGC TTC CTG CTA ATC AGT GCC TCA   48
Met His Phe Gln Val Gln Ile Phe Ser Phe Leu Leu Ile Ser Ala Ser
 1               5                  10                  15
```

```
GTC ATA ATG TCC AGA GGA GAT ATC CAG CTG ACC CAG AGC CCA AGC AGC   96
Val Ile Met Ser Arg Gly Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser
            20                  25                  30

CTG AGC GCT AGC CCA GGT GAC AGA GTG ACC ATC ACG TGC AGT GCC AGC  144
Leu Ser Ala Ser Pro Gly Asp Arg Val Thr Ile Thr Cys Ser Ala Ser
        35                  40                  45

TCA AGT GTA AGT TAC ATG CAC TGG TAT CAG CAG AAA CCA GGT AAG GCT  192
Ser Ser Val Ser Tyr Met His Trp Tyr Gln Gln Lys Pro Gly Lys Ala
    50                  55                  60

CCA AAG CTT TGG ATC TAC AGC ACA TCC AAC CTG GCT TCT GGT GTG CCA  240
Pro Lys Leu Trp Ile Tyr Ser Thr Ser Asn Leu Ala Ser Gly Val Pro
65                  70                  75                  80

TCT AGA TTC AGC GGT AGC GGT AGC GGT ACA TCT TAC TCT CTC ACC ATC  288
Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile
                85                  90                  95

AGC CGA CTC CAG CCA GAG GAC ATC GCT ACA TAC TAC TGC CAG CAA AGG  336
Ser Arg Leu Gln Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Arg
            100                 105                 110

AGT AGT TAC CCG TAC ACG TTC GGC GGG GGG ACC AAG GTG GAA ATC AAA  384
Ser Ser Tyr Pro Tyr Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
        115                 120                 125

CGT ACG                                                         390
Arg Thr
    130
```

(2) INFORMATION FOR SEQ ID NO:56:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 94 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

```
CAGGAAACAG CTATGACGAA TTCCACCATG CATTTTCAAG TGCAGATTTT CAGCTTCCTG 60

CTAATCAGTG CCTCAGTCAT AATGTCCAGA GGAG                             94
```

(2) INFORMATION FOR SEQ ID NO:57:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 88 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

ACAAGTGATG GTGACTCTGT CTCCTGGAGA TGCAGACATG GAGGATGGAG ACTGGGTCAG 60

CTGGATGTCT CCTCTGGACA TTATGACT 88

(2) INFORMATION FOR SEQ ID NO:58:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 92 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

ACAGAGTCAC CATCACTTGT AGTGCAAGTT CAAGTGTAAG TTACATGCAC TGGTTTCAGC 60

AGAAACCAGG GAAATCACCT AAGCTCTGGA TC 92

(2) INFORMATION FOR SEQ ID NO:59:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 87 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

AAGATGTACC GCTACCGCTA CCGCTGAATC TAGATGGCAC ACCAGAAGCT AAATTTGAAG 60

TTGAGTAGAT CCAGAGCTTA GGTGATT 87

(2) INFORMATION FOR SEQ ID NO:60:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 89 base pairs
(B) TYPE: nucleic acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

TAGCGGTAGC GGTACATCTT ACTCTCTCAC CATCAGCAGC ATGCAGCCTG AAGATTTTGC 60

AACTTATTAC TGTCAGCAAA GGAGTAGTT 89

(2) INFORMATION FOR SEQ ID NO:61:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 84 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

GTTTTCCCAG TCACGACCGT ACGTTTGATT TCCAGCTTGG TCCCCTGGCC GAACGTGTAC 60

GGGTAACTAC TCCTTTGCTG ACAG 84

(2) INFORMATION FOR SEQ ID NO:62:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

ACTCGAGGCT CTTTCCAGGG CTCTGCTTCA CCCAG 35

(2) INFORMATION FOR SEQ ID NO:63:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

CTGGGTGAAG CAGAGCCCTG GAAAGAGCCT CGAGT 35

(2) INFORMATION FOR SEQ ID NO:64:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 433 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(ix) FEATURES:
(A) NAME/KEY: sig peptide
(B) LOCATION: -19..-1
(C) IDENTIFICATION METHOD: S
(A) NAME/KEY: domain
(B) LOCATION: 31..35
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 1
(A) NAME/KEY: domain
(B) LOCATION: 50..66
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 2
(A) NAME/KEY: domain
(B) LOCATION: 99..109
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

```
ATG GGA TGG AGC TGG ATC TTT CTC TTC CTC CTG TCA GGA ACT GCA GGT   48
Met Gly Trp Ser Trp Ile Phe Leu Phe Leu Leu Ser Gly Thr Ala Gly
 1               5                  10                  15

GTC CTC TCT GAG GTG CAG CTG GTG CAG TCT GGA GCA GAG GTG AAG AAG   96
Val Leu Ser Glu Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys
            20                  25                  30

CCT GGG GCC TCA GTG AAG GTC TCC TGC AAG GCT TCC GGA TAC ACC TTC  144
Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe
        35                  40                  45

ACT GAC TAC AAC ATG GAC TGG GTG AAG CAG AGC CCT GGA AAG AGC CTC  192
Thr Asp Tyr Asn Met Asp Trp Val Lys Gln Ser Pro Gly Lys Ser Leu
    50                  55                  60

GAG TGG ATG GGA TAT ATT TAT CCT AAC AAT GGT GGT ACT GGC TAC AAC  240
Glu Trp Met Gly Tyr Ile Tyr Pro Asn Asn Gly Gly Thr Gly Tyr Asn
65                  70                  75                  80

CAG AAG TTC AAG AGC AAG GTC ACC ATT ACC GTA GAC ACA TCC ACG AGC  288
Gln Lys Phe Lys Ser Lys Val Thr Ile Thr Val Asp Thr Ser Thr Ser
                85                  90                  95

ACA GCC TAC ATG GAG CTG CAC AGC CTG AGA TCT GAG GAC ACG GCC GTG  336
Thr Ala Tyr Met Glu Leu His Ser Leu Arg Ser Glu Asp Thr Ala Val
            100                 105                 110

TAT TAC TGT GCG ACC TAC GGT CAT TAC TAC GGC TAC ATG TTT GCT TAC  384
Tyr Tyr Cys Ala Thr Tyr Gly His Tyr Tyr Gly Tyr Met Phe Ala Tyr
        115                 120                 125

TGG GGC CAG GGA ACC CTG GTC ACC GTC TCC TCA GCC TCC ACC AAG GGC  432
Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly
    130                 135                 140                 144

C                                                               433
```

(2) INFORMATION FOR SEQ ID NO:65:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

TGTCCAGGGC TCTGCTTCAC CCAG 24

(2) INFORMATION FOR SEQ ID NO:66:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

CTGGGTGAAG CAGAGCCCTG GACA 24

(2) INFORMATION FOR SEQ ID NO:67:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

TCTACGGTCA AGGTGGCCTT GCTCT 25

(2) INFORMATION FOR SEQ ID NO:68:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

AGAGCAAGGC CACCTTGACC GTAGA 25

(2) INFORMATION FOR SEQ ID NO:69:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 433 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(ix) FEATURES:
(A) NAME/KEY: sig peptide
(B) LOCATION: -19..-1
(C) IDENTIFICATION METHOD: S
(A) NAME/KEY: domain
(B) LOCATION: 31..35
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 1
(A) NAME/KEY: domain
(B) LOCATION: 50..66
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 2
(A) NAME/KEY: domain
(B) LOCATION: 99..109
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

```
ATG GGA TGG AGC TGG ATC TTT CTC TTC CTC CTG TCA GGA ACT GCA GGT   48
Met Gly Trp Ser Trp Ile Phe Leu Phe Leu Leu Ser Gly Thr Ala Gly
 1               5                  10                  15

GTC CTC TCT GAG GTG CAG CTG GTG CAG TCT GGA GCA GAG GTG AAG AAG   96
Val Leu Ser Glu Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys
            20                  25                  30

CCT GGG GCC TCA GTG AAG GTC TCC TGC AAG GCT TCC GGA TAC ACC TTC  144
Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe
        35                  40                  45

ACT GAC TAC AAC ATG GAC TGG GTG CGA CAG GCC CCT GGA CAA GGG CTC  192
Thr Asp Tyr Asn Met Asp Trp Val Arg Gln Ala Pro Gly Gln Gly Leu
    50                  55                  60

GAG TGG ATG GGA TAT ATT TAT CCT AAC AAT GGT GGT ACT GGC TAC AAC  240
Glu Trp Met Gly Tyr Ile Tyr Pro Asn Asn Gly Gly Thr Gly Tyr Asn
65                  70                  75                  80

CAG AAG TTC AAG AGC AAG GCC ACC TTG ACC GTA GAC ACA TCC ACG AGC  288
Gln Lys Phe Lys Ser Lys Ala Thr Leu Thr Val Asp Thr Ser Thr Ser
                85                  90                  95

ACA GCC TAC ATG GAG CTG CAC AGC CTG AGA TCT GAG GAC ACG GCC GTG  336
Thr Ala Tyr Met Glu Leu His Ser Leu Arg Ser Glu Asp Thr Ala Val
            100                 105                 110

TAT TAC TGT GCG ACC TAC GGT CAT TAC TAC GGC TAC ATG TTT GCT TAC  384
Tyr Tyr Cys Ala Thr Tyr Gly His Tyr Tyr Gly Tyr Met Phe Ala Tyr
        115                 120                 125

TGG GGC CAG GGA ACC CTG GTC ACC GTC TCC TCA GCC TCC ACC AAG GGC  432
Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly
    130                 135                 140                 144

C                                                               433
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(2) INFORMATION FOR SEQ ID NO:70:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 433 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(ix) FEATURES:
(A) NAME/KEY: sig peptide
(B) LOCATION: -19..-1
(C) IDENTIFICATION METHOD: S
(A) NAME/KEY: domain
(B) LOCATION: 31..35
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 1
(A) NAME/KEY: domain
(B) LOCATION: 50..66
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 2
(A) NAME/KEY: domain
(B) LOCATION: 99..109
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

```
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Met Gly Trp Ser Trp Ile Phe Leu Phe Leu Leu Ser Gly Thr Ala Gly
 1               5                  10                  15

GTC CTC TCT GAG GTG CAG CTG GTG CAG TCT GGA GCA GAG GTG AAG AAG   96
Val Leu Ser Glu Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys
            20                  25                  30

CCT GGG GCC TCA GTG AAG GTC TCC TGC AAG GCT TCC GGA TAC ACC TTC  144
Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe
        35                  40                  45

ACT GAC TAC AAC ATG GAC TGG GTG AAG CAG AGC CCT GGA AAG AGC CTC  192
Thr Asp Tyr Asn Met Asp Trp Val Lys Gln Ser Pro Gly Lys Ser Leu
    50                  55                  60

GAG TGG ATG GGA TAT ATT TAT CCT AAC AAT GGT GGT ACT GGC TAC AAC  240
Glu Trp Met Gly Tyr Ile Tyr Pro Asn Asn Gly Gly Thr Gly Tyr Asn
65                  70                  75                  80

CAG AAG TTC AAG AGC AAG GCC ACC TTG ACC GTA GAC ACA TCC ACG AGC  288
Gln Lys Phe Lys Ser Lys Ala Thr Leu Thr Val Asp Thr Ser Thr Ser
                85                  90                  95

ACA GCC TAC ATG GAG CTG CAC AGC CTG AGA TCT GAG GAC ACG GCC GTG  336
Thr Ala Tyr Met Glu Leu His Ser Leu Arg Ser Glu Asp Thr Ala Val
            100                 105                 110

TAT TAC TGT GCG ACC TAC GGT CAT TAC TAC GGC TAC ATG TTT GCT TAC  384
Tyr Tyr Cys Ala Thr Tyr Gly His Tyr Tyr Gly Tyr Met Phe Ala Tyr
        115                 120                 125
```

```
TGG GGC CAG GGA ACC CTG GTC ACC GTC TCC TCA GCC TCC ACC AAG GGC  432
Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly
    130                 135                 140             144

C                                                               433
```

(2) INFORMATION FOR SEQ ID NO:71:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 433 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(ix) FEATURES:
(A) NAME/KEY: sig peptide
(B) LOCATION: -19..-1
(C) IDENTIFICATION METHOD: S
(A) NAME/KEY: domain
(B) LOCATION: 31..35
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 1
(A) NAME/KEY: domain
(B) LOCATION: 50..66
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 2
(A) NAME/KEY: domain
(B) LOCATION: 99..109
(C) IDENTIFICATION METHOD: S
(D) OTHER INFORMATION: hypervariable region 3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

```
ATG GGA TGG AGC TGG ATC TTT CTC TTC CTC CTG TCA GGA ACT GCA GGT   48
Met Gly Trp Ser Trp Ile Phe Leu Phe Leu Leu Ser Gly Thr Ala Gly
 1               5                  10                  15

GTC CTC TCT GAG GTG CAG CTG GTG CAG TCT GGA GCA GAG GTG AAG AAG   96
Val Leu Ser Glu Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys
            20                  25                  30

CCT GGG GCC TCA GTG AAG GTC TCC TGC AAG GCT TCC GGA TAC ACC TTC  144
Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe
        35                  40                  45

ACT GAC TAC AAC ATG GAC TGG GTG AAG CAG AGC CCT GGA CAA GGG CTC  192
Thr Asp Tyr Asn Met Asp Trp Val Lys Gln Ser Pro Gly Gln Gly Leu
    50                  55                  60

GAG TGG ATG GGA TAT ATT TAT CCT AAC AAT GGT GGT ACT GGC TAC AAC  240
Glu Trp Met Gly Tyr Ile Tyr Pro Asn Asn Gly Gly Thr Gly Tyr Asn
65                  70                  75                  80

CAG AAG TTC AAG AGC AAG GCC ACC TTG ACC GTA GAC ACA TCC ACG AGC  288
Gln Lys Phe Lys Ser Lys Ala Thr Leu Thr Val Asp Thr Ser Thr Ser
                85                  90                  95

ACA GCC TAC ATG GAG CTG CAC AGC CTG AGA TCT GAG GAC ACG GCC GTG  336
Thr Ala Tyr Met Glu Leu His Ser Leu Arg Ser Glu Asp Thr Ala Val
            100                 105                 110
```

```
TAT TAC TGT GCG ACC TAC GGT CAT TAC TAC GGC TAC ATG TTT GCT TAC  384
Tyr Tyr Cys Ala Thr Tyr Gly His Tyr Tyr Gly Tyr Met Phe Ala Tyr
        115                 120                 125

TGG GGC CAG GGA ACC CTG GTC ACC GTC TCC TCA GCC TCC ACC AAG GGC  432
Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly
    130                 135                 140             144

C                                                               433
```

(2) INFORMATION FOR SEQ ID NO:72:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

TGAATCTAGC TGGCACACCA 20

(2) INFORMATION FOR SEQ ID NO:73:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

TGGTGTGCCA GCTAGATTCA 20

Claims

1. A human CDR-grafted antibody which specifically reacts with ganglioside GM_2, wherein said antibody comprises CDR 1, CDR 2 and CDR 3 of heavy chain (H chain) variable region (V region) comprising amino acid sequences of SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3 or functional equivalents thereof, and CDR 1, CDR 2 and CDR 3 of light chain (L chain) V region comprising amino acid sequences of SEQ ID NO:4, SEQ ID NO:5 and SEQ ID NO:6 or functional equivalents thereof, and wherein at least one of the frameworks (FR) of said H chain and L chain V regions comprises an amino acid sequence selected from common sequences (HMHCS; human most homologous consensus sequence) derived from human antibody subgroups.

2. The human CDR-grafted antibody according to claim 1, wherein said FR is an amino acid sequence of an FR of an HMHCS having a high homology with an FR of a monoclonal antibody originated from nonhuman animal which specifically reacts with ganglioside GM_2.

3. The human CDR-grafted antibody according to claim 1 or 2, wherein said FR of H chain or L chain V region of the human CDR-grafted antibody comprises an amino acid sequence in which at least one amino acid is replaced by

an other amino acid, and wherein said antibody has antigen-binding activity and binding specificity comparable to those of a human chimeric antibody having a V region of a monoclonal antibody derived from nonhuman animal which specifically reacts with ganglioside GM_2.

4. The human CDR-grafted antibody according to any one of claims 1 to 3, wherein said FR of H chain or L chain V region of the human CDR-grafted antibody comprises an amino acid sequence in which at least one amino acid is replaced by an other amino acid, and wherein said antibody has antibody dependent cell mediated cytotoxicity (ADCC) comparable to that of a human chimeric antibody having a V region of a monoclonal antibody derived from nonhuman animal which specifically reacts with ganglioside GM_2

5. The human CDR-grafted antibody according to any one of claims 1 to 4, wherein said FR of H chain or L chain V region of the human CDR-grafted antibody comprises an amino acid sequence in which at least one amino acid is replaced by an other amino acid, and wherein said antibody has complement dependent cytotoxicity (CDC) comparable to that of a human chimeric antibody having a V region of a monoclonal antibody originated from nonhuman animal which specifically reacts with ganglioside GM_2.

6. The human CDR-grafted antibody according to any one of claims 3 to 5, wherein said other amino acid is selected from amino acids in a position corresponding to the FR of the monoclonal antibody derived from nonhuman animal.

7. The human CDR-grafted antibody according to any one of claims 3 to 6, wherein at least one amino acid of positions 38, 40, 67, 72, 84 and 98 in the FR of H chain V region and positions 4, 11, 15, 35, 42, 46, 59, 69, 70, 71, 72, 76, 77 and 103 in the FR of L chain V region is replaced by an other amino acid.

8. The human CDR-grafted antibody according to any one of claims 1 to 7, therein said H chain C region of the antibody is derived from an antibody belonging to the human antibody IgG class.

9. The human CDR-grafted antibody according to any one of claims 1 to 4 and 6 to 8, which is KM8966 comprising the H chain V region of the antibody having an amino acid sequence of SEQ ID NO:7 and the L chain V region of the antibody having an amino acid sequence of SEQ ID NO:8.

10. The human CDR-grafted antibody according to any one of claims 1 to 4 and 6 to 8, which is KM8967 comprising the H chain V region of the antibody having an amino acid sequence of SEQ ID NO:7 and the L chain V region of the antibody having an amino acid sequence of SEQ ID NO:9.

11. The human CDR-grafted antibody and according to any one of claims 1 to 4 and 6 to 8, which is KM8970 comprising the H chain V region of the antibody having an amino acid sequence of SEQ ID NO:10 and the L chain V region of the antibody having an amino acid sequence of SEQ ID NO:8.

12. The human CDR-grafted antibody according to any one of claims 1 to 8, which is KM8969 comprising the H chain V region of the antibody having an amino acid sequence of SEQ ID NO:10 and the L chain V region of the antibody having an amino acid sequence of SEQ ID NO:11.

13. A DNA fragment encoding an amino acid sequence of the H chain V region and L chain V region of the antibody according to any one of claims 1 to 12.

14. A recombinant vector comprising the DNA fragment according to claim 13 or a part thereof.

15. The recombinant vector according to claim 14, which is derived from a tandem cassette vector, pKANTEX 93, for expressing a human chimeric antibody and a human CDR-grafted antibody.

16. A transformant comprising the recombinant vector according to claim 14 or 15.

17. A transformant cell line KM8966 (FERM BP-5105), which produces the antibody KM8966 according to claim 9.

18. A transformant cell line KM8967 (FERM BP-5106), which produces the antibody KM8967 according to claim 10.

19. A transformant cell line KM8970 (FERM BP-5528), which produces the antibody KM8970 according to claim 11.

20. A transformant cell line KM8969 (FERM BP-5527), which produces the antibody KM8969 according to claim 12.

21. A method for producing the antibodies according to any one of claims 1 to 12 using said transformant according to any one of claims 17 to 20.

22. An anti-tumor agent comprising the antibody of any one of claims 1 to 12 as an active ingredient.

23. A diagnostic agent for cancer comprising the antibody of any one of claims 1 to 12 as an active ingredient.

FIG. 1

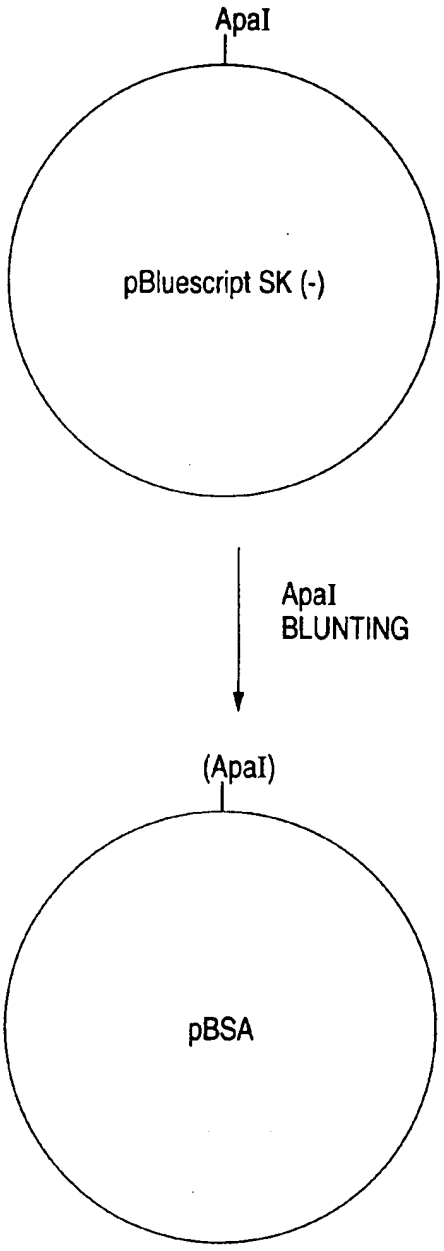


FIG. 2

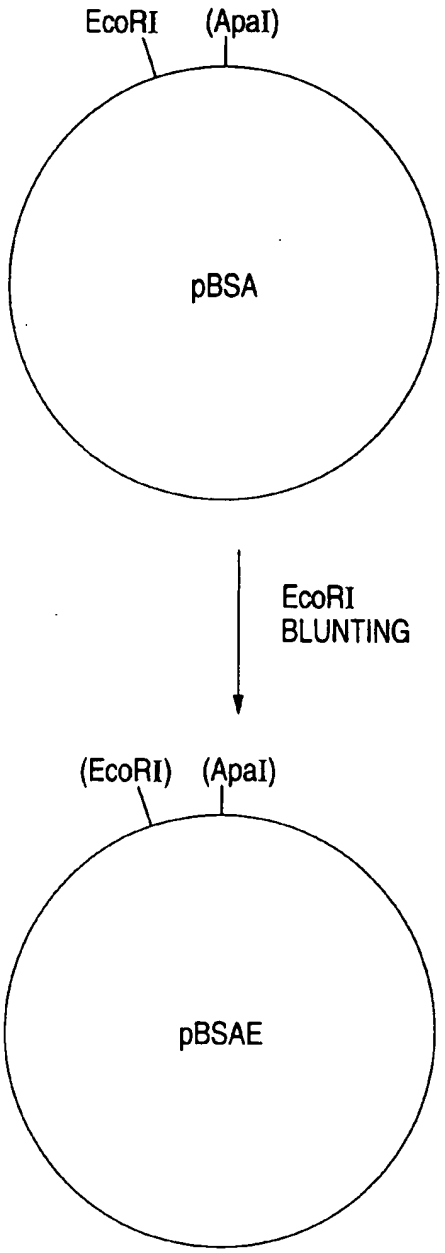


FIG. 3

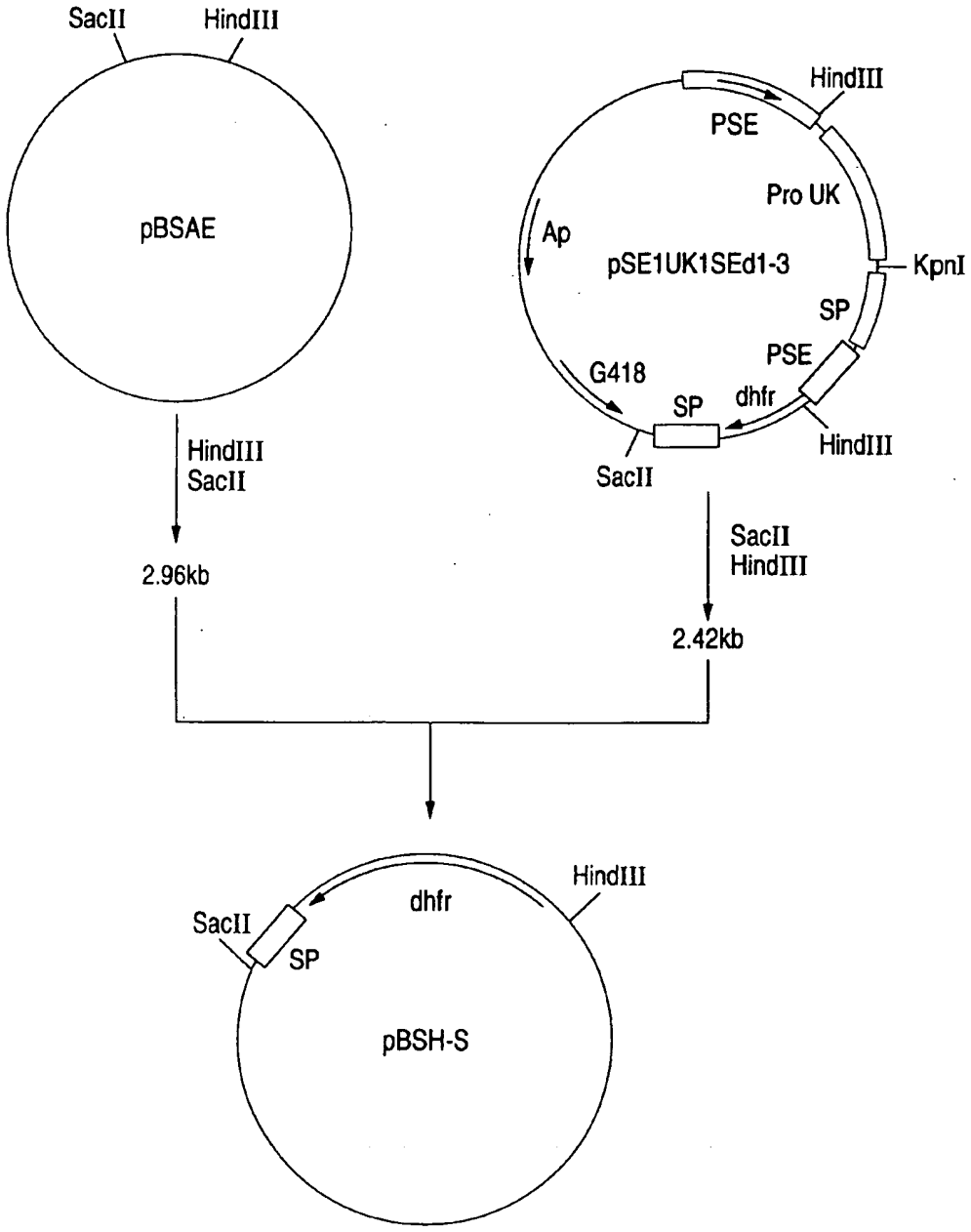


FIG. 4

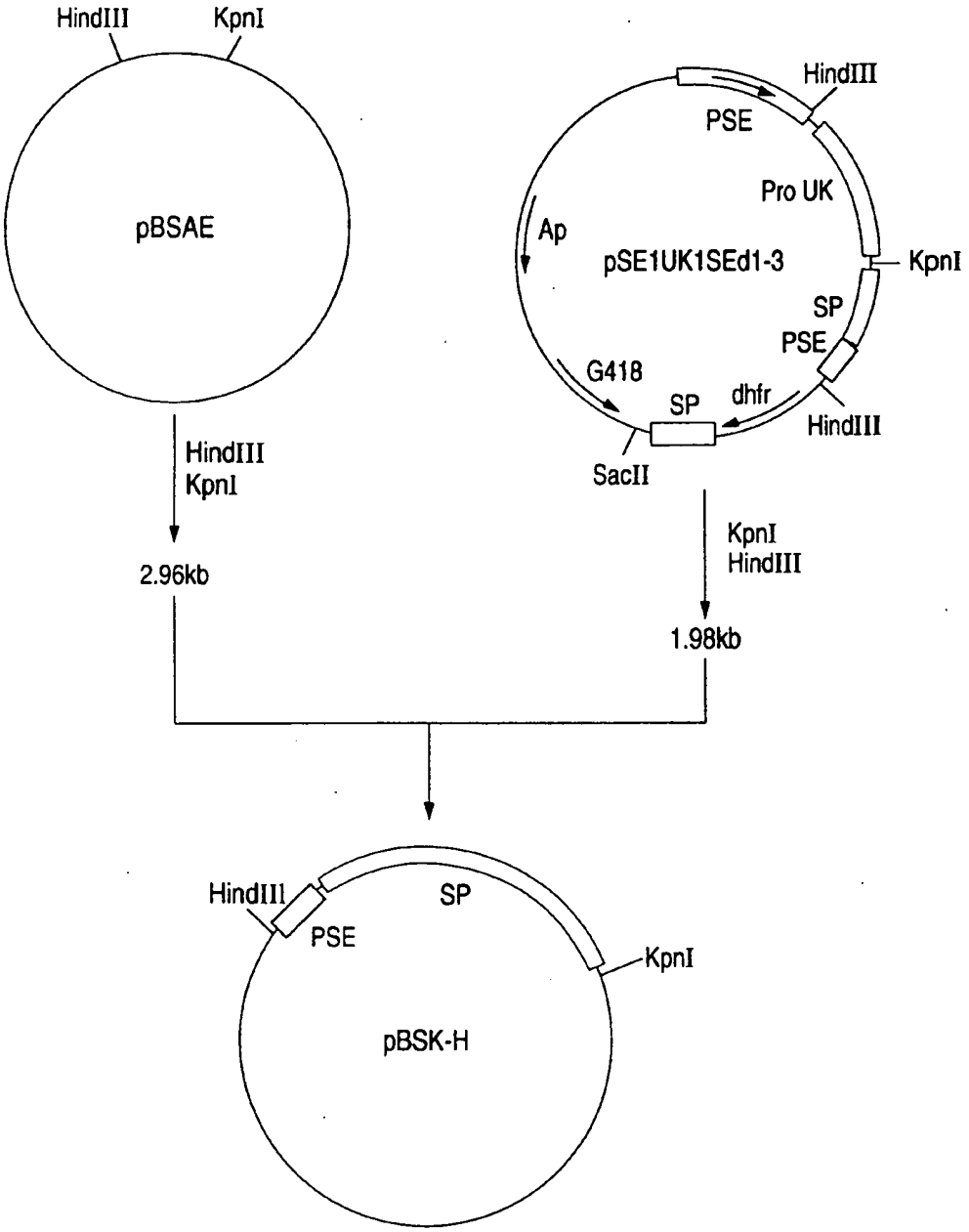


FIG. 5

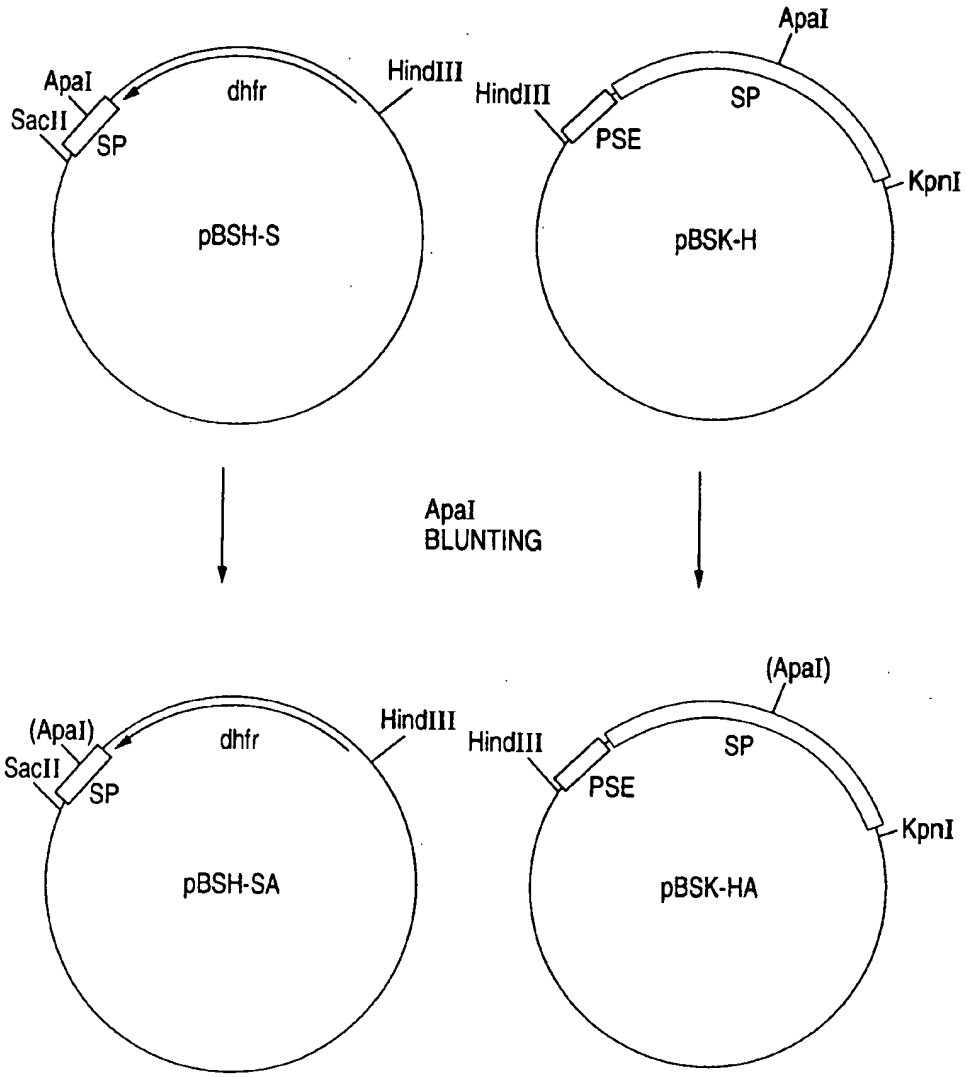


FIG. 6

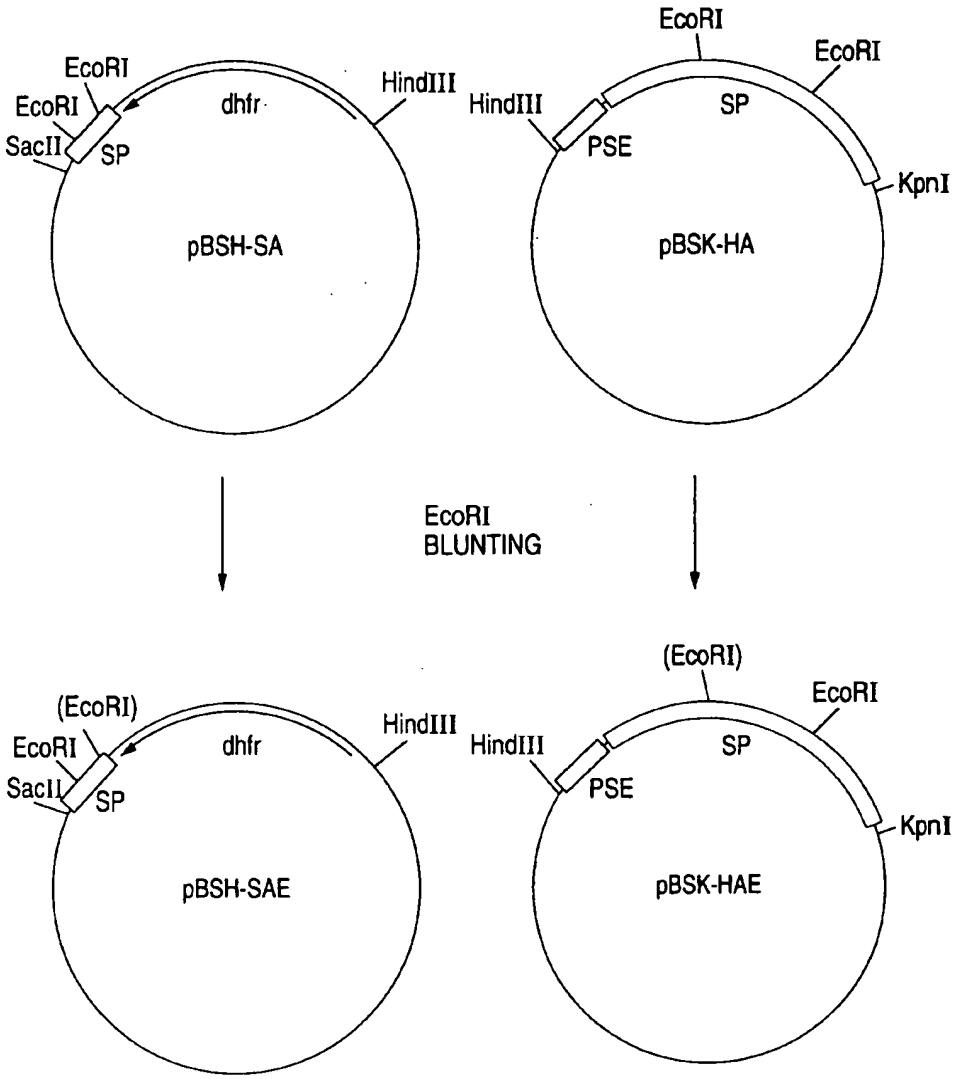


FIG. 7

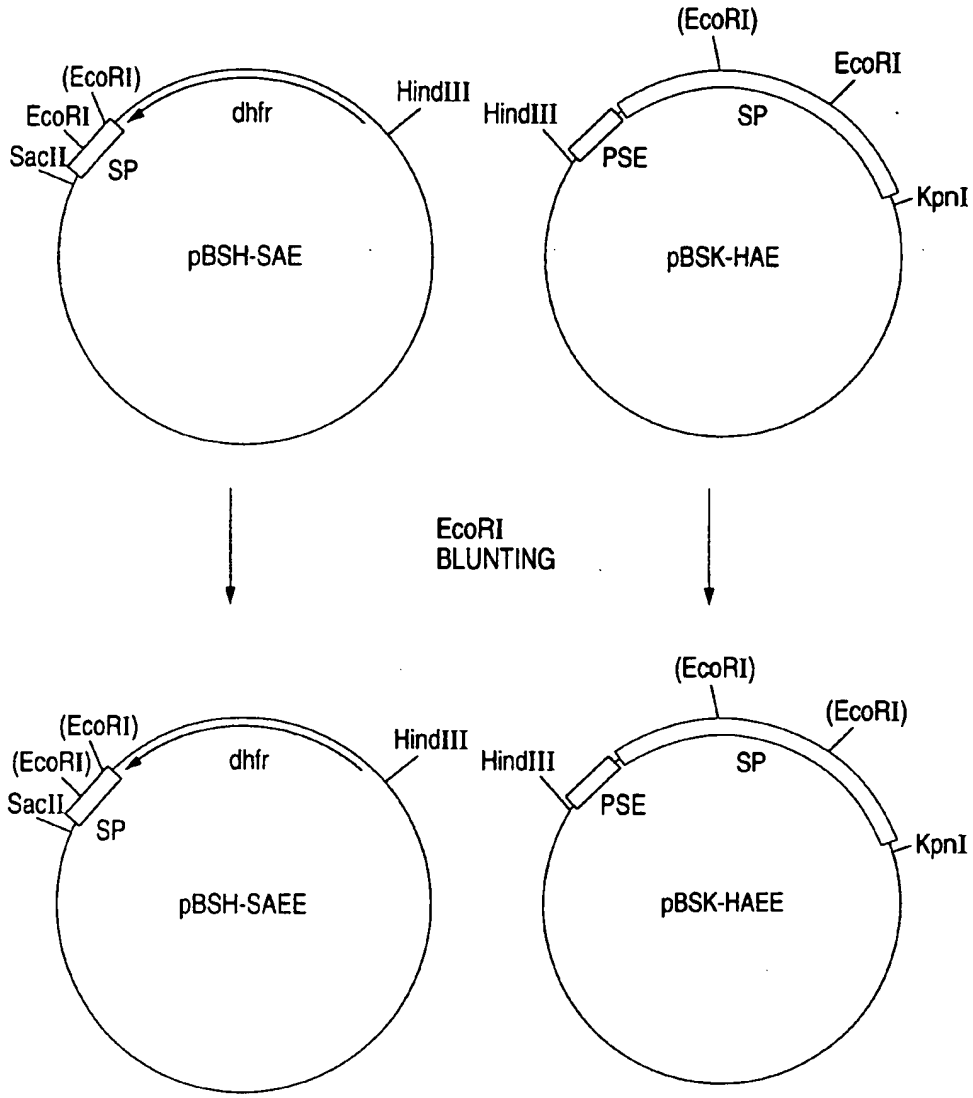


FIG. 8

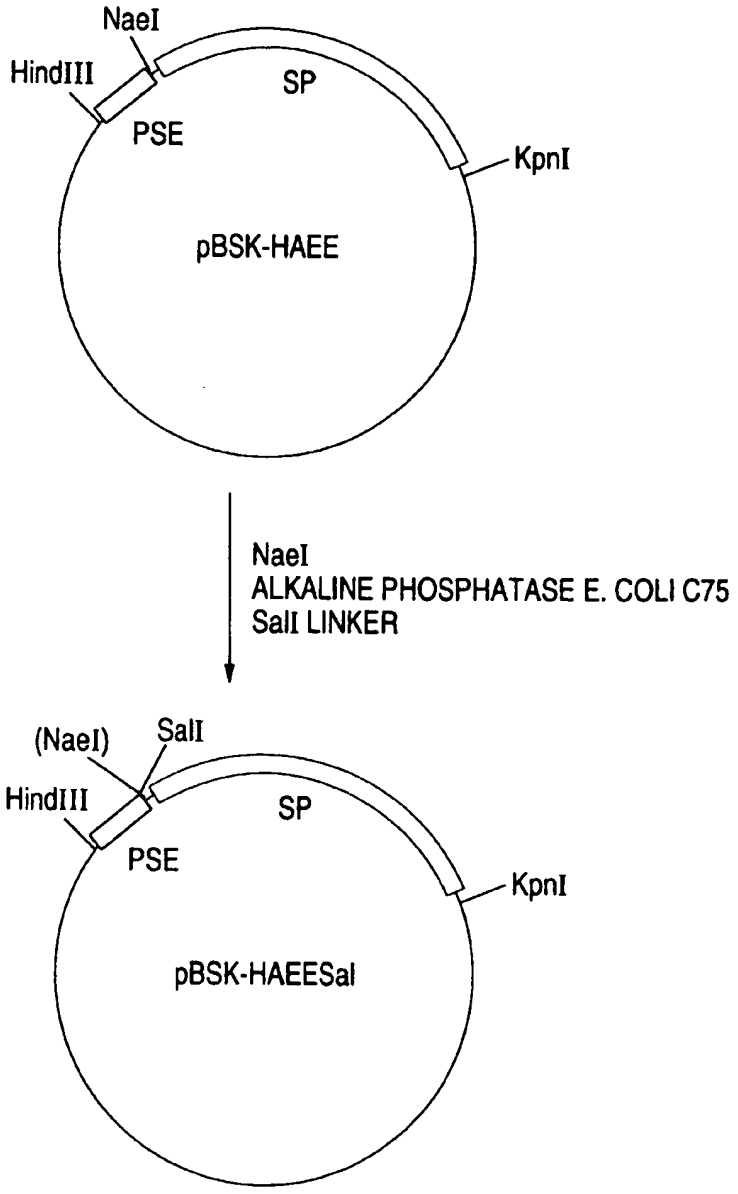


FIG. 9

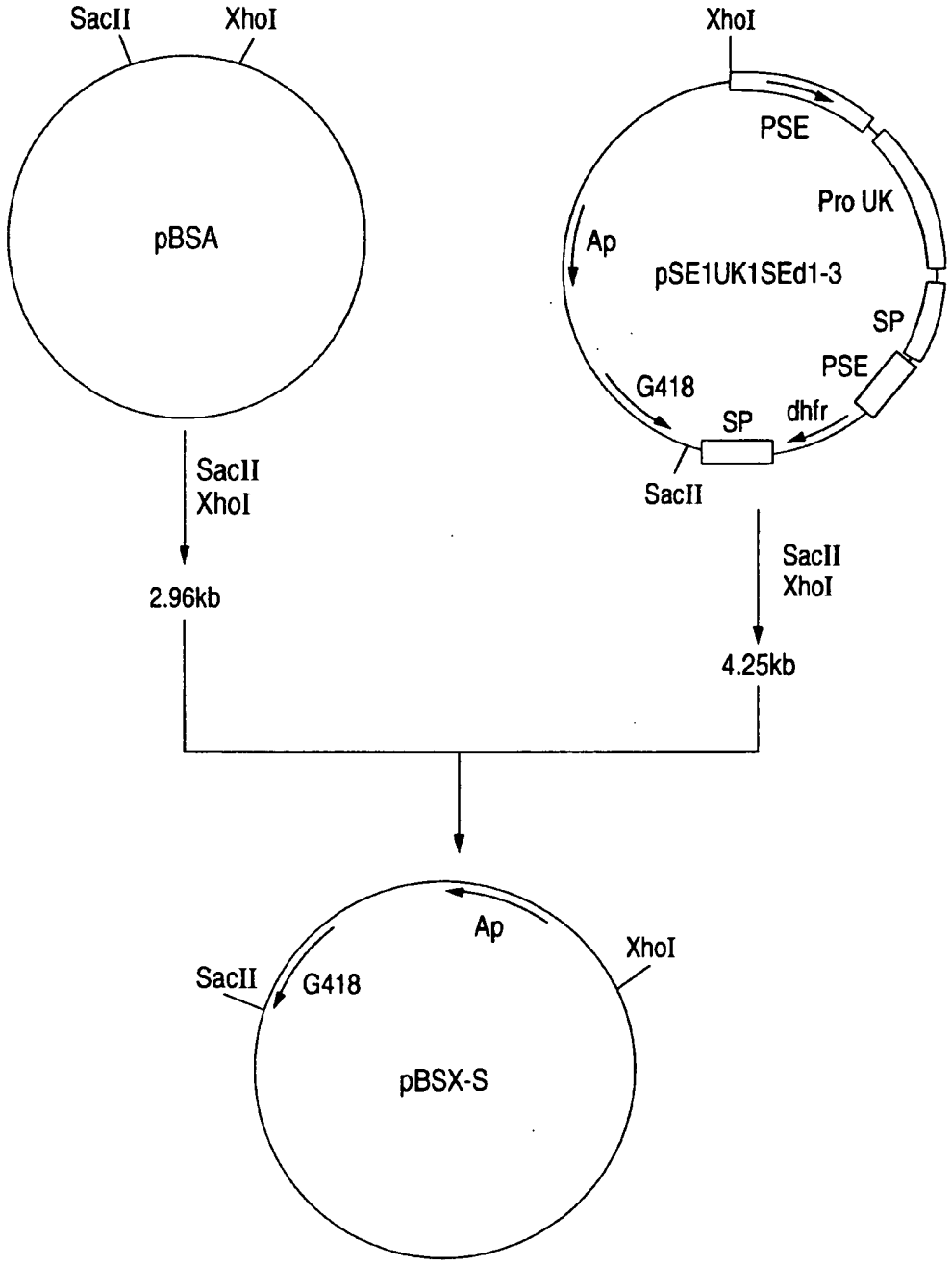


FIG. 10

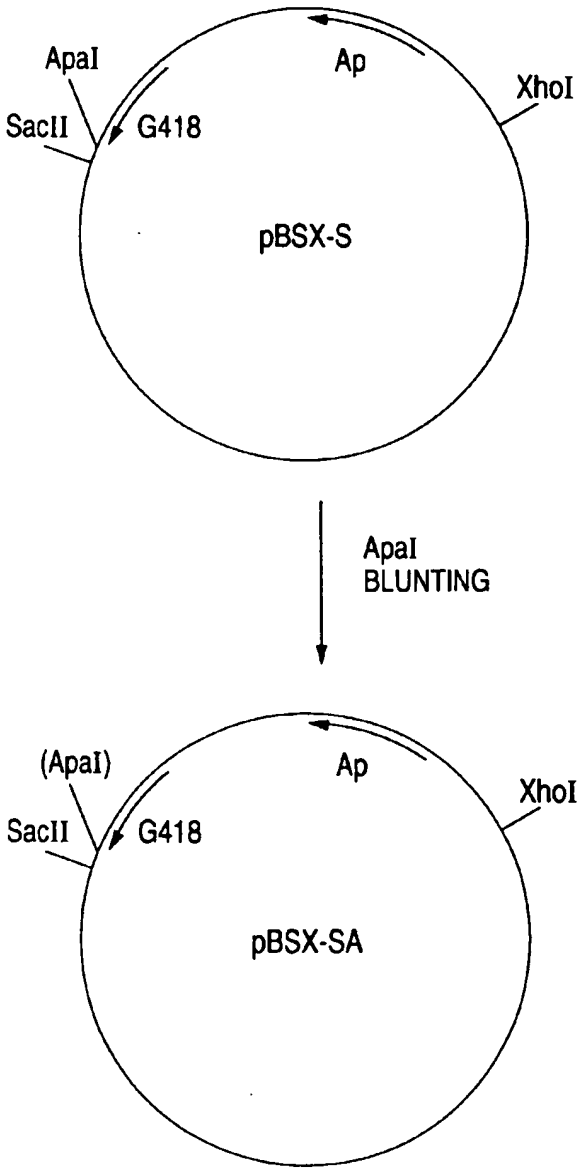


FIG. 11

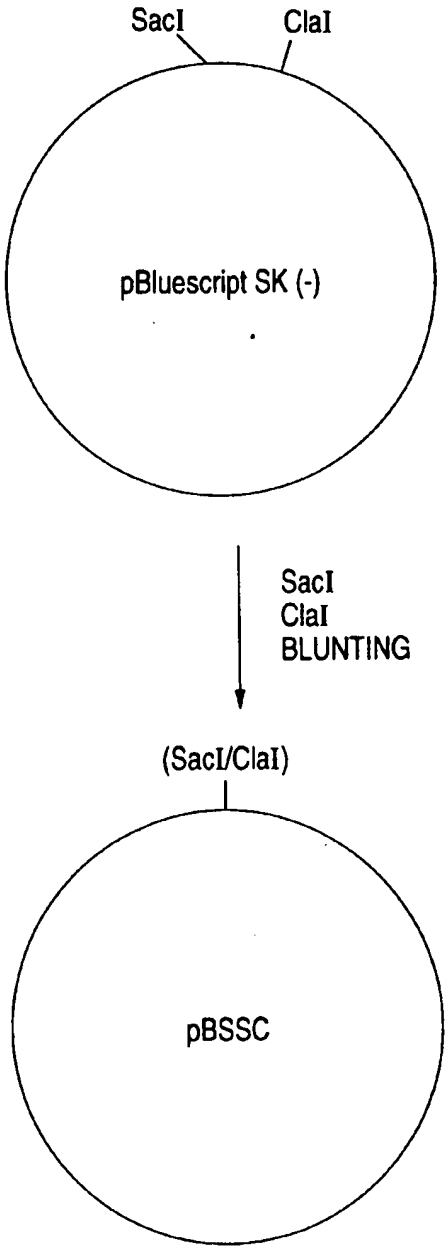


FIG. 12

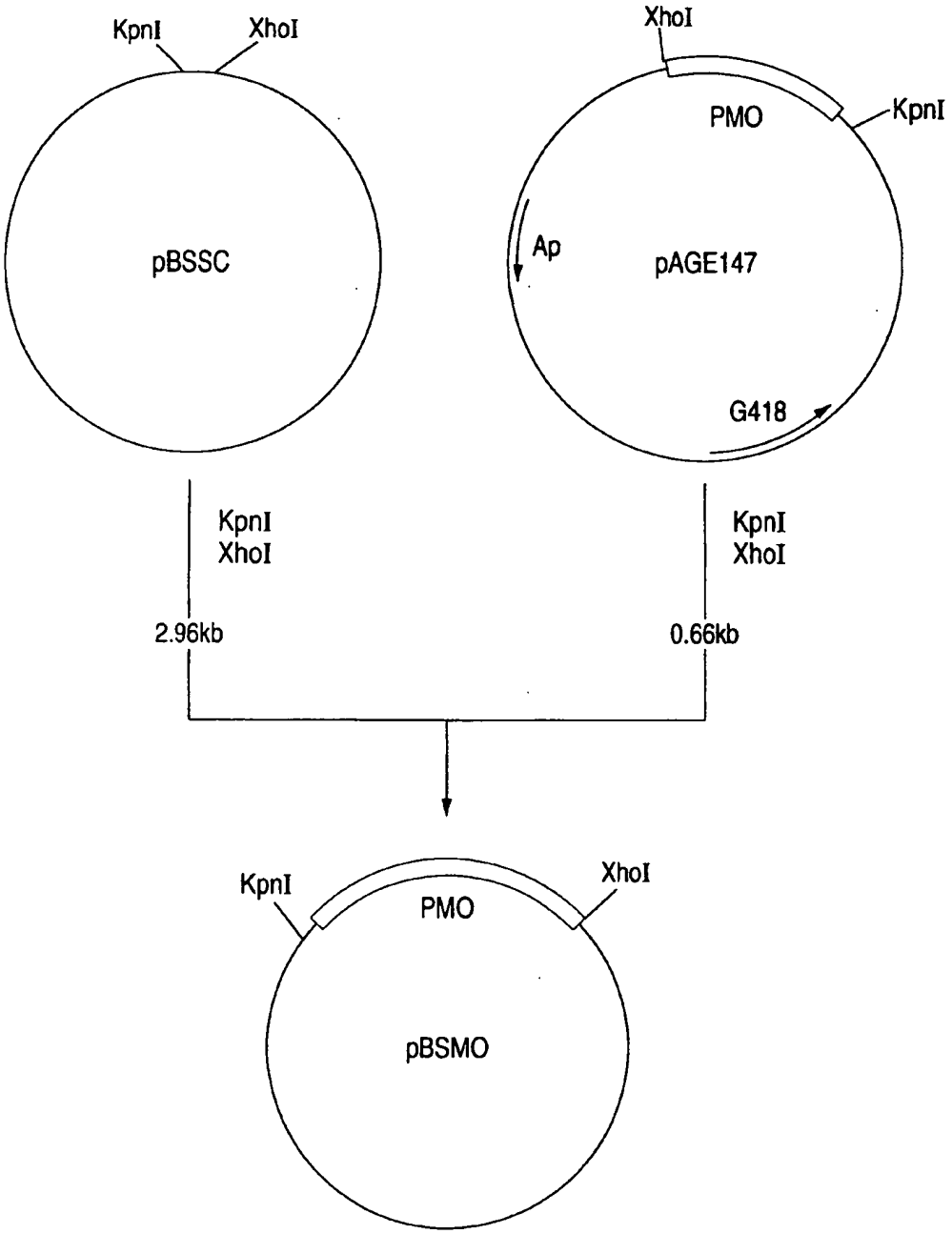


FIG. 13

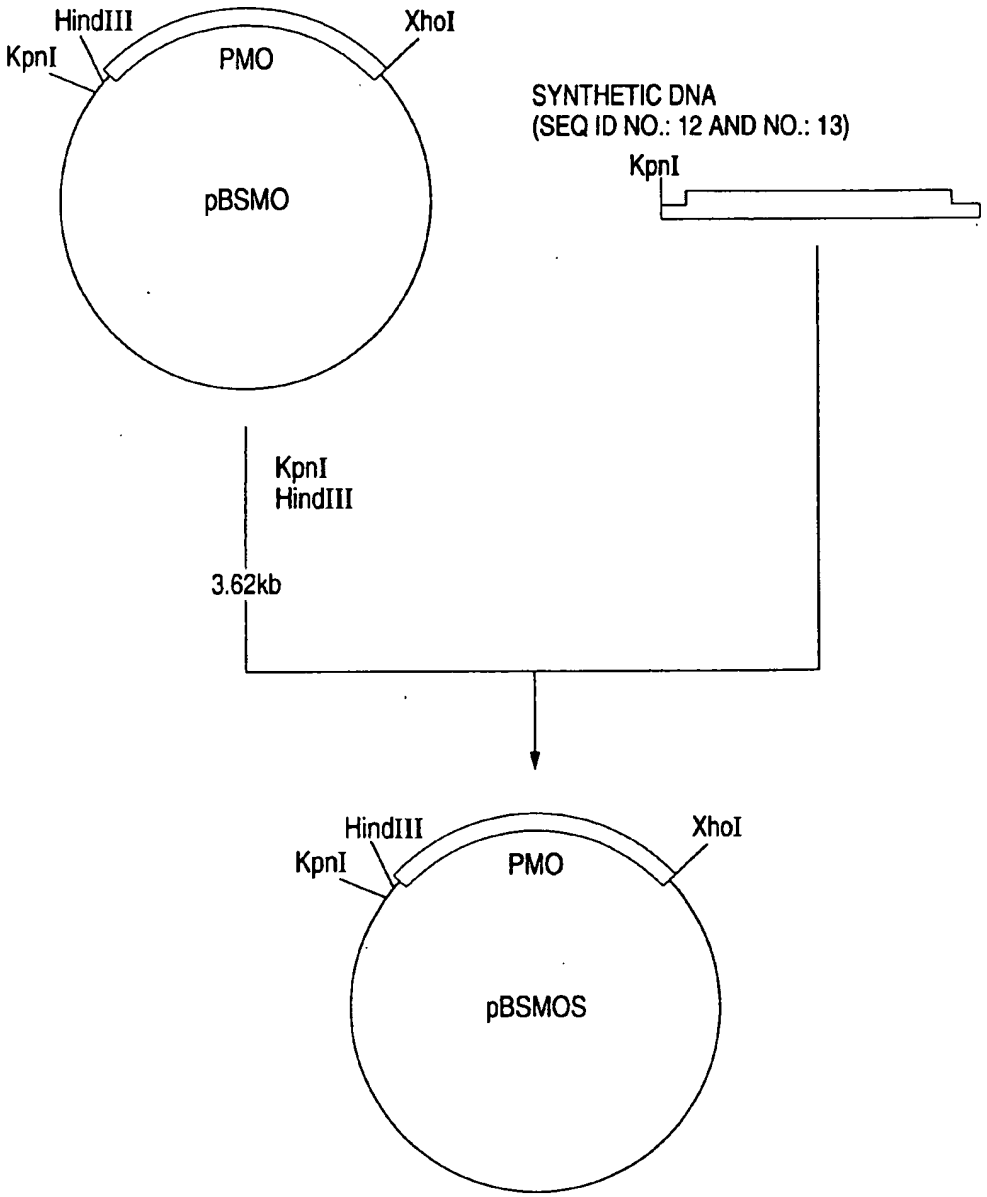


FIG. 14

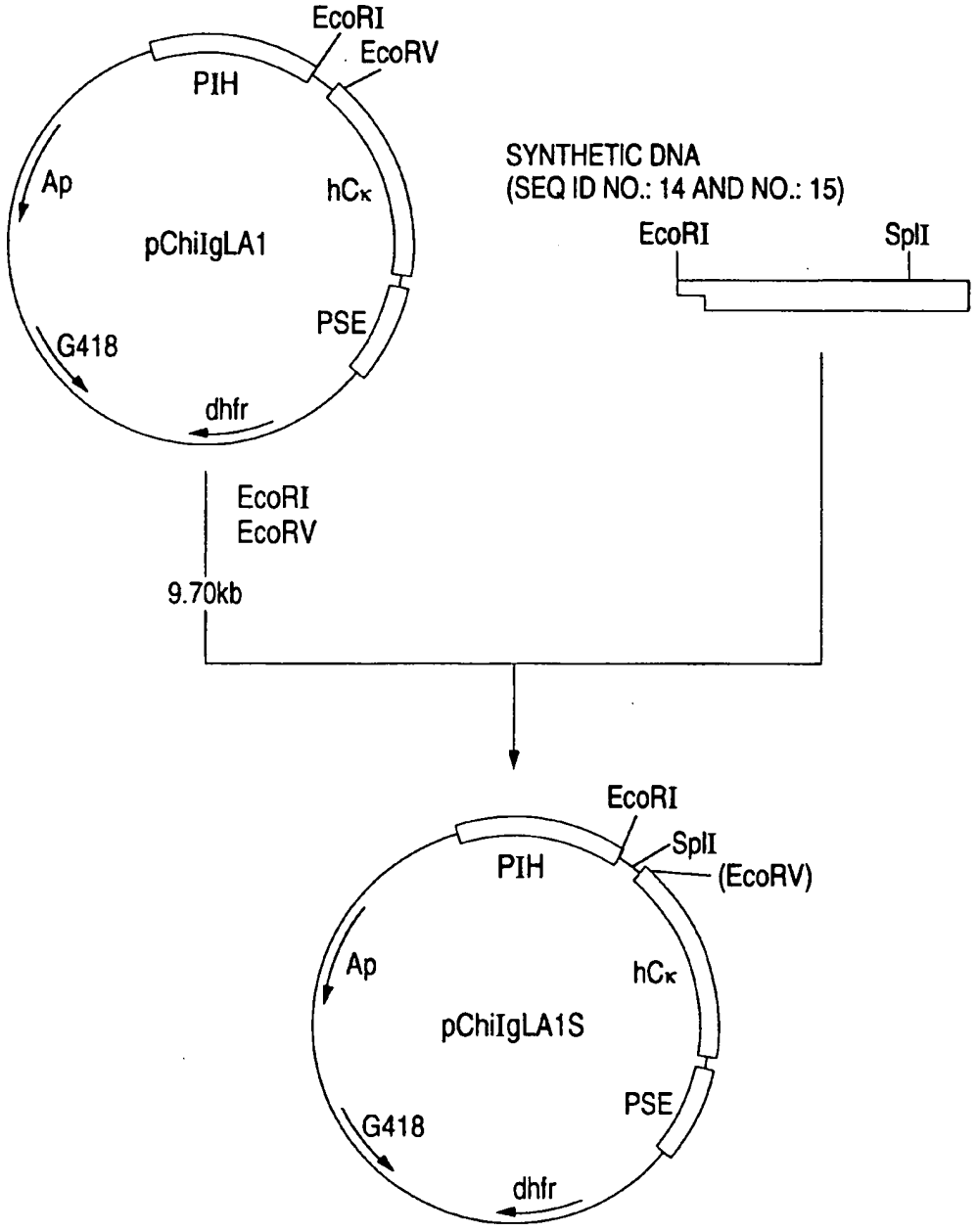


FIG. 15

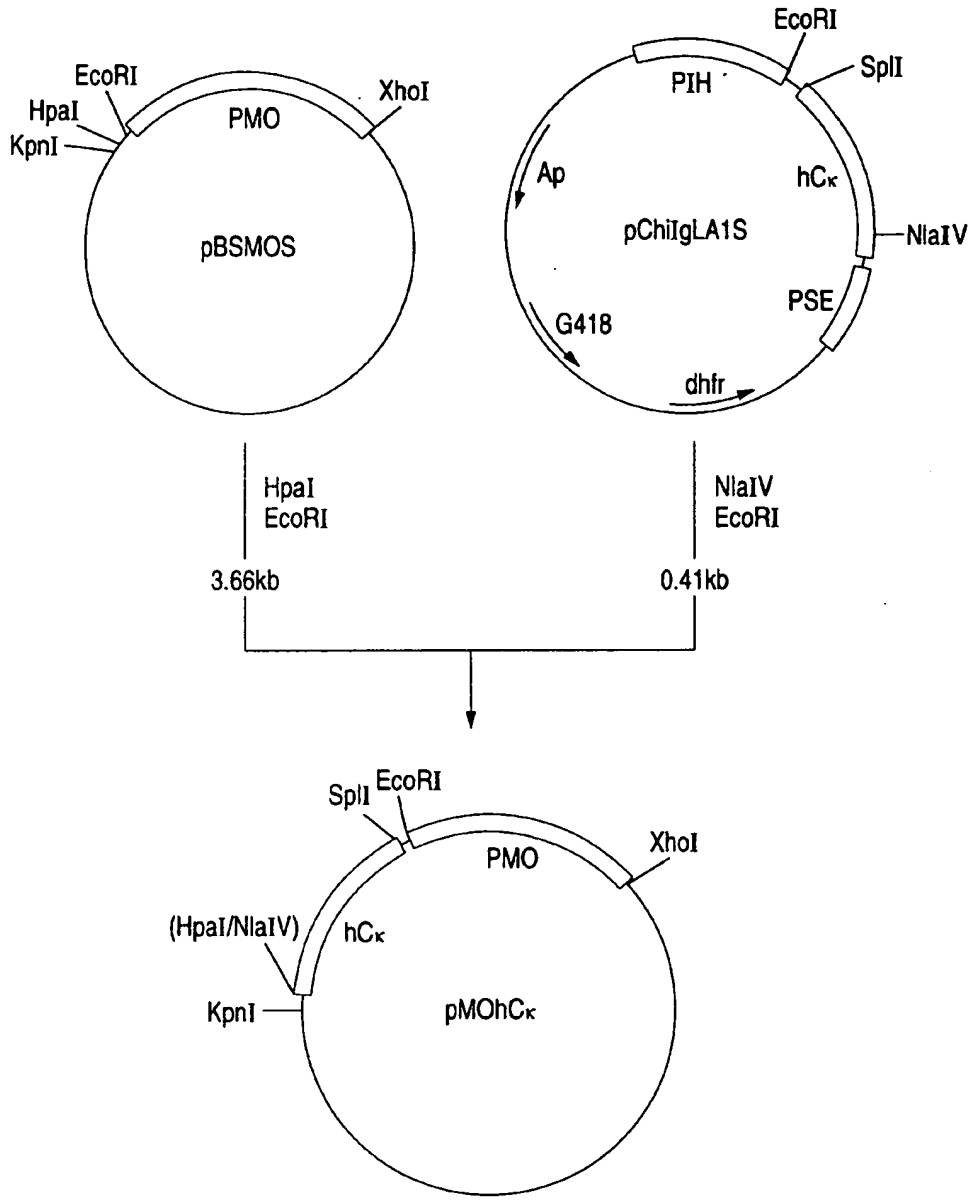


FIG. 16

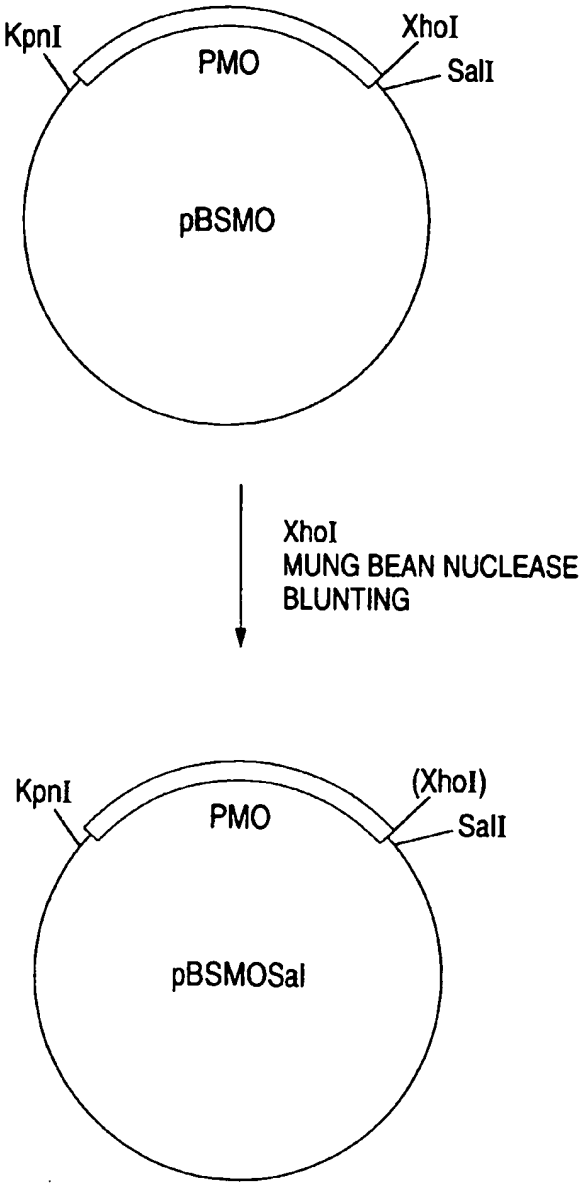


FIG. 17

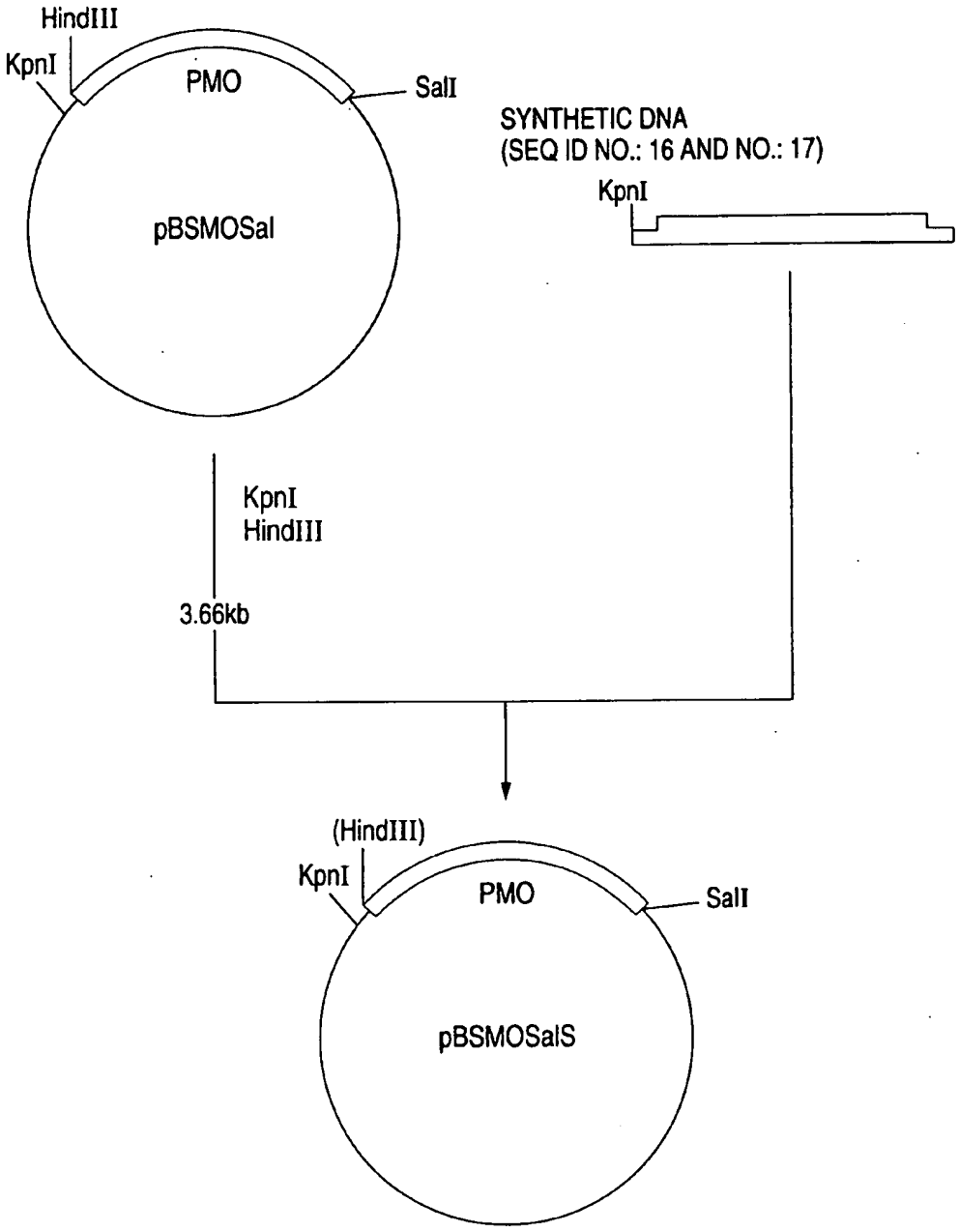


FIG. 18

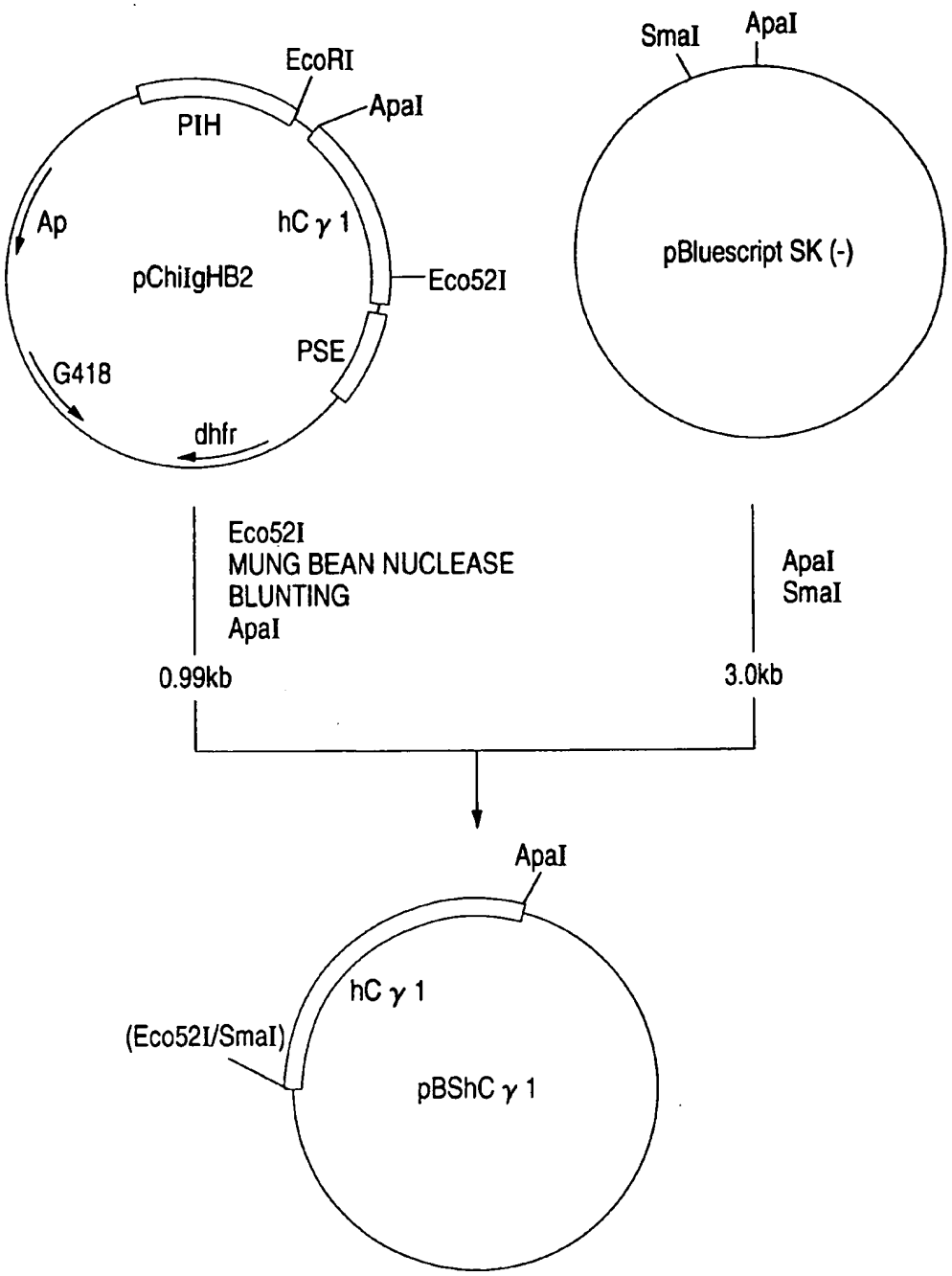


FIG. 19

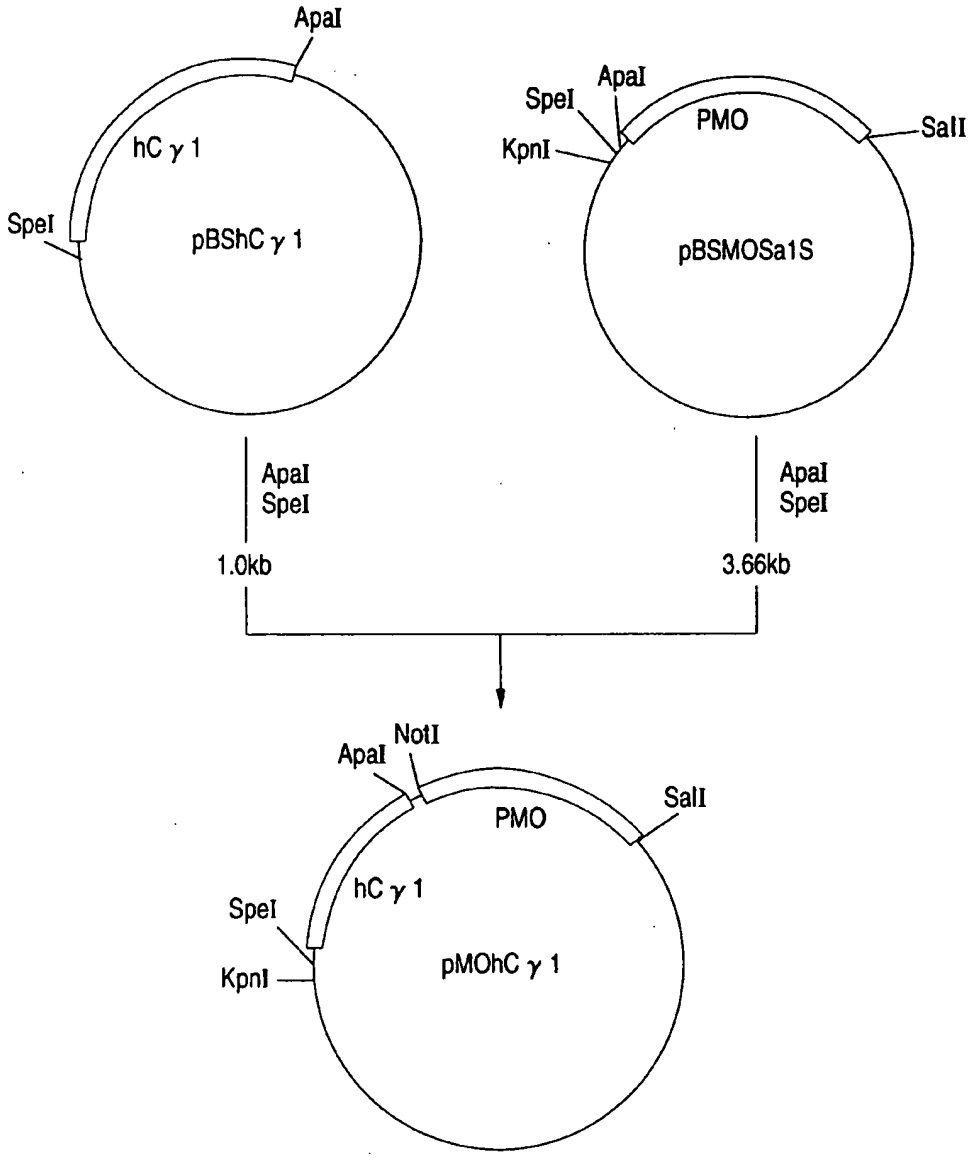


FIG. 20

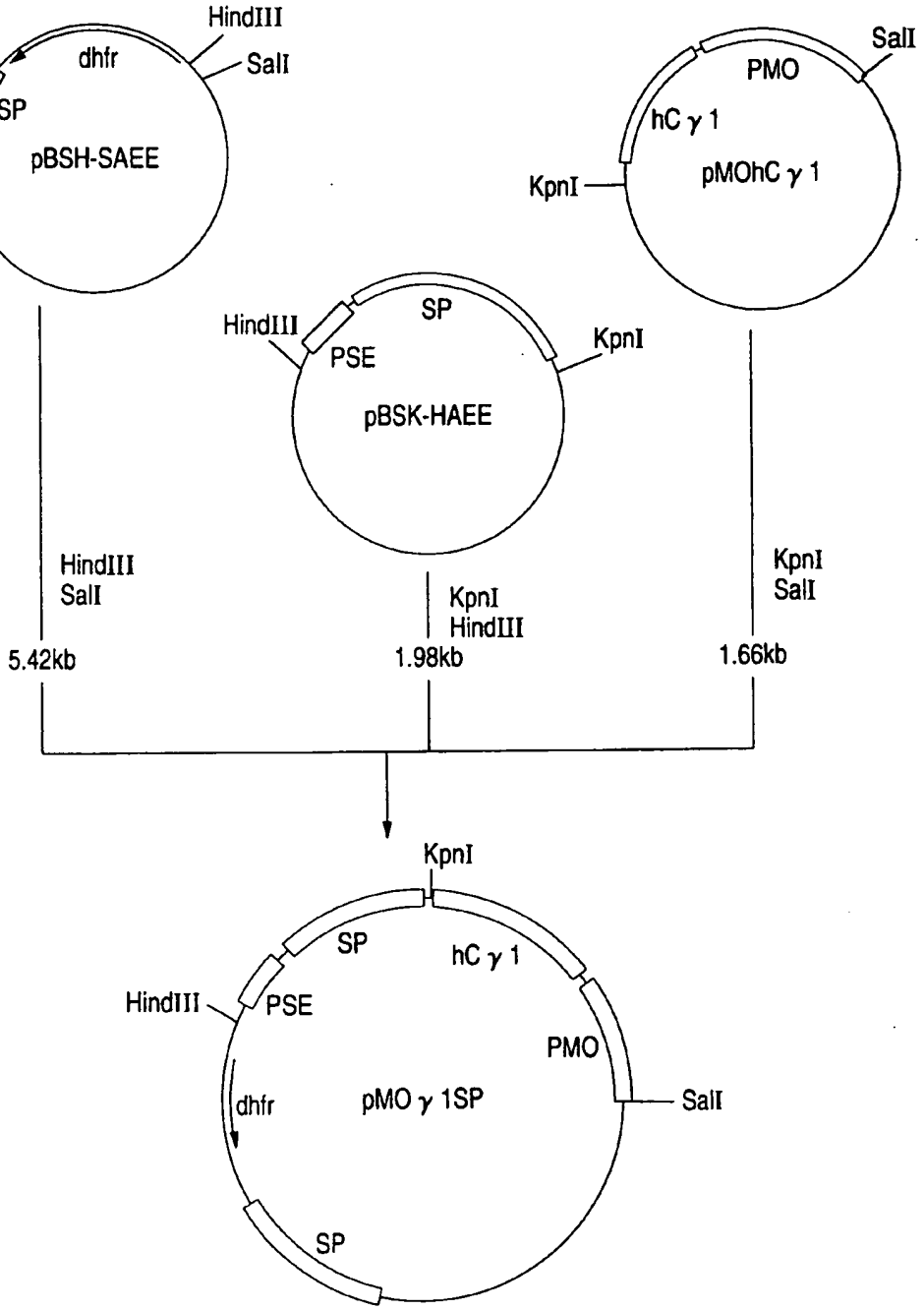


FIG. 21

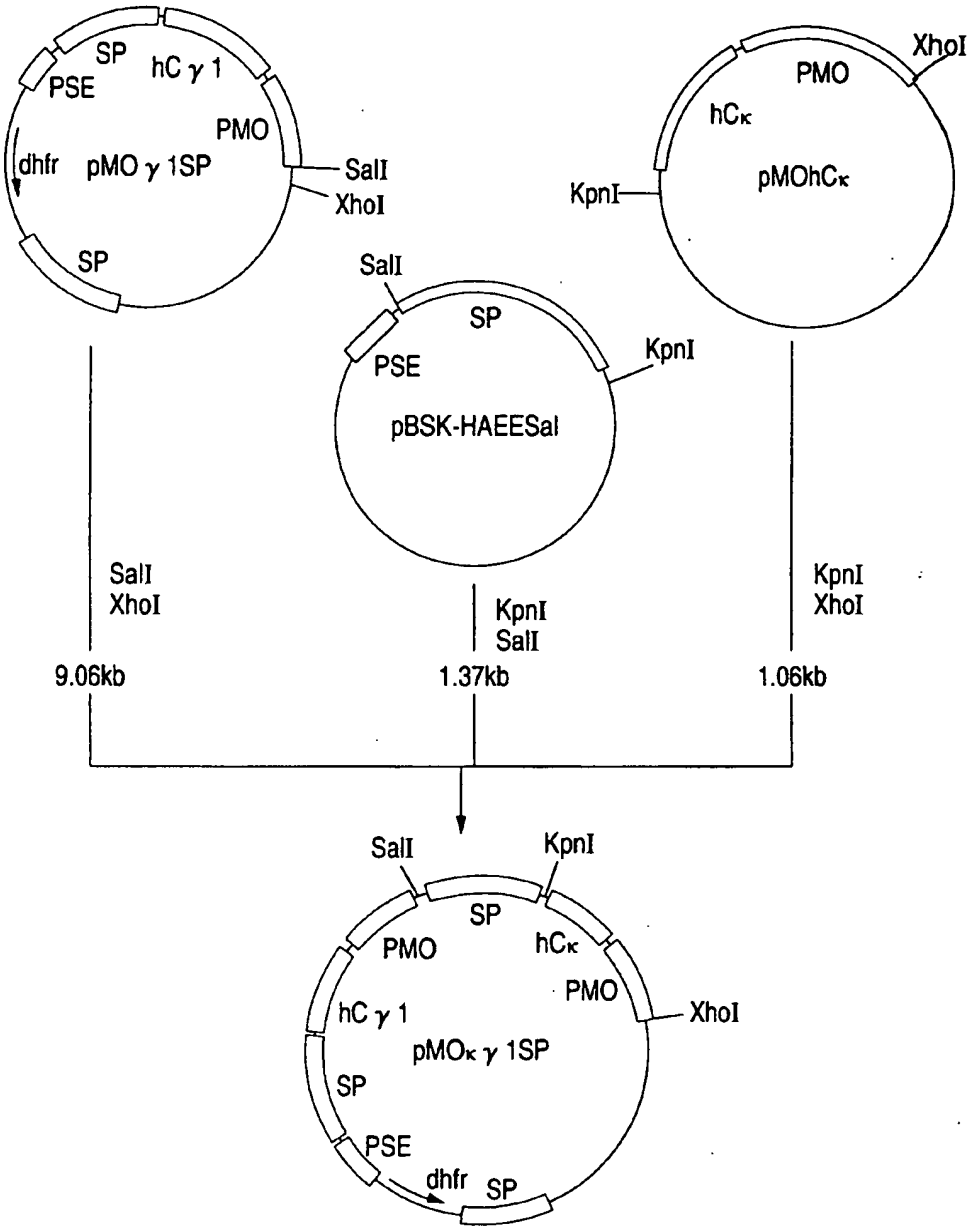


FIG. 22

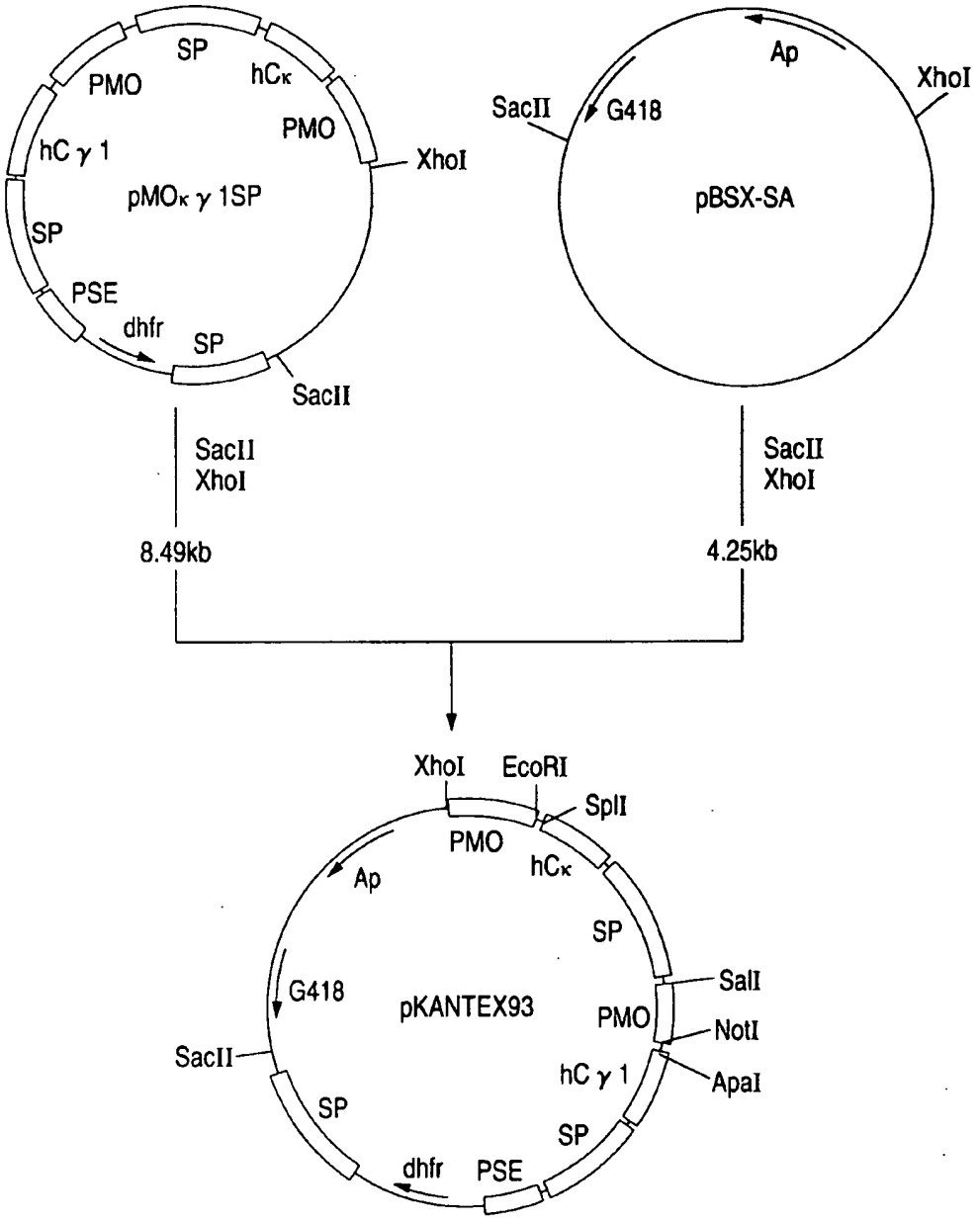


FIG. 23

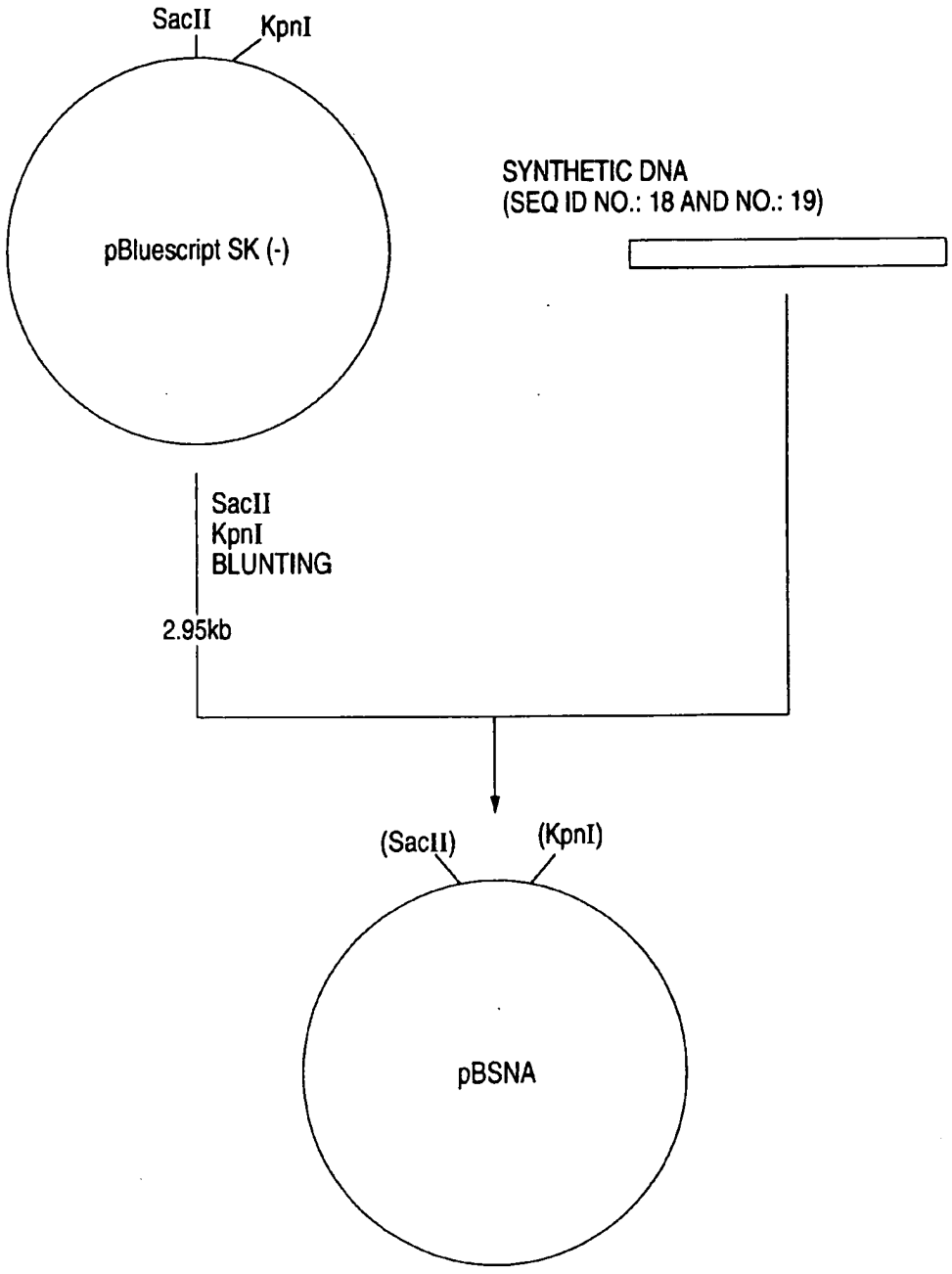


FIG. 24

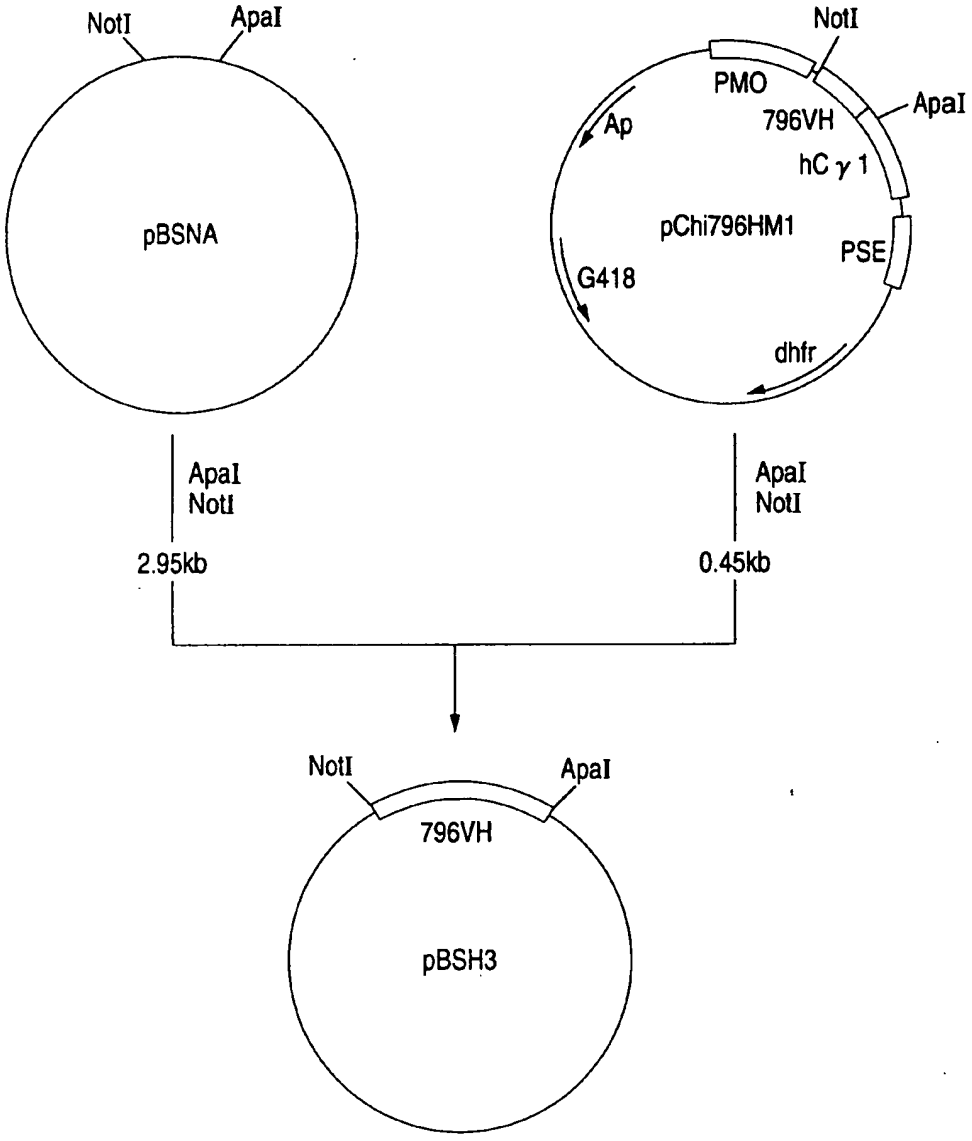


FIG. 25

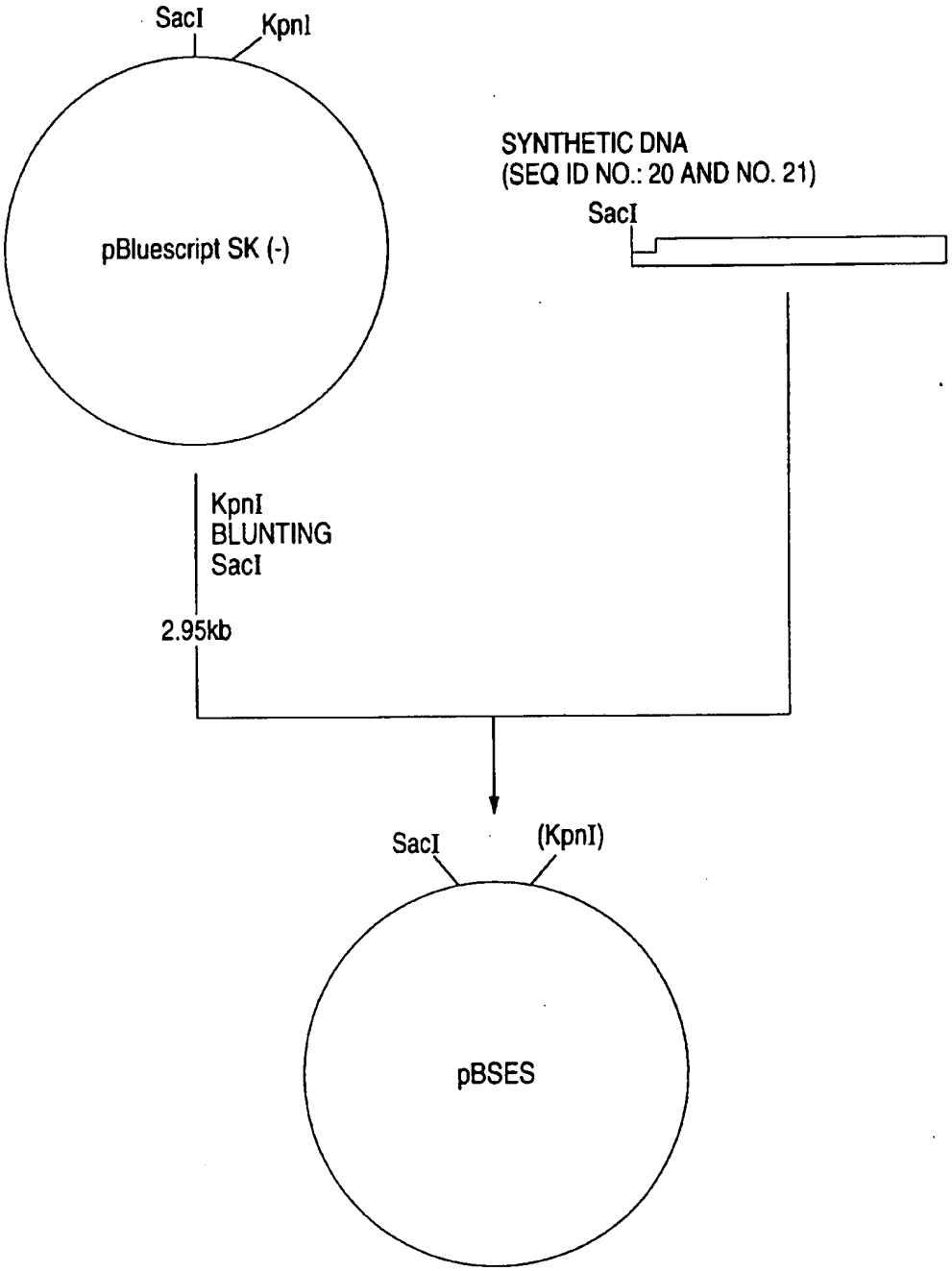


FIG. 26

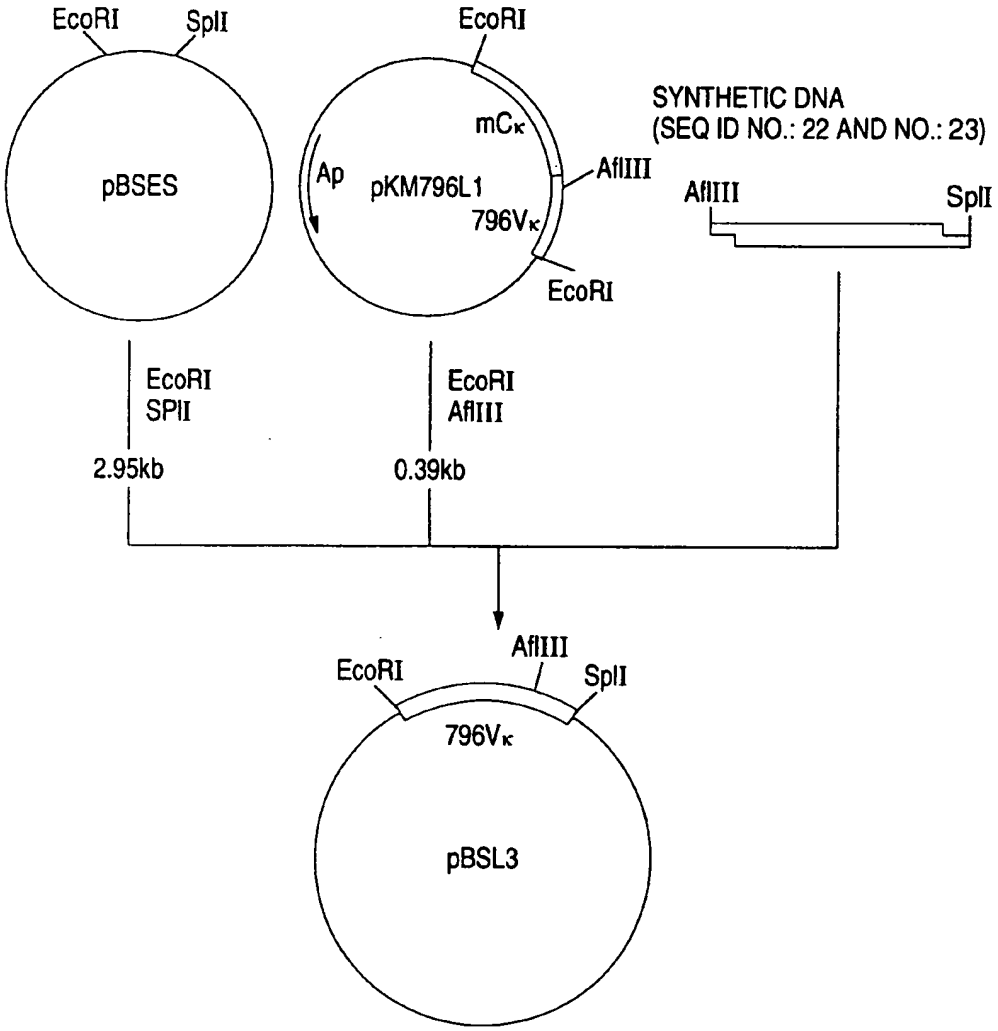


FIG. 27

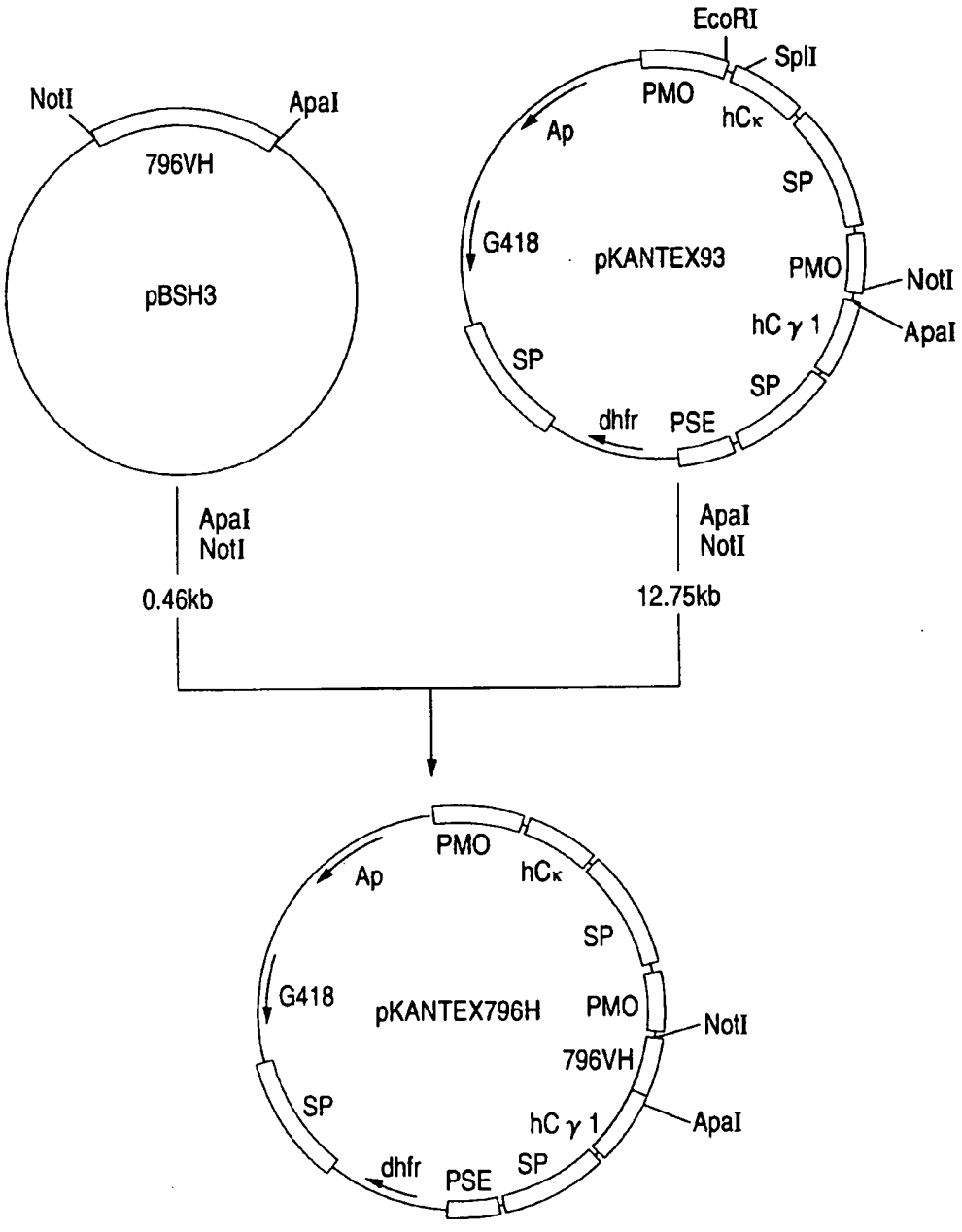


FIG. 28

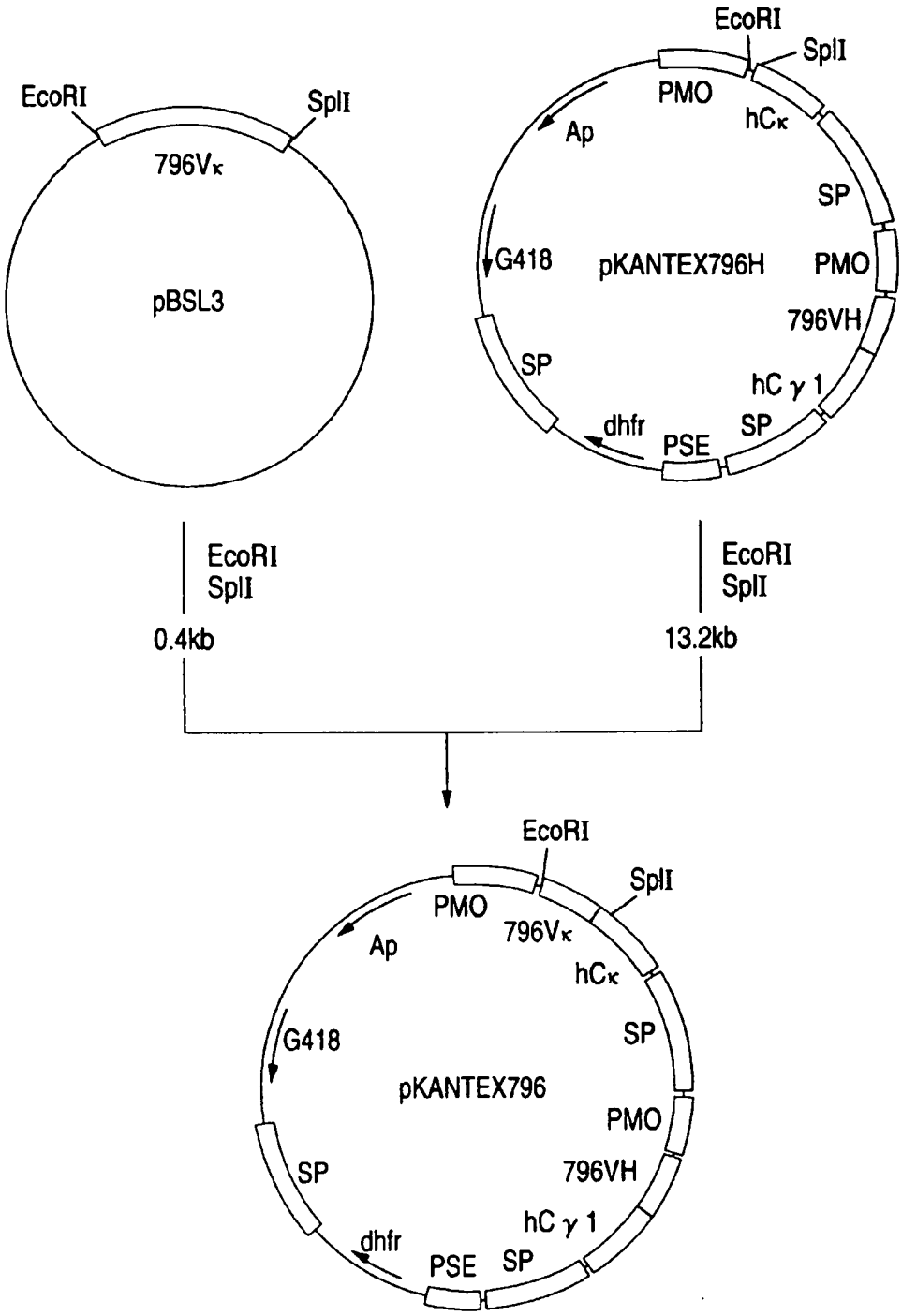


FIG. 29

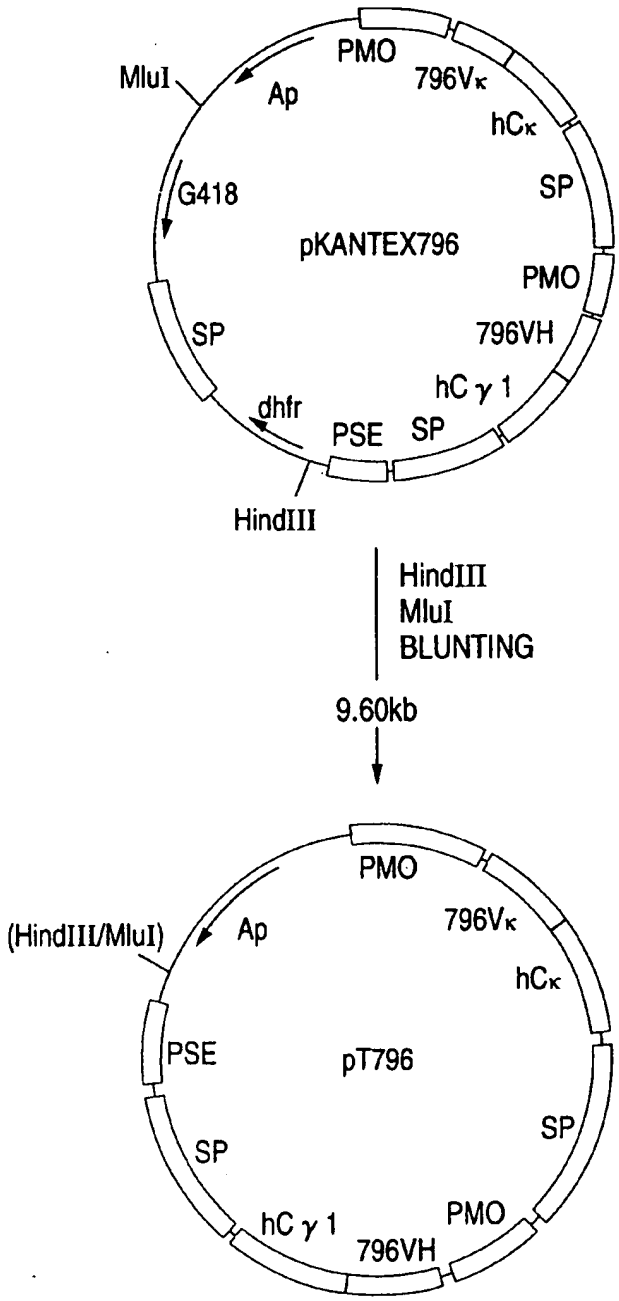


FIG. 30

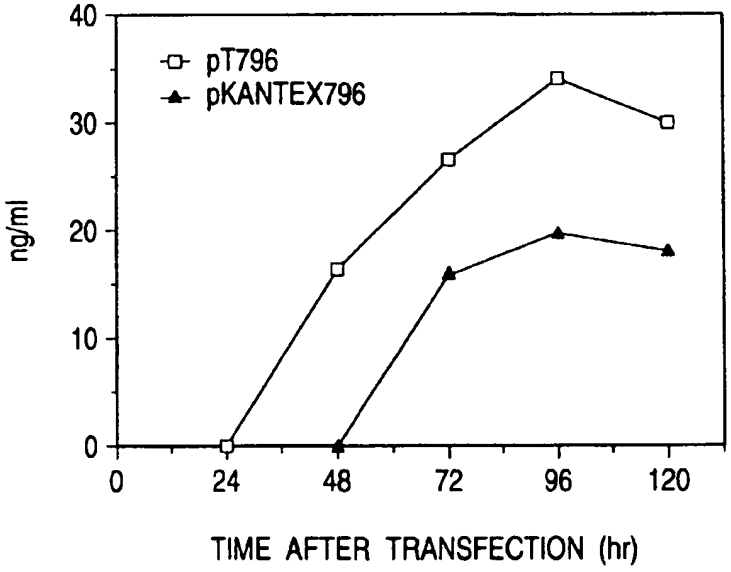

40
30
20
10
0
ng/ml
pT796
pKANTEX796
0
24
48
72
96
120
TIME AFTER TRANSFECTION (hr)

FIG. 31

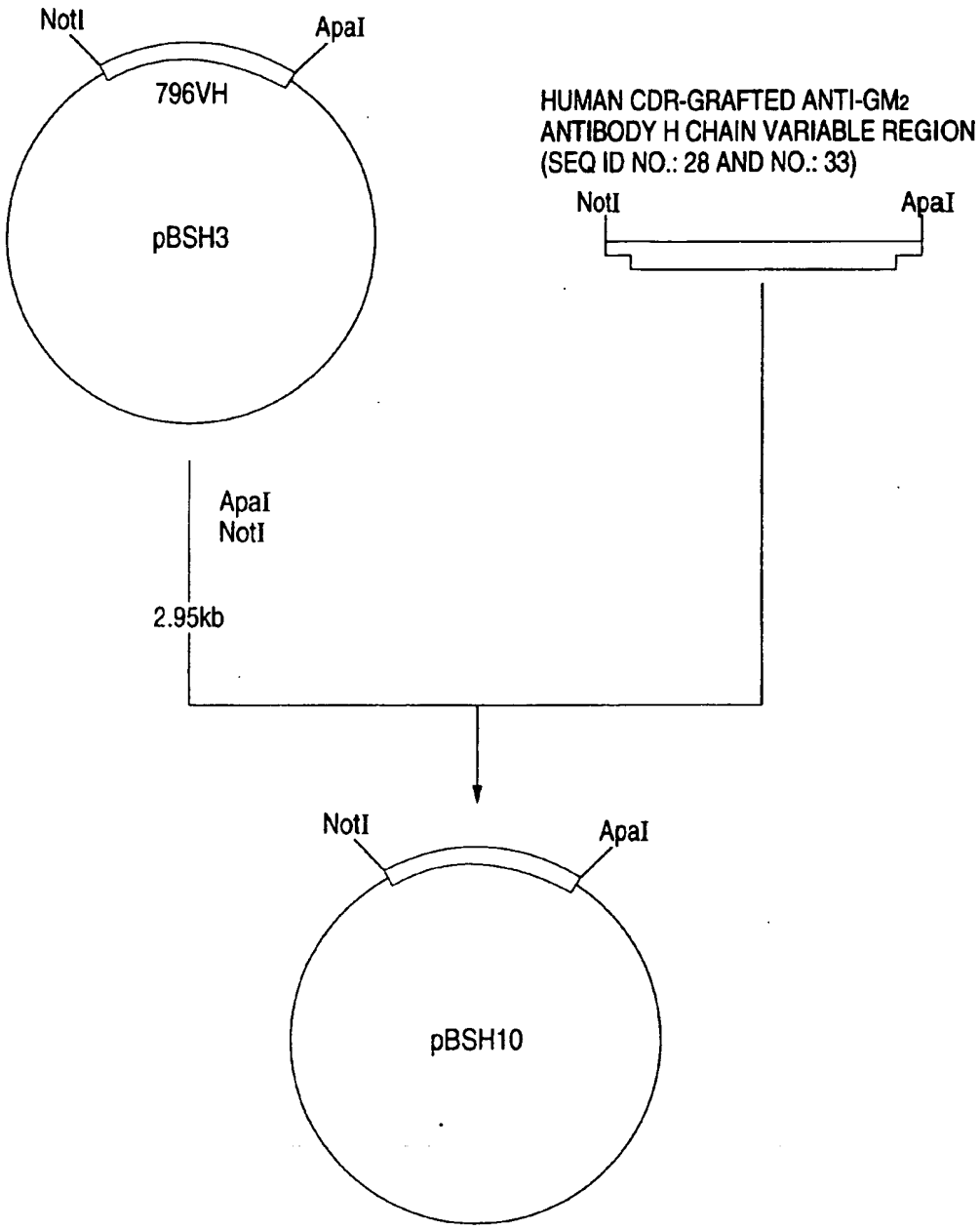


FIG. 32

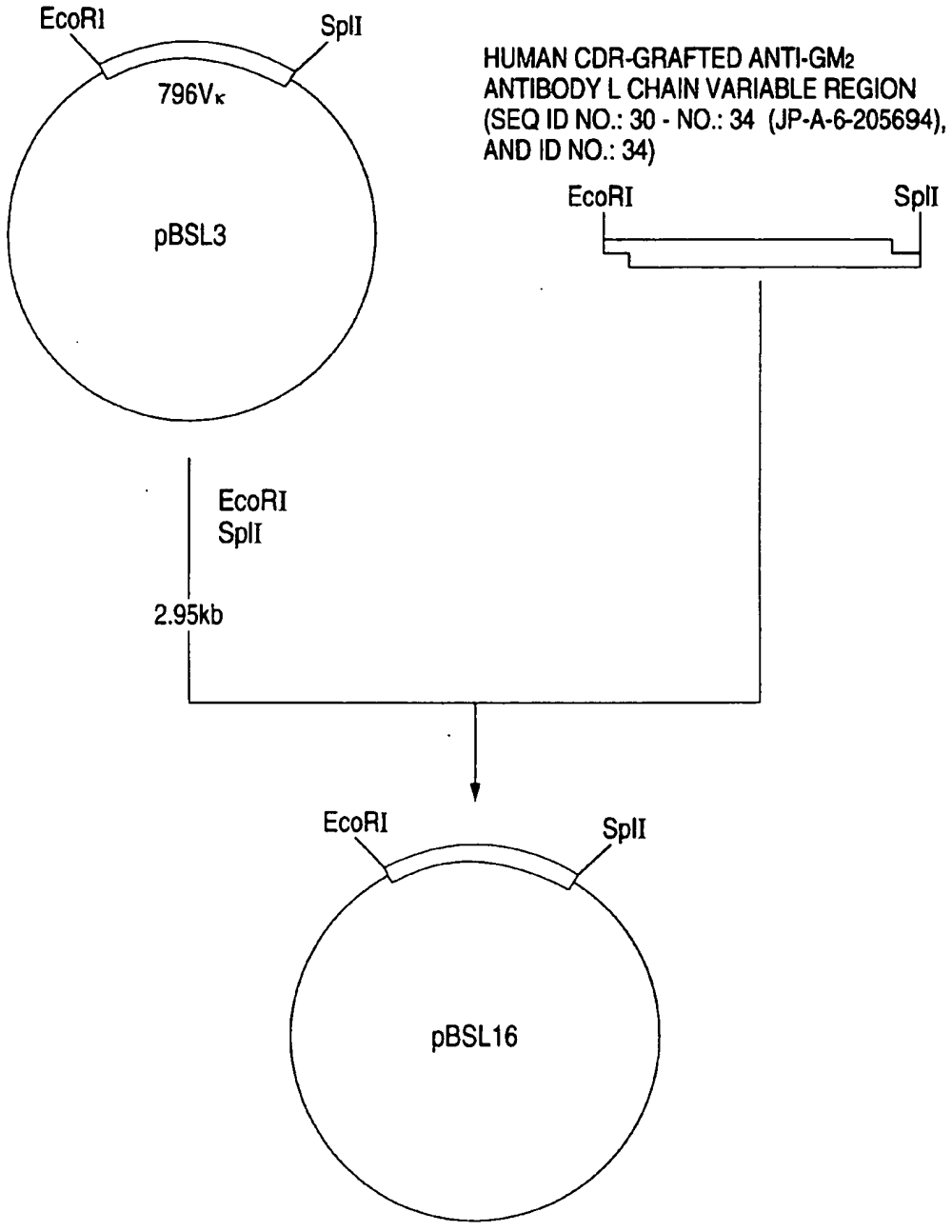


FIG. 33

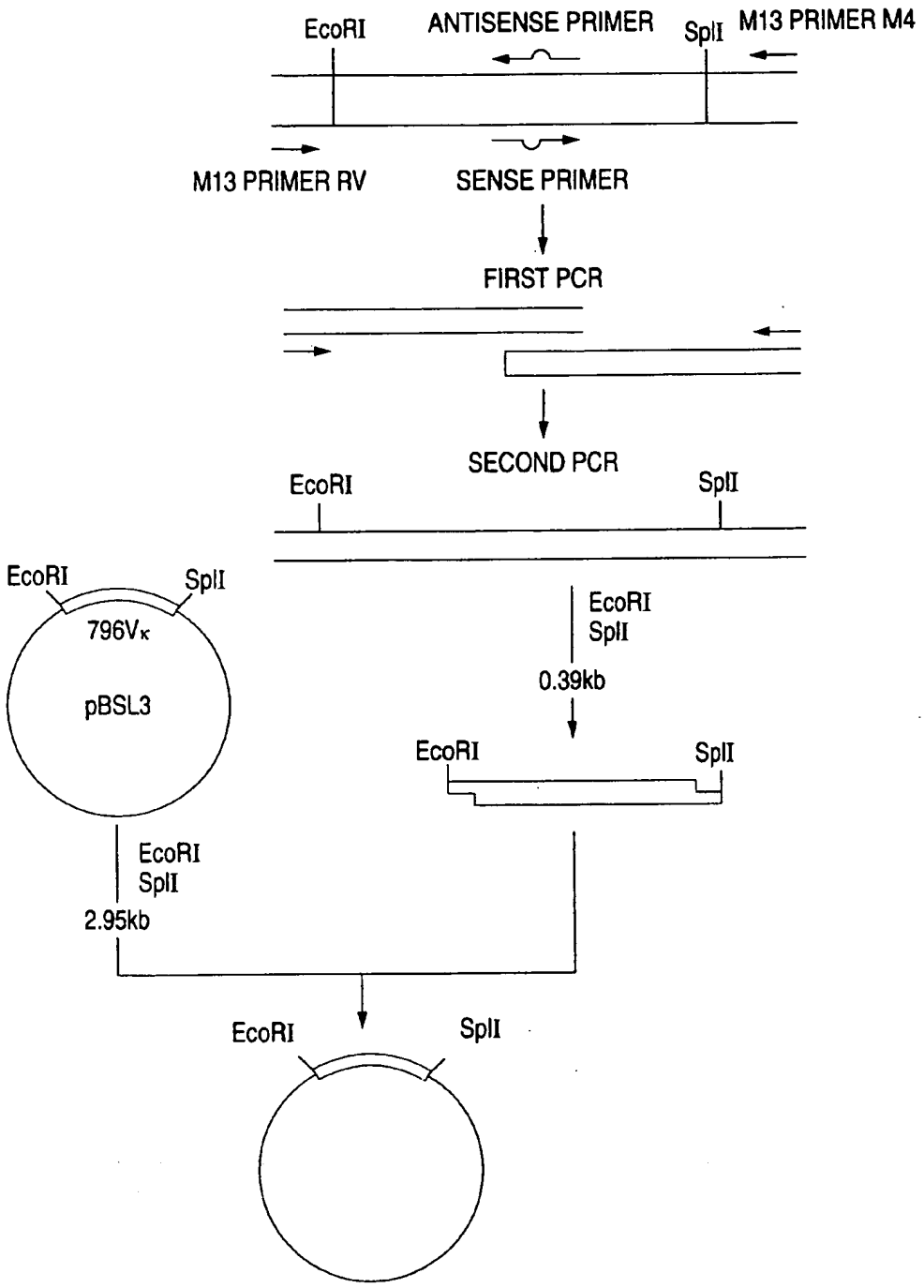


FIG. 34

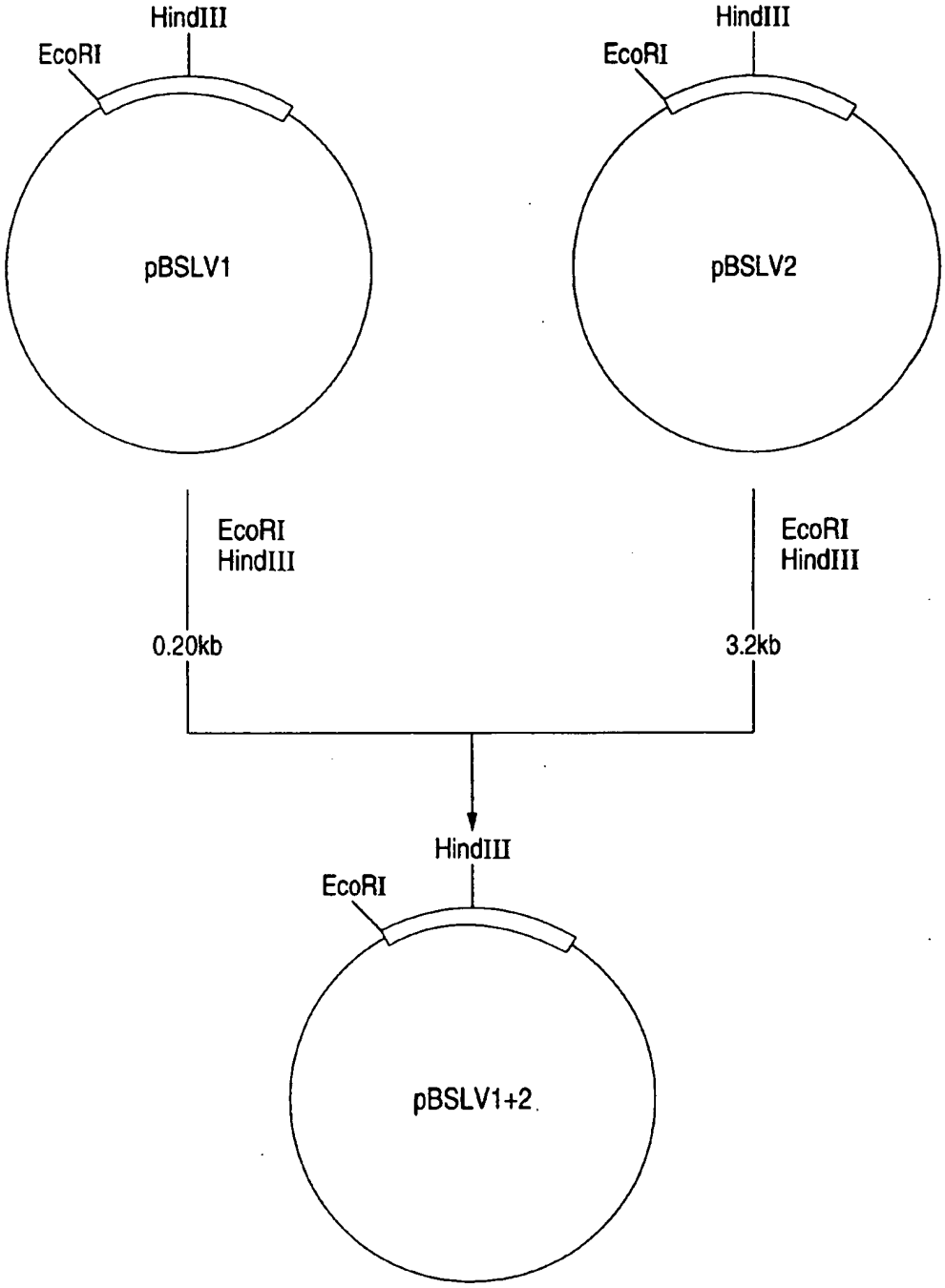


FIG. 35

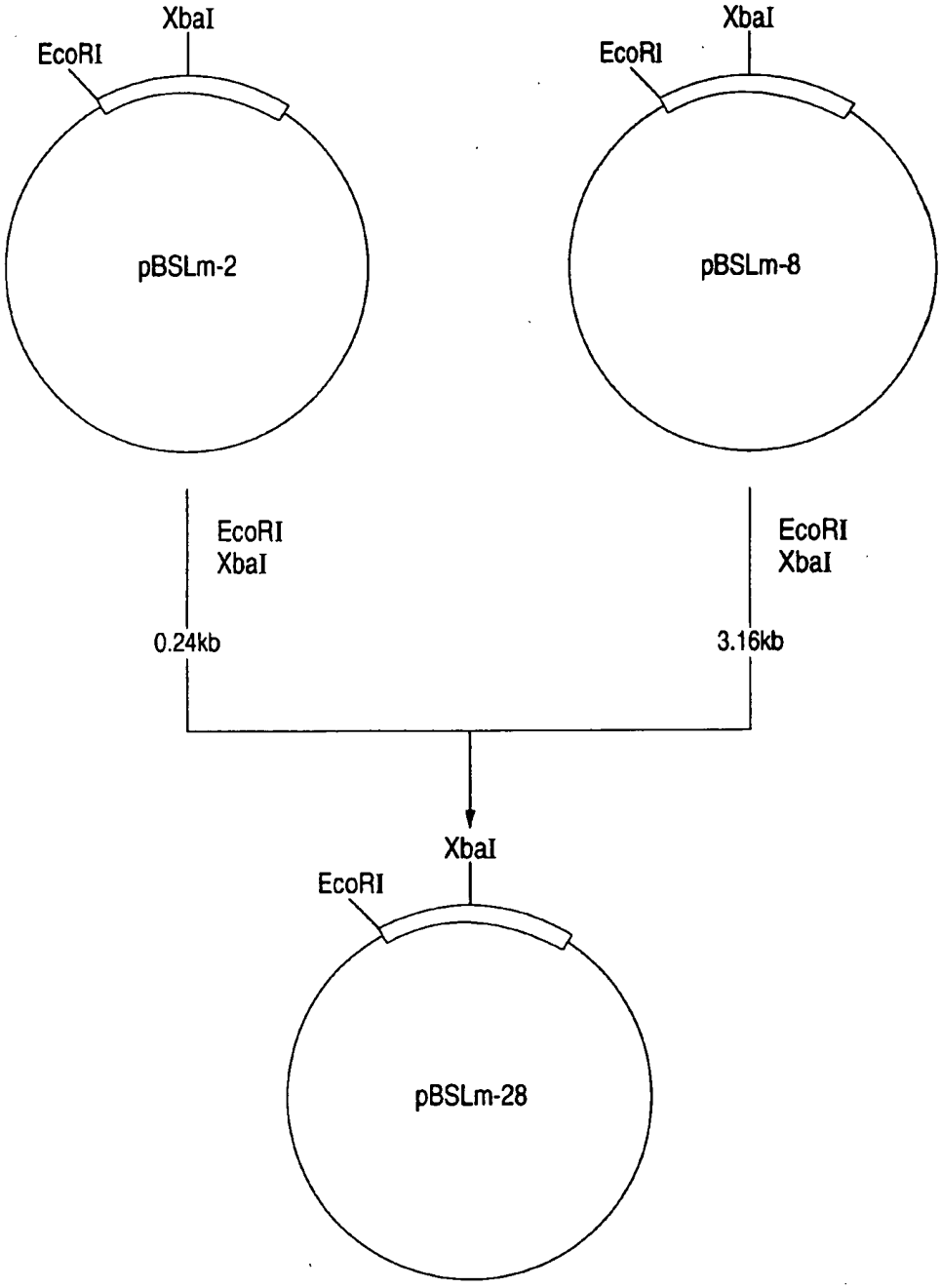


FIG. 36

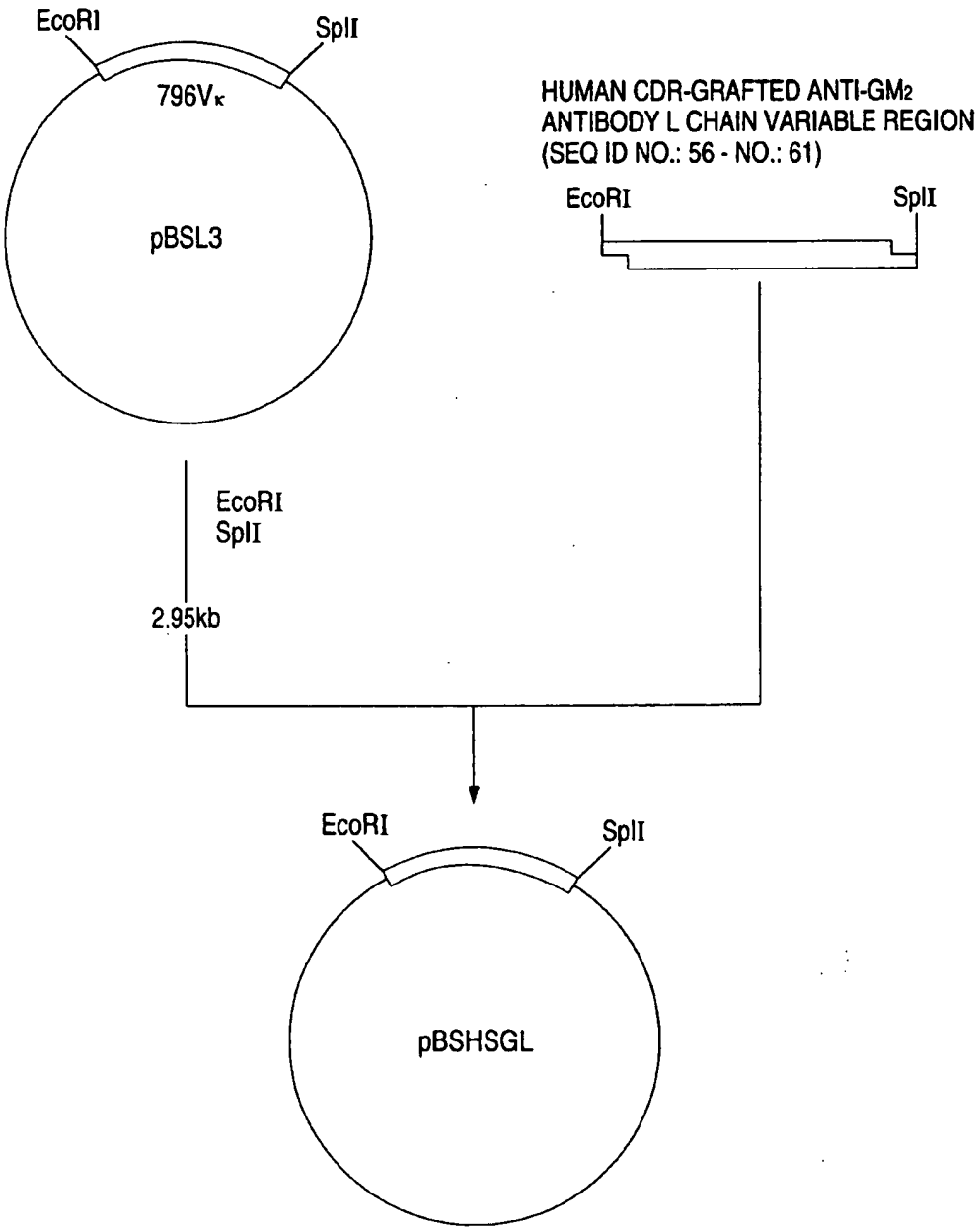


FIG. 37

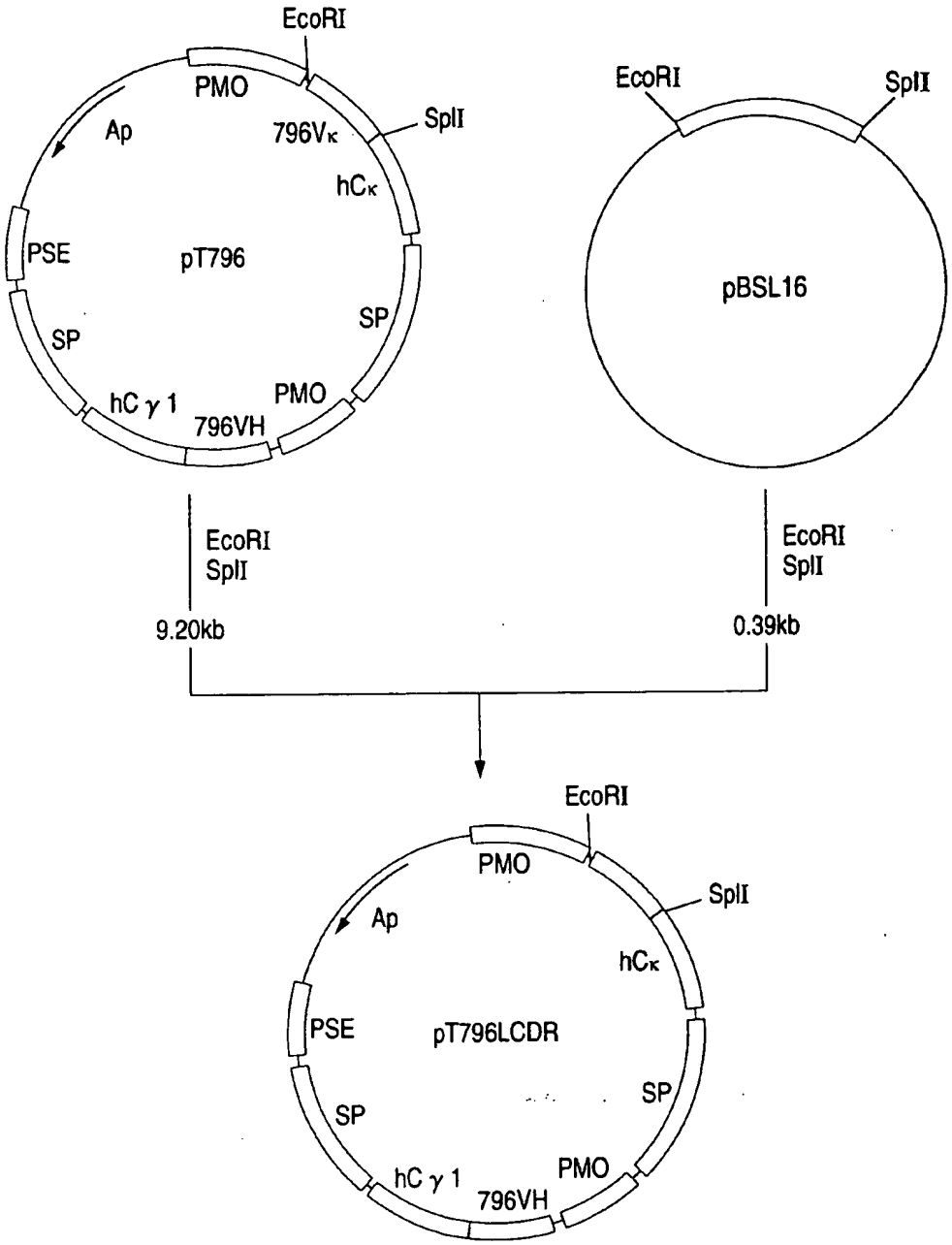


FIG. 38

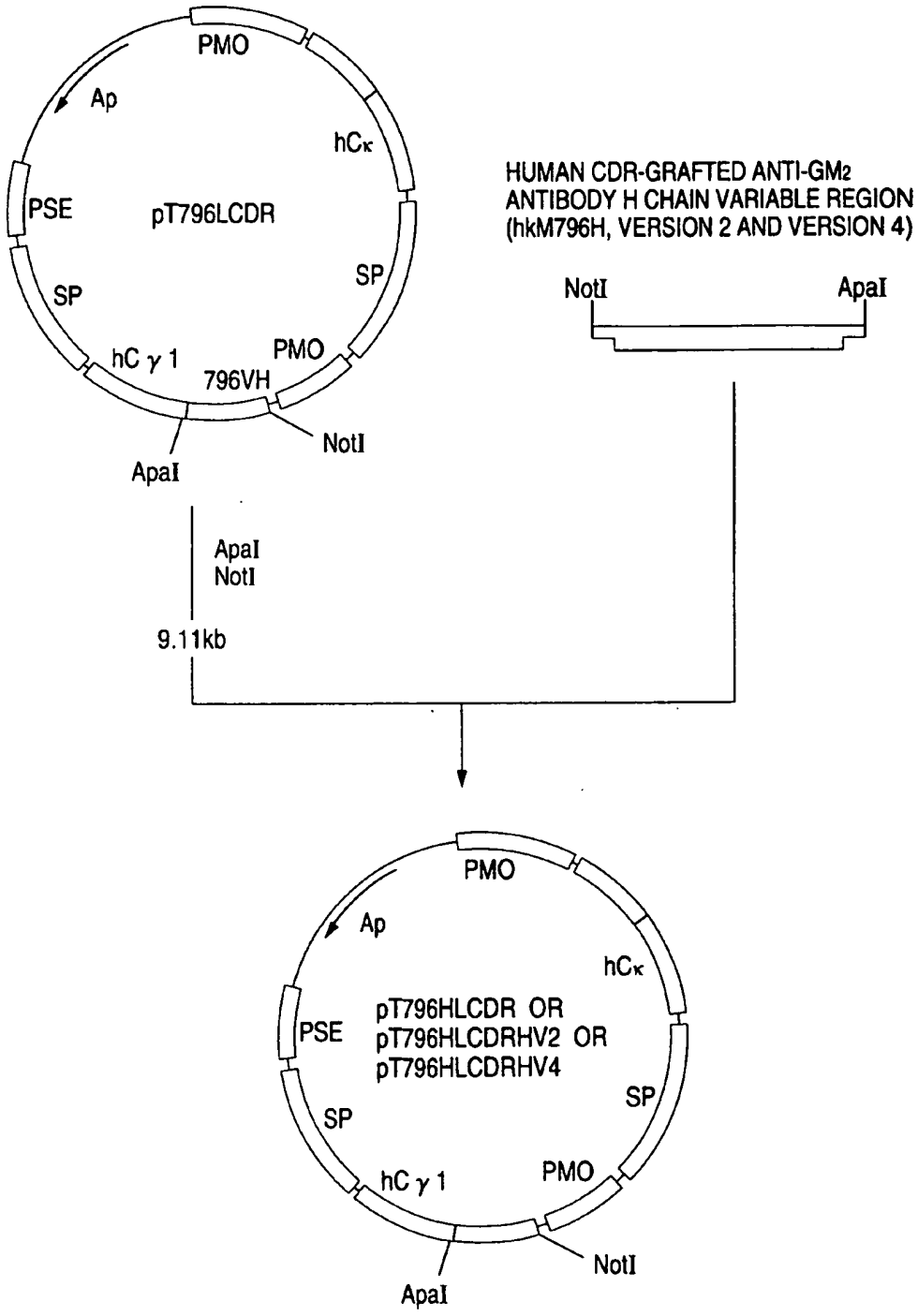


FIG. 39

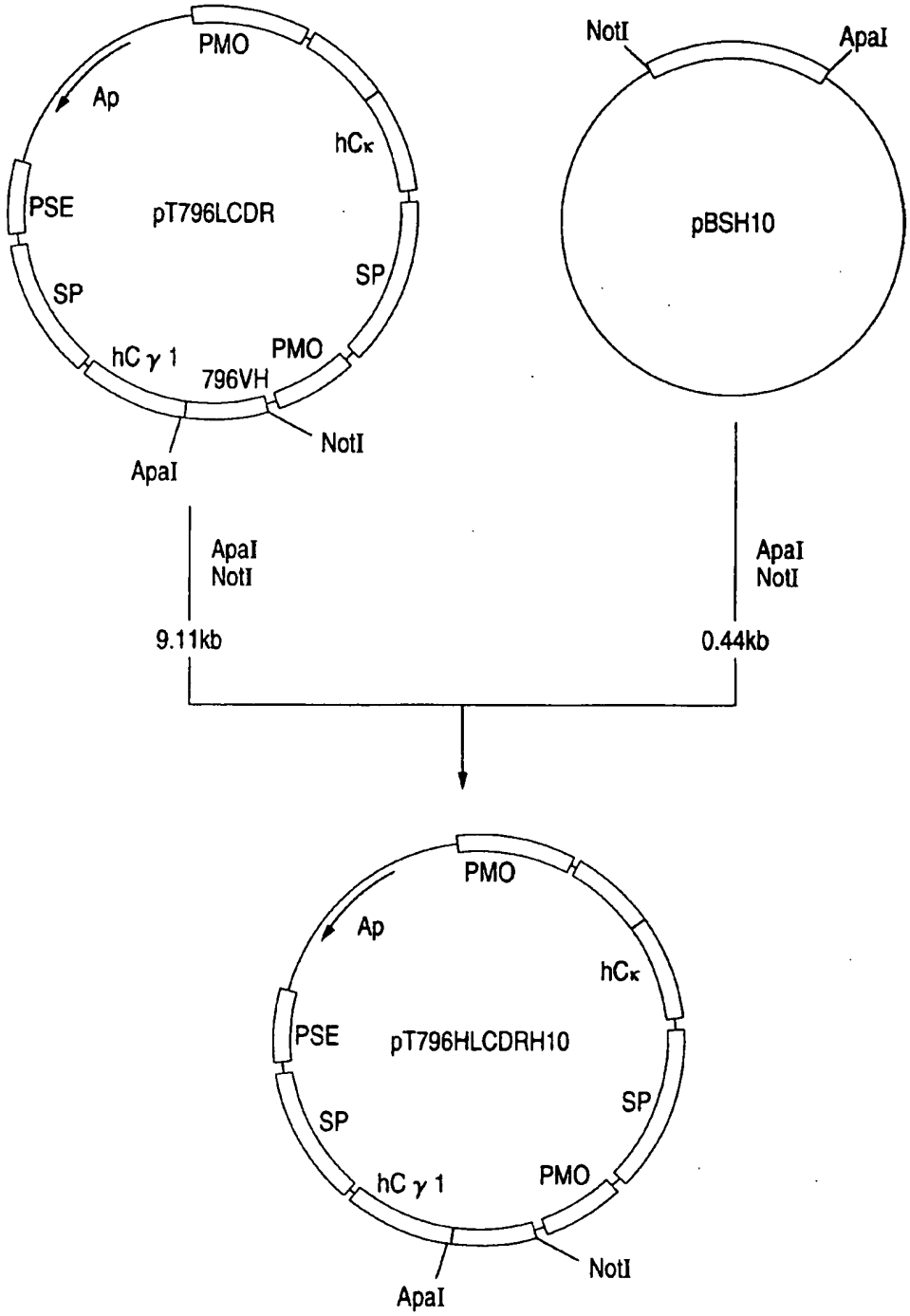


FIG. 40

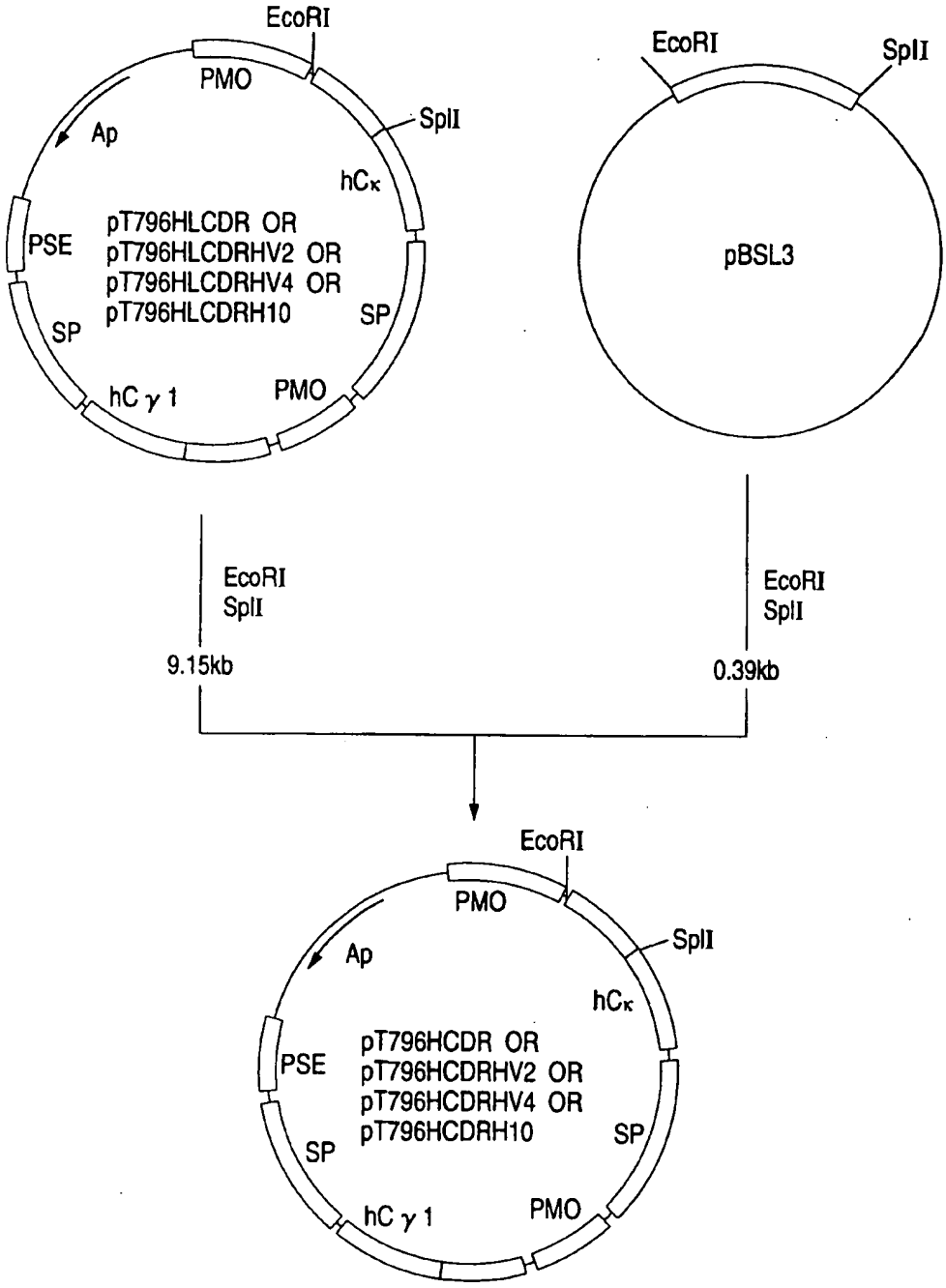


FIG. 41

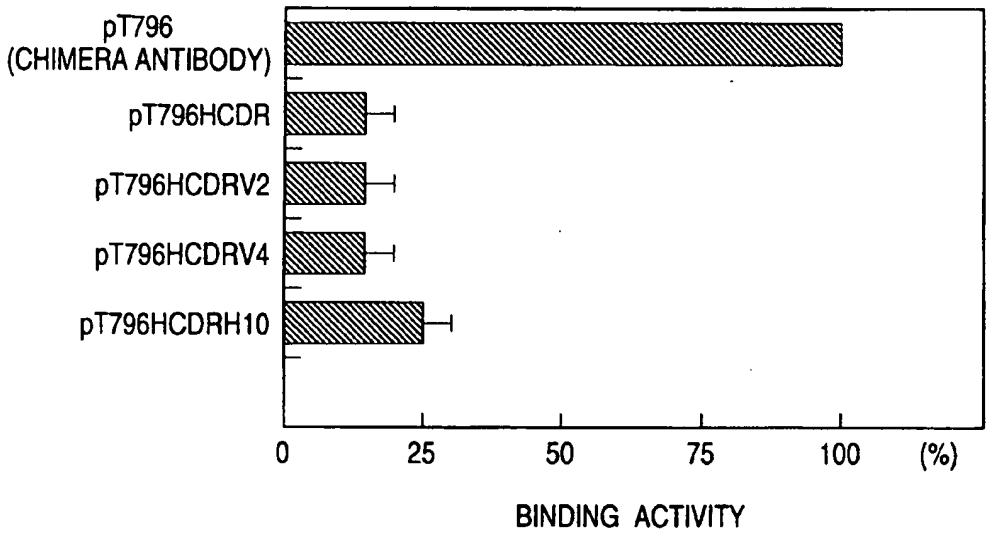


FIG. 42

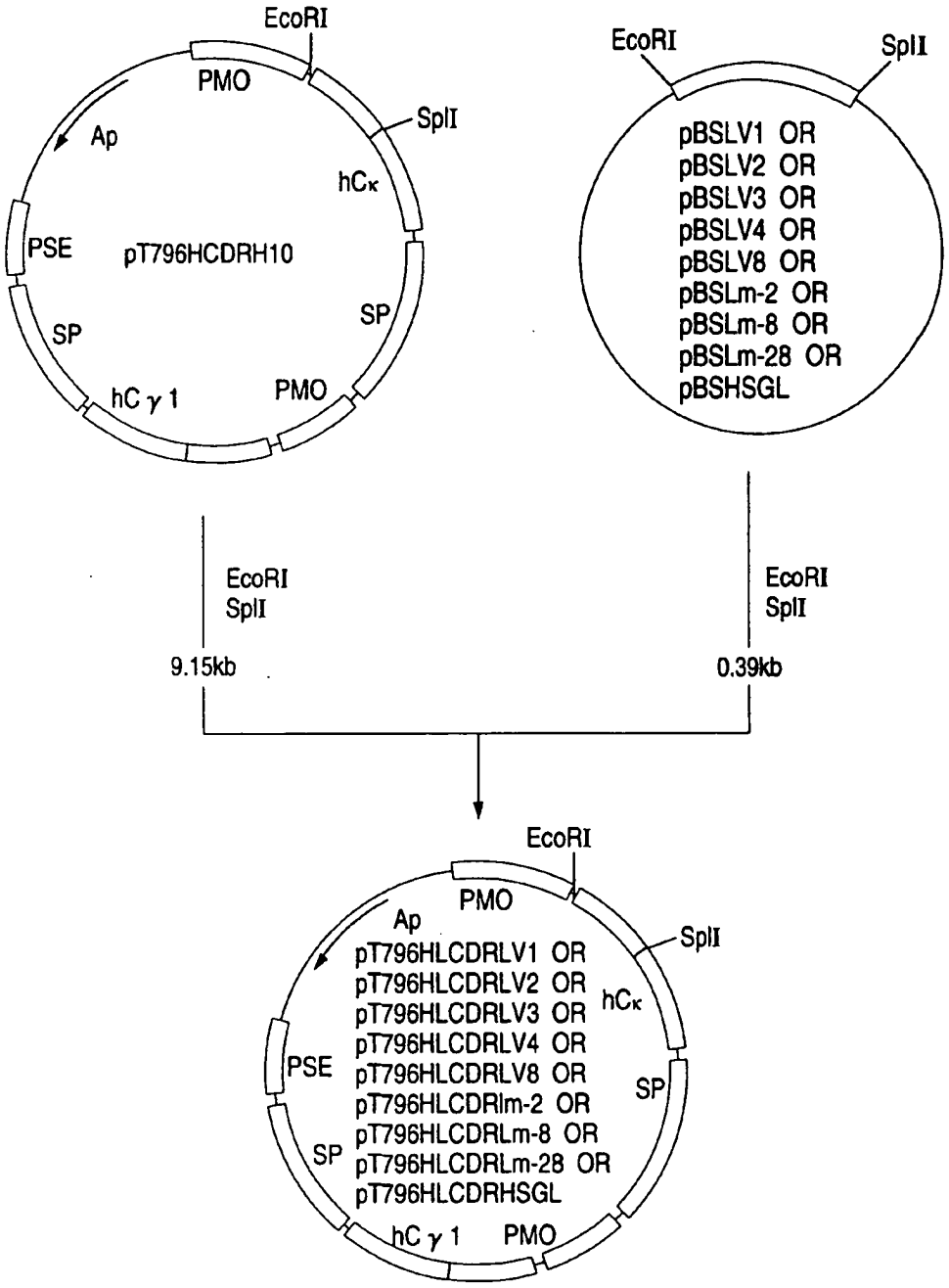


FIG. 43

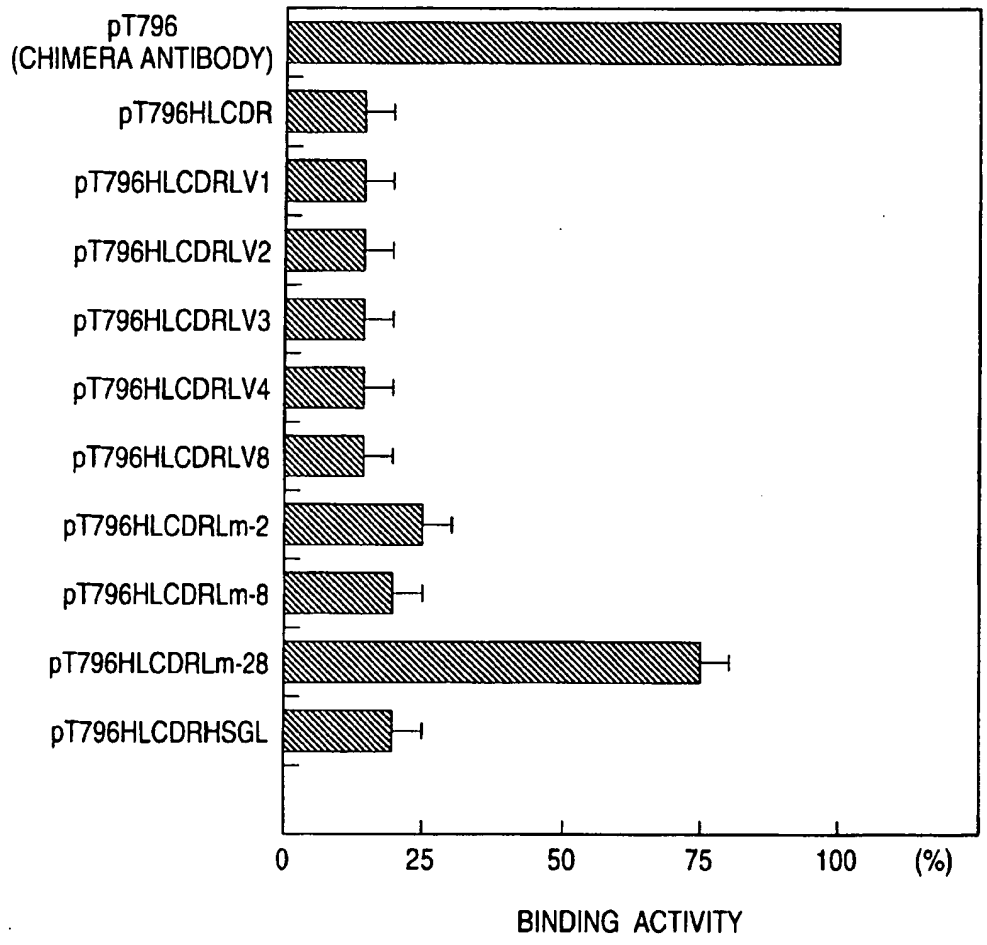


FIG. 44

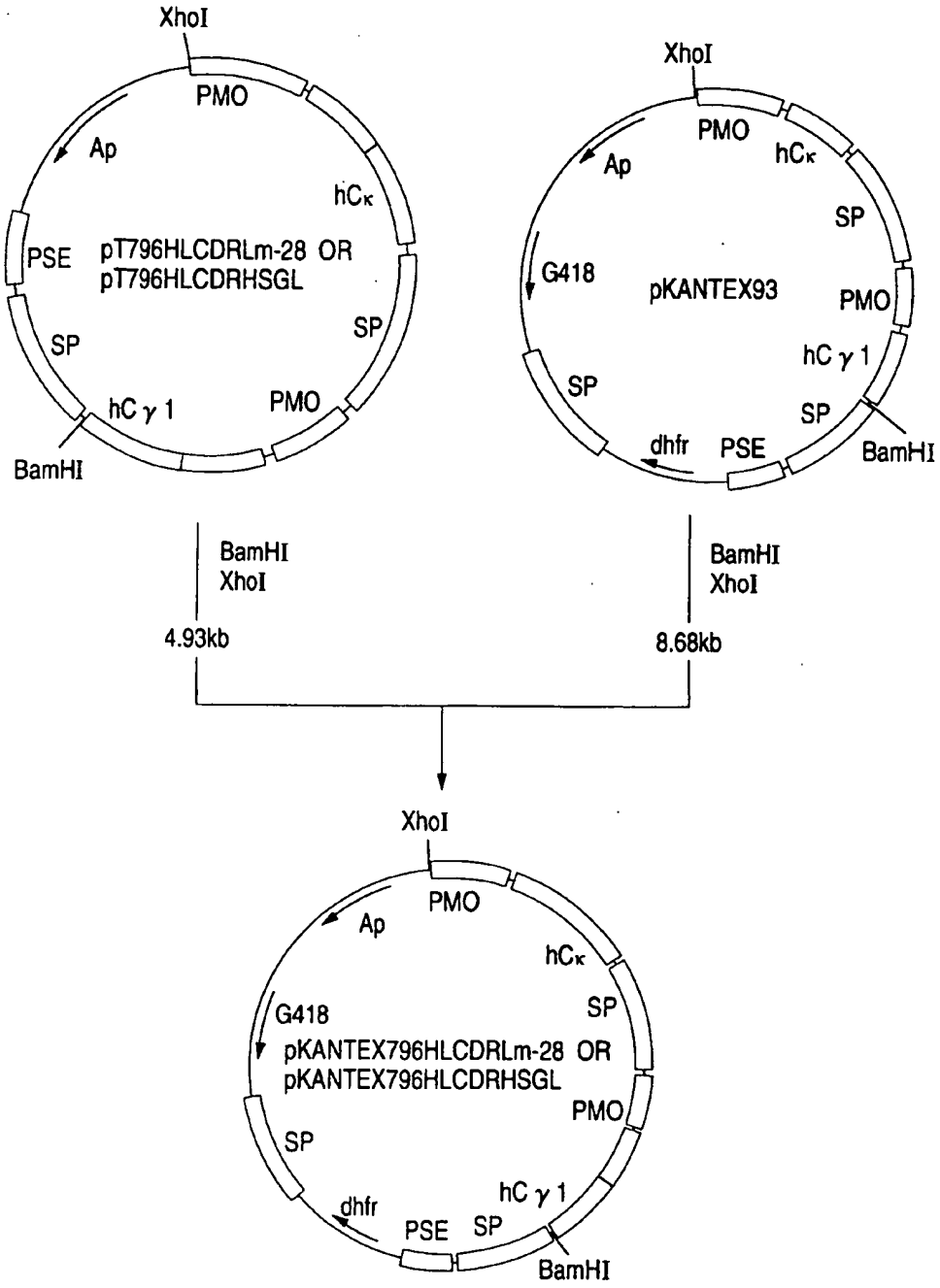


FIG. 45

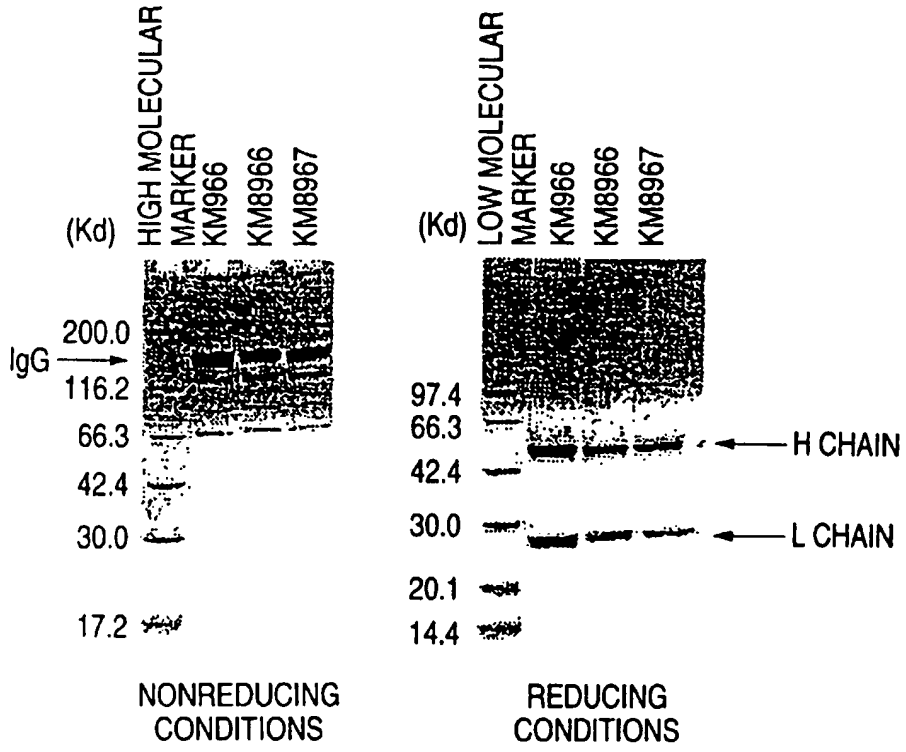


FIG. 46

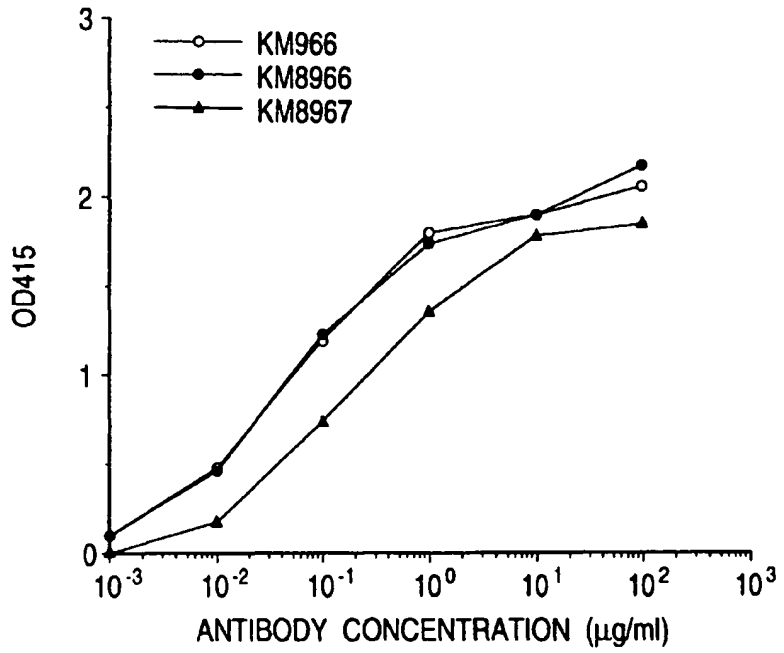


FIG. 47

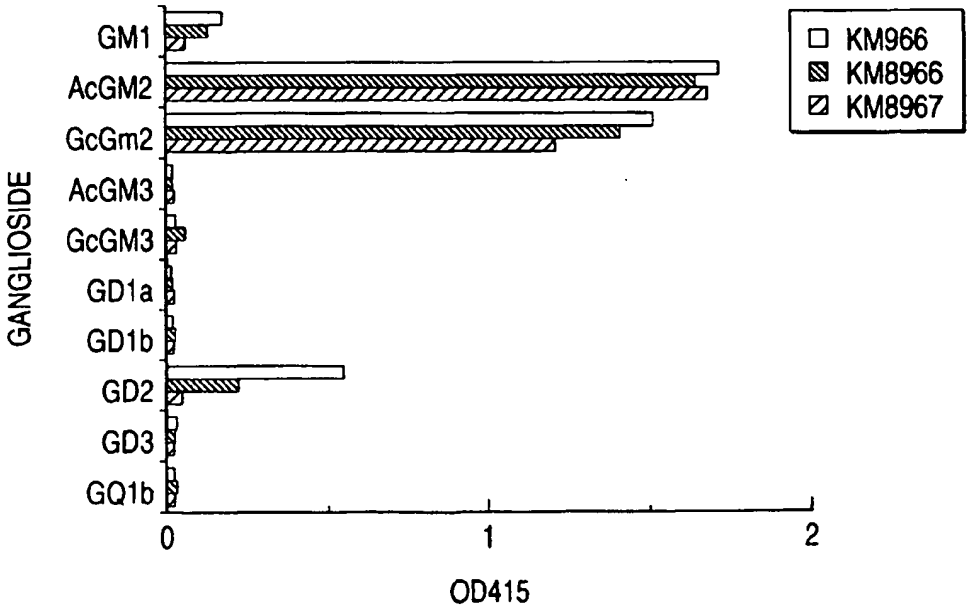


FIG. 48

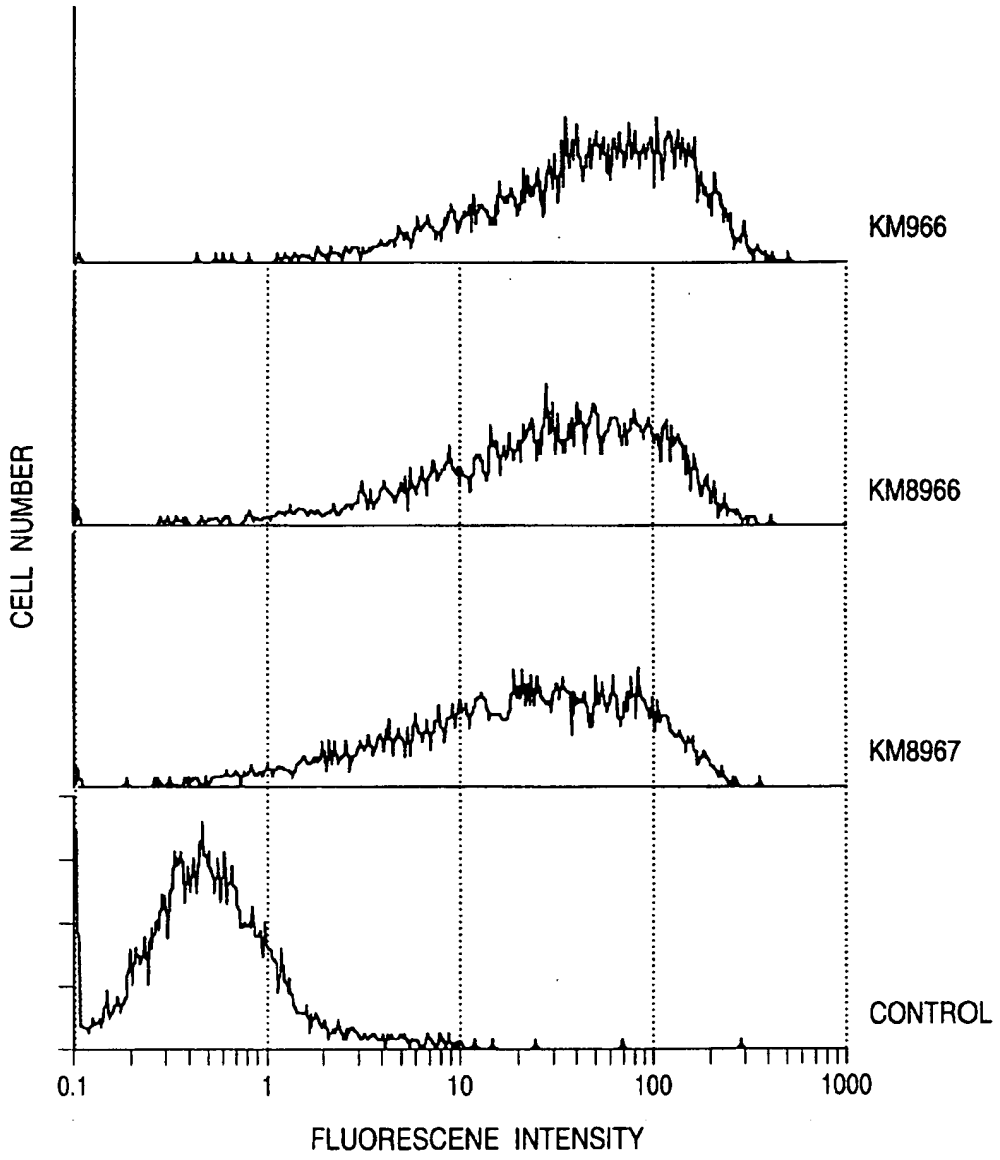


FIG. 49

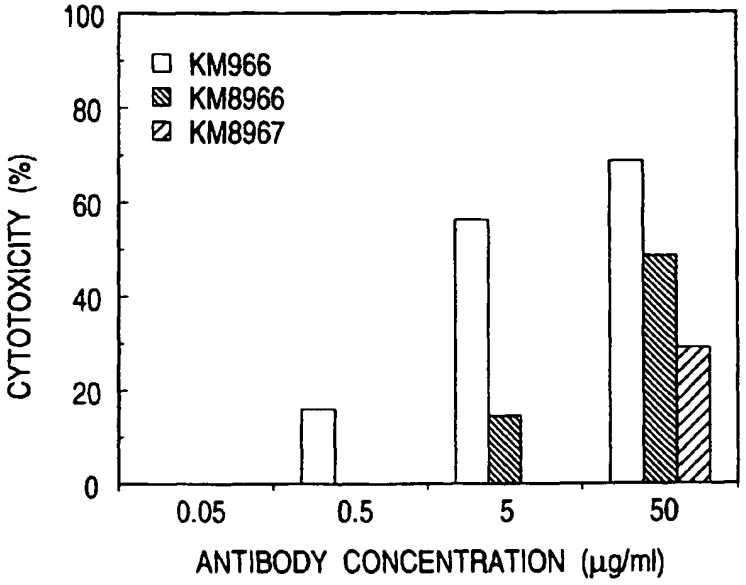


FIG. 50

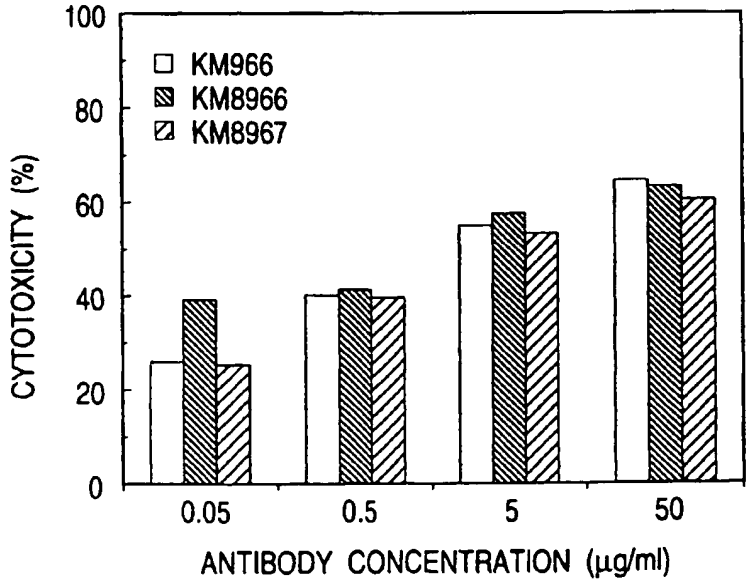


FIG. 51

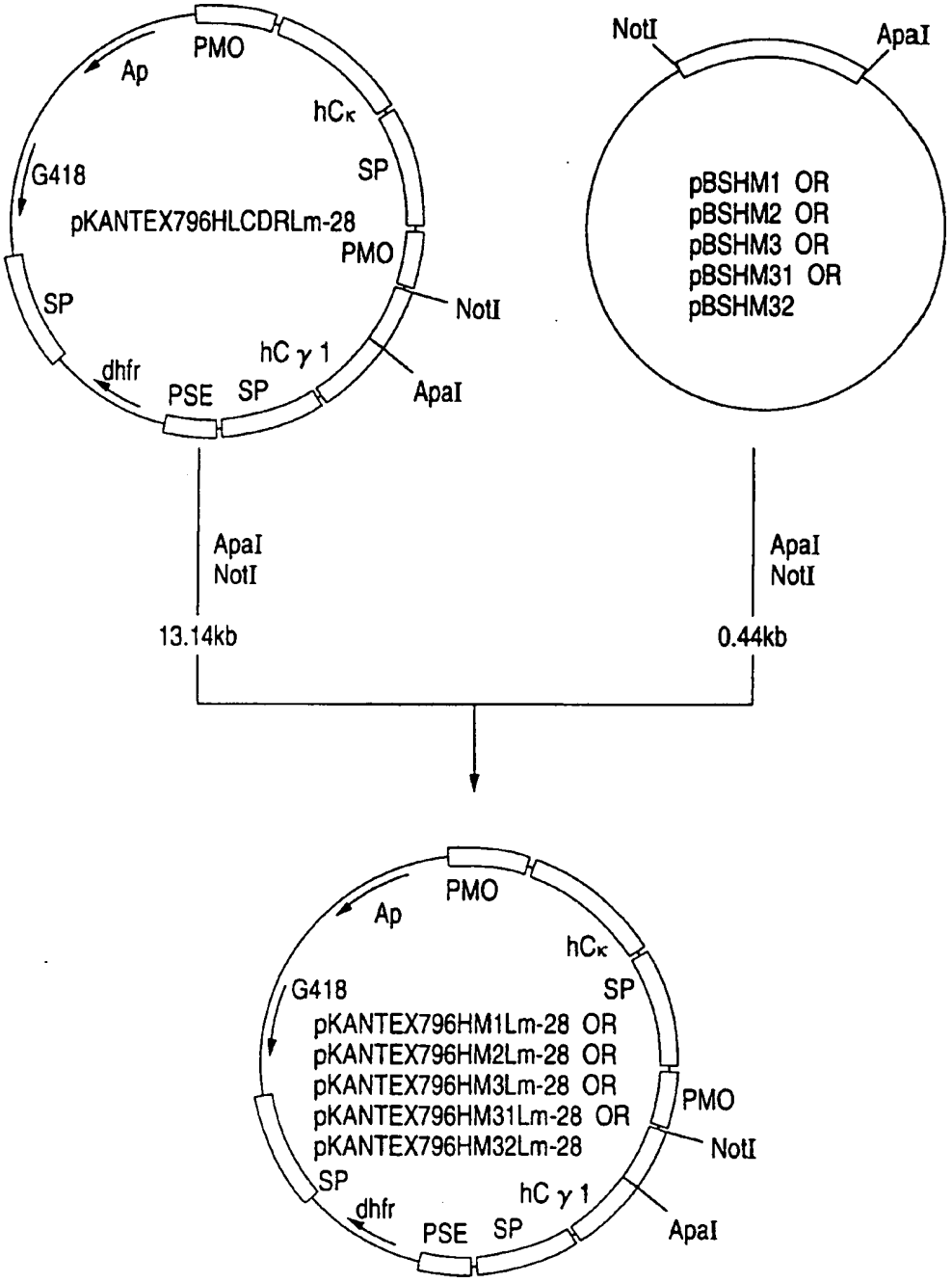


FIG. 52

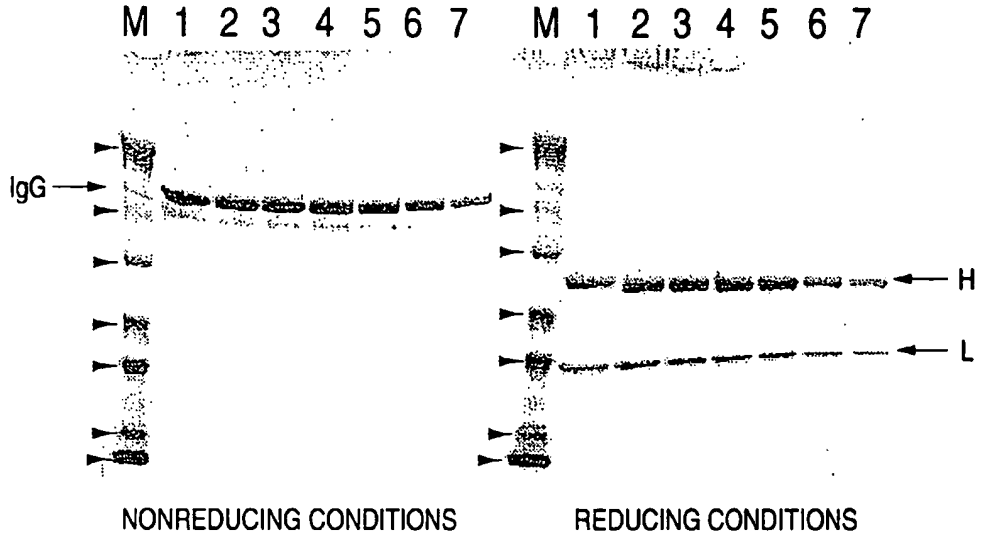


FIG. 53

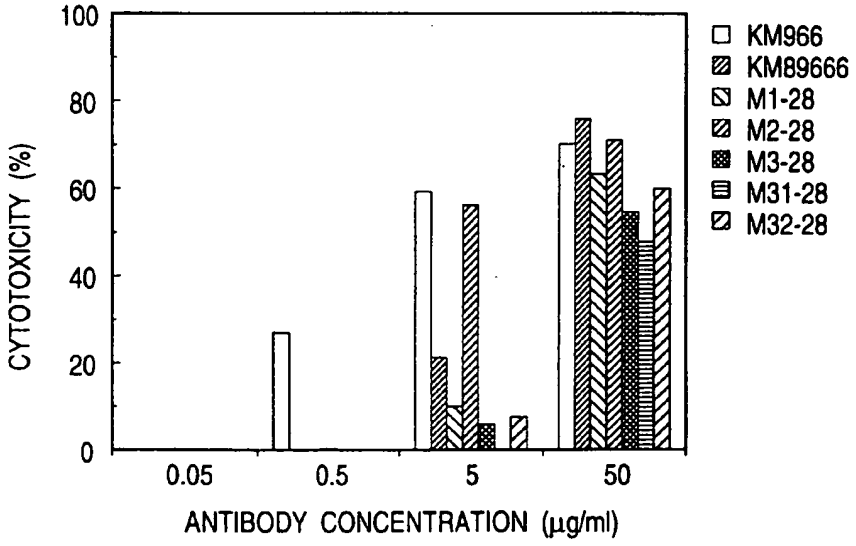


FIG. 54

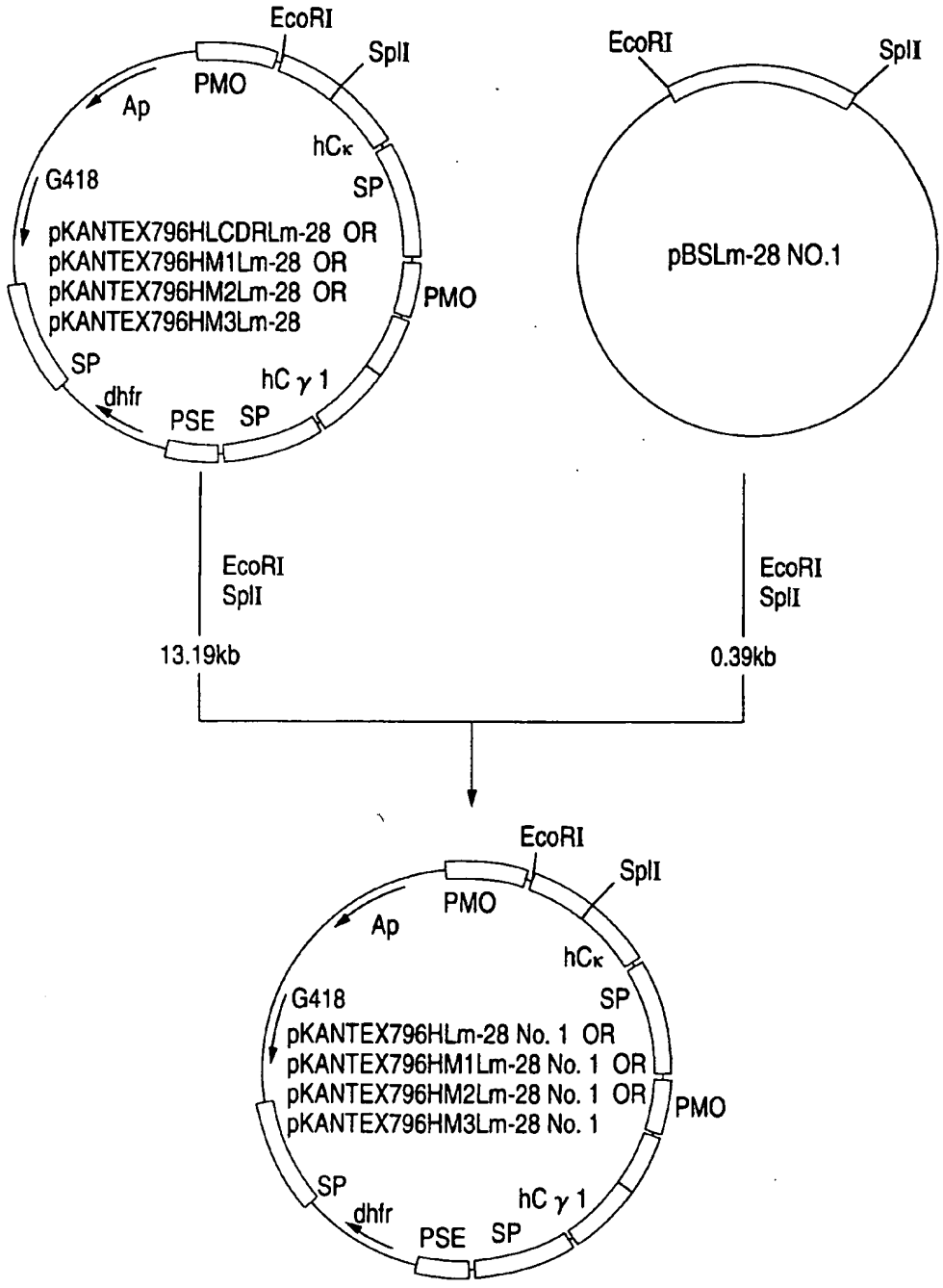


FIG. 55

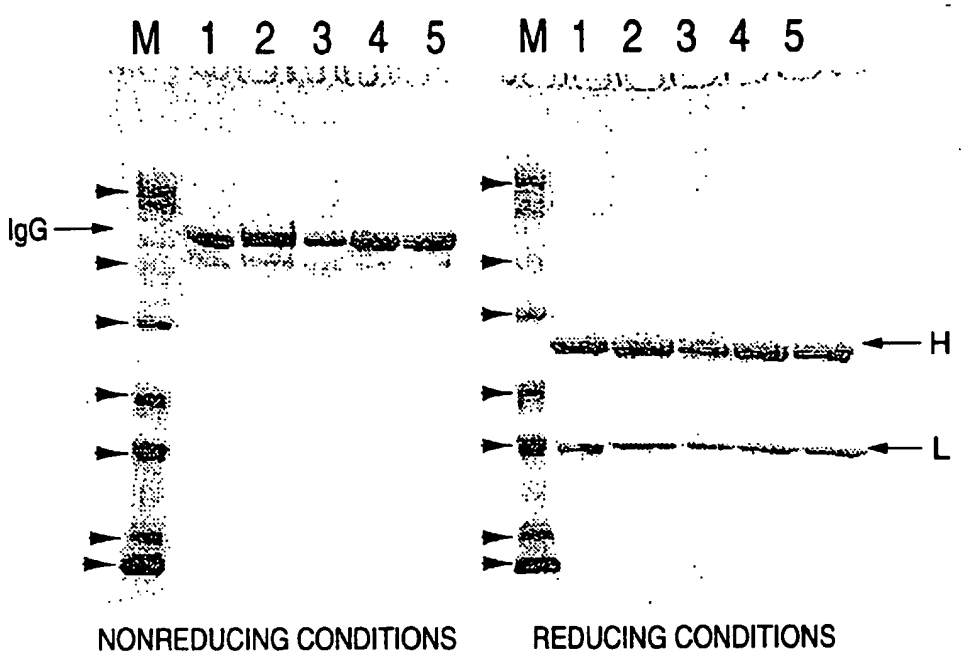


FIG. 56

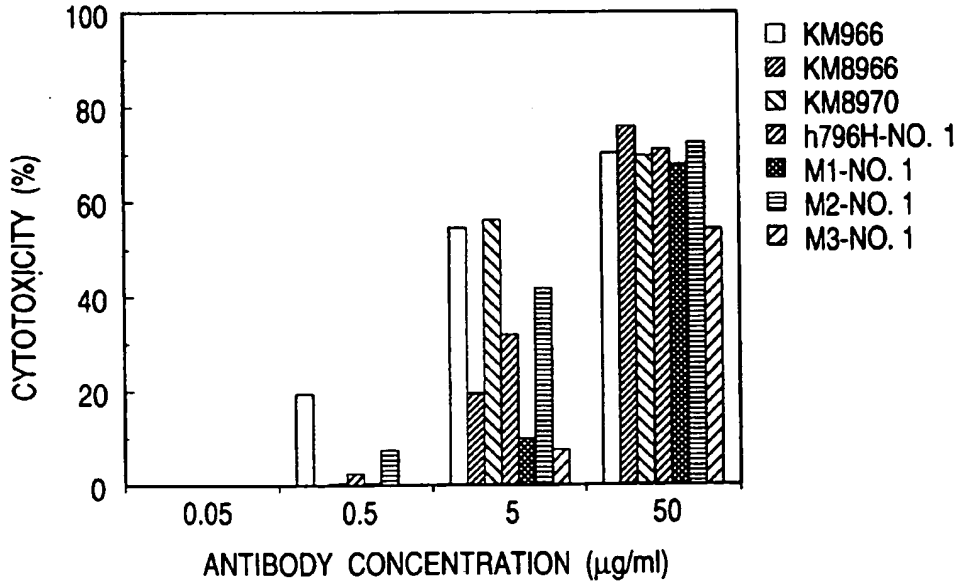


FIG. 57

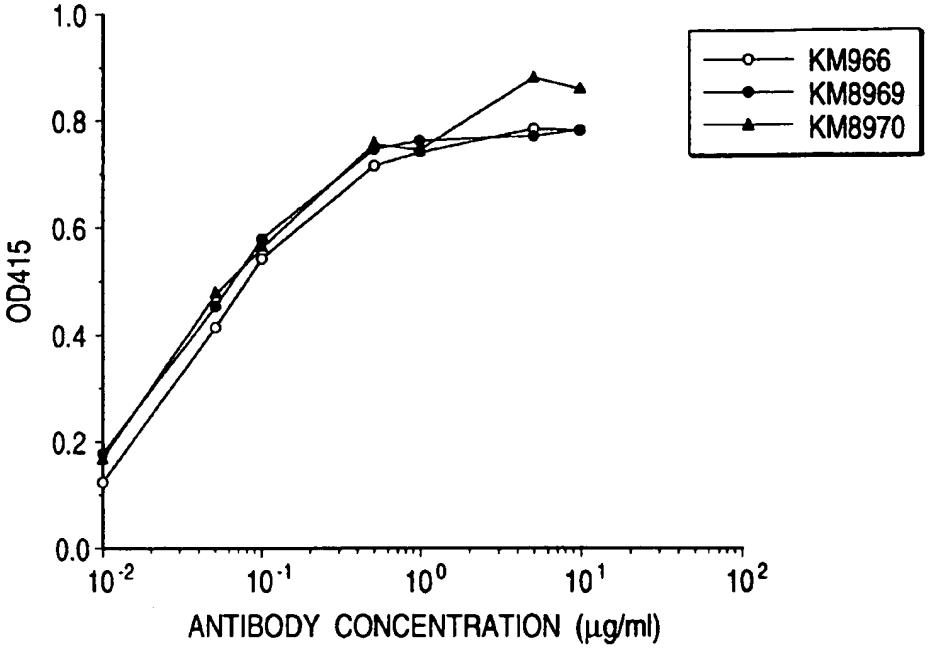


FIG. 58

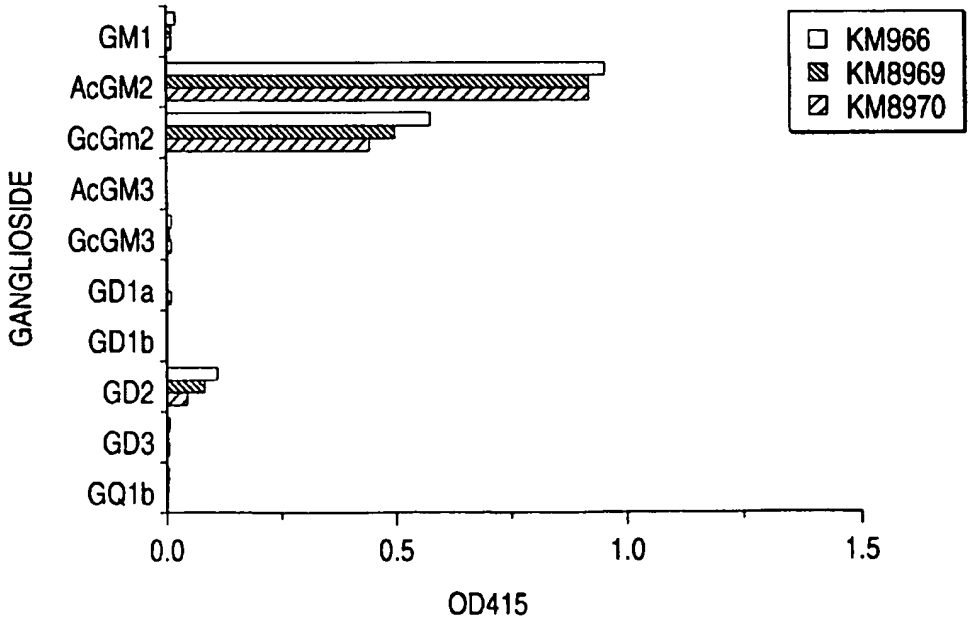


FIG. 59

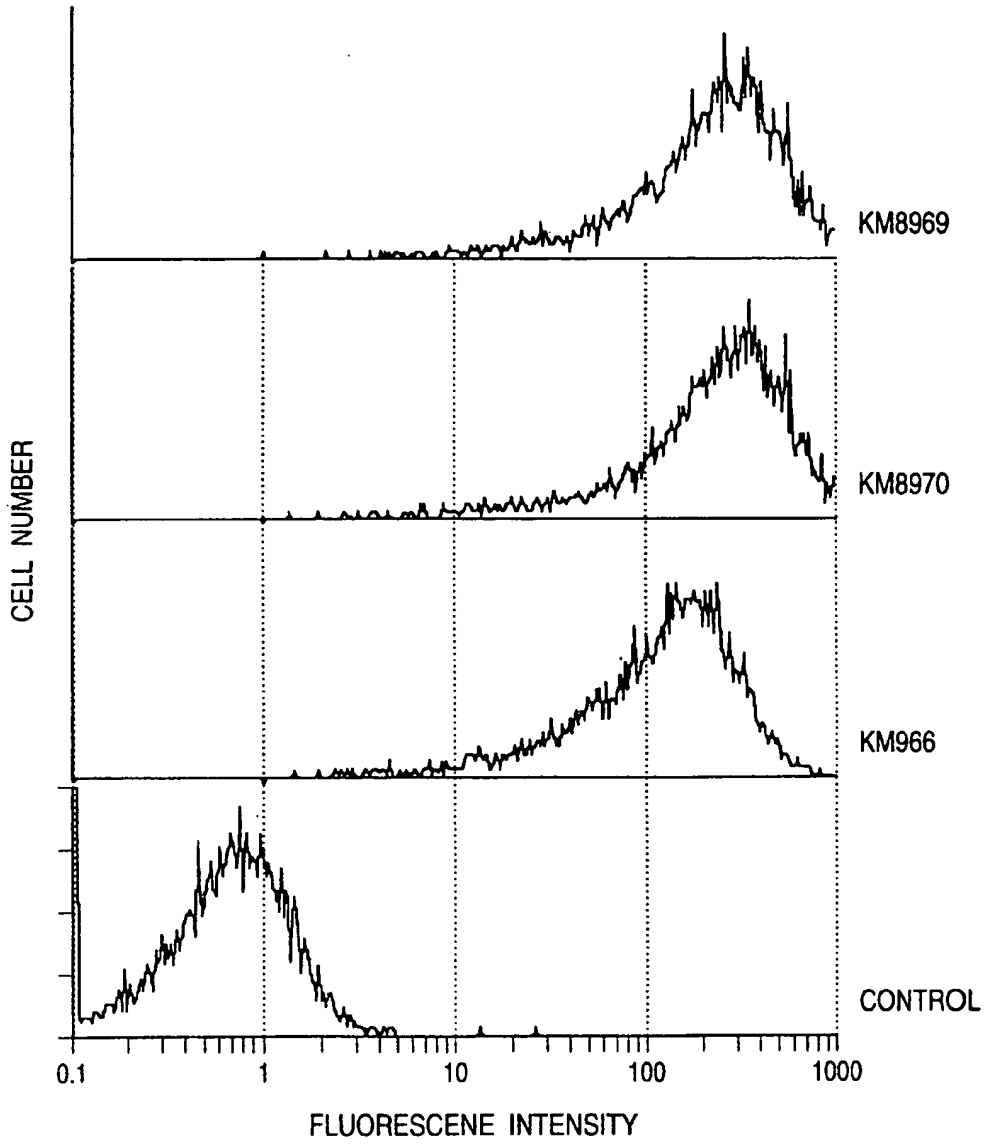


FIG. 60

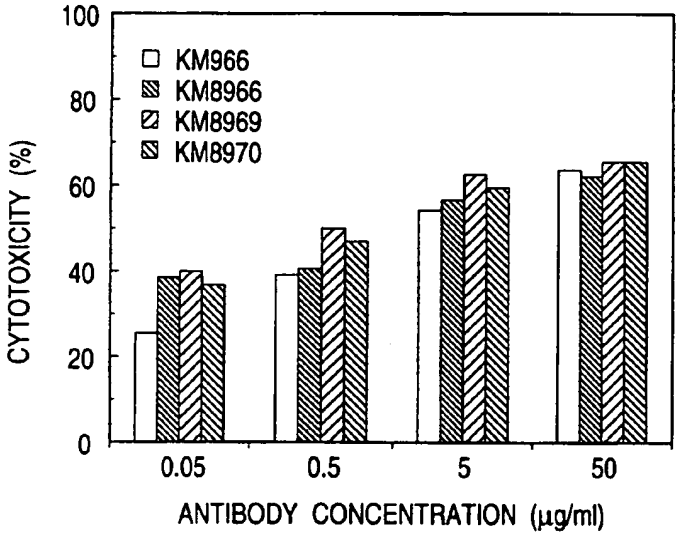
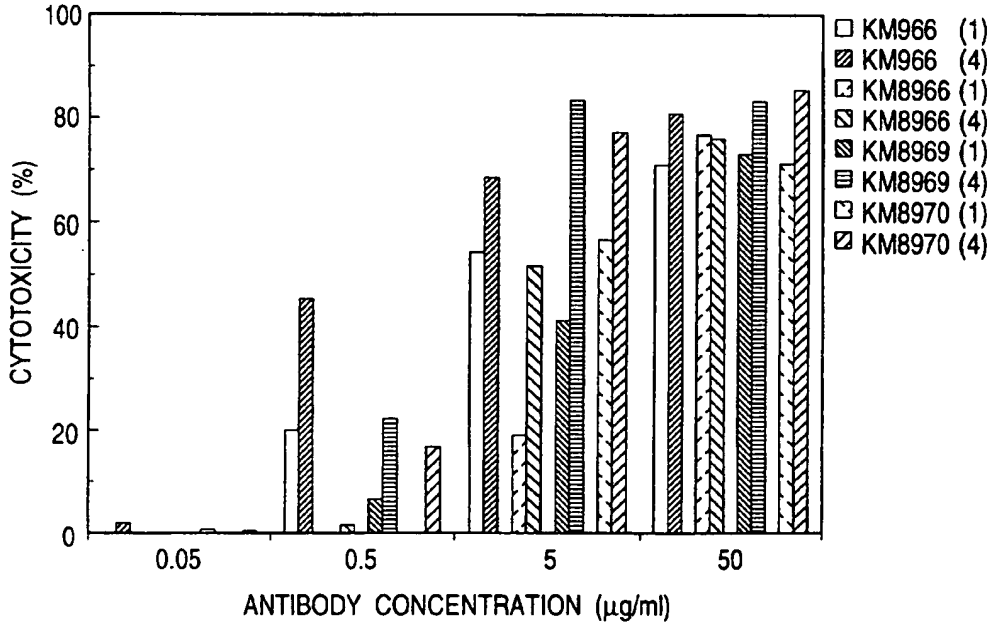


FIG. 61



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(54) **Bifunctional glycoproteins having a modified carbohydrate complement, and their use in tumorselective therapy.**

(57) Provided herein are carbohydrate complement-modified bifunctional glycoproteins, and their use in tumor-selective therapy. The bifunctional glycoproteins comprise a first component that specifically binds to a tumor-specific antigen and a second component having enzymatic activity by means of which a non-toxic prodrug is cleaved into a cytotoxic drug. The carbohydrate complement comprises at least one exposed carbohydrate residue selected from the group consisting of mannose, galactose, N-acetylglucosamine, N-acetyllactose, glucose and fucose. The modified carbohydrate complement contributes to increased relative concentration of the glycoproteins at the site of the tumor, and enhanced clearance from the general circulation and non-tumor sites.

EP 0 623 352 A2

Background of the Invention

Field of the Invention

The invention relates to bifunctional glycoproteins having targeting protein and enzyme properties. More particularly, the invention relates to such proteins whose complements of carbohydrate residues have been modified in a manner that enhances the clearance of such proteins from the circulation and increases the relative binding of the proteins at the tumor site. The enzymatic portion is capable of converting a prodrug into a cytotoxic drug that attacks tumor cells.

This invention also relates to the treatment of tumors with such proteins, and the production of such proteins, including recombinant production and production by transgenic animals.

Description of the Background Art

In efforts to control tumors, attempts have been made in the last twenty years to achieve selective therapeutic effects based on the specificity of antibodies. However, important therapeutic successes still have not been achieved in the case of solid tumors. Although highly specific tumor-selective monoclonal antibodies are available for targeting purposes, the lack of success in immunotherapy is primarily due to the small quantities of monoclonal antibody molecules that can be localized to solid tumors. One reason for this low degree of localization, which is generally insufficient for therapeutic purposes, is the presence of diffusion barriers in the tumor (Jain, R.K., *Cancer Res.* 47: 3039 (1987)). Prior attempts to compensate by increasing the dosage of the drug have encountered problems of widespread non-specific binding in non-tumor structures, and generalized toxic side effects.

Prior art compounds have sought to utilize (i) the specificity of a monoclonal antibody or tumor-binding protein partner and (ii) the catalytic amplification potential of an enzyme. Such antibody-enzyme conjugates can be administered to a patient and given time to bind to the tumor. Thereafter, a non-toxic prodrug, which can be cleaved by the enzyme portion of the conjugate to yield a cytotoxic drug, is administered to the patient. In theory, the enzyme portion of the molecules bound to the tumor converts the prodrug in the vicinity of the tumor into a drug which is cytotoxic to the tumor. In reality, however, such compounds suffer several drawbacks.

First, such antibody-enzyme conjugates are highly immunogenic in humans, since they represent chemical conjugates composed, as a rule, of mouse antibodies and xenogeneic enzymes. Repeated use of the same antibody-enzyme conjugate on the same patient is therefore not possible clinically (Bagshawe *et al., Disease Markers*, 9: 233 (1991)).

Second, the conjugates are only relatively slowly removed from the plasma, so that selective and effective prodrug activation is only possible if the elimination of the unwanted non-bound enzyme activity from the plasma is significantly enhanced.

The above-mentioned problem of the immunogenicity of xenogeneic antibody-enzyme conjugates is largely solved by using a recombinant fusion protein that is composed of purely human components. Details for the production of such fusion proteins are described in European Patent Application EP-A-0 501 215, which is incorporated by reference to the extent that it discloses such fusion proteins. In that publication, proteins are described, for example, of the general formula hutuMab-L-β-gluc, with hutuMab being a humanized, or human, tumor-specific monoclonal antibody, or a part thereof which still binds to the tumor, L representing a linker moiety, and β-gluc denoting human β-glucuronidase.

However, in carrying out pharmacological tests on such a fusion protein, it was unexpectedly found that, even at very short periods of time (1 -3 minutes) after i.v. injection of the fusion protein into human tumor-carrying nude mice, significant quantities of the protein were bound to tumor cells in regions which are close to the blood vessels (easily accessible sites = EAS). Further, at these early times, large quantities of the fusion protein were still present in the plasma, so that selective and effective activation of a suitable prodrug in the tumor was not possible at this early time point after injection.

One proposed solution to the above problem is described in the International Patent Application WO 89/10140, which discloses a three component system for treatment of malignant diseases. The first component localizes at the tumor and has enzymatic activity, e.g., an antibody-enzyme conjugate. The second component is able to bind to the first component and inactivate the catalytic site and/or accelerate the clearance of the first component from the plasma. The third component is a prodrug which can be converted by the enzymatic activity of the first component to form the cytotoxic substance used to treat the tumor.

The three components of 89/10140 are designed to be administered sequentially, not simultaneously. That is, the first component is administered and given sufficient time to localize at the tumor site. The second component is then administered after the first component has localized. Later, the third component is given.

The process of WO 89/10140 has drawbacks, however. First, it has the disadvantage of being a more complex process than the two step processes of the prior art. Moreover, by introducing an additional substance to the human or animal body, particularly the second compound, the risk of side effects and/or adverse reactions, such as the development of an unwanted immune reaction, is increased.

Accordingly, there remains a need for improved compounds and methods for selectively targeting tumors with cytotoxic drugs.

SUMMARY OF THE INVENTION

The present inventors have developed solutions to the foregoing problems that make it possible to achieve the desired therapeutic effects using a simpler, two step approach. In their investigation the present inventors prepared bifunctional compounds having both a tumor binding moiety and a catalytic moiety, which compounds are cleared very rapidly from the plasma. In doing so, it was expected that the incidence of binding to the tumor would be decreased due to the short time the compounds were in the plasma. Surprisingly, however, despite the very short presence of the compounds in the plasma, the relative binding of the compounds to the tumor increased. Moreover, to achieve the enhanced clearance, no additional compounds were administered either simultaneously with or subsequently to administration of the bifunctional compounds.

Thus, one aspect of the invention involves, in a first treatment step, administering intravenously ("i.v.") to tumor patients a compound comprising a bifunctional glycoprotein or bifunctional glycoprotein conjugate, the compound comprising a first portion that possesses an enzyme activity and a second portion that preferentially binds to a tumor-specific antigen. The carbohydrate complement of the compound comprises at least one exposed carbohydrate residue selected from the group consisting of mannose, galactose, N-acetylglucosamine, N-acetyllactose, glucose and fucose, which exposed residue is responsible for the advantageous binding and clearance characteristics of the compound. The enzyme activity of the first portion cleaves a non-toxic drug, which is administered to the subject either concurrently with or subsequently to the administration of the compound, to a form that is cytotoxic to the tumor cells.

For convenience, the term modified carbohydrate complement or the like will be used herein to denote a carbohydrate complement of the glycoprotein that comprises at least one exposed carbohydrate residue selected from the group consisting of mannose, galactose, N-acetylglucoseamine, N-acetyllactose, glucose and fucose.

Thus, in one aspect of the invention there is provided a bifunctional fusion glycoprotein ("FUP") containing a tumor targeting portion, an enzyme portion, and a modified carbohydrate complement. The modified carbohydrate complement contributes to an increased relative concentration of the FUP bound to a tumor and an enhanced clearance of the FUP from the general circulation and non-specific binding sites. The enzyme portion of the FUP is capable of cleaving a non-toxic drug into a tumor cytotoxic drug.

In another aspect of the invention, methods are provided for producing the FUPs having modified carbohydrate complements by colony selection, recombinant DNA and transgenic animal techniques, and chemical or enzymatic reactions.

In yet another aspect of the invention, a bifunctional antibody-enzyme conjugate ("AEC") having a modified carbohydrate complement is provided, wherein the antibody moiety is directed to an epitope on a tumor-specific antigen, and the enzyme is capable of converting a non-toxic drug into a tumor cytotoxic drug.

In still another aspect of the invention there are provided methods for appropriately modifying the carbohydrate complement of an AEC.

These and other aspects of the invention will become readily apparent by reference to the description of the invention and appended claims.

DETAILED DESCRIPTION OF THE FIGURES

Fig. 1 shows the amplification of the V_H and V_L genes. The V_H gene, including its own signal sequence, is amplified (Güssow et al., *Meth. Enzymology*, 203: 99 (1991)) from pABstop 431/26 hum V_H using the oligonucleotides pAB-Back and Linker-Anti (Table 1). The V_L gene is amplified from pABstop 431/26 hum V_L using the oligonucleotides Linker-Sense and $V_{L-(Mul)}$-For (Table 2).

Fig. 2 shows a PCR fragment composed of the V_H that is connected to the V_L gene via a linker.

Fig. 3a shows the removal of the *Hind* III to *Bgl*II restriction fragment from the plasmid pAB 431 VH to produce a vector.

Fig. 3b shows the insertion of the PCR fragment from Fig. 2 into the vector from Fig. 3A to produce the plasmid pMCG-E1, which clone contains the humanized sFv 431/26, a hinge exon, and the complete β-glucuronidase, which clone is transfected into BHK cells.

Fig. 4 shows the plasmid pRMH 140 that carries a neomycin resistance gene into transfected BHK cells.

Fig. 5 shows the plasmid pSV2 that carries the methotrexate resistance gene into transfected BHK cells.

Fig. 6 shows the PCR amplification scheme. The sFv 431/26 fragment (a) is employed as the template for a PCR using the oligos pAB-Back (Table 2) and sFv-For (Table 5). This results in *Bgl*II and *Hind*III cleavage sites being introduced at the 3' end of the newly generated sFv 431/26 fragment (b). The PCR fragment is purified and digested with *Hind*III, and then ligated into a pUC18 vector which has been cut with *Hind*III and treated with alkaline phosphatase. The plasmid clone pKBO1 is isolated, containing the sFv fragment with the *Bgl*II cleavage site.

Fig. 7 shows the amplification of the gene encoding the *E. coli* β-glucuronidase from the vector pRAJ275 by PCR using the oligos *E. coli* β-gluc-Back1 (Table 6) and *E. coli* β-gluc.-For (Table 7), and at the same time provided with a *Bgl*II cleavage site, an *Xba*I cleavage site and, at the 5' end, with a sequence encoding a linker. The resulting fragment is purified and digested with *Bgl*II/*Xba*I, and then cloned into the vector pKBO1, which has likewise been digested with *Bgl*II/*Xba*I. The plasmid clone pKBO2 is isolated, containing sFv 431/26 linked to the *E. coli* β-glucuronidase via a linker sequence.

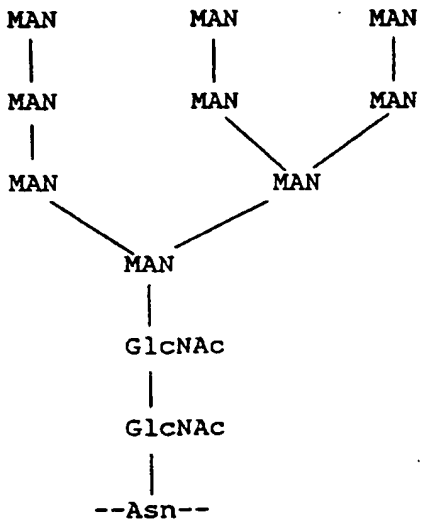
Fig. 8 shows the sFv-*E.* coli β-gluc. fragment, obtained from vector pKBO2 by digesting with *Hind*III/*Xba*I, is purified and then ligated into the expression vector pABstop, which has also been cut with *Hind*III/*Xba*I. The plasmid clone pKBO3 is isolated, containing the humanized sFv 431/26, a linker and the complete *E. coli* β-glucuronidase.

DETAILED DESCRIPTION OF THE INVENTION

It has been discovered that solid tumors in a subject may be treated efficiently *in vivo* with cytotoxic drugs, with no or lessened deleterious effect of the cytotoxic drugs on non-tumor tissues, by administering a carbohydrate complement-modified FUP or AEC of this invention with a prodrug. The targeting portion of the FUP or the targeting antibody of the AEC directs the fusion glycoprotein or glycoprotein conjugate to specific sites in or on a tumor cell, and the enzyme portion of the FUP or the AEC is capable of cleaving a prodrug to a tumor cytotoxic drug. As mentioned above, the modified carbohydrate complement enhances both the relative concentration of the FUP or AEC at the tumor site and increase the clearance of these proteins from non-specific sites and from the general circulation.

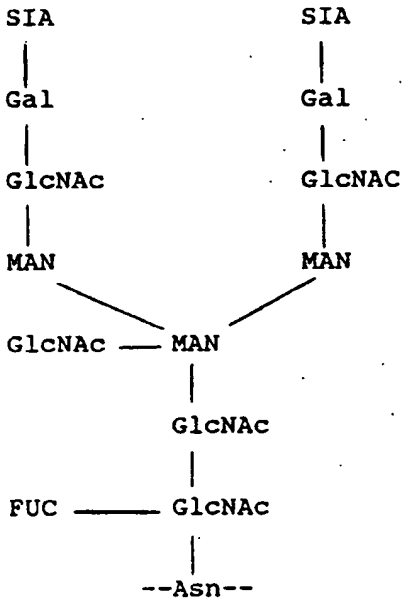
Once the FUP or AEC has been substantially cleared from the plasma and the normal tissues, while remaining bound on the tumor, a prodrug (advantageously hydrophilic), which is non-toxic and which disseminates extracellularly, is administered i.v. at appropriate (e.g., high) concentration. The prodrug is then cleaved by the FUP or AEC which is bound to the tumor to yield a tumor cytotoxic drug, which is advantageously lipophilic.

Glycoproteins are composed of oligosaccharide units linked to the protein chain(s) either through the side chain oxygen atom of serine or threonine by O-glycosidic linkages, or to the side chain nitrogen of asparagine residues by N-glycosidic linkages. The sum total of oligosaccharide units of a glycoprotein is referred to as the carbohydrate complement. The N-linked oligosaccharides contain a common pentasaccharide core consisting of 3 mannose (MAN) and 2 N-acetylglucoseamine (GlcNAc) residues, as shown in Sketch I (high mannose type) below.



I

A complex type of oligosaccharide core is shown in Sketch II (see below), showing N-acetylneuraminic acid (sialic acid, SIA) residues as terminal carbohydrates, fucose (FUC) residues as side chains, and galactose (Gal) residues as penultimate sugars. Skilled artisans will appreciate that configurations other than those shown in sketches I and II are possible.



II

Additional sugars are attached to this common core in many different ways to form a great variety of oligosaccharide patterns. The nature of the terminal sugars in glycoproteins is part of a complex recognition system that is known to influence, *inter alia*, the uptake of glycoproteins by organs, macrophages and other tissues. See, e.g., Steer *et al.*, *Prog. Liver Dis.*, 8:99 (1986); Stahl, *Curr. Opin. Immunol.*, 4: 49 (1992); Brady *et al.*, *J. Inherit. Metab. Dis.*, in press (1994). These influences are highly tissue and glycoprotein specific, and it is not yet known *a priori* to predict patterns of enhanced clearance of particular circulating glycoproteins by specific tissues.

By galactosylating the FUP, or by eliminating terminal neuraminic acid residues from the protein by treating it with neuraminidase, the half life of the FUP in the plasma is shortened. It has been found, surprisingly, that the fusion protein which has been modified in this way continues to bind to the EAS, even at early time points, while retaining its specificity, avidity and enzymic activity. Further, the fusion protein is cleared from the plasma within 1-3 hours to such an extent that efficient, tumor-selective activation of a suitable prodrug is effectively made possible without the need to inject a clearing second antibody as in WO 89/10140 and Sharma et al., Brit. J. Cancer 61:659 (1990). In addition, the present inventors have succeeded, by admixing, for example, galactose with the galactosylated FUP, in achieving still more efficient tumor localization. Further, it was possible successfully to extend these observations, within the scope of the invention, to additional FUPs and AECs, which were galactosylated or treated with neuraminidase, while preserving their biological properties.

Those skilled in the art will appreciate that different amounts of exposed residues may be utilized in the compounds of this invention. The number of exposed residues may be expressed as an average of exposed residues per molecule. For example, the average number of exposed residues per molecule generally will be at least about one, although averages of less than one are possible. Hence, for example, averages of less than 1, 1 to 2, 2 to 3, 3 to 4, 4 to 5, 5 to 6, 6 to 7, 7 to 8, 8 to 9, 9 to 10, 10 to 20, 20 to 50, 50 to 100, or greater than 100, are contemplated.

The general utility of the invention was verified using four different chemical compositions, namely, a xenogeneic antibody-enzyme conjugate, a humanized two-chain fusion protein, a humanized single-chain fusion protein and a xenogeneic single-chain fusion protein. Also useful are antibody fragment-enzyme conjugates, as well as to sFv-enzyme conjugates I and ligand-enzyme conjugates. The disclosure of WO 89/10140 is incorporated herein by reference to the extent that it discloses bifunctional proteins whose carbohydrate complement may be modified in accordance with this invention.

A representative AEC is composed of an intact monoclonal mouse antibody (e.g. as described in EP-A-0 388 914 which is incorporated by reference herein in its entirety) which is linked chemically to the enzyme E. *coli* glucuronidase by means of a heterobifunctional reagent according to Haisma *et al. (Brit. J. Cancer 66:* 474 (1992)) or to Wang *et al. (Cancer Res.* 52: 4484 (1992)), which are incorporated herein by reference in their entirety. Additional linkage possibilities, which can likewise lead to functional AECs, have been summarized by Means et al (*Bioconjugate Chem.* 1: 2 (1990), which is also incorporated by reference.

A humanized, two-chain fusion protein is described in detail in EP-A-0 501 215. It is a protein which is composed of two polypeptide chains and which has been prepared by genetic manipulation. One chain was prepared by linking the nucleotide sequences that encode a humanized V_HC_{H1} hinge S region to the nucleotide sequence which encodes a human β-glucuronidase (S = oligonucleotide encoding a polypeptide spacer). Following transfection and expression in suitable expression systems, preferably BHK or CHO cells, the nucleotide sequence which encodes the humanized V_LC_L chain, together with the above-mentioned nucleotide sequence, produces the humanized two-chain fusion protein.

The humanized single-chain fusion protein was produced, following expression in suitable expression systems, preferably in BHK or CHO cells, by linking the nucleotide sequences which encode the humanized V_HSV_L hinge S region (single chain Fv, sFv) and the nucleotide sequence which encodes human β-glucuronidase. The construction of a representative humanized single-chain fusion protein is described in Examples 1-4 below. A xenogeneic single-chain fusion protein is described in Example 5 below.

After recloning into suitable vectors, the constructs which are described in the examples below can also be expressed in other expression systems, such as, for example. *E. coli, Saccharomyces cerevisiae and Hansenula polymorpha*, insect cells or transgenic animals.

Non-human transgenic mammalian animals can be genetically engineered to secrete into readily accessible body fluids such as milk, blood and urine recombinant human FUPs of the invention in amounts and in forms that are suitable for treating humans with tumors.

The term "animal" here denotes all mammalian animals except humans. It also includes an individual animal in all stages of development, including embryonic and fetal stages. A "transgenic" animal is any animal containing cells that bear genetic information received, directly or indirectly, by deliberate genetic manipulation at the subcellular level, such as by microinjection or infection with recombinant virus.

"Transgenic" in the present context does not encompass classical crossbreeding or *in vitro* fertilization, but rather denotes animals in which one or more cells receive a recombinant DNA molecule. Although it is highly preferred that this molecule be integrated within the animal's chromosomes, the invention also contemplates the use of extrachromosomally replicating DNA sequences, such as might be engineered into yeast artificial chromosomes.

The term "germ cell line transgenic animal" refers to a transgenic animal in which the genetic information has been taken up and incorporated into a germ line cell, therefore conferring the ability to transfer the information to offspring. If such offspring, in fact, possess some or all of that information, then they, too, are transgenic animals.

The information to be introduced into the animal is preferably foreign to the species of animal to which the recipient belongs (i.e., "heterologous"), but the information may also be foreign only to the particular individual recipient, or genetic information already possessed by the recipient. In the last case, the introduced gene may be differently expressed than is the native gene.

The transgenic animals of this invention may be any, other than human, that produce milk, blood serum, and urine. Farm animals (pigs, goats, sheep, cows, horses, rabbits and the like), rodents (such as mice), and domestic pets (for example, cats and dogs) are included in the scope of this invention. It is preferred to select a transgenic animal that secrets into its milk a recombinant fusion protein, whose carbohydrate complement is modified to expose at least one mannose, galactose, N-acetylglucosamine, N-acetyllactose, glucose or fucose residue.

It is highly preferred that the transgenic animals of the present invention be produced by introducing into single cell embryos appropriate polynucleotides that encode the inventive FUPs in a manner such that these polynucleotides are stably integrated into the DNA of germ line cells of the mature animal and inherited in normal mendelian fashion.

Advances in technologies for embryo micromanipulation now permit introduction of heterologous DNA into fertilized mammalian ova. For instance, totipotent or pluripotent stem cells can be transformed by microinjection, calcium phosphate mediated precipitation, liposome fusion, retroviral infection or other means, the transformed cells are then introduced into the embryo, and the embryo then develops into a transgenic animal.

In a preferred method, developing embryos are infected with a retrovirus containing the desired DNA, and transgenic animals produced from the infected embryo. In a most preferred method, however, the appropriate DNAs are coinjected into the pronucleus or cytoplasm of embryos, preferably at the single cell stage, and the embryos allowed to develop into mature transgenic animals. Those techniques as well known. For instance, reviews of standard laboratory procedures for microinjection of heterologous DNAs into mammalian fertilized ova include: Hogan *et al.*, *Manipulating the Mouse Embryo*, Cold Spring Harbor Press, 1986; Krimpenfort *et al.*, *Bio/Technology* 9: (1991); Palmiter *et al.*, *Cell*, 41:343 (1985); Kraemer *et al.*, *Genetic Manipulation of the Early Mammalian Embryo*, Cold Spring Harbor Laboratory Press, 1985; Hammer *et al.*, *Nature*, 315:680 (1985); Meade *et al.*, U.S. 4,873,316; Wagner *et al.*, U.S. 5,175,385; Krimpenfort *et al.*, U.S. 5,175,384, all of which are incorporated by reference in their entirety. The procedure of Meade *et al.*, U.S. 4,873,316 is believed to provide one advantageous method of production, for example, using transgenic goats expressing the fusion protein under the control of the β-casein promoter in the mammary gland.

Genes for insertion into the genomes of transgenic animals so as to produce the FUPs of the invention can be obtained as described in the above-incorporated references and in the examples below. The gene encoding humanized two chain fusion glycoporteins are described in EP-A-0 501 215. The disclosure of which is incorporated herein by reference. The construction of a gene for a representative single-chain fusion glycoprotein is described in Examples 1-4 below. The gene for a single chain fusion glycoprotein is described in Example 5. Within the scope of the recombinantly produced modifications described herein, one can prepare constructs that include genes that encode proteins controlling posttranslational modification of expressed fusion glycoproteins. For example, constructs can be prepared, in which the sialyl transferase synthesis cycle is lacking or defective, thus producing fusion proteins in which terminal sialic acid residues are reduced in number or absent.

The cDNAs encoding desired FUPs can be fused, in proper reading frame, with appropriate regulatory signals to produce a genetic construct that is then amplified, for example, by preparation in a bacterial vector, according to conventional methods (see, Sambrook *et al, Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, 1989 which is incorporated herein by reference in its entirety). The amplified construct is thereafter excised from the vector and purified for use in producing transgenic animals. Purification can be accomplished by means of one or more cycles of anionic HPLC; alternate techniques include ultracentrifugation through a sucrose or NaCl gradient, gel electrolution followed by agarose treatment and ethanol precipitation, or low pressure chromatography. Purification by several cycles of HPLC allows for remarkably high transformation frequencies, on the order of 20% or more in both mice and pigs.

The regulatory signals referred to above include *cis*-acting signals necessary for mammary gland-specific expression of the fusion proteins and their post-translational glycosylation, secretion of the expressed fusion glycoprotein into milk or other body fluids, and expression of full biological activity.

Such regulatory signals include the promoter that drives expression of the fusion genes. Highly preferred are promoters that are specifically active in mammary gland cells and that involve milk proteins. Among such promoters, preferred are those for the whey acidic protein (WAP), short and long α, β and kappa caseins, α-lactalbumin and β-lactoglobulin (BLG) promoters.

Promoters may be selected on the basis of the native protein compositions of the various animals' milks. For example, the WAP and BLG promoters are particularly useful with transgenic rodents, pigs and sheep.

The genes for these promoters have been isolated and characterized. Clark *et al., TIBTECH* 5:20 (1987); Henninghausen, *Protein Expression and Purification* 1:3 (1990), which are incorporated by reference. The promoters can be isolated by conventional restriction endonuclease and subcloning steps. A mouse WAP promoter, isolated as a 2.6 kb EcoR1-Kpn1 fragment immediately 5' to the WAP signal sequence can be used, although the "long" WAP promoter (the 5' 4.2 kb Sau 3A-Kpn1 promoter of the mouse gene is also suitable.

Important to the transgenic animal embodiment are regulatory sequences that direct secretion of proteins into milk and/or other body fluids. Generally, homologous or heterologous regulators sequences known to direct the secretion of milk proteins, such as either signal peptides from milk or nascent target polypeptides, can be used, although the scope of this invention includes signal sequences that direct the secretion of proteins into fluids other than milk.

Among the useful sequences that regulate transcription, in addition to those described above, are enhancers, splice signals, transcription termination codons, and polyadenylation sites.

The injected DNA sequences may also include a 3' untranslated region downstream of the DNA encoding the desired fusion protein, or the milk protein gene used for regulation. This region may stabilize the RNA transcript of the expression system and thus increase the yield of the desired fusion protein. Among these 3'untranslated regions useful in this regard are sequences that provide a poly A signal. Such sequences can be derived from, for example, the SV40 small t antigen, the casein 3' untranslated region, and others well known in this art.

Obtaining milk from transgenic female animals is done conventionally. McBurney *et al., J. Lab. Clin. Med.*, 64:485 (1964) ; Velander *et al., Proc Natl. Acad. Sci. USA* 89: 12003 (1992).

Within the scope of recombinantly produced modifications, there are employed those, for example, in which the gene for sialyl transferase is inactive or is lacking, or in which other enzymes of the sialyl transferase synthesis cycle are deficient or are lacking. Other preferred expression systems exhibit overexpression of galactosyl transferase or mannose-6-phosphate synthetases/transferases. In addition, it has been found that clones which have been produced from CHO cells having a very high ability to express fusion protein, for example, by means of double selection, in accordance with EP-A-0 330 977 (which is incorporated by reference herein in its entirety), are deficient in sialyation. Such clones, that may generally be produced by a process known as "homologous recombination" (Pomerantz et al., Progress in Cancer Res. and Therapy, 30:37-45 (1975), incorporated by reference herein in its entirety), are thus very suitable for use as expression systems.

These proteins (antibody-enzyme conjugate, humanized two-chain fusion protein, humanized single-chain fusion protein and xenogeneic single-chain fusion protein, which have been described by way of example), were, once they had been purified by anti-idiotype and/or anti-β-glucuronidase immunaffinity chromatography, chemically galactosylated in accordance with the method described by Krantz *et al. (Biochemistry* 15: 3963 (1976) which is incorporated by reference herein in its entirety) or, alternatively, treated with carrier-bound neuraminidase. In that which follows, they are termed modified glycoproteins.

The modified proteins were compared *in vitro* and *in vivo* to the control unmodified starting proteins which had been expressed in BHK cells. The *in vitro* tests for specificity, affinity, quantitative immunoreactivity and quantitative enzyme activity demonstrated that the modified proteins did not differ significantly in these respects from the control proteins. In contrast, the half life ($t_{1/2\beta}$) of the modified proteins in mouse and rat plasma (*in vivo*) was dramatically shortened (Tables 6, 7).

As a result of this dramatic shortening of the $t_{1/2\beta}$, at 1-3 hours after injecting the galactosylated proteins i.v. into tumor-bearing nude mice, modified proteins could no longer be detected in the plasma. In the case of the desialylated proteins, the $t_{1/2\beta}$ was shortened to such an extent that desialylated protein was no longer detectable in the plasma after 48 hours. At the same time, the concentration of functionally active modified proteins in the tumor was in the range from 200-400 ng/g of tumor (a very high specificity ratio > 100:1 was consequently obtained on injecting ≈ 400 µg of modified protein per mouse).

Viewed in absolute terms, the concentrations of modified proteins can be two to three times higher than those which are achieved, after appreciably longer times, for a comparable specificity ratio using unmodified starting proteins *in vivo*. Furthermore, the above-mentioned high specificity ratio (µg of modified

protein/g of tumor: µg of modified protein/g of normal tissue) for modified proteins, is attained after only a few hours (1-3 hours or 48 hours, respectively), whereas, in the case of the unmodified starting proteins, a comparable specificity ratio (µg of unmodified starting protein/g of tumor: µg of unmodified starting protein/g of normal tissue) is only reached after several days (7-8 days), or even requires the use of a second antibody to accelerate the rate of clearance from normal tissue.

The rapid removal of the modified proteins from the plasma and the extracellular region of the organism by means of internalization via sugar-binding receptors (chiefly the galactose receptor in the liver, Thornburg *et al., J. Biol. Chem.* 255: 6820 (1980)) should also lead to the modified proteins having reduced immunogenicity in humans, particularly in the case of the antibody-enzyme conjugate and the xenogeneic single-chain fusion protein. This therefore also facilitates the use of xenogeneic or humanized FUPs in anti-tumor therapy, or at the least makes such use appear feasible for the first time.

A particularly useful humanized two-chain fusion protein has been expressed in CHO cells that had been selected for a very high level of expression, and purified by anti-idiotype affinity chromatography. Three or seven days after i.v. injection, this FUP was concentrated in the tumor to an extent 2-3-fold higher than that of the analogous fusion protein that is expressed in the BHK cells (Table 8). In addition, the FUP that has been expressed in CHO cells is removed from the plasma appreciably more efficiently than the fusion protein expressed in BHK cells, so that tumor: plasma ratios of > 15 are reached by day 3 in the case of the CHO fusion protein. In the case of the BHK fusion protein, the corresponding ratios are < 1 (Table 8). On day 7, the tumor:plasma ratios for the CHO fusion protein are in the region of 130 while those for the BHK fusion protein are in the region of 20 (Table 8).

These highly significant pharmacokinetic differences between the humanized two-chain fusion protein expressed in CHO cells or expressed in BHK cells can be explained by differences in the carbohydrate content of the fusion proteins. An analysis of the monosaccharide components in the carbohydrate content of the fusion protein expressed in BHK or CHO cells is given in Table 1a. Differences are observed mainly in the content of galactose, mannose and N-acetylneuraminic acid.

Table 1a

An analysis of the monosaccharide components in the carbohydrate content of the fusion proteins					
	mol monosaccharide / mol fusion protein monomer (125kDa)				
	Fucose	N-acetyl glucosamine	Galactose	Mannose	N-acetyl neuraminic acid
CHO fusion protein	0.6	4.35	1.40	7.04	0.54
BHK fusion protein	0.68	4.46	1.59	6.31	0.69

Method:

Neuraminic acid was determined by the method of Hermentin and Seidat (1991) GBF Monographs Volume 15, pp. 185-188 (after hydrolysis of 30 minutes in the presence of 0.1 N sulfuric acid at 80° C and an subsequent neutralization with 0.4 N sodium hydroxide solution) by high-pH anion exchange chromatography with pulsed amperometric detection (HPAE-PAD).

The monosaccharide components were determined (after hydrolysis of 4 hours in the presence of 2 N trifluoracetic acid at 100° C and evaporation to dryness in a SpeedVac) likewise by HPAE-PAD in a motivation of the method described by Hardy *et al.* (1988) *Analytical Biochemistry* 170, pp. 54-62.

Particularly, the increased amounts of mannose or mannose-6-phosphate in combination with reduced amounts of N-acetylneuraminic acid, as observed in the fusion protein expressed in CHO cells, might be responsible for its faster elimination from plasma and normal tissues due to more efficient binding to mannose and galactose receptors (compare Table 8, pharmacokinetics of unmodified fusion proteins). Furthermore, glycan mapping showed higher contents of high mannose/asialo-structures (see Sketch II above) in the CHO-expression product compared to the normal BHK-expression product.

The high β-glucuronidase concentrations, which were determined in the enzyme activity test, represent the activity of the endogenous murine β-glucuronidase and that of the FUP, as well as that of any human β-glucuronidase which may have been liberated from the latter by the cleavage which can potentially occur. Using enzyme-histochemical methods (Murray *et al., J. Histochem. Cytochem.* 37: 643 (1989)), it was demonstrated that this enzyme activity was present as intracellular activity in the normal tissues. Thus, this

catalytic potential either does not contribute, or only contributes unimportantly, to the cleavage of a hydrophilic prodrug which is disseminated extracellularly.

The several embodiments exemplified below are not to be taken as in any way limiting the scope of the invention which is described in the specification and in the appended claims.

EXAMPLES

Examples 1 - 4:

Recombinant preparation of a humanized single-chain fusion protein from a humanized tumor antibody moiety and human β-glucuronidase.

Example 1

Using the oligonucleotides pAB-Back and Linker-Anti (Table 1), the V_H gene, including its own signal sequence, is amplified from pABstop 431/26 hum V_H (Güssow *et al*, 1991, above). Using the oligonucleotides Linker-Sense and V_L(Mut)-For (Table 2), the V_L gene is amplified (Fig. 1) from pABstop 431/26 hum V_L (Güssow *et al.*, 1991, above).

Table 1

pAB-Back: SEQUENCE ID NO. 1

```
5'                                    3'
ACC AGA AGC TTA TGA ATA TGC AAA TC
```

Linker-Anti: SEQUENCE ID NO. 2

```
5'
GCC ACC CGA CCC ACC ACC GCC CGA TCC ACC GCC TCC TGA
                          3'
GGA GAC GGT GAC CGT GGT C
```

Table 2

Linker-Sense: SEQUENCE ID NO. 3

```
5'
GGT GGA TCG GGC GGT GGT GGG TCG GGT GGC GGC GGA TCT
                             3'
GAC ATC CAG CTG ACC CAG AGC
```

VL(Mut)-For: SEQUENCE ID NO. 4

```
5'
TGC AGG ATC CAA CTG AGG AAG CAA AGT TTA AAT TCT ACT
            3'
CAC CTT TGA TC
```

Example 2

The oligonucleotides Linker-Anti and Linker-Sense are partially complementary to each other and encode a polypeptide linker which is intended to link the V_H and V_L domains to form an sFv fragment. In order to fuse the amplified V_H and V_L fragments, they are purified and introduced into a 10-cycle reaction as follows:

H_20:	37.5 µl
dNTP's (2.5 mM)	5.0 µl
PCR buffer (10x)	5.0 µl
Taq polymerase (Perkin-Elmer Corp., Emmeryville, CA) (2.5 U/µl)	0.5 µl
0.5 µg/pl DNA of the $V_L$frag.	1.0 µl
0.5 µg/pi DNA of the V_H frag.	1.0 µl
PCR buffer (10x): 100 mM Tris, pH 8.3, 500 mM KCl, 15 m $MgCl_2$, 0.1% (w/v) gelatin.	

The surface of the reaction mixture is sealed off with paraffin and the 10-cycle reaction is subsequently carried out in a PCR apparatus using the program 94 °C, 1 min; 55 °C, 1 min; 72 °C, 2 min. After that, 2.5 pM of the flanking primers pAB-Back and V_L(Mut)-For are added and a further 20 cycles are carried out. A PCR fragment is obtained which is composed of the V_H gene, which is connected to the V_L gene via a linker (Fig. 4). The V_H gene's own signal sequence is also located prior to the V_H gene. As a result of using the oligonucleotide V_L(Mut)-For, the last nucleotide base of the V_L gene, a C, is at the same time replaced by a G. This PCR fragment encodes a humanized single-chain Fv (sFv).

Example 3

The sFv fragment from Example 2 is restricted with HindIII and BamHI and ligated into the vector pABstop 431/26$V_H$huβgluc1H, which has been completely cleaved with HindIII and partially cleaved with BglII. The vector pABstop 1/26$V_H$huβgluc1H contains a V_H exon, including the V_H-specific signal sequence, followed by a CH1 exon, the hinge exon of a human IgG3 C gene and the complete cDNA of human β-glucuronidase. The plasmid clone pMCG-E1 is isolated, which clone contains the humanized sFv 431/26, a hinge exon and the complete β-glucuronidase (Fig. 3a). Vector pABstop 431/26$V_H$huβgluc is described in Bosslet *et al., Brit. J. Cancer* 65: 234 (1992), which is incorporated by reference herein in its entirety and where information on the remaining individual components can be obtained from the references listed therein.

Example 4

The clone pMCG-E1 is transfected, together with the plasmid pRMH 140 (Fig. 4), which carries a neomycin resistance gene, and the plasmid pSV2 (Fig. 5), which carries a methotrexate resistance gene, into BHK cells. The BHK cells then express a fusion protein which possesses both the antigen-binding properties of Mab BW 431/26hum and the enzymic activity of human β-glucuronidase (see Examples 8 and 9).

Example 5

Construction of xenogeneic single-chain fusion protein

The xenogeneic single-chain fusion protein was produced, following expression in suitable expression systems, preferably in BHK cells, by linking the nucleotide sequences which encode the humanized V_H, S, V_L hinge and S regions (see Examples 1-4 and below) to the nucleotide sequence which encodes *E. coli* β-glucuronidase. The construction of a single-chain fusion protein from a humanized sFv (antiCEA) and *E. coli* β-glucuronidase is described in detail below.

The sFv 431/26 fragment (a) is employed as the template for a PCR using the oligos pAB-Back (Table 1) and sFv-For (Table 3). In this way, BglII and HindIII cleavage sites are introduced at the 3' end of the newly generated sFv 431 26 fragment (b). The PCR fragment is purified and digested with HindIII, and then ligated into a pUC18 vector which has been cut with *Hind*III and treated with alkaline phosphatase. The plasmid clone pKBO1 is isolated, containing the sFv fragment with the BglII cleavage site (Fig. 6).

The gene encoding the *E. coli* (β-glucuronidase is amplified from the vector pRAJ260 (Jefferson *et al., Proc. Natl. Acad. Sci. USA*, 83:8447 (1986)) by PCR using the oligos *E. coli* (β-gluc-Back1 (Table 4) and *E. coli* β-gluc-For (Table 5), and at the same time provided at the 5' end with a *Bgl*II cleavage site, at the 3' end with an XbaI cleavage site and, additionally at the 5' end, with a sequence encoding a linker. The resulting fragment is purified and digested with BglII/XbaI, and then cloned into the vector pKBOI which has likewise been digested with *Bgl*II/*Xba*I. The plasmid clone pKBO2 is isolated, containing sFv 431/26 linked to *E. coli* β-glucuronidase via a linker sequence (Fig. 7).

The sFv-*E. coli* β-gluc. fragment, obtained from vector pKBO2 by HindIII/XbaI digestion, is purified and then ligated into the expression vector pABstop (Zettlmeissl *et al., Behring Institute Mitteilungen (Communications)* 82: 26 (1988)) which has likewise been cut with HindIII/XbaI. The plasmid clone pKBO3 is isolated, containing the humanized sFv 431/26, a linker and the complete E. coli β-glucuronidase (Fig. 8).

Table 3

sFv For: SEQUENCE ID NO. 5

5' 3'

TTT TTA AGC TTA GAT CTC CAC CTT GGT C

Table 4

E. coli β-gluc-Back 1: SEQUENCE ID NO. 6

5'

AAA AAG ATC TCC GCG TCT GGC GGG CCA CAG TTA CGT GTA GAA

3'

ACC CCA

Table 5

E. coli β-gluc-For: SEQUENCE ID NO. 7

5' 3'

GCT TCT AGA TCA TTG TTT GCC TCC CTG

Tab.6

Pharmacokinetic comparison between unmodified and modified humanized two-chain fusion proteins in CD-1 nude mice bearing a human tumor xenograft (MzSto1)

unmodified fusion protein produced in BHK cells

	μg fusion protein /g of tumor or /g of organ measured in an OFAT						
	tumor	spleen	liver	intestine	kidney	lung	heart	plasma
0.05 hr	3	21	61.4	6.5	35.6	77.6	60.1	456.2
1 hr	4.9	15	26.1	8.6	17.3	33.9	19.5	199.9
1.5 hr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2 hr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3 hr	5.7	4.8	14.8	3.5	6.5	7.7	2.5	122
5.5 hr	3.8	3.8	8.4	3.8	7.7	8.8	2.9	84.9
24 hr	4.7	1	2.1	0.6	2.5	2.1	0.5	19
48 hr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
168 hr	0.19	0.005	0.003	0	0	0.002	0	0

unmodified fusion protein produced in BHK cells

	μg of ß-glucuronidase /g of tumor or /g of organ measured in an EAT							
	tumor	spleen	liver	gut	kidney	lung	heart	plasma
0.05 hr	n.d.	48.5	81.7	18.6	45	83.7	60.2	n.d.
1 hr	n.d.	58.9	126.2	20	24.1	39.7	21.1	n.d.
1.5 hr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2 hr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3 hr	n.d.	50.2	125.2	15.6	11.4	13.3	4.2	n.d.
5.5 hr	n.d.	78	177.8	17	9.3	14.5	5.4	n.d.
24 hr	n.d.	90.7	267.5	15.9	6.7	8.5	4	n.d.
48 hr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
168 hr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

modified fusion protein produced in BHK cells: (galactosylated)

	μg galactosylated fusion protein /g of tumor or /g of organ measured in an OFAT							
	tumor	spleen	liver	intestine	kidney	lung	heart	plasma
0.05 hr	1.6	7.1	51.9	2.15	7.78	21.6	15.7	83.3
1 hr	0.5	0.19	1.9	0.29	1.16	0.26	0.17	0.06
1.5 hr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2 hr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3 hr	0.16	0.27	0.09	0.03	0	0.3	0	0
5.5 hr	0.27	0.02	0.11	0.05	0.02	0.08	0	0
24 hr	0.05	0.02	0.04	0	0	0	0	0
48 hr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
168 hr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

modified fusion protein produced in BHK cells (galactosylated)

	μg of ß-glucuronidase /g of tumor or /g of organ measured in an EAT							
	tumor	spleen	liver	gut	kidney	lung	heart	plasma
0.05 hr	n.d.	35.5	75.1	14	13	39	17.9	n.d.
1 hr	n.d.	27.6	167.5	17.4	5.5	7.2	1.4	n.d.
1.5 hr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2 hr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3 hr	n.d.	23.8	132.7	8.9	4.4	6.3	0.86	n.d.
5.5 hr	n.d.	28.3	164.5	10.7	4.1	6	1.1	n.d.
24 hr	n.d.	31.5	126.2	12.8	4.7	5.6	1.4	n.d.
48 hr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
168 hr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Tab. 6 – continuation

	modified fusion protein produced in BHK cells (desialylated)										modified fusion protein produced in BHK cells (desialylated)									
	μg desialylated fusion protein /g of tumor or /g of organ measured in an OFAT										μg of β-glucuronidase /g of tumor or /g of organ measured in an EAT									
	tumor	spleen	liver	intestine	kidney	lung	heart	plasma			tumor	spleen	liver	gut	kidney	lung	heart	plasma		
0.05 hr	1.3	14.6	38.9	10.2	20.6	49.8	24.2	234.9			1.3	43.2	63.2	48.7	23.8	56.1	25.7	250		
1 hr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
1.5 hr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
2 hr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
3 hr	3.3	1.1	2.8	0.37	1.5	1.8	1.3	13.8			3.3	69.8	199.2	9.2	7.4	6.1	3.9	13.5		
5.5 hr	2.9	0.1	0.22	0.02	0.04	0.19	0.06	0.65			2.9	68.7	342.1	10.6	4	4.1	2.5	0.76		
24 hr	0.4	0.1	0.04	0.002	0.004	0.16	0.001	0.004			0.4	178	641.1	34.6	15.8	16.9	7	0.058		
48 hr	0.1	0	0.04	0	0	0.55	0	0			0.1	185.9	639.7	32.7	8.8	21	12.7	0.013		
168 hr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		

Tab.6

Tab.7

Pharmacokinetic comparison between unmodified and modified humanized two-chain fusion proteins in CD-1 nude mice bearing a human tumor xenograft (LoVo)

unmodified fusion protein produced in BHK cells

	µg fusion protein /g of tumor or /g of organ measured in an OFAT							
	tumor	spleen	liver	intestine	kidney	lung	heart	plasma
0.05 hr	0.86	13.5	56.8	1.9	7.6	15.5	3.4	602
1 hr	n.d	7.9	25.2	2.7	3.9	10.9	2.6	171.7
1.5 hr	4.05	7.3	19.5	3.3	10.3	23.9	14.2	138
2 hr	n.d	5.4	15.1	2.5	4.8	7.9	1.9	119
3 hr	1.8	4.9	12.4	3	8.5	17.3	9.2	105
5.5 hr	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
24 hr	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
48 hr	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
168 hr	0.409	n.d	n.d	n.d	n.d	n.d	n.d	0.023

unmodified fusion protein produced in BHK cells

	µg of ß-glucuronidase /g of tumor or /g of organ measured in an EAT							
	tumor	spleen	liver	gut	kidney	lung	heart	plasma
0.05 hr	n.d	42.8	96.5	12.5	12.4	21.6	3.9	n.d
1 hr	n.d	57.2	202.6	10.7	8.4	15.5	9.2	n.d
1.5 hr	n.d	51.5	164.6	12.3	16.1	30.6	6.8	n.d
2 hr	n.d	59.5	136.9	12	8.6	12.9	5.4	n.d
3 hr	n.d	51.9	181	14.1	13.1	23.4	8.9	n.d
5.5 hr	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
24 hr	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
48 hr	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
168 hr	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d

modified fusion protein produced in BHK cells (galactosylated)

	µg galactosylated fusion protein /g of tumor or /g of organ measured in an OFAT							
	tumor	spleen	liver	intestine	kidney	lung	heart	plasma
0.05 hr	0.45	6.3	41.2	1.43	2.78	7.7	0.82	257.6
1 hr	n.d	0.16	0.63	0.05	0.03	0.033	0	0.032
1.5 hr	1.45	0.09	0.42	0.05	0.05	0.164	0.036	0.006
2 hr	n.d	0.008	0.11	0.03	0	0.1	0	0
3 hr	0.48	0	0.08	0.006	0	0.05	0.002	0
5.5 hr	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
24 hr	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
48 hr	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
168 hr	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d

modified fusion protein produced in BHK cells (galactosylated)

	µg of ß-glucuronidase /g of tumor or /g of organ measured in an EAT							
	tumor	spleen	liver	gut	kidney	lung	heart	plasma
0.05 hr	n.d	29.3	48.2	12.4	8.2	14.1	1.7	n.d
1 hr	n.d	24.6	132.1	13.1	4.6	5.2	0.95	n.d
1.5 hr	n.d	19.6	120.8	15.3	4.2	4.7	1.7	n.d
2 hr	n.d	10.7	111.4	16.6	3.6	5.6	0.88	n.d
3 hr	n.d	22	164.9	13.6	3.8	4.9	0.95	n.d
5.5 hr	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
24 hr	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
48 hr	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
168 hr	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d

Tab. 8

Pharmacokinetic comparison between unmodified humanized two-chain fusion proteins, produced in BHK cells and CHO cells, in CD-1 nude mice bearing a human tumor xenograft (MzSto1)

	unmodified fusion protein produced in BHK cells							
	µg of fusion protein /g of tumor or /g of organ measured in an OFAT							
	tumor	spleen	liver	intestine	kidney	lung	heart	plasma
0.05 hr	3.807	22.779	44.411	7.732	27.792	53.59	33.941	413
3hr	6.166	8.847	20.099	4.125	12.609	26.363	12.93	147
24hr	4.944	0.935	1.416	0.249	1.315	2.779	1.493	12.3
72hr	0.818	0.094	0.14	0.058	0.112	0.241	0.136	1.152
168hr	0.314	0.002	0.005	0.002	0.002	0.008	0	0.015

	unmodified fusion protein produced in BHK cells							
	µg of β-glucuronidase /g of tumor or /g of organ measured in an EAT							
	tumor	spleen	liver	intestine	kidney	lung	heart	plasma
0.05 hr	6.473	27.927	44.748	13.045	27.419	59.345	30.897	n.d.
3hr	9.982	70.009	445.32	15.714	16.415	27.014	14.427	n.d.
24hr	28.384	41.799	33.834	6.605	2.422	3.99	2.498	n.d.
72hr	9.929	20.458	12.391	12.416	1.051	2.283	1.306	n.d.
168hr	7.423	7.393	6.083	5.164	0.94	1.483	0.502	n.d.

	unmodified fusion protein produced in CHO cells							
	µg of fusion protein /g of tumor or /g of organ measured in an OFAT							
	tumor	spleen	liver	intestine	kidney	lung	heart	plasma
0.05 hr	3.583	19.179	33.992	7.96	23.089	61.279	24.018	308
3hr	6.526	8.555	17.787	7.098	11.613	26.755	10.824	157.9
24hr	4.668	1.002	1.225	0.299	1.218	2.926	1.361	12.082
72hr	2.176	0.036	0.028	0.013	0.023	0.059	0.029	0.144
168hr	0.653	0.003	0.002	0.003	0	0.003	0	0.005

	unmodified fusion protein produced in CHO cells							
	µg of β-glucuronidase /g of tumor or /g of organ measured in an EAT							
	tumor	spleen	liver	intestine	kidney	lung	heart	plasma
0.05 hr	6.475	23.883	29.696	14	23.223	56.864	23.042	n.d.
3hr	10.96	89.594	204.82	15.928	13.66	26.994	12.556	n.d.
24hr	29.279	72.999	23.754	7.011	2.513	5.003	3.089	n.d.
72hr	50.13	25.828	7.527	7.613	1.542	2.568	1.719	n.d.
168hr	5.515	14.251	4.172	4.172	1.202	1.588	1.193	n.d.

Example 6

Galactosylation of the two-chain fusion protein

The galactosylation of the fusion protein was carried out using a modification of the method of Mattes (*J. Natl. Cancer Inst.*, 79: 855 (1987) which is incorporated herein in its entirety):

Cyanomethyl-2,3,4,6-tetra-O-acetyl-1-thio-β-D-galactopyranoside (Sigma; 250 mg) was dissolved in dried methanol (Merck; 6.25 ml), and 625 µl of a methanolic sodium methoxide solution (5.4 mg/ml) were then pipetted in. After incubating at room temperature for 48 h, an aliquot of 5 ml of the activated galactose derivative was removed and the methanol evaporated off in a stream of nitrogen 100 ml of a fusion protein solution (1 mg/ml in 0.25 M sodium borate buffer, pH 8.5) were added to the remaining residue, and the mixture incubated at R.T. for 24 h. This was followed by dialysis overnight against PBS.

Galactosylation of the preformed BW 431/26-E. coli β-glucuronidase conjugates and the monoclonal antibody BW 431/26 was carried out in a similar manner. Using similar chemistry, lactosilation, N-acetyl-lactosilation and glucosilation of AEC and FUP can be performed.

Example 7

Working up organs/tumors for FUP determination

The following sequential steps were carried out:

1. Nude mice (CD1), which possess a subcutaneous tumor and which have been treated with fusion protein or antibody-enzyme conjugate, are bled retroorbitally and then sacrificed.
2. The blood is immediately added to an Eppendorf tube which already contains 10 µl of Liquemin 25000 (from Hoffman LaRoche AG).
3. The treated blood from 2. above is centrifuged (in a Megafuge 1.0 centrifuge, from Heraeus) at 2500 rpm for 10 min; the plasma is then isolated and frozen down until testing.
4. The organs, or the tumor, are removed, weighed and then completely homogenized with 2 ml of 1% BSA in PBS, pH 7.2.
5. The tumor homogenates are adjusted to pH 4.2 with O.1N HCl (the sample must not be overtitrated, or the β-glucuronidase will be activated prematurely at pH < 3.8!)
6. Homogenates are centrifuged at 16000 g for 30 min; the clear supernatant fluids are removed and neutralized with 0.1 N NaOH.
7. The supernatants and the plasma can now be tested in an OFAT (measures FUP concentration) or an EAT (measures β-glucuronidase concentration), as described in the examples below.

Example 8

OFAT (organ fusion protein activity test)

The test proceeds in the following manner:

1. 75 µl of a goat anti-human-kappa antibody (from Southern Biotechnology Associates, Order No. 2060-01), diluted 1:300 in PBS, pH 7.2, are added to each well of a microtitration plate (polystyrene U form, type B, from Nunc, Order No. 4-60445).
2. The microtitration plates are covered and incubated at room temperature overnight.
3. The microtitration plates are then washed 3 times with 250 µl of 0.05 M Tris-citrate buffer, pH 7.4, per well.
4. These microtitration plates, which have been coated in this manner, are incubated with 250 µl of blocking solution (1% casein in PBS, pH 7.2) per well at room temperature for 30 mins (blocking of non-specific binding sites)

(coated microtitration plates which are not required are dried at room temperature for 24 hours and then sealed, together with desiccator cartridges, in coated aluminium bags for long-term storage).

5. The substrate is prepared while the blocking is proceeding (fresh substrate for each test: 2.5 mM 4-methylumbelliferyl β-D-glucuronide, Order No.: M-9130, from Sigma, in 200 mM Na acetate + 0.01% BSA, pH 4.5).
6. Thereafter, 10 samples + 1 positive control + 1 negative control are diluted in 1% casein in PBS, pH 7.2, 1:2 in 8 steps (starting from 150 µl of sample, 75 µl of sample are pipetted into 75 µl of casein recipient solution, etc.) in an untreated 96-well U-shape bottomed microtiter plate (polystyrene, from Renner, Order No. 12058).
7. The blocking solution is sucked off from the microtitration plate coated with anti-human-kappa antibody, and 50 µl of the diluted samples are transferred to each well of the test plate from the dilution plate, and the test plate is incubated at room temperature for 30 min.

8. The test plate is washed 3 times with ELISA washing buffer (Behringwerke, Order No. OSEW96);
9. 50 µl of substrate are applied per well and the test plates are covered and incubated at 37 °C for 2 h.
10. 150 µl of stock solution (0.2 M glycine + 0.2% SDS, pH 11.7) are then added to each well.
11. Fluorometric evaluation is carried out in a Fluoroscan II (ICN Biomedicals, Cat. No. 78-611-00) at an excitation wavelength of 355 nm and an emission wavelength of 460 nm.
12. With the aid of the fluorescence values for the positive control (dilution series with purified fusion protein as the standard curve) included in the identical experiment, the unknown concentration of fusion protein is determined in the sample.

Example 9: EAT (enzyme activity test)

The test is carried out in the following manner:
1. 10 samples + 1 positive control + 1 negative control are diluted 1:2 in 1% casein in PBS, pH 7.2, in 8 dilution steps in a 96-well microtiter plate (polystyrene, from Renner, Order No. 12058) so that each well contains 50 µl of sample.
2. 50 µl of substrate (2.5 mM 4-methylumbelliferyl β-D-glucuronide (from Sigma, Order No. M-9130, in 200 mM Na acetate + 0.01% BSA, pH 4.5) are added to each well.
3. The microtiter plate is covered and incubated at 37 °C for 2 h.
4. 150 µl of stock solution (0.2 M glycine + 0.2% SDS, pH 11.7) are then added per well.
5. Fluorometric evaluation is carried out in a Fluoroscan II (ICN Biomedicals, Cat. No. 78-611-00) at an excitation wavelength of 355 nm and an emission wavelength of 460 nm;
6. With the aid of the positive control (dilution series with purified fusion protein as the standard curve) which has been included, the sample concentrations can now be calculated.

Example 10

Desialylation of the two-chain fusion glycoprotein

The two-chain fusion protein was desialylated according to Murray (*Methods in Enzymology* 149: 251 (1987)). Eight units of neuraminidase (Sigma, type X-A from *Clostridium perfringens*) coupled to agarose were washed 3x with 40 ml of 100 mM sodium acetate buffer, pH 5, and then taken up as a 1:1 suspension. One hundred milliliters of two-chain fusion protein (1 mg/ml in sodium acetate buffer, pH 5) were added to this suspension, which was then incubated with gentle shaking at 37 °C for 4 h. The immobilized neuraminidase was removed by centrifuging off, and the fusion protein was dialyzed overnight against PBS.

Example 11

Demonstrating rapid elimination of modified FUP

100 mg of humanized two-chain fusion glycoprotein were purified from BHK transfectant supernatant, as described in EP-A-0 501 215, pages 10-11. The purified protein was galactosylated or desialylated, as described in the preceding examples.

400 µg of the modified protein thus obtained, in this case the galactosylated humanized two-chain fusion protein, were injected i.v. into nude mice. The mice had been injected subcutaneously, 10 days previously, with 10^6 CEA-expressing human stomach carcinoma cells (Mz-Sto-1). At various time intervals, the mice were killed, and the concentration of functionally active modified protein was determined in the tumor, the plasma and the normal tissues using the OFAT or the EAT (see Examples 7, 8 and 9).

Nude mice which in each case had been provided with 1 x 10^6 CEA-negative human tumors (Oat-75) were used as the antigen control. In addition, identical quantities of the humanized two-chain fusion protein (starting protein) or of a humanized two-chain fusion protein sample which had been treated with solid phase neuraminidase (desialylated protein) were injected i.v., as the protein control (see Example 10). The quantities of the functionally active proteins found in the organs in this representative experiment are given in Tables 6 and 7.

Comparable results were found in the identical animal model system using the example of a CEA-positive colon carcinoma, a CEA-positive rectal carcinoma, a CEA-positive adenocarcinoma of the lung, a CEA-positive pancreatic carcinoma, a CEA-positive thyroid gland carcinoma, and a CEA-positive mammary carcinoma.

Therapeutic effects which are superior to those of the standard chemotherapy can be achieved when suitable non-toxic prodrugs, e.g. those described in EP-A-0 511 917, are used which are applied at a point in time at which the modified proteins have been largely eliminated from the plasma or have been internalized and degraded in the normal tissues. These effects can be improved still further by adding large quantities of galactose to the relevant modified protein, leading to optimization of the pharmacokinetics.

Further improvements can be achieved in accordance with the method described by Jähde *et al.* (*Cancer Res.* 52: 6209 (1992)) by adding glucose, phosphate ions or metaiodobenzylguanidine to the relevant modified protein, or injecting these compounds prior to the protein. This method leads to a decline in the pH within the tumor. This results in more efficient catalysis of the prodrugs by the enzymes used in the modified and non-modified proteins according to the invention: Alternatively, HCO_3^- can also be employed for lowering the pH in the tumor (Gullino *et al., J. Nat. Cancer Inst.* 34: 857, (1965)).

It will be apparent to those skilled in the art that various modifications and variations can be made to the compositions and processes of this invention. Thus, it is intended that the present invention cover such modifications and variations, provided they come within the scope of the appended claims and their equivalents.

The disclosure of all publications cited above are expressly incorporated herein by reference in their entireties to the same extent as if each were incorporated by reference individually. The disclosure of German Patent Application P 43 14 556.6 for which benefit under 35 USC §119 is claimed, is expressly incorporated herein in its entirety.

Claims

1. A compound comprising a bifunctional fusion glycoprotein or bifunctional glycoprotein conjugate, the compound comprising a carbohydrate complement, and:
   a. at least one first portion which possesses enzymatic activity;
   b. at least one second portion which binds specifically to an epitope of a tumor-specific antigen;
   wherein the carbohydrate complement comprises at least one exposed terminal carbohydrate residue selected from the group consisting of mannose, galactose, N-acetylglucosamine, N-acetyllactose, glucose and fucose.

2. A compound as claimed in claim 1, wherein the exposed carbohydrate residue is produced by enzymatic degradation.

3. A compound as claimed in claim 2, wherein the enzymatic degradation is effected by an enzyme selected from the group consisting of endoglycosidases, exoglycosidases, and neuraminidases, and a combination thereof.

4. A compound as claimed in claim 1, wherein the exposed carbohydrate residue is produced by chemical degradation.

5. A compound as claimed in claim 1 wherein the exposed carbohydrate residue is added to the compound by chemical means.

6. A compound as claimed in claim 1, wherein the first portion consists essentially of an enzyme.

7. A compound as claimed in claim 6, wherein the enzyme is selected from the group consisting of penicillin G amidase, penicillin V amidase, β-lactamase, alkaline phosphatase, carboxypeptidase G2, carboxypeptidase A, cytosine deaminase, nitroreductase, diaphorase, arylsulfatase, glycosidase, β-glucosidase, and β-glucuronidase.

8. A compound as claimed in claim 6 wherein the enzyme is a catalytic antibody.

9. A compound as claimed in claim 1, wherein the tumor cell marker to which the second portions binds comprises a tumor associated antigen selected from the group consisting of CEA, N-CAM, N-cadherin, PEM, GICA, TAG-72, TFβ, GM3, GD3, GM2, GD2, GT3, HMWMAA, pMel17, gp113 (Muc18), p53, p97, MAGE-1, gp105, erbB2, EGF-R, PSA, transferrin-R, P-glycoprotein and cytokeratin.

10. A compound as claimed in claim 1, wherein the second portion consists essentially of an antibody or a fragment thereof.

11. A compound as claimed in claim 11, wherein the antibody is the monoclonal antibody BW 431/26 or a fragment thereof.

12. A compound as claimed in claim 1, wherein the first portion and the second portion are connected by a linker molecule.

13. A compound of claim 12 having the formula huTuMab-L-β-Gluc, wherein huTuMab is a human tumor specific monoclonal antibody or a tumor binding fragment thereof, L is the linker molecule and β-Gluc is a human β-glucuronidase.

14. A compound as claimed in claim 1, comprising a fusion glycoprotein that has been synthesized in CHO cells, the cells having been selected for a high level of expression of the glycoprotein.

15. A compound as claimed in claim 1, wherein the exposed carbohydrate is a galactose or a mannose.

16. A pharmaceutical preparation containing a compound as claimed in claim 1 in a pharmaceutically acceptable vehicle.

17. A pharmaceutical preparation containing a compound as claimed in claim 1, and an agent capable of lowering the pH in a tumor to be treated, in a pharmaceutically acceptable vehicle.

18. A pharmaceutical preparation, containing a compound as claimed in claim 1, and galactose, in a pharmaceutically acceptable vehicle.

19. A process of making a fusion glycoprotein comprising the steps of:
    a. preparing a DNA encoding a fusion glycoprotein according to claim 1;
    b. inserting the DNA in an expression vector;
    c. expressing the DNA in a eukaryote expression system; and,
    d. isolating the expressed fusion glycoprotein.

20. A process according to claim 19 wherein the expression system is a transgenic non-human mammalian animal.

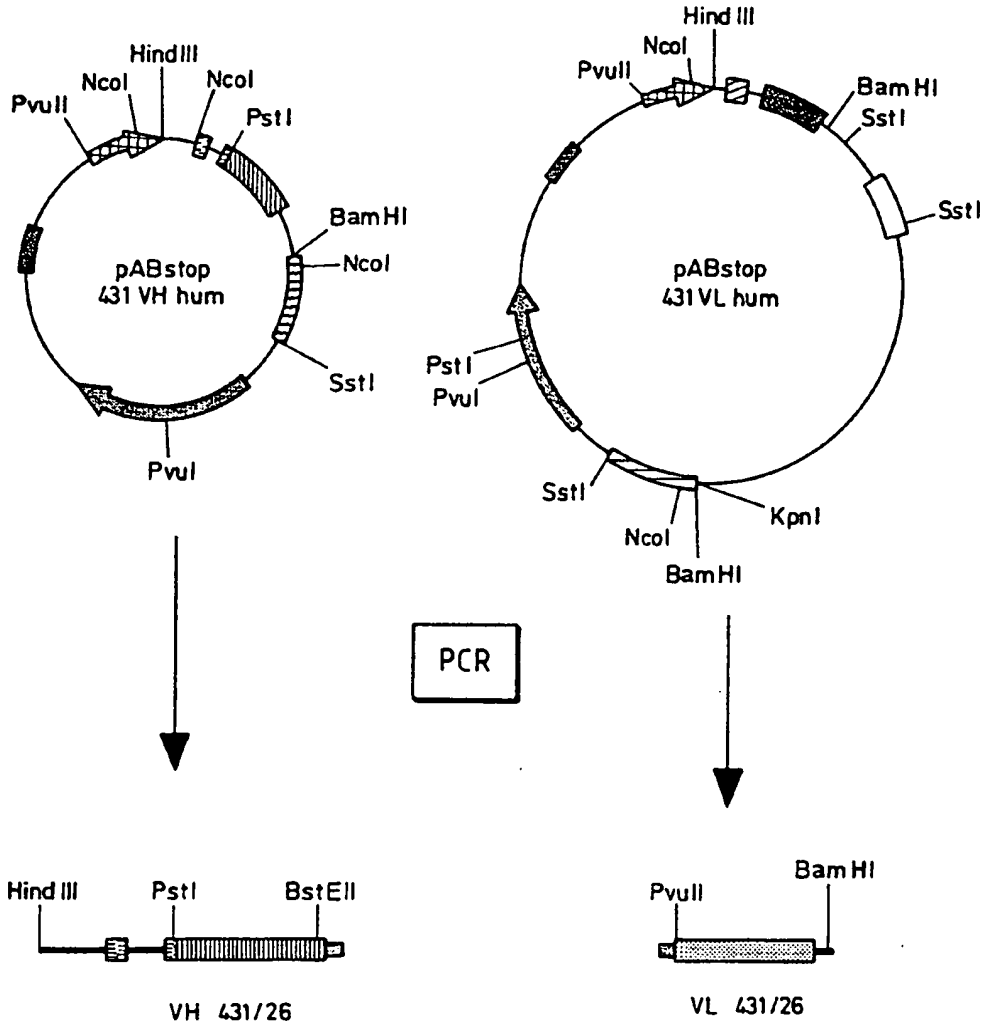


FIG.1

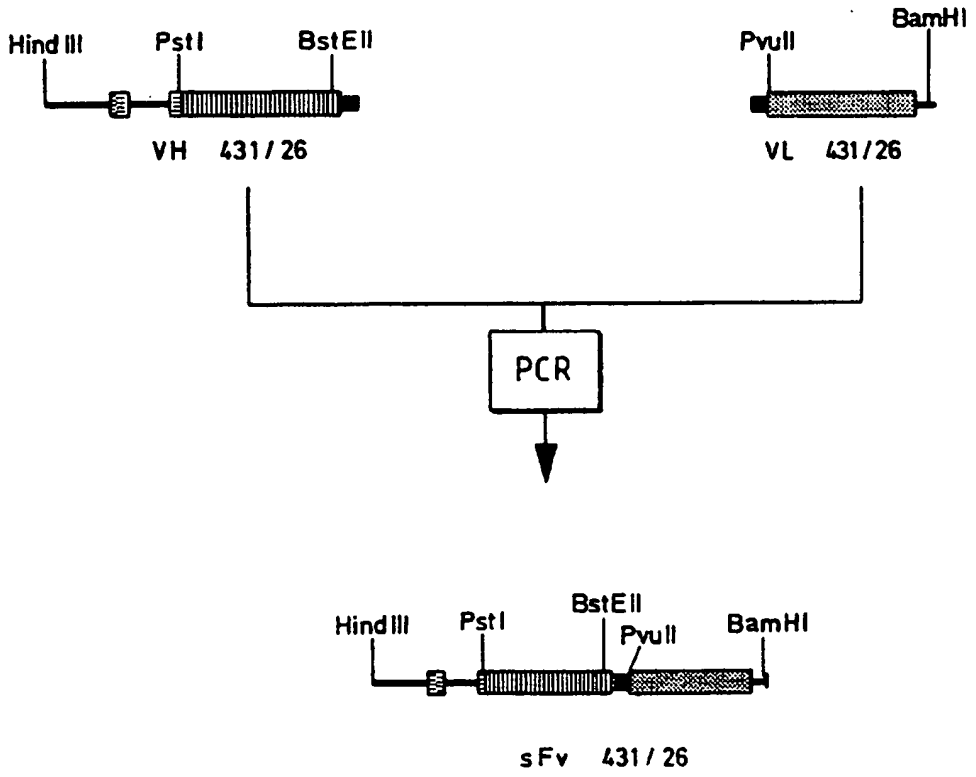


FIG.2

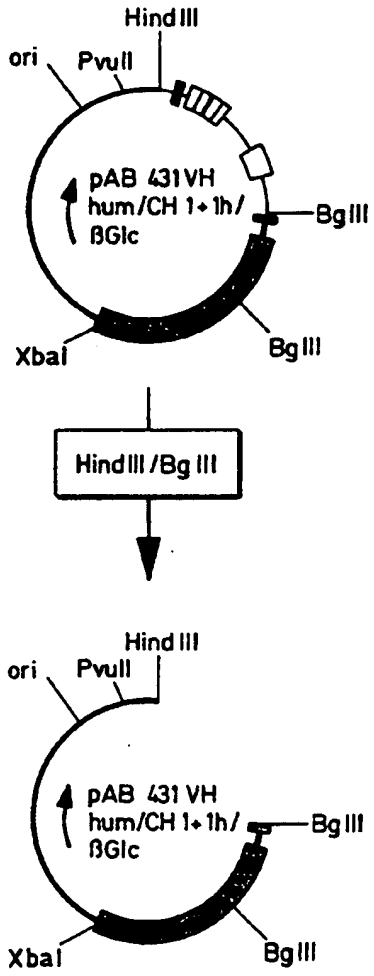
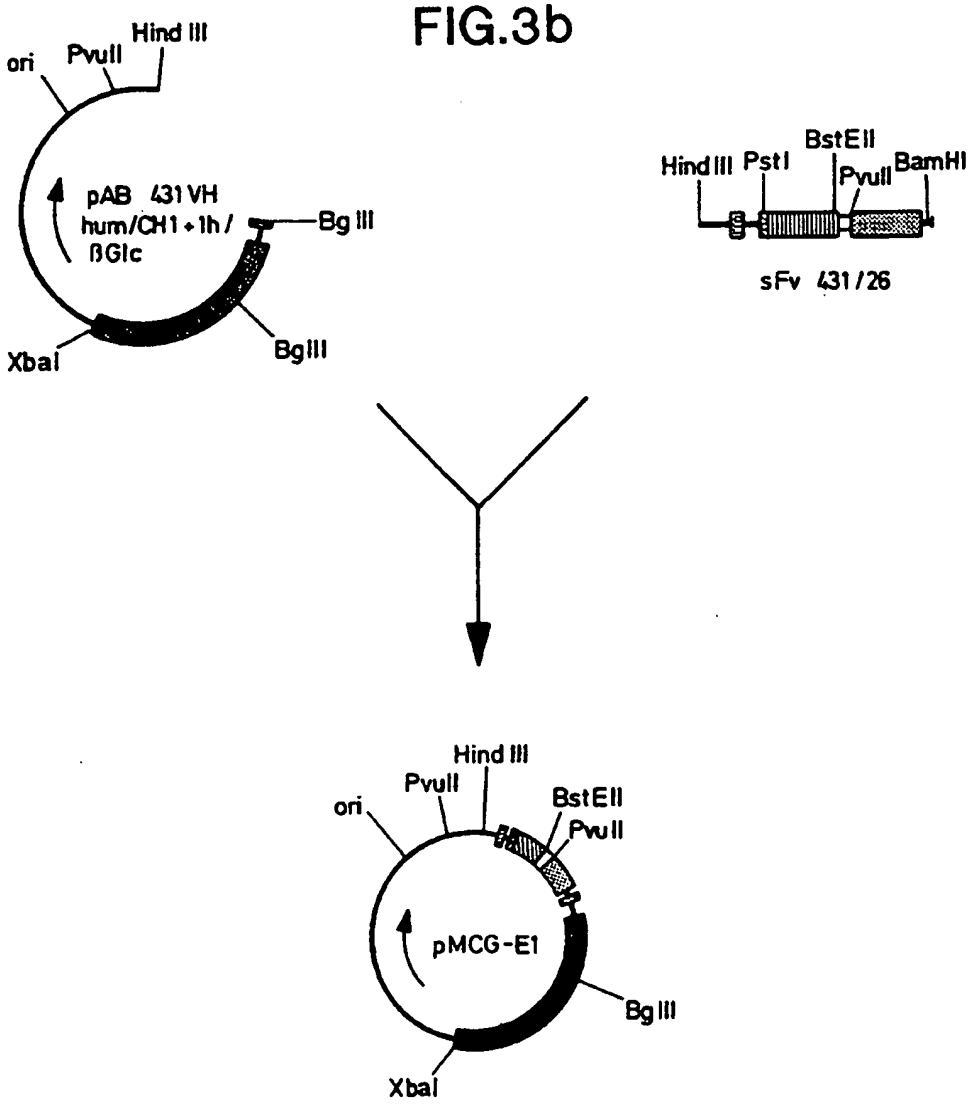


FIG.3a

FIG.3b



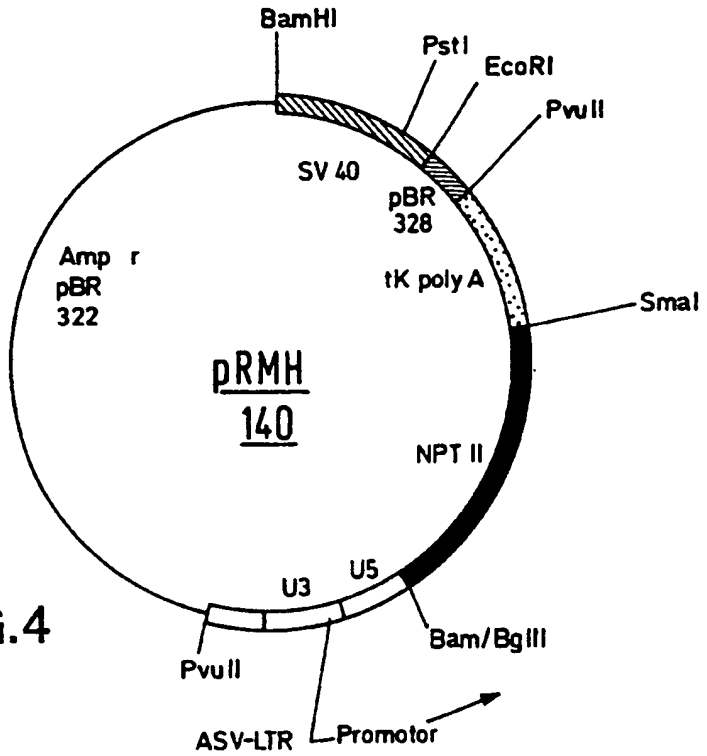


FIG.4

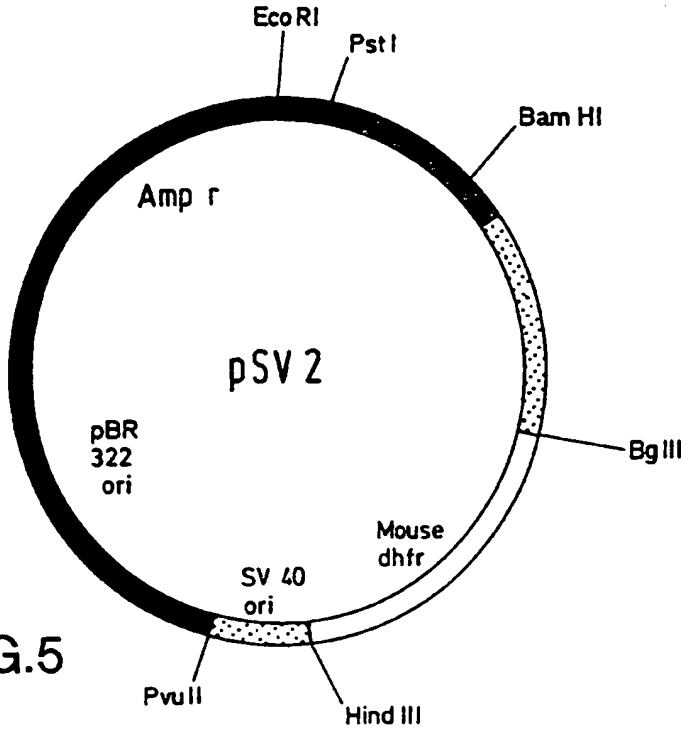


FIG.5

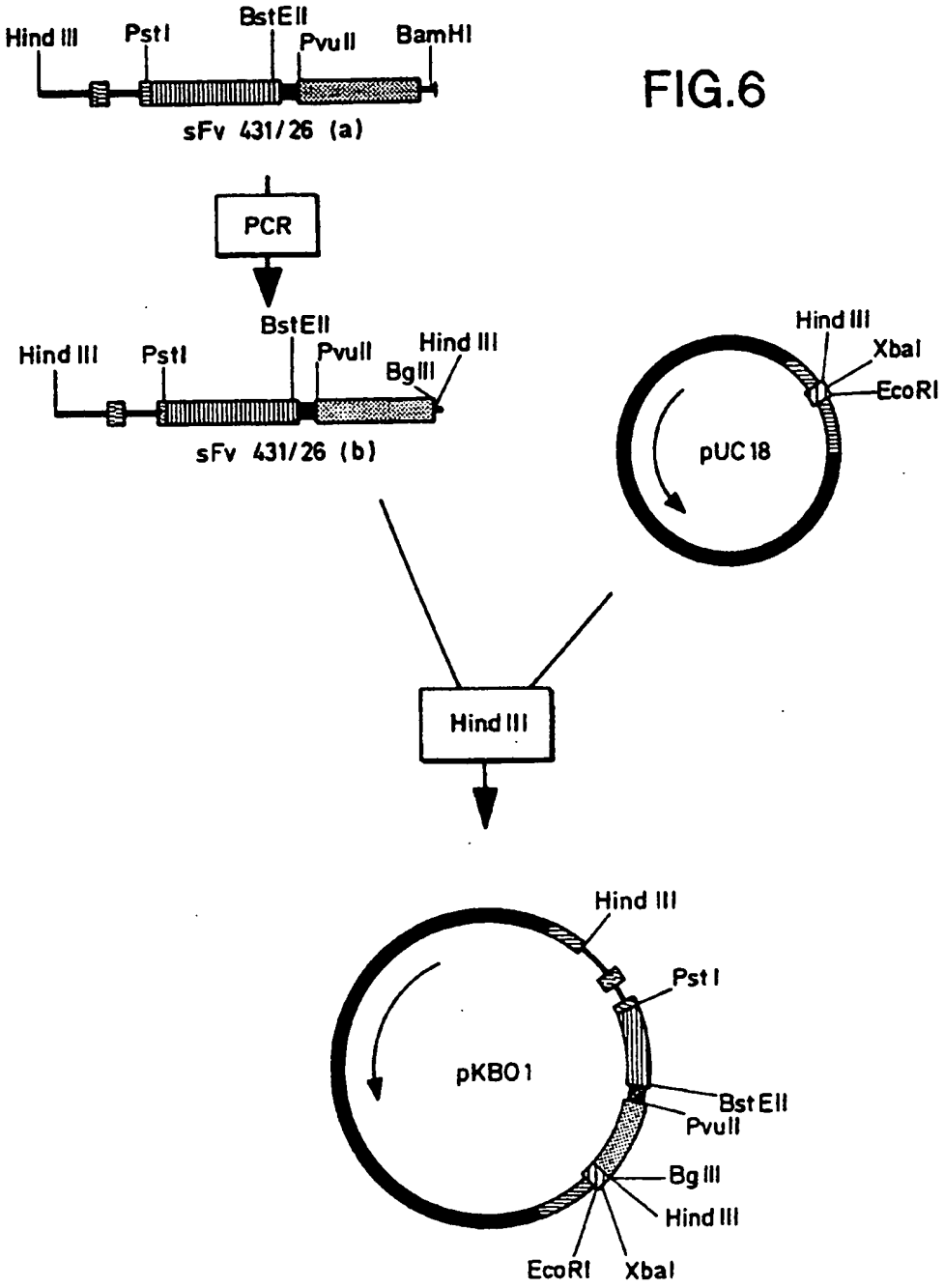


FIG.6

FIG.7

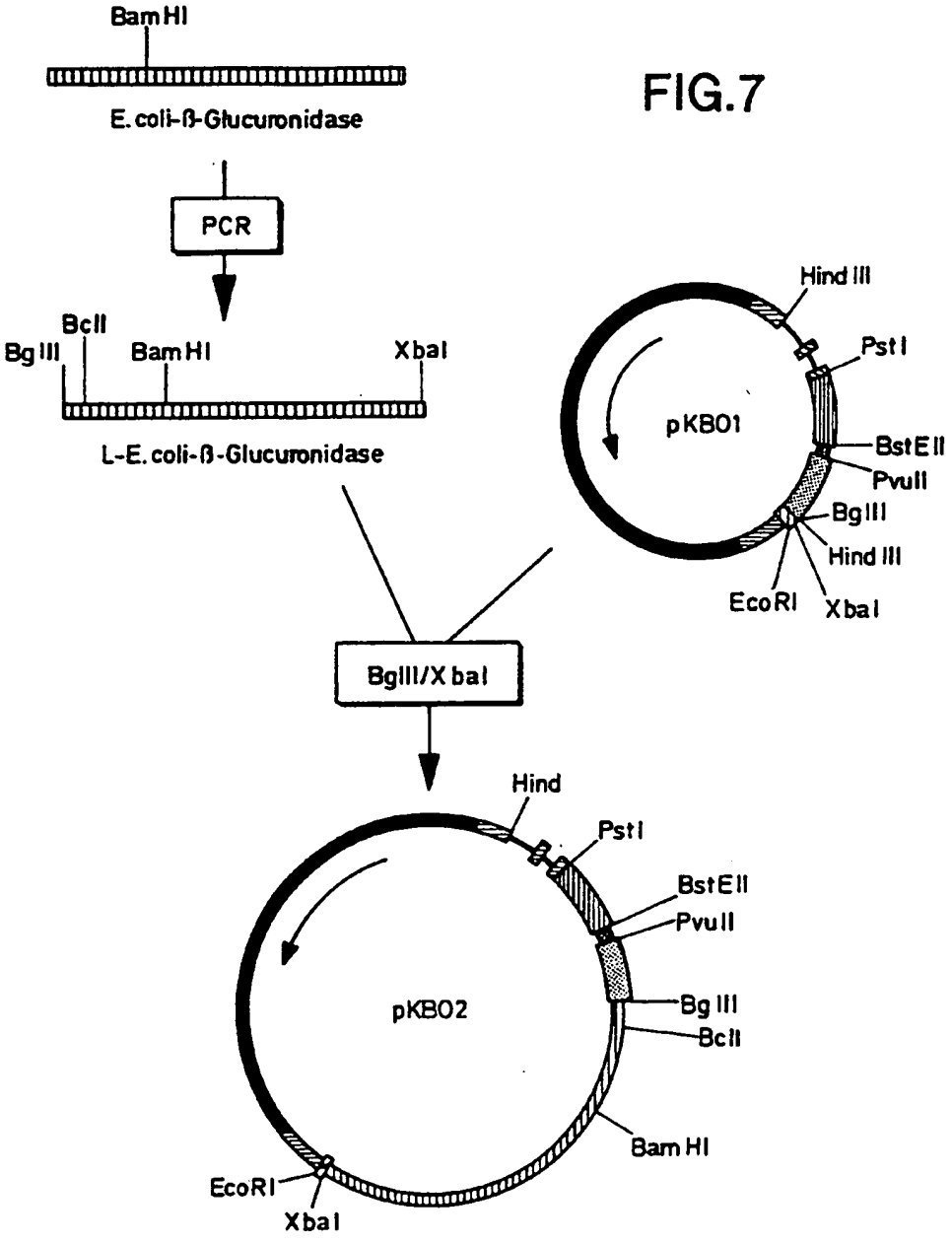
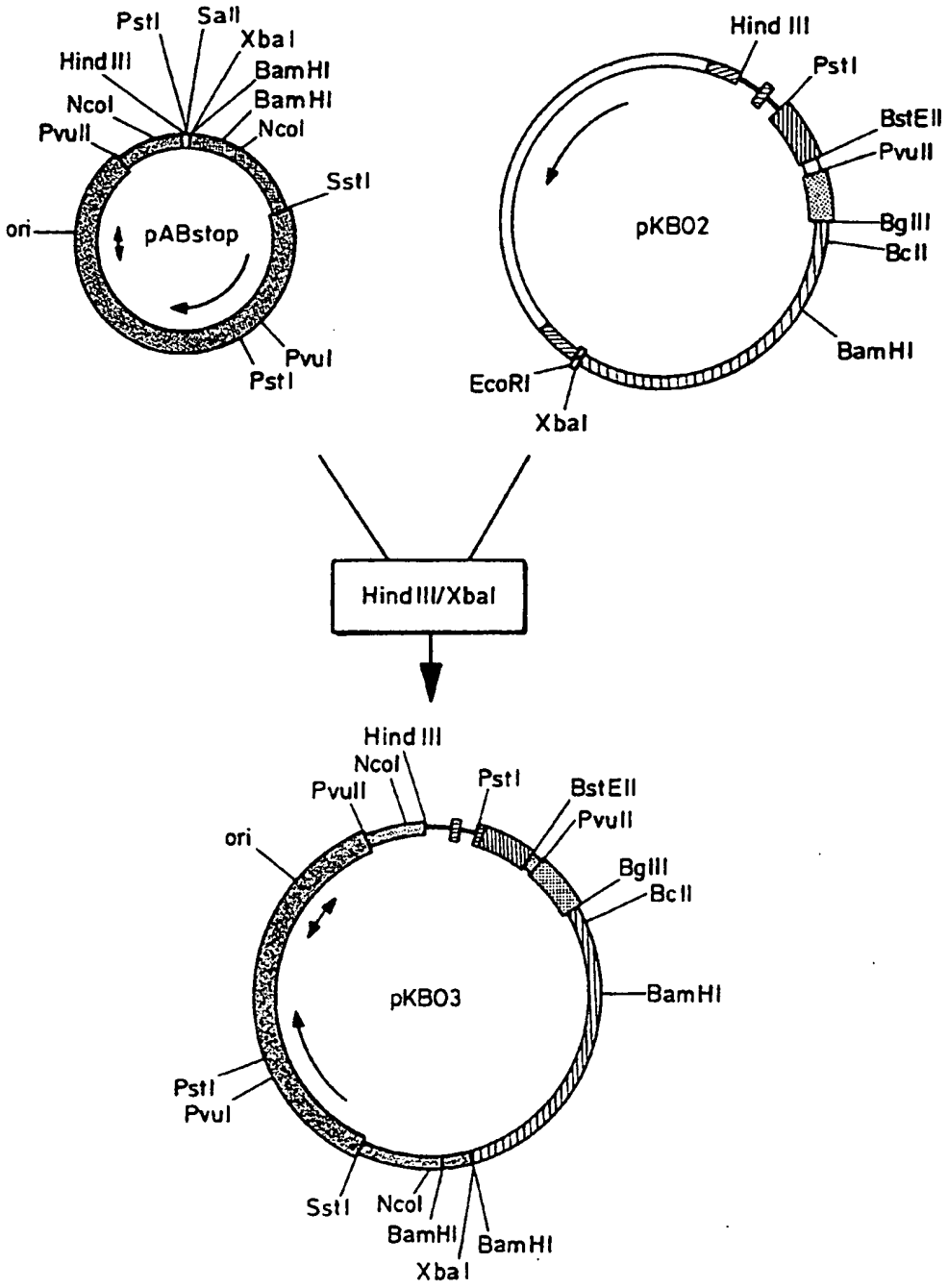


FIG.8



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(54) Title: RECOMBINANT ANTI-VLA4 ANTIBODY MOLECULES

(57) Abstract

The present invention discloses recombinant anti-VLA4 antibody molecules, including humanized recombinant anti-VLA4 antibody molecules. These antibodies are useful in the treatment of specific and non-specific inflammation, including asthma and inflammatory bowel disease. In addition, the humanized recombinant anti-VLA4 antibodies disclosed can be useful in methods of diagnosing and localizing sites of inflammation.

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RECOMBINANT ANTI-VLA4 ANTIBODY MOLECULES

FIELD OF THE INVENTION

The present invention relates to recombinant anti-VLA4 antibody molecules, including humanized recombinant anti-VLA4 antibody molecules.

BACKGROUND OF THE INVENTION

A. Immunoglobulins and Monoclonal Antibodies

Natural immunoglobulins have been known for many years, as have the various fragments thereof, such as the Fab, $(Fab')_2$ and Fc fragments, which can be derived by enzymatic cleavage. Natural immunoglobulins comprise generally a Y-shaped molecule having an antigen-binding site towards the free end of each upper arm. The remainder of the structure, and particularly the stem of the Y, mediates the effector functions associated with immunoglobulins.

Specifically, immunoglobulin molecules are comprised of two heavy (H) and two light (L) polypeptide chains, held together by disulfide bonds. Each chain of an immunoglobulin chain is divided into regions or domains, each being approximately 110 amino acids. The light chain has two such domains while the heavy chain has four domains. The amino acid sequence of the amino-terminal domain of each polypeptide chain is highly variable (V region), while the sequences of the remaining domains are conserved or constant (C regions). A light chain is therefore composed of one variable (V_L) and one constant domain (C_L) while a heavy chain contains one variable (V_H) and three constant domains (CH_1, CH_2 and CH_3). An arm of the Y-shaped molecule consists of a light chain (V + C_L) and the variable domain (V_H) and one constant domain (CH_1) of a heavy chain. The tail of the Y is composed of

the remaining heavy chain constant domains (CH_2 + CH_3). The C-terminal ends of the heavy chains associate to form the Fc portion. Within each variable region are three hypervariable regions. These hypervariable regions are also described as the complementarity determining regions (CDRs) because of their importance in binding of antigen. The four more conserved regions of the variable domains are described as the framework regions (FRs). Each domain of an immunoglobulin consists of two beta-sheets held together by a disulfide bridge, with their hydrophobic faces packed together. The individual beta strands are linked together by loops. The overall appearance can be described as a beta barrel having loops at the ends. The CDRs form the loops at one end of the beta barrel of the variable region.

Natural immunoglobulins have been used in assay, diagnosis and, to a more limited extent, therapy. However, such uses, especially in therapy, have been hindered by the polyclonal nature of natural immunoglobulins. A significant step towards the realization of the potential of immunoglobulins as therapeutic agents was the discovery of techniques for the preparation of monoclonal antibodies (MAbs) of defined specificity, Kohler et al., 1975 [1]. However, most MAbs are produced by fusions of rodent (i.e., mouse, rat) spleen cells with rodent myeloma cells. They are therefore essentially rodent proteins.

By 1990, over 100 murine monoclonal antibodies were in clinical trials, particularly in the U.S. and especially for application in the treatment of cancer. However, by this time it was recognized that rejection of murine monoclonal antibodies by the undesirable immune response in humans termed the HAMA (Human Anti-Mouse Antibody) response was a severe limitation, especially for the treatment of chronic disease. Therefore, the use of rodent MAbs as therapeutic agents in humans is

inherently limited by the fact that the human subject will mount an immunological response to the MAb and either remove the MAb entirely or at least reduce its effectiveness. In practice MAbs of rodent origin may not be used in a patient for more than one or a few treatments as a HAMA response soon develops rendering the MAb ineffective as well as giving rise to undesirable reactions. In fact, a HAMA response has been observed in the majority of patients following a single injection of mouse antibody, Schroff et al., 1985 [2]. A solution to the problem of HAMA is to administer immunologically compatible human monoclonal antibodies. However, the technology for development of human monoclonal antibodies has lagged well behind that of murine antibodies (Borrebaeck et al., 1990 [3] such that very few human antibodies have proved useful for clinical study.

Proposals have therefore been made for making non-human MAbs less antigenic in humans. Such techniques can be generically termed "humanization" techniques. These techniques generally involve the use of recombinant DNA technology to manipulate DNA sequences encoding the polypeptide chains of the antibody molecule. The use of recombinant DNA technology to clone antibody genes has provided an alternative whereby a murine monoclonal antibody can be converted to a predominantly human-form (i.e., humanized) with the same antigen binding properties (Riechmann et al., 1988 [4]). Generally, the goal of the humanizing technology is to develop humanized antibodies with very little or virtually no murine component apart from the CDRs (see, e.g., Tempest et al., 1991 [5]) so as to reduce or eliminate their immunogenicity in humans.

Early methods for humanizing MAbs involved production of chimeric antibodies in which an antigen binding site comprising the complete variable domains of one antibody is linked to constant domains derived from

another antibody. Methods for carrying out such chimerization procedures have been described, for example, in EP 120694 [6], EP 125023 [7], and WO 86/01533 [8]. Generally disclosed are processes for preparing antibody molecules having the variable domains from a non-human MAb such as a mouse MAb and the constant domains from a human immunoglobulin. Such chimeric antibodies are not truly humanized because they still contain a significant proportion of non-human amino acid sequence, i.e., the complete non-human variable domains, and thus may still elicit some HAMA response, particularly if administered over a prolonged period, Begent et al., 1990 [9]. In addition, it is believed that these methods in some cases (e.g., EP 120694 [6]; EP 125023 [7] and U.S. Patent No. 4,816,567 [10] did not lead to the expression of any significant quantities of Ig polypeptide chains, nor the production of Ig activity without *in vitro* solubilization and chain reconstitution, nor to the secretion and assembly of the chains into the desired chimeric recombinant antibodies. These same problems may be noted for the initial production of non-chimeric recombinant antibodies (e.g., U.S. Patent No. 4,816,397 [11].

B. Humanized Recombinant Antibodies and CDR-Grafting Technology

Following the early methods for the preparation of chimeric antibodies, a new approach was described in EP 0239400 [12] whereby antibodies are altered by substitution of their complementarity determining regions (CDRs) for one species with those from another. This process may be used, for example, to substitute the CDRs from human heavy and light chain Ig variable region domains with alternative CDRs from murine variable region domains. These altered Ig variable regions may subsequently be combined with human Ig constant regions to created antibodies which are totally human in composition except for the substituted murine CDRs. Such

murine CDR-substituted antibodies would be predicted to be less likely to elicit a considerably reduced immune response in humans compared to chimeric antibodies because they contain considerably less murine components.

The process for humanizing monoclonal antibodies via CDR grafting has been termed "reshaping". (Riechmann et al., 1988 [4]; Verhoeyen et al., 1988 [13]. Typically, complementarity determining regions (CDRs) of a murine antibody are transplanted onto the corresponding regions in a human antibody, since it is the CDRs (three in antibody heavy chains, three in light chains) that are the regions of the mouse antibody which bind to a specific antigen. Transplantation of CDRs is achieved by genetic engineering whereby CDR DNA sequences are determined by cloning of murine heavy and light chain variable (V) region gene segments, and are then transferred to corresponding human V regions by site-directed mutagenesis. In the final stage of the process, human constant region gene segments of the desired isotype (usually gamma 1 for C_H and kappa for C_L) are added and the humanized heavy and light chain genes are coexpressed in mammalian cells to produce soluble humanized antibody.

The transfer of these CDRs to a human antibody confers on this antibody the antigen binding properties of the original murine antibody. The six CDRs in the murine antibody are mounted structurally on a V region "framework" region. The reason that CDR-grafting is successful is that framework regions between mouse and human antibodies may have very similar 3-D structures with similar points of attachment for CDRs, such that CDRs can be interchanged. Nonetheless, certain amino acids within framework regions are thought to interact with CDRs and to influence overall antigen binding affinity. The direct transfer of CDRs from a murine antibody to produce a recombinant humanized antibody

without any modifications of the human V region frameworks often results in a partial or complete loss of binding affinity.

In Riechmann et al., 1988 [4] and WO 89/07454 [14], it was found that transfer of the CDR regions alone (as defined by Kabat et al., 1991 [15] and Wu et al., 1970 [16] was not sufficient to provide satisfactory antigen binding activity in the CDR-grafted product. Riechmann et al. 1988 [4] found that it was necessary to convert a serine residue at position 27 of the human sequence to the corresponding rat phenylalanine residue to obtain a CDR-grafted product having satisfactory antigen binding activity. This residue at position 27 of the heavy chain is within the structural loop adjacent to CDR1. A further construct which additionally contained a human serine to rat tyrosine change at position 30 of the heavy chain did not have a significantly altered binding activity over the humanized antibody with the serine to phenylalanine change at position 27 alone. These results indicate that changes to residues of the human sequence outside the CDR regions, for example, in the loop adjacent to CDR1, may be necessary to obtain effective antigen binding activity for CDR-grafted antibodies which recognize more complex antigens. Even so, the binding affinity of the best CDR-grafted antibodies obtained was still significantly less than the original MAb.

More recently, Queen et al., 1989 [17] and WO 90/07861 [18] have described the preparation of a humanized antibody that binds to the interleukin 2 receptor, by combining the CDRs of a murine MAb (anti-Tac) with human immunoglobulin framework and constant regions. They have demonstrated one solution to the problem of the loss of binding affinity that often results from direct CDR transfer without any modifications of the human V region framework residues; their solution involves two key steps. First, the human

V framework regions are chosen by computer analysis for optimal protein sequence homology to the V region framework of the original murine antibody, in this case, the anti-Tac MAb. In the second step, the tertiary structure of the murine V region is modelled by computer in order to visualize framework amino acid residues which are likely to interact with the murine CDRs and these murine amino acid residues are then superimposed on the homologous human framework. Their approach of employing homologous human frameworks with putative murine contact residues resulted in humanized antibodies with similar binding affinities to the original murine antibody with respect to antibodies specific for the interleukin 2 receptor (Queen et al., 1989 [17]) and also for antibodies specific for herpes simplex virus (HSV) (Co. et al., 1991 [19]). However, the reintroduction of murine residues into human frameworks (at least 9 for anti-interleukin 2 receptor antibodies, at least 9 and 7 for each of two anti-HSV antibodies) may increase the prospect of HAMA response to the framework region in the humanized antibody. Bruggemann et al., 1989 [20] have demonstrated that human V region frameworks are recognized as foreign in mouse, and so, conversely, murine modified human frameworks might give rise to an immune reaction in humans.

According to the above described two step approach in WO 90/07861 [18], Queen et al. outlined four criteria for designing humanized immunoglobulins. The first criterion is to use as the human acceptor the framework from a particular human immunoglobulin that is usually homologous to the non-human donor immunoglobulin to be humanized, or to use a consensus framework from many human antibodies. The second criterion is to use the donor amino acid rather than the acceptor if the human acceptor residue is unusual and the donor residue is typical for human sequences at a specific residue of the

framework. The third criterion is to use the donor framework amino acid residue rather than the acceptor at positions immediately adjacent to the CDRs. The fourth criterion is to use the donor amino acid residue at framework positions at which the amino acid is predicted to have a side chain atom within about 3 Å of the CDRs in a three-dimensional immunoglobulin model and to be capable of interacting with the antigen or with the CDRs of the humanized immunoglobulin. It is proposed that criteria two, three or four may be applied in addition or alternatively to criterion one, or each criteria may be applied singly or in any combination.

In addition, WO 90/07861 [18] details the preparation of a single CDR-grafted humanized antibody, a humanized antibody specificity for the p55 Tac protein of the IL-2 receptor, by employing the combination of all four criteria, as above, in designing this humanized antibody. The variable region frameworks of the human antibody EU (see, Kabat et al., 1991 [15]) were used as acceptor. In the resultant humanized antibody, the donor CDRs were as defined by Kabat et al., 1991 [15] and Wu et al., 1970 [16] and, in addition, the mouse donor residues were used in place of the human acceptor residues, at positions 27, 30, 48, 66, 67, 89, 91, 94, 103, 104, 105 and 107 in heavy chain and at positions 48, 60 and 63 in the light chain, of the variable region frameworks. The humanized anti-Tac antibody obtained was reported to have an affinity for p55 of 3×10^9 M^{-1}, about one-third of that of the murine MAb.

Several other groups have demonstrated that Queen et al.'s approach of first choosing homologous frameworks followed by reintroduction of mouse residues may not be necessary to achieve humanized antibodies with similar binding affinities to the original mouse antibodies (Riechmann et al., 1988 [4]; Tempest et al., 1991 [5]; Verhoeyen, et al. 1991 [21]). Moreover, these groups

have used a different approach and have demonstrated that it is possible to utilize, as standard, the V region frameworks derived from NEWM and REI heavy and light chains respectively for CDR-grafting without radical introduction of mouse residues. However, the determination of which mouse residues should be introduced to produce antibodies with binding efficiencies similar to the original murine MAb can be difficult to predict, being largely empirical and not taught by available prior art. In the case of the humanized CAMPATH-IH antibody, the substitution of a phenylalanine for a serine residue at position 27 was the only substitution required to achieve a binding efficiency similar to that of the original murine antibody (Riechmann, et al., 1988 [4]; WO92/04381 [22]). In the case of a humanized (reshaped) antibody specific for respiratory syncytial virus (RSV) for the inhibition of RSV infection *in vivo*, substitution of a block of 3 residues adjacent to CDR3 in the CDR-grafted NEWM heavy chain was required to produce biological activity equivalent to the original mouse antibody (Tempest et al., 1991 [5]; WO 92/04381 [22]). The reshaped antibody in which only the mouse CDRs were transferred to the human framework showed poor binding for RSV. An advantage of using the Tempest et al., 1991 [5] approach to construct NEWM and REI based humanized antibodies is that the 3-dimensional structures of NEWM and REI variable regions are known from x-ray crystallography and thus specific interactions between CDRs and V region framework residues can be modelled.

Regardless of the approach taken, the examples of the initial humanized antibodies prepared to date have shown that it is not a straightforward process to obtain humanized antibodies with the characteristics, in particular, the binding affinity, as well as other desirable properties, of the original murine MAb from

which the humanized antibody is derived. Regardless of the approach to CDR grafting taken, it is often not sufficient merely to graft the CDRs from a donor Ig onto the framework regions of an acceptor Ig (see, e.g., Tempest et al., 1991 [5], Riechmann et al., 1988 [4], etc., cited herein). In a number of cases, it appears to be critical to alter residues in the framework regions of the acceptor antibody in order to obtain binding activity. However, even acknowledging that such framework changes may be necessary, it is not possible to predict, on the basis of the available prior art, which, if any, framework residues will need to be altered to obtain functional humanized recombinant antibodies of the desired specificity. Results thus far indicate that changes necessary to preserve specificity and/or affinity are for the most part unique to a given antibody and cannot be predicted based on the humanization of a different antibody.

In particular, the sets of residues in the framework region which are herein disclosed as being of critical importance to the activity of the recombinant humanized anti-VLA4 antibodies constructed in accordance with the teachings of the present invention do not generally coincide with residues previously identified as critical to the activity of other humanized antibodies and were not discovered based on the prior art.

C. Therapeutic Applications of Humanized Antibodies

To date, humanized recombinant antibodies have been developed mainly for therapeutic application in acute disease situations (Tempest, et al., 1991 [5]) or for diagnostic imaging (Verhoeyen, et al., 1991 [21]). Recently, clinical studies have begun with at least two humanized antibodies with NEWM and REI V region frameworks, CAMPATH-IH (Riechmann et al., 1988 [4]) and

humanized anti-placental alkaline phosphatase (PLAP) (Verhoeyen et al., 1991 [21]) and these studies have initially indicated the absence of any marked immune reaction to these antibodies. A course of treatment with CAMPATH-IH provided remission for two patients with non-Hodgkins lymphoma thus demonstrating efficacy in a chronic disease situation (Hale et al., 1988 [23]). In addition, the lack of immunogeneicity of CAMPATH-1H was demonstrated after daily treatment of the two patients for 30 and 43 days. Since good tolerance to humanized antibodies has been initially observed with CAMPATH-IH, treatment with humanized antibody holds promise for the prevention of acute disease and to treatment of diseases with low mortality.

D. The VCAM-VLA4 Adhesion Pathway and Antibodies to VLA4

Vascular endothelial cells constitute the lining of blood vessels and normally exhibit a low affinity for circulating leukocytes (Harlan, 1985 [24]). The release of cytokines at sites of inflammation, and in response to immune reactions, causes their activation and results in the increased expression of a host of surface antigens. (Collins et al., 1986 [25]; Pober et al., 1986 [26]; Bevilacqua et al., 1987 [27]; Leeuwenberg et al., 1989 [28]). These include the adhesion proteins ELAM-1, which binds neutrophils (Bevilacqua et al., 1989 [29], ICAM-1 which interacts with all leukocytes (Dustin et al., 1986 [30]; Pober et al. 1986, [26]; Boyd et al., 1988 [31]; Dustin and Springer, 1988 [32]), and VCAM-1 which binds lymphocytes (Osborn et al., 1989 [33]). These cytokine-induced adhesion molecules appear to play an important role in leukocyte recruitment to extravascular tissues.

The integrins are a group of cell-extracellular matrix and cell-cell adhesion receptors exhibiting an alpha-beta heterodimeric structure, with a widespread cell distribution and a high degree of conservation throughout evolution (Hynes, 1987 [34]; Marcantonio and

Hynes, 1988 [35]). The integrins have been subdivided into three major subgroups; the β_2 subfamily of integrins (LFA-1, Mac-1, and p150,95) is mostly involved in cell-cell interactions within the immune system (Kishimoto et al., 1989 [36]), whereas members of the β_1 and β_3 integrin subfamilies predominantly mediate cell attachment to the extracellular matrix (Hynes, 1987 [34]; Ruoslahti, 1988 [37]). In particular, the β_1 integrin family, also termed VLA proteins, includes at least six receptors that specifically interact with fibronectin, collagen, and/or laminin (Hemler, 1990 [38]). Within the VLA family, VLA4 is atypical because it is mostly restricted to lymphoid and myeloid cells (Hemler et al., 1987 [39]), and indirect evidence had suggested that it might be involved in various cell-cell interactions (Clayberger et al., 1987 [40]; Takada et al., 1989 [41]; Holtzmann et al., 1989 [42]; Bendarczyk and McIntyre, 1990 [43]). In addition, VLA4 has been shown to mediate T and B lymphocyte attachment to the heparin II binding fragment of human plasma fibronectin (FN) (Wayner et al., 1989 [44]).

VCAM-1, like ICAM-1, is a member of the immunoglobulin gene superfamily (Osborn et al., 1989 [33]). VCAM-1 and VLA4 were demonstrated to be a ligand-receptor pair that allows attachment of lymphocytes to activated endothelium by Elices et al., 1990 [45]. Thus, VLA4 represents a singular example of a β_1 integrin receptor participating in both cell-cell and cell-extracellular matrix adhesion functions by means of the defined ligands VCAM-1 and FN.

VCAM1 (also known as INCAM-110) was first identified as an adhesion molecule induced on endothelial cells by inflammatory cytokines (TNF and IL-1) and LPS (Rice et al., 1989 [46]; Osborn et al., 1989 [33]). Because VCAM1 binds to cells exhibiting the integrin VLA4 ($\alpha_4\beta_1$), including T and B lymphocytes, monocytes, and

eosinophils, but not neutrophils, it is thought to participate in recruitment of these cells from the bloodstream to areas of infection and inflammation (Elices et al, 1990 [45]; Osborn, 1990 [33]). The VCAM1/VLA4 adhesion pathway has been associated with a number of physiological and pathological processes. Although VLA4 is normally restricted to hematopoietic lineages, it is found on melanoma cell lines, and thus it has been suggested that VCAM1 may participate in metastasis of such tumors (Rice et al., 1989 [46]).

In vivo, VCAM1 is found on areas of arterial endothelium representing early atherosclerotic plaques in a rabbit model system (Cybulsky and Gimbrone, 1991 [47]). VCAM1 is also found on follicular dendritic cells in human lymph nodes (Freedman et al., 1990 [48]). It is also present on bone marrow stromal cells in the mouse (Miyake et al., 1991 [49]), thus VCAM1 appears to play a role in B-cell development.

The major form of VCAM1 in vivo on endothelial cells, has been referred to as VCAM-7D, and has seven Ig homology units or domains; domains 4, 5 and 6 are similar in amino acid sequence to domains 1, 2 and 3, respectively, suggesting an intergenic duplication event in the evolutionary history of the gene (Osborn et al., 1989 [33]; Polte et al. 1990 [50]; Hession et al., 1991 [51]; Osborn and Benjamin, U.S. Ser. No. 07/821,712 filed September 30, 1991, [52]). A 6-domain form (referred to as VCAM-6D herein) is generated by alternative splicing, in which the fourth domain is deleted (Osborn et al., 1989 [33]; Hession et al. 1991 [51], Cybulsky et al., 1991 [47]; Osborn and Benjamin, U.S. Ser. No. 07/821,712 filed September 30, 1991 [52]). The VCAM-6D, was the first sequenced of these alternate forms, however, later in vivo studies showed that the VCAM-7D form was dominant in vivo. The biological significance of the alternate splicing is not known, however as shown by Osborn and

Benjamin, U.S. Ser. No. 07/821,712 filed September 30, 1991 [52], VCAM-6D can bind VLA4-expressing cells and thus clearly has potential functionality in vivo.

The apparent involvement of the VCAM1/VLA4 adhesion pathway in infection, inflammation and possibly atherosclerosis has led to continuing intensive research to understand the mechanisms of cell-cell adhesion on a molecular level and has led investigators to propose intervention in this adhesion pathway as a treatment for diseases, particularly inflammation (Osborn et al., 1989 [33]). One method of intervention in this pathway could involve the use of anti-VLA4 antibodies.

Monoclonal antibodies that inhibit VCAM1 binding to VLA4 are known. For example, anti-VLA4 MAbs HP2/1 and HP1/3 have been shown to block attachment of VLA4-expressing Ramos cells to human umbilical vein cells and VCAM1-transfected COS cells (Elices et al., 1990 [45]). Also, anti-VCAM1 antibodies such as the monoclonal antibody 4B9 (Carlos et al., 1990 [53]) have been shown to inhibit adhesion of Ramos (B-cell-like), Jurkat (T-cell-like) and HL60 (granulocyte-like) cells to COS cells transfected to express VCAM-6D and VCAM-7D (Hession et al., 1991 [51]).

The monoclonal antibodies to VLA4 that have been described to date fall into several categories based on epitope mapping studies (Pulido, et al., 1991 [54]). Importantly one particular group of antibodies, to epitope "B", are effective blockers of all VLA4-dependent adhesive functions (Pulido et al., 1991, [54]). The preparation of such monoclonal antibodies to epitope B of VLA 4, including, for example the HP1/2 MAb, have been described by Sanchez-Madrid et al., 1986, [55]. Antibodies having similar specificity and having high binding affinities to VLA4 comparable to that of HP1/2, would be particularly promising candidates for the

preparation of humanized recombinant anti-VLA4 antibodies useful as assay reagents, diagnostics and therapeutics.

As stated above, inflammatory leukocytes are recruited to sites of inflammation by cell adhesion molecules that are expressed on the surface of endothelial cells and which act as receptors for leukocyte surface proteins or protein complexes. In particular, eosinophils have recently been found to participate in three distinct cell adhesion pathways to vascular endothelium, binding to cells expressing intercellular adhesion molecule-1 (ICAM-1), endothelial cell adhesion molecule-1 (ELAM-1), and vascular cell adhesion molecule-1 (VCAM-1) (Weller et al., 1991 [56]; Walsh et al., 1991 [57]; Bochner et al., 1991 [58]; and Dobrina et al., 1991 [59]). That eosinophils express VLA4 differentiates them from other inflammatory cells such as neutrophils, which bind to ELAM-1 and ICAM-1 but not VCAM-1.

The VLA4-mediated adhesion pathway has been investigated in an asthma model to examine the possible role of VLA4 in leukocyte recruitment to inflamed lung tissue (Lobb, U.S. Ser. No. 07/821,768 filed January 13, 1992 [60]). Administering anti-VLA4 antibody inhibited both the late phase response and airway hyperresponsiveness in allergic sheep. Surprisingly, administration of anti-VLA4 led to a reduction in the number of both neutrophils and eosinophils in the lung at 4 hours after allergen challenge, even though both cells have alternate adhesion pathways by which they can be recruited to lung tissues. Also surprisingly, inhibition of hyperresponsiveness in the treated sheep was observed which continued to 1 week, even though infiltration of leukocytes, including neutrophils and eosinophils, was not significantly reduced over time.

The VLA4-mediated adhesion model has also been investigated in a primate model of inflammatory bowel

disease (IBD) (Lobb, U.S. Ser. No, 07/835,139 filed February 12, 1992 [61]). The administration of anti-VLA4 antibody surprisingly and significantly reduced acute inflammation in that model, which is comparable to ulcerative colitis in humans.

More recently, anti-VLA4 antibodies have been used in methods for the peripheralizing of $CD34^+$ cells, including hematopoietic stem cells as described in Papyannopoulou, U.S. Ser. No. 07/977,702, filed November 13, 1992 [62].

Thus, anti-VLA4 antibodies having certain epitopic specificities and certain binding affinities may be therapeutically useful in a variety of inflammatory conditions, including asthma and IBD. In particular, humanized recombinant versions of such anti-VLA4 antibodies, if they could be constructed, might be especially useful for administration in humans. Such humanized antibodies would have the desired potency and specificity, while avoiding or minimizing an immunological response which would render the antibody ineffective and/or give rise to undesirable side effects.

SUMMARY OF THE INVENTION

The present invention provides a method of constructing a recombinant anti-VLA4 antibody molecule. Specifically, recombinant antibodies according to the present invention comprise the antigen binding regions derived from the heavy and/or light chain variable regions of an anti-VLA4 antibody.

The present invention provides a method for the construction of humanized recombinant antibody molecule using as a first step CDR grafting or "reshaping" technology. Specifically, the humanized antibodies according to the present invention have specificity for VLA4 and have an antigen binding site wherein at least one or more of the complementarity determining regions (CDRs) of the variable domains are derived from a donor non-human anti-VLA4 antibody, and in which there may or may not have been minimal alteration of the acceptor antibody heavy and/or light variable framework region in order to retain donor antibody binding specificity. Preferably, the antigen binding regions of the CDR-grafted heavy chain variable domain comprise the CDRs corresponding to positions 31-35 (CDR1), 50-65 (CDR2) and 95-102 (CDR3). Preferably, the antigen binding regions of the CDR-grafted light chain variable domain comprise CDRs corresponding to positions 24-34 (CDR1), 50-56 (CDR2) and 89-97 (CDR3). These residue designations are numbered according to the Kabat numbering (Kabat et al., 1991 [15]). Thus, the residue/position designations do not always correspond directly with the linear numbering of the amino acid residues shown in the sequence listing. In the case of the humanized V_K sequence disclosed herein, the Kabat numbering does actually correspond to the linear numbering of amino acid residues shown in the sequence listing. In contrast, in the case of the humanized V_H sequences disclosed herein, the Kabat numbering does not correspond to the linear numbering of

amino acid residues shown in the sequence listing (e.g., for the humanized V_H regions disclosed in the sequence listing, CDR2 = 50-66, CDR3 = 99-110).

The invention further provides the recombinant and humanized anti-VLA4 antibodies which may be detectably labelled.

The invention additionally provides a recombinant DNA molecule capable of expressing the recombinant and humanized anti-VLA4 antibodies of the present invention.

The invention further provides host cells capable of producing the recombinant and humanized anti-VLA4 antibodies of the present invention.

The invention additionally relates to diagnostic and therapeutic uses for the recombinant and humanized anti-VLA4 antibodies of the present invention.

The invention further provides a method for treating inflammation resulting from a response of the specific defense system in a mammalian subject, including humans, which comprises providing to a subject in need of such treatment an amount of an anti-inflammatory agent sufficient to suppress the inflammation wherein the anti-inflammatory agent is a recombinant and humanized anti-VLA4 antibody of the present invention.

The invention further provides a method for treating non-specific inflammation in a mammalian subject, including humans using the recombinant and humanized anti-VLA4 antibodies.

The invention further concerns the embodiment of the above-described methods wherein the recombinant and humanized anti-VLA4 antibodies of the present invention are derived from the murine monoclonal antibody HP1/2.

DETAILED DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The technology for producing monoclonal antibodies is well known. Briefly, an immortal cell line (typically myeloma cells) is fused to lymphocytes (typically splenocytes) from a mammal immunized with whole cells expressing a given antigen, e.g., VLA4, and the culture supernatants of the resulting hybridoma cells are screened for antibodies against the antigen (see, generally, Kohler et al., 1975 [1]).

Immunization may be accomplished using standard procedures. The unit dose and immunization regimen depend on the species of mammal immunized, its immune status, the body weight of the mammal, etc. Typically, the immunized mammals are bled and the serum from each blood sample is assayed for particular antibodies using appropriate screening assays. For example, anti-VLA4 antibodies may be identified by immunoprecipitation of ^{125}I-labeled cell lysates from VLA4-expressing cells (see, Sanchez-Madrid et al., 1986 [55] and Hemler et al., 1987 [39]). Anti-VLA-4 antibodies may also be identified by flow cytometry, e.g., by measuring fluorescent staining of Ramos cells incubated with an antibody believed to recognize VLA4 (see, Elices et al., 1990 [45]). The lymphocytes used in the production of hybridoma cells typically are isolated from immunized mammals whose sera have already tested positive for the presence of anti-VLA4 antibodies using such screening assays.

Typically, the immortal cell line (e.g., a myeloma cell line) is derived from the same mammalian species as the lymphocytes. Preferred immortal cell lines are mouse myeloma cell lines that are sensitive to culture medium containing hypoxanthine, aminopterin and thymidine ("HAT medium").

Typically, HAT-sensitive mouse myeloma cells are fused to mouse splenocytes using 1500 molecular weight polyethylene glycol ("PEG 1500"). Hybridoma cells resulting from the fusion are then selected using HAT medium, which kills unfused and unproductively fused myeloma cells (unfused splenocytes die after several days because they are not transformed). Hybridomas producing a desired antibody are detected by screening the hybridoma culture supernatants. For example, hybridomas prepared to produce anti-VLA4 antibodies may be screened by testing the hybridoma culture supernatant for secreted antibodies having the ability to bind to a recombinant α_4-subunit-expressing cell line, such as transfected K-562 cells (see, e.g., Elices et al., 1990 [45]).

To produce anti VLA4-antibodies, hybridoma cells that tested positive in such screening assays are cultured in a nutrient medium under conditions and for a time sufficient to allow the hybridoma cells to secrete the monoclonal antibodies into the culture medium. Tissue culture techniques and culture media suitable for hybridoma cells are well known. The conditioned hybridoma culture supernatant may be collected and the anti-VLA4 antibodies optionally further purified by well-known methods.

Alternatively, the desired antibody may be produced by injecting the hybridoma cells into the peritoneal cavity of an unimmunized mouse. The hybridoma cells proliferate in the peritoneal cavity, secreting the antibody which accumulates as ascites fluid. The antibody may be harvested by withdrawing the ascites fluid from the peritoneal cavity with a syringe.

Several anti-VLA4 monoclonal antibodies have been previously described (see, e.g., Sanchez-Madrid et al., 1986 [55]; Hemler et al., 1987 [39]; Pulido et al., 1991 [54]). HP1/2, for example, is one such murine monoclonal antibody which recognizes VLA4. VLA4 acts as a leukocyte

receptor for plasma fibronectin and VCAM-1. Other monoclonal antibodies, such as HP2/1, HP2/4, L25 and P4C2, have been described that also recognize VLA4.

Recombinant antibodies have been constructed and are described herein in which the CDRs of the variable domains of both heavy and light chains were derived from the murine HP1/2 sequence. Preferred starting materials for constructing recombinant humanized antibodies according to the present invention are anti-VLA4 antibodies, such as HP1/2, that block the interaction of VLA4 with both VCAM1 and fibronectin. Particularly preferred are those antibodies, such as HP1/2, which in addition, do not cause cell aggregation. Some anti-VLA4 blocking antibodies have been observed to cause such aggregation. The HP1/2 MAb (Sanchez-Madrid et al., 1986 [55]) is a particularly excellent candidate for humanization since it has an extremely high potency, blocks VLA4 interaction with both VCAM1 and fibronectin, but does not cause cell aggregation, and has the specificity for epitope B on VLA4. In the initial experiments, V_H and V_K DNA were isolated and cloned from an HP1/2-producing hybridoma cell line. The variable domain frameworks and constant domains for humanization were initially derived from human antibody sequences.

The three CDRs that lie on both heavy and light chains are composed of those residues which structural studies have shown to be involved in antigen binding. Theoretically, if the CDRs of the murine HP1/2 antibody were grafted onto human frameworks to form a CDR-grafted variable domain, and this variable domain were attached to human constant domains, the resulting CDR-grafted antibody would essentially be a human antibody with the specificity of murine HP1/2 to bind human VLA4. Given the highly "human" nature of this antibody, it would be expected to be far less immunogenic than murine HP1/2 when administered to patients.

However, following testing for antigen binding of a CDR-grafted HP1/2 antibody in which only the CDRs were grafted onto the human framework, it was shown that this did not produce a CDR-grafted antibody having reasonable affinity for the VLA4 antigen. It was therefore decided that additional residues adjacent to some of the CDRs and critical framework residues needed to be substituted from the human to the corresponding murine HP1/2 residues in order to generate an antibody with binding affinity in the range of 10% to 100% of the binding affinity of the murine HP1/2 MAb. Empirically, changes of one or more residues in the framework regions of V_H and V_K were made to prepare antibodies of the desired specificity and potency, but without making so many changes in the human framework so as to compromise the essentially human nature of the humanized V_H and V_K region sequences.

Furthermore, VLA4-binding fragments may be prepared from the recombinant anti-VLA4 antibodies described herein, such as Fab, Fab', $F(ab')_2$, and F(v) fragments; heavy chain monomers or dimers; light chain monomers or dimers; and dimers consisting of one heavy chain and one light chain are also contemplated herein. Such antibody fragments may be produced by chemical methods, e.g., by cleaving an intact antibody with a protease, such as pepsin or papain, or via recombinant DNA techniques, e.g., by using host cells transformed with truncated heavy and/or light chain genes. Heavy and light chain monomers may similarly be produced by treating an intact antibody with a reducing agent such as dithiothreitol or β-mercaptoethanol or by using host cells transformed with DNA encoding either the desired heavy chain or light chain or both.

The following examples are intended to further illustrate certain preferred embodiments of the invention and are not intended to be limiting in nature. In the following examples, the necessary restriction enzymes, plasmids, and other reagents and materials may be obtained from commercial sources and cloning, ligation and other recombinant DNA methodology may be performed by procedures well-known in the art.

Example 1

Isolation of DNA Sequences Encoding Murine Anti-VLA4 Variable Regions

A. Isolation of the HP1/2 heavy and light chain cDNA

To design a humanized recombinant antibody with specificity for VLA4, it was first necessary to determine the sequence of the variable domain of the murine HP1/2 heavy and light chains. The sequence was determined from heavy and light chain cDNA that had been synthesized from cytoplasmic RNA according to methods referenced in Tempest et al., 1991 [5].

1. Cells and RNA isolation

Cytoplasmic RNA (~200 μg) was prepared by the method of Favaloro et al., 1980 [63], from a semi-confluent 150cm^2 flask of HP1/2-producing hybridoma cells (about 5 X 10^5 logarithmic phase cells). The cells were pelleted and the supernatant was assayed for the presence of antibody by a solid phase ELISA using an Inno-Lia mouse monoclonal antibody isotyping kit (Innogenetics, Antwerp, Belgium) using both the kappa conjugate and the lambda conjugate. The antibody was confirmed to be IgG1/κ by this method.

2. cDNA Synthesis

cDNAs were synthesized from the HP1/2 RNA via reverse transcription initiated from primers based on the 5′ end of either the murine IgG1 CH_1 or the murine kappa constant domains using approximately 5 μg RNA and 25 pmol primer in reverse transcriptase buffer containing 1 μl/50 μl Pharmacia (Milton Keynes, United Kingdom) RNA Guard™ and 250 micromolar dNTPs. The sequence of these primers, CG1FOR and CK2FOR are shown as SEQ ID NO: 1 and SEQ ID NO: 2, respectively. The mixture was heated to 70°C, then allowed to cool slowly to room temperature. Then, 100 units/50 μl MMLV reverse transcriptase (Life Technologies, Paisley, United Kingdom) was added and the reaction was allowed to proceed at 42°C for one hour.

3. Amplification of V_H and V_K cDNA

Polymerase chain reaction (PCR) of murine MAb variable regions can be achieved using a variety of procedures, for example, anchored PCR or primers based on conserved sequences (see, e.g., Orlandi et al., 1989 [64]). Orlandi et al. [64], Huse et al., 1989 [65] and Jones and Bendig, 1991 [66], have described some variable region primers. We have been unsuccessful, however, in using a number of such primers, particularly those for the light chain PCR of HP1/2 derived V_K sequences.

HP1/2 Ig V_H and V_K cDNAs were amplified by PCR as described by Saiki et al., 1988 [67] and Orlandi et al., 1989 [64]. Reactions were carried out using 2.5 units/50 μl Amplitaq™ polymerase (Perkin Elmer Cetus, Norwalk, CT) in 25 cycles of 94°C for 30 seconds followed by 55°C for 30 seconds and 75°C for 45 seconds. The final cycle was followed by five minute incubation at 75°C. The same 3′ oligonucleotides used for cDNA synthesis were used in conjunction with appropriate 5′ oligonucleotides based on consensus sequences of relatively conserved regions at the 5′ end of each V region. V_H cDNA was successfully amplified using the primers VH1BACK [SEQ ID NO: 3] and CG1FOR [SEQ ID NO: 1] and yielded an amplification product of approximately 400 bp. V_K cDNA was successfully amplified using the primers VK5BACK [SEQ ID NO: 4] and CK2FOR [SEQ ID NO: 2] and yielded an amplification product of approximately 380 bp.

4. Cloning and Sequencing V_H DNA

The primers used for the amplification of V_H DNA, contain the restriction enzyme sites PstI and HindIII which facilitate cloning into sequencing vectors. The general cloning and ligation methodology was as described in Molecular Cloning, A Laboratory Manual 1982, [68]. The

amplified DNA was digested with PstI to check for internal PstI sites and an internal PstI site was found. Therefore,the V_H DNA was cloned as PstI-PstI and PstI-HindIII fragments into M13mp18 and 19. The resulting collection of clones from two independent cDNA preparations were sequenced by the dideoxy method (Sanger, et al., 1977, [69] using Sequenase™ (United States Biochemicals, Cleveland, Ohio, USA). The sequence of a region of ~100-250 bp was determined from each of 25 clones. Out of more than 4000 nucleotides sequenced, there were three PCR-induced transition mutation in three separate clones. The HP1/2 V_H DNA sequence and its translated amino acid sequence are set forth in SEQ ID NO: 5 and SEQ ID NO: 6, respectively. It should be noted that the first eight amino acids are dictated by the 5′ primer used in the PCR. Computer-assisted comparisons indicate that HP1/2 V_H [SEQ ID NOS: 5 and 6] is a member of family IIC (Kabat et al., 1991, [15]. A comparison between HP1/2 V_H [SEQ ID NOS: 5 and 6] and a consensus sequence of family IIC revealed that the only unusual residues are at amino acid positions 80, 98 and 121 (79, 94 and 121 in Kabat numbering). Although Tyr 80 is invariant in subgroup IIC other sequenced murine V_H regions have other aromatic amino acids at this position although none have Trp. The majority of human and murine V_Hs have an arginine residue at Kabat position 94. The presence of Asp 94 in HP1/2 V_H is extremely rare; there is only one reported example of a negatively charged residue at this position. Proline at Kabat position 113 is also unusual but is unlikely to be important in the conformation of the CDRs because of its distance from them. The amino acids making up CDR1 have been found in three other sequenced murine V_H regions. However, CDR2 and CDR3 are unique to HP1/2 and are not found in any other reported murine V_H.

5. Cloning and Sequencing V_K DNA

The primers used for the amplification of V_K DNA contain restriction sites for the enzymes EcoRI and HindIII. The PCR products obtained using primers VK1BACK [SEQ ID NO: 7], VK5BACK [SEQ ID NO: 4] and VK7BACK [SEQ ID NO: 8] were purified and cloned into M13. Authentic kappa sequences were obtained only with VK5BACK [SEQ ID NO: 4]. The sequence of a region of ~200-350 bp was determined by the dideoxy method (Sanger et al., 1977, [69] using Sequenase™ (United States Biochemicals, Cleveland, Ohio, USA) from each of ten clones from two independent cDNA preparations. Out of more than 2 kb sequenced, there were only two clones which each contained one PCR-induced transition mutation.

The HP1/2 V_K DNA sequence and its translated amino acid sequence are set forth in SEQ ID NO: 9 and SEQ ID NO: 10, respectively. The first four amino acids are dictated by the 5' PCR primer but the rest of the sequence is in total agreement with partial protein sequence data. HP1/2 V_K is a member of Kabat family V (Kabat et al., 1991 [15]) and has no unusual residues. The amino acids of CDR1 and CDR3 are unique. The amino acids making up CDR2 have been reported in one other murine V_K.

Example 2

Design of a CDR-grafted Anti-VLA4 Antibody

To design a CDR-grafted anti-VLA4 antibody, it was necessary to determine which residues of murine HP1/2 comprise the CDRs of the light and heavy chains.

Three regions of hypervariability amid the less variable framework sequences are found on both light and heavy chains (Wu and Kabat, 1970 [16]; Kabat et al., 1991 [15]). In most cases these hypervariable regions correspond to, but may extend beyond, the CDR. The amino acid sequences of the murine HP1/2 V_H and V_K chains are

set forth in SEQ ID NO: 6 and SEQ ID NO: 10, respectively. CDRs of murine HP1/2 were elucidated in accordance with Kabat et al., 1991 [15] by alignment with other V_H and V_K sequences. The CDRs of murine HP1/2 V_H were identified and correspond to the residues identified in the humanized V_H sequences disclosed herein as follows:

CDR1	AA_{31}-AA_{35}
CDR2	AA_{50}-AA_{66}
CDR3	AA_{99}-AA_{110}

These correspond to AA_{31}-AA_{35}, AA_{50}-AA_{65}, and AA_{95}-AA_{102}, respectively, in Kabat numbering. The CDRs of murine HP1/2 V_K were identified and correspond to the residues identified in the humanized V_K sequences disclosed herein as follows:

CDR1	AA_{24}-AA_{34}
CDR2	AA_{50}-AA_{56}
CDR3	AA_{89}-AA_{97}

These correspond to the same numbered amino acids in Kabat numbering. Thus, only the boundaries of the V_K, but not V_H, CDRs corresponded to the Kabat CDR residues. The human frameworks chosen to accept the HP1/2 CDRs were NEWM and REI for the heavy and light chains respectively. The NEWM and the REI sequences have been published in Kabat et al., 1991 [15].

An initial stage of the humanization process may comprise the basic CDR grafting with a minimal framework change that might be predicted from the literature. For example, in Riechmann et al., 1988 [4], the MAb CAMPATH-1H was successfully humanized using direct CDR grafting with only one framework change necessary to obtain an antibody with a binding efficiency similar to that of the original murine antibody. This framework change was the substitution of a Phe for a Ser at position 27. However, using the same humanization strategy by CDR grafting and the single framework change discovered by Riechmann et

al., 1988 [4] for the preparation of humanized antibodies having other specificities did not yield antibodies with affinities comparable to the murine antibodies from which they were derived. In such cases, the humanization process must necessarily include additional empirical changes to achieve the desired specificity and potency. Such changes may be related to the unique structure and sequence of the starting murine antibody but are not predictable based upon other antibodies of different specificity and sequence. For example, analysis of the murine V_H amino acid sequence from HP1/2 as set forth in SEQ ID NO: 6 as compared with the other known sequences indicated that residues 79, 94 and 113 (Kabat numbering) were unusual. Of these, only Asp 94 is likely to be important in CDR conformation. Most V_H regions that have been sequenced have an arginine at this position which is able to form a salt bridge with a relatively conserved Asp 101 in CDR3. Because NEWM has an Arg 94 and V_H CDR3 of HP1/2 has an Asp 101, there remains the possibility that a salt bridge would form which would not normally occur. The presence of a negatively charged residue at position 94 is very unusual and therefore it was decided to include the Asp 94 into the putative humanized V_H.

A chimeric (murine V/human IgG1/κ) HP1/2 antibody may be useful, but not a necessary, intermediate in the initial stages of preparing a CDR grafted construct because (i) its antigen-binding ability may indicate that the correct V regions have been cloned; and (ii) it may act as a useful control in assays of the various humanized antibodies prepared in accordance with the present invention.

For V_H, an M13 clone containing full-length HP1/2 V_H was amplified using VH1BACK [SEQ ID NO: 3] and VH1FOR [SEQ ID NO: 11] which contain PstI and BstEII sites respectively at the 5′ and 3′ ends of the V_H domain. The amplified DNA was cut with BstEII and partially cut with

PstI, full-length DNA purified and cloned into M13VHPCR1 (Orlandi et al., 1989 [64]) which had been cut with PstI and BstEII. For V_K an M13 clone containing full-length HP1/2 V_K was amplified using VK3BACK [SEQ ID NO: 12] and VK1FOR [SEQ ID NO: 13] to introduce PvuII and BglII sites respectively at the 5′ and 3′ ends of the V_K domain. The amplified DNA was cut with PvuII and BglII and cloned into M13VKPCR1 (Orlandi et al., 1989 [64]) which had been cut with PvuII and BclI.

In sum, the 5′ primers used for the amplification of the murine V_H and V_K regions contain convenient restriction sites for cloning into our expression vectors. The 3′ primers used in the PCRs were from the constant regions. Restriction sites at the 3′ end of the variable regions were introduced into cloned murine variable region genes with PCR primers which introduced BstII or BglII sites in the heavy and light (kappa) variable regions, respectively. Additionally, the V_H primer changed Pro 113 to Ser.

The murine V_H and V_K DNAs were cloned into vectors containing the gpt and hygromycin resistance genes respectively, such as pSVgpt and pSVhyg as described by Orlandi, et al. [64], and appropriate human IgG1, IgG4 or κ constant regions were added, for example, as described by Takahashi et al., 1982 [70], Flanagan and Rabbitts, 1982 [71], and Hieter et al., 1980 [72], respectively. The vectors were cotransfected into the rat myeloma YB2/0 and mycophenolic acid resistant clones screened by ELISA for secretion of chimeric IgG/κ antibody. The YB2/0 cell line was described by Kilmartin et al., 1982 [73] and is available from the American Type Culture Collection (ATCC, Rockville, MD). ELISA positive clones were expanded and antibody purified from culture medium by protein A affinity chromatography. The chimeric antibody purified from the transfected cells was assayed for anti-

VLA4 antibody activity as described in Example 7 and was found to be equipotent with the murine HP1/2 antibody.

Example 3

Transplantation of CDR Sequences and Mutagenesis of Selected Framework Residues

Transplantation of the CDRs into human frameworks was performed using M13 mutagenesis vectors. The human frameworks chosen to accept the CDR sequences outlined in Example 2 were derived from NEWM for V_H and REI for V_K, each in an M13 mutagenesis vector. The M13 mutagenesis vectors used for V_H and V_K, were M13VHPCR1 and M13VKPCR2, respectively. M13VKPCR2 is identical to M13VKPCR1 as described by Orlandi et al., 1989 [64], except for a single amino acid change from valine (GTG) to glutamine (GAA) in framework 4 of the REI V_K coding sequence. M13VHPCR1 described by Orlandi et al., 1989 [64] is M13 that contains the coding sequence for a V_H region that is an NEWM framework sequence with CDRs derived from an anti-hapten (4-hydroxy-3-nitrophenyl acetyl caproic acid) antibody; the irrelevant V_H CDRs are replaced by site-directed mutagenesis with the CDRs derived from HP1/2 V_H as described below. The V_H region sequence (DNA and amino acid) encoded by M13VHPCR1 is shown as SEQ ID NOS: 14 and 15. M13VKPCR2, like M13VKPCR1 described by Orlandi et al. [64], is M13 that contains the coding sequence for a V_K region that is N-terminal modified REI framework sequence with CDRs derived from an anti-lysozyme antibody; these irrelevant V_K CDRs are replaced by site-directed mutagenesis with the CDRs derived from HP1/2 V_K as described below. The V_K region sequence (DNA and amino acid) encoded by M13PCR2 is shown as SEQ ID NOS: 16 and 17.

Synthetic oligonucleotides were synthesized containing the HP1/2-derived V_H and V_K CDRs flanked by short sequences drawn from NEWM and REI frameworks,

respectively, and grafted into the human frameworks by oligonucleotide site-directed mutagenesis as follows. For CDR grafting into the human V_H framework, mutagenizing oligonucleotides 598 [SEQ ID NO: 18], 599 [SEQ ID NO: 19] and 600 [SEQ ID NO: 20] were used. For CDR grafting into the human V_K framework, the mutagenizing oligonucleotides were 605 [SEQ ID NO: 21], 606 [SEQ ID NO: 22] and 607 [SEQ ID NO: 23]. To 5 μg of V_H or V_K single-stranded DNA in M13 was added a 2-fold molar excess of each of the three V_H or V_K phosphorylated oligonucleotides together with flanking primers based on M13 sequences, oligo 10 [SEQ ID NO: 24] for V_H and oligo 385 [SEQ ID NO: 25] for V_K. Primers were annealed to the template by heating to 70°C and slowly cooling to 37°C. The annealed DNA was extended and ligated with 2.5 U T7 DNA polymerase (United States Biochemicals) and 1 U T4 DNA ligase (Life Technologies) in 10 mM Tris HCl pH 8.0, 5 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 250 μM dNTPs in a reaction volume of 50 μl at 16°C for 1-2 hours.

The newly extended mutagenic strand was preferentially amplified using 1 U Vent DNA polymerase (New England Biolabs) and 25 pmol oligo 11 [SEQ ID NO: 26] or oligo 391 [SEQ ID NO: 27] (for V_H or V_K, respectively) in 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris HCl pH 8.8, 2 mM $MgSO_4$, 0.1% Triton X-100, 25 μM dNTPs in a reaction volume of 50 μl and subjecting the sample to 30 cycles of 94°, 30s; 50°, 30s; 75°, 90s.

A normal PCR was then performed by adding 25 pmol oligo 10 [SEQ ID NO: 24] (for V_H) or oligo 385 [SEQ ID NO: 25] (for V_K) with 10 thermal cycles. The product DNAs were digested with HindIII and BamHI and cloned into M13mp19. Single-stranded DNA was prepared from individual plaques, sequenced and triple mutants were identified.

The resulting Stage 1 V_H construct with the DNA sequence and its translated product set forth in SEQ ID

NO: 28 and SEQ ID NO: 29, respectively. In addition to the CDR grafting, the Stage 1 V_H construct contained selected framework changes. Just prior to CDR1, a block of sequences was changed to the murine residues Phe 27, Asn 28, Ile 29 and Lys 30 [compare AA_{27}-AA_{30} of SEQ ID NO: 29 with that of murine V_H sequence [SEQ ID NO: 6]]. This included Phe-27 as substituted in the humanization of the rat CAMPATH1-H antibody (Riechmann et al., 1988 [4]), but then also substitutes the next three residues found in the murine sequence. Although these four residues are not nominally included in CDR1 (i.e., are not hypervariable in the Kabat sense), structurally they are a part of the CDR1 loop (i.e., structural loop residues), and therefore included empirically as part of CDR1. In addition, the change from Arg to Asp at residue 94 was made based on the rationale discussed in Example 2. An alignment of the CDR-grafted Stage 1 framework sequences as compared with the NEWM framework is shown in Table I. The resulting VK1 (DQL) construct with the DNA sequence and its translated product are set forth in SEQ ID NO: 30 and SEQ ID NO: 31, respectively. An alignment of the CDR-grafted VK1 (DQL) framework sequences as compared with the REI framework is shown in Table II.

The CDR replaced V_H (Stage 1) and V_K (VK1) genes were cloned in expression vectors according to Orlandi, et al., 1989 [64] to yield the plasmids termed pHuVHHuIgG1, pHuVHHuIgG4 and pHuVKHuCK. For pHuVHHuIgG1 and pHuVHHuIgG4, the Stage 1 V_H gene together with the Ig heavy chain promoter, appropriate splice sites and signal peptide sequences were excised from the M13 mutagenesis vector by digestion with HindIII and BamHI, and cloned into an expression vector such as pSVgpt as described by Orlandi et al. [64], containing the murine Ig heavy chain enhancer, the SV40 promoter, the gpt gene for selection in mammalian cells and genes for replication and selection in E. coli. A human IgG1 constant region as

described in Takahashi et al., 1982 [70] was then added as a BamHI fragment. Alternatively, a human IgG4 construct region as described by Flanagan and Rabbitts, 1982 [71] is added. The construction of the pHuVKHuCK plasmid, using an expression vector such as pSVhyg as described by Orlandi et al. [64], was essentially the same as that of the heavy chain expression vector except that the gpt gene for selection was replaced by the hygromycin resistance gene (hyg) and a human kappa chain constant region as described by Hieter, 1980, [72] was added. The vectors were cotransfected into the rat myeloma YB2/0 and mycophenolic acid resistant clones screened by ELISA for secretion of human IgG/κ antibody. The YB2/0 cell line was described by Kilmartin et al., 1982 [73] and is available from the American Type Culture Collection (ATCC, Rockville, MD). ELISA positive clones were expanded and antibody purified from culture medium by protein A affinity chromatography. The transfected cells are assayed for anti-VLA4 antibody activity as described in Example 7.

Example 4

Modification of a CDR grafted Antibody

Beyond the stages of design and preparation to yield anti-VLA4 antibodies as described above in Examples 2 and 3, additional stages of empirical modifications were used to successfully prepare humanized recombinant anti-VLA4 antibodies. The Stage 1 modifications as described in Example 3 were based on our analysis of primary sequence and experience in attempting to successfully humanize antibodies. The next modifications, designated as Stage 2, were empirical, based in part on our analysis of 3D modelling data. For the V_H region, further modifications, designated Stage 3, were so-called "scanning" modifications empirically made to correct any remaining defects in affinities or other antibody

properties. The modifications that were made in these several stages were empirical changes of various blocks of amino acids with the goal of optimizing the affinity and other desired properties of humanized anti-VLA4 antibodies. Not every modification made during the various stages resulted in antibodies with desired properties.

1. Additional heavy chain modifications

a. Stage 2 Modification

An additional empirical change in the V_H framework was made with the use of computer modelling, to generate a Stage 2 construct with the DNA sequence and its translated product set forth in SEQ ID NO: 32 and SEQ ID NO: 33, respectively. Using computer modelling of the Stage 1 V_H region, we determined to make a single change in the framework for Stage 2, namely a substitution of a Ser for Lys at position 75 (Kabat numbering), that is position 76 in SEQ ID NO: 33. This determination was in part based on the possibility that Lys-75 might project into CDR1 and alter its conformation. The M13 vector containing the Stage 1 CDR grafted HuVH, as described in Example 3, was used as template for two-step PCR-directed mutagenesis using the overlap/extension method as described by Ho et al., 1989 [74]. In the first step, two separate PCRs were set up, one with an end primer, oligo 10, [SEQ ID NO: 24] and a primer containing the desired mutation, 684 [SEQ ID NO: 34], and the other with the opposite end primer, oligo 11 [SEQ ID NO: 26], and a primer, 683 [SEQ ID NO: 35], that is complementary to the first mutagenic primer. The amplification products of this first pair of PCRs were then mixed together and a second PCR step was carried out using only the end primers oligos 10 and 11, SEQ ID NO: 24 and SEQ ID NO: 26, respectively. The mutagenized amplification product of this PCR was then cloned into M13mp19 and

sequenced, and a mutant bearing the Lys to Ser change (Stage 2 or "S mutant") was identified.

This turned out to be a critical change in the humanized heavy chain derived from HP1/2 (see Example 7). However, this critical change in the preparation of humanized recombinant anti-VLA4 antibodies according to the present invention was not similarly critical in the preparation of other humanized antibodies. Specifically, using the same rationalization and analysis as outlined above, a change in that position was not found to be a beneficial change in the humanization of antibodies of 2 different specificities. An alignment of the CDR-grafted Stage 2 framework sequences as compared with the NEWM, as well as Stage 1 sequences, is shown in Table I.

b. Stage 3 Modifications

Additional empirical changes were made as Stage 3 constructs. In Stage 3, a series of 5 different block changes of amino acids, for largely empirical reasons, were made to try to improve potency. These constructs are designated STAW, KAITAS, SSE, KRS, and AS. All contain the position 75 Ser (Kabat numbering) changed in Stage 2 [position 76 of SEQ ID NO: 35], with other changes as noted. Each of these constructs was prepared by two-step PCR directed mutagenesis using the overlap/extension method of Ho et al., 1989 [74], as described for the Stage 2 Ser mutant, above. For STAW, the additional changes were Gln to Thr at position 77, Phe to Ala at position 78 and Ser to Trp at position 79 (Kabat numbering). These changes were accomplished using end primers, oligos 10 [SEQ ID NO: 24] and 11 [SEQ ID NO: 26] in conjunction with mutagenizing primers 713 [SEQ ID NO: 36] and 716 [SEQ ID NO: 37]. The STAW V_H DNA sequence and its translated amino acid sequence are set forth in SEQ ID NO: 38 and SEQ ID NO: 39, respectively. KAITAS was prepared with additional changes of Arg to Lys

(position 66), Val to Ala (67), Met to Ile (69), Leu to Thr (70) and Val to Ala (71) (Kabat numbering), using oligos 10 [SEQ ID NO: 24] and 11 [SEQ ID NO: 26] in conjunction with oligos 706 [SEQ ID NO: 40] and 707 [SEQ ID NO: 41]. The KAITAS V_H DNA sequence and its translated amino acid sequence are set forth in SEQ ID NO: 42 and SEQ ID NO: 43, respectively. SSE had additional changes of Ala to Ser (84) and Ala to Glu (85) (Kabat numbering), effected by oligos 10 and 11 with oligos 768 [SEQ ID NO: 44] and 769 [SEQ ID NO: 45]. The SSE V_H DNA sequence and its translated amino acid sequence are set forth in SEQ ID NO: 46 and SEQ ID NO: 47, respectively. KRS had additional changes of Arg to Lys (38) and Pro to Arg (40) (Kabat numbering), from oligos 10 [SEQ ID NO: 24] and 11 [SEQ ID NO: 26] with oligos 704 [SEQ ID NO: 48] and 705 [SEQ ID NO: 49]. The KRS V_H DNA sequence and its translated amino acid sequence are set forth in SEQ ID NO: 50 and SEQ ID NO: 51, respectively. AS had additional change Val to Ala at position 24 (Kabat numbering) from oligos 10 [SEQ ID NO: 24] and 11 [SEQ ID NO: 26] with oligos 745 [SEQ ID NO: 52] and 746 [SEQ ID NO: 53]. The AS V_H DNA sequence and its translated amino acid sequence are set forth in SEQ ID NO: 54 and SEQ ID NO: 55, respectively. An alignment of the CDR-grafted Stage 3 framework sequences with the NEWM, Stage 0 (see below), Stage 1, and Stage 2 sequences is shown in Table I. Importantly, as shown in Example 7, the potency of STAW and AS humanized antibodies were improved, while KAITAS and KRS humanized antibodies were not of better potency. This could not be predicted.

c. Reverse (Stage 0) Modifications

The two blocks of changes made to generate Stage 1 at positions 28-30 (NIK) and 94 (D) were mutated back to the NEWM sequences at positions 28-30 (TFS), 94 (R), or both positions 27-30 (TFS) and 94 (R). These constructs

were designated Stage 0-A, 0-B and 0-C, respectively. Each of these constructs was prepared by two-step PCR directed mutagenesis using the overlap/extension method of Ho et al., 1989 [74], as described for the Stage 2 Ser mutant, above. Stage 0-A and 0-B were generated from Stage 1; Stage 0-C was generated from Stage 0-A, as follows. For Stage 0-A, the change was from Asp to Arg at position 94. This change was accomplished using end primers, oligos 10 [SEQ ID NO: 24] and 11 [SEQ ID NO: 26] in conjunction with mutagenizing primers 915 [SEQ ID NO: 56] and 917 [SEQ ID NO: 57]. For stage 0-B, the changes were from Asn-Ile-Lys to Thr-Phe-Ser at positions 28-30. These changes were accomplished by using end primers 10 [SEQ ID NO: 24] and 11 [SEQ ID NO: 26] in conjunction with mutagenizing primers 918 [SEQ ID NO: 58] and 919 [SEQ ID NO: 59]. Finally, for stage 0-C, to the change of Asp to Arg at position 94 in Stage 0-A were added the changes were from Asn-Ile-Lys to Thr-Phe-Ser at positions 28-30. These changes were accomplished with the same end primers and mutagenizing primers described above for the Stage 0-B construct.

TABLE I

HEAVY CHAIN SEQUENCES

FR1

NEWM		?VQLXXSGPGLVRPSQTLSLTCTVSGSTFS
Humanized Anti-VLA4:		
STAGE O-A		QVQLQE.....FNIK
STAGE O-B		QVQLQE.....F...
STAGE O-C		QVQLQE.....F...
STAGE 1		QVQLQE.....FNIK
STAGE 2		QVQLQE.....FNIK
STAGE 3	(STAW)	QVQLQE.....FNIK
	(KAITAS)	QVQLQE.....FNIK
	(SSE)	QVQLQE.....FNIK
	(KRS)	QVQLQE.....FNIK
	(AS)	QVQLQE.....A..FNIK

FR2

NEWM		WVRQPPGRGLEWIG
Humanized Anti-VLA4:		
STAGE O-A		.....
STAGE O-B		.....
STAGE O-C		.....
STAGE 1		.....
STAGE 2		.....
STAGE 3	(STAW)	.....
	(KAITAS)	.....
	(SSE)	.....
	(KRS)	..K.R.....
	(AS)	.....

TABLE I (Cont'd)

FR3

NEWM			RVTMLVDTSKNQFSLRLSSVTAADTAVYYCAR
Humanized Anti-VLA4:			
STAGE O-A			.....
STAGE O-B			.....D
STAGE O-C			.....
STAGE 1			.....D
STAGE 2			.....S.....D
STAGE 3	(STAW)		.....S.TAW.....D
	(KAITAS)		KA.ITA...S.....D
	(SSE)		.....S.....SE.....D
	(KRS)		.....S.....D
	(AS)		.....S.....D

FR4

NEWM		WGQGSLVTVSS
Humanized Anti-VLA4:		
STAGE O-A		....TT.....
STAGE O-B		....TT.....
STAGE O-C		....TT.....
STAGE 1		....TT.....
STAGE 2		....TT.....
STAGE 3	(STAW)	....TT.....
	(KAITAS)	....TT.....
	(SSE)	....TT.....
	(KRS)	....TT.....
	(AS)	....TT.....

Note: X denotes Glx., ? denotes Q or E.

2. Light Chain Modifications

In our experience, the humanized light chain generally requires few, if any, modifications. However, in the preparation of humanized anti-VLA4 antibodies, it became apparent that the light chain of HP1/2 did require several empirical changes. For example, humanized heavy chain of the Stage 2 construct (the Ser mutant) with murine light chain was about 2.5 fold lower potency than murine HP1/2, while the same humanized heavy chain with humanized light chain was about 4-fold lower potency. The Stage 1 humanized V_K construct was designated VK1 (DQL) and the DNA sequence and its translated amino acid sequence are set forth in SEQ ID NO: 30 and SEQ ID NO: 31, respectively. The DQL mutations arose from the PCR primer used in the initial cloning of the V_K region (see Example 1). Alterations were made in the light chain, generating two mutants, SVMDY and DQMDY (VK2 and VK3, respectively). The SVMDY mutant was prepared from the DQL sequence using oligos 10 [SEQ ID NO: 24] and 11 [SEQ ID NO: 26] for DY sequences with oligos 697 [SEQ ID NO: 60 and 698 [SEQ ID NO: 61] for SVM sequences. The VK2 (SVMDY) DNA sequence and its translated amino acid sequence are set forth in SEQ ID NO: 62 and SEQ ID NO: 63, respectively. The DQMDY sequences were restored to the original REI framework sequences by two-step PCR-directed mutagenesis using end primers 10 [SEQ ID NO: 24] and 11 [SEQ ID NO: 26] with mutagenic primers 803 [SEQ ID NO: 64] and 804 [SEQ ID NO: 65], and using the SVMDY sequence as template. The VK3 (DQMDY) DNA sequence and its translated amino acid sequence are set forth in SEQ ID NO: 66 and SEQ ID NO: 67, respectively. The change in the amino terminus (SVM versus DQM) is not relevant, and relates to the amino terminus of the murine light chain. The other two changes, D and Y, were made to improve potency, and did indeed do so as described in Example 7. An alignment of the CDR-grafted DQL (VK1), SVMDY (VK2)

and DQMDY (VK3) framework sequences as compared with the REI sequence is shown in Table II.

When the AS mutant heavy chain was combined with the improved light chain (SVMDY), the resulting humanized antibody was equipotent with murine HP1/2 as shown in Table III.

3. Alternative Humanized V_H and V_K Regions

Alternatively, a humanized V_H region sequence based on HP1/2 V_H region [SEQ ID NO: 5] may be prepared. One such alternative is designated V_H-PDLN. The DNA sequence of PDLN V_H and its translated amino acid sequence are set forth as SEQ ID NO: 68 and SEQ ID NO: 69, respectively.

In addition, an alternative humanized V_K region sequence based on the HP1/2 V_K region [SEQ ID NO: 9] may be prepared. One such alternative V_K sequence is designated V_K-PDLN and its translated amino acid sequence are set forth as SEQ ID NO: 70 and SEQ ID NO: 71, respectively.

The humanized V_H-PDLN was prepared by ligating 12 oligonucleotides, which together span the entire humanized variable region, and by screening for constructs having the correct sequence. The protocol is described in more detail below.

Oligonucleotides 370-119 through 370-130 (SEQ ID NO:72 through SEQ ID NO:83, respectively) (20 pmoles each) were dried down, and separately resuspended in 20 μl 1x Kinase Buffer containing 1 mM ATP and 1 μl T4 polynucleotide kinase (10 U/μl). The kinase reaction mixture was incubated for 1 hour at 37°C. The reaction was terminated by incubating at 70°C for 5 minutes.

The kinase-treated oligonucleotides were combined with each other (240 μl total) and ligated together with 26 μl 10 mM ATP and 2 μl T4 DNA ligase (10 U/μl), and the reaction mixture was incubated at room temperature for 6 hours. The ligation reaction mixture was extracted

with phenol:chloroform (1:1) saturated with TE buffer, and then ethanol precipitated and washed 5 times with 70% ethanol.

The dried and washed ethanol precipitate was resuspended in 50 µl 1x 150 mM Restriction Enzyme Buffer (10x 150 mM Restriction Enzyme Buffer is 100 mM Tris-HCl, pH 8.0, 1.5 M NaCl, 100 mM $MgCl_2$, 1 mg/ml gelatin, 10 mM dithiothreitol) and incubated with restriction enzymes BstE2 and PstI for 16 hours at 37°C. The digestion products were electrophoresed through a 2% agarose gel, and the band corresponding to 330 bp was excised. The fragment was eluted using GENECLEAN II® and the eluate was ethanol precipitated. The ethanol precipitate was resuspended in 20 µl TE buffer.

Next, the 330 bp fragment was ligated into vector pLCB7 which was prepared for ligation by digesting with PstI and BstE2, dephosphorylating the 5' ends with calf alkaline phosphatase, fractionating on a low melting temperature agarose (LMA) gel, and excising the pLCB7/PstI/BstE2 LMA fragment. The pLCB7 LMA fragment was then ligated to the 330 bp oligonucleotide fragment encoding the humanized V_H region using T4 DNA ligase.

The ligation mixture was used to transform E. coli JA221(Iq) to ampicillin resistance. Colonies were grown up and mini-prep DNA was prepared. The recombinant plasmids were screened for the presence of an approximately 413 bp NotI/BstE2 fragment. DNA sequence analysis identified vector pMDR1023 as having the designed humanized V_H-PDLN sequence.

The humanized V_K-PDLN was prepared by ligating 12 oligonucleotides, which together span the entire humanized V_K-PDLN variable region, and by screening for constructs having the correct sequence. The protocol is described in more detail below.

Oligonucleotides 370-131 through 370-142 (SEQ ID NO:84 through SEQ ID NO:95, respectively)

(20 pmoles each) were dried down, and separately resuspended in 20 μl 1x Kinase Buffer containing 1 mM ATP and 1 μl T4 polynucleotide kinase (10 U/μl). The kinase reaction mixture was incubated for 1 hour at 37°C. The reaction was terminated by incubating at 70°C for 5 minutes.

The kinase-treated oligonucleotides were combined with each other (240 μl total) and ligated together with 26 μl 10 mM ATP and 2 μl T4 DNA ligase (10 U/μl), and the reaction mixture was incubated at room temperature for 6 hours. The ligation reaction mixture was extracted with phenol:chloroform (1:1) saturated with TE buffer, and then ethanol precipitated and washed 5 times with 70% ethanol.

The dried and washed ethanol precipitate was resuspended in 40 μl TE, then electrophoresed through a 2% agarose gel, and the band corresponding to 380 bp was excised. The fragment was eluted using GENECLEAN II® and the eluate was ethanol precipitated. The ethanol precipitate was resuspended in 20 μl TE buffer.

Next, the 380 bp fragment was ligated into vector pNN03, which was prepared for ligation by linearizing with HindIII and BamHI, dephosphorylating the 5' ends with calf alkaline phosphatase, fractionating on a low melting temperature agarose gel, and excising the band corresponding to linearized pNN03 (2.7 kb). The linearized, dephosphorylated pNN03 was then ligated to the 380 bp oligonucleotide fragment encoding the humanized V_K region using T4 DNA ligase.

The ligation mixture was used to transform E. coli JA221(Iq) to ampicillin resistance. Colonies were grown up and mini-prep DNA was prepared. The recombinant plasmids were screened for the presence of the variable

region fragment. DNA sequence analysis identified vector pMDR1025 as having the designed humanized V_K-PDLN sequence.

When an antibody with a V_H-PDLN containing heavy chain and with a V_K-PDLN containing light chain was assayed for potency according to Example 7, the resulting humanized antibody was approximately equipotent with the murine HP1/2 antibody.

TABLE II

LIGHT CHAIN SEQUENCES

	FR1
REI	DIQMTQSPSSLSASVGDRVTITC
Humanized Anti-VLA4:	
Construct VK1 (DQL)	...L.....
Construct VK2 (SVMDY)	S.VM.....
Construct VK3 (DQMDY)	D.QM.....

	FR2
REI	WYQQTPGKAPKLLIY
Humanized Anti-VLA4:	
VK1 (DQL)	....K.....
VK2 (SVMDY)	....K.....
VK3 (DQMDY)	....K.....

	FR3
REI	GVPSRFSGSGSGTDYTFTISSLQPEDIATYYC
Humanized Anti-VLA4:	
VK1 (DQL)	.....F.....
VK2 (SVMDY)	...D.....Y...F.....
VK3 (DQMDY)	...D.....Y...F.....

	FR4
REI	FGQGTKLQIT
Humanized Anti-VLA4:	
VK1 (DQL)	.....VE.K
VK2 (SVMDY)	.....VE.K
VK3 (DQMDY)	.....VE.K

Example 5

Expression of Recombinant Anti-VLA4 Antibodies

Each of the V_H region sequences and V_K region sequences prepared according to Examples 1-4, are transferred into expression vectors with constant region sequences, and the vectors are transfected, preferably via electroporation, into mammalian cells. The heavy and light chain sequences may be encoded on separate vectors and co-transfected into the cells or alternatively heavy and light chain sequences may be encoded by and transfected as a single vector. Such a single vector will contain 3 expression cassettes: one for Ig heavy chain, one for Ig light chain and one for a selection marker. Expression levels of antibody are measured following transfection, as described below, or as described in Example 7.

V_H and V_K region sequences as described in Example 4, were inserted into various cloning and expression vectors. For the anti-VLA4 V_H region sequences, plasmids containing such sequences [as described in Examples 1-4] were digested with PstI and BstE2. The plasmid DNA after digestion with PstI and BstE2, was dephosphorylated and electrophoresed through 2% agarose gel. The band for ligation was excised and the DNA elected using the GENECLEAN™ technique (Bio101 Inc., LaJolla, California), ethanol precipitated and resuspended in 20 µl TE buffer (10mM Tris-HCl, 1mM Na_2 EDTA). Then, 10 µl of the resuspended DNA was used for ligation with the PstI/BstE2 digested V_H region sequence.

The ligation mixture was used to transform E. coli K 12 JA221 (Iq) to ampicillin resistance. E. coli K12 JA221 (Iq) cells have been deposited with the ATCC (accession number 68845). Recombinant colonies were screened for the presence of the V_H insert. Some of the plasmids containing such fragments were sequenced. The

V_H-containing plasmids were designated pBAG 184 (V_H-STAW), pBAG 183 (V_H-KAITAS), pBAG 185 (V_H-KRS), pBAG 207 (V_H-SSE) and pBAG 195 (V_H-AS), and were deposited in E. coli K12 J221 (Iq) cells with the ATCC as accession nos. 69110, 69109, 69111, 69116 and 69113, respectively. The plasmid containing alternative V_H-PDLN region was designated pMDR1023.

For the V_K region sequences, the DNA encoding these sequences were amplified for cloning and transformation using PCR. Prior to amplification, 20 pmoles of each of the V_K chain primers were kinased by incubation with T4 polynucleotide kinase at 37°C for 60 minutes by a conventional protocol. The kinase reactions were stopped by heating at 70°C for 10 minutes.

The PCR reactions each contained 10 µl 10X PCR buffer (10X PCR buffer is 100 mM Tris/HCl, pH 8.3., 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin, 20 pmoles each of the appropriate kinased primers, 20 µl cDNA, 0.5 µl Taq polymerase (5 U/µl, Perkin Elmer-Cetus) and 49.5 µl H_2O. The PCR conditions were 30 cycles of incubation for: 1 minute at 94°C; 2 minutes at 40°C (for heavy chain PCR) or at 55°C (for light chain PCR); and 2 minutes at 72°C. For VK1-DQL, primers were 370-247 [SEQ ID NO: 96] and 370-210 [SEQ ID NO: 97]. For VK2-SVMDY, primers were 370-269 [SEQ ID NO: 98] and 370-210 [SEQ ID NO: 97]. For VK3-DQMDY, primers were 370-268 [SEQ ID NO: 99] and 370-210 [SEQ ID NO: 97].

The reaction mixtures were electrophoresed through 2% agarose gel, and the bands corresponding to the expected sizes of the light chain variable region (~330 bp) were excised with AgeI and BamHI. The DNA in those bands were eluted using the GENECLEAN™ technique (Bio101 Inc., LaJolla, California), ethanol precipitated and subsequently each resuspended in 20 µl TE buffer (10 mM Tris-HCl, 1 mM Na_2EDTA).

Klenow fragment of DNA polymerase (New England Biolabs, 5 U/μl) (1 μl) was added to the purified PCR fragments in a reaction volume of 25 μl containing 1x ligation buffer (10x ligation buffer is 0.5 M Tris/HCl, pH 7.5, 100 mM $MgCl_2$ and 40 mM DTT) and 0.125 mM each of dXTPs and the reaction incubated at room temperature for 15 minutes. The reaction was terminated by incubation at 70°C for 5 minutes, and then stored on ice.

The fragment from each PCR reaction is ligated to a plasmid such as pNN03 or a plasmid derived from pNN03 such as pLCB7, which had been previously linearized by EcoRV, dephosphorylated and fractionated through low temperature melting agarose. Such plasmids, including pNN03 and pLCB7 have been described in co-pending and co-assigned (Burkly et al., U.S. Ser. No. 07/916,098, filed July 24, 1992 [75]).

The ligation mixture was used to transform E.coli K12 JA221(Iq) to ampicillin resistance. E.coli K12 JA221(Iq) cells are deposited with American Type Culture Collection (accession number 68845). Recombinant colonies were screened for the presence of the V_K insert. Some of the plasmids containing such fragments were sequenced. The V_K-containing plasmids were designated pBAG 190 (VK1-DQL), pBAG 198 (VK2-SVMDY) and pBAG 197 (VK3-DQMDY), and were deposited in E. coli K12 JA 221 (Iq) cells with the ATCC as accession nos. 69112, 69115 and 69114, respectively. The plasmid containing the alternative V_K (PDLN) region was designated pMDR 1025.

In a series of experiments, the expression vectors encoding recombinant anti-VLA4 heavy and light chains are transfected via electroporation and the cells are then cultured for 48 hours. After 48 hours of culture, the cells are radiolabelled using ^{35}S-cysteine overnight and then the cell extracts and conditioned media are immunoprecipitated by incubation with protein A-Sepharose. The protein A-Sepharose is washed and the

bound proteins are eluted with SDS-PAGE loading buffer. The samples are analyzed via electrophoresis through 10% SDS-PAGE gels under reducing conditions. In this way, light chain expression is detected only as a consequence of the light chains being associated with the heavy chains. The expected sizes of the heavy and light chains as visualized in the 10% gels are 50 kD and 25 kD, respectively.

Since recombinant anti-VLA4 antibody molecules, prepared as described in Examples 1-4, may be stably expressed in a variety of mammalian cell lines, it is possible to express recombinant antibody genes in nonsecreting myeloma or hybridoma cell lines under the control of Ig-gene promoters and enhancers or in non-lymphoid cells, such as Chinese hamster ovary (CHO) cells, in conjunction with vector amplification using DHFR selection. Recently, Bebbington et al., 1992 [76] have described a method for the high-level expression of a recombinant antibody from myeloma cells using a glutamine synthetase gene as an amplifiable marker. This GS expression system is most preferred for the production of recombinant anti-VLA4 antibody molecules according to the present invention. The methods, vectors with hCMV promoters and with 5′ untranslated sequences from the hCMV-MIE genes including cell lines (most preferably NSO) and media for GS expression of recombinant antibodies is described in detail in Bebbington et al., 1992 [76], WO86/05807 [77], WO87/04462 [78], WO89/01036 [79] and WO89/10404 [80].

In accordance with the teachings of these publications, NSO cells were transfected with a heavy chain sequence having the VH-AS region sequence [SEQ ID NO: 54] and a light chain sequence having the VK-SVMDY sequence [SEQ ID NO: 66] to obtain a stable cell line secreting a humanized recombinant anti-VLA4 antibody with high potency comparable to the murine HP1/2 antibody.

This cell line has been deposited with the ATCC on November 3, 1992 and given accession no. CRL 11175. The AS/SVMDY humanized antibody is at least equipotent with or perhaps more potent than the murine HP1/2 antibody.

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Example 6

Purification of MAbs from Conditioned Media for Assay

To obtain accurate values for half-maximal binding or inhibition, stock solutions of purified antibodies are needed at known concentrations. Stable cell lines secreting the antibodies of interest were made and the humanized recombinant anti-VLA4 antibodies were purified from conditioned medium using conventional protein A chromatography. The concentration of the purified antibodies is assessed by their absorption coefficient at 280 nm, which is well established for antibodies.

A cell line producing a humanized anti-VLA4 antibody is grown in roller bottles in Dulbecco's modified Eagle medium containing 10% fetal bovine serum. A 2 liter batch of conditioned medium is used for each purification run. Cells are removed from the medium by centrifugation in a RC-3B preparative centrifuge (4K, 30 minutes, H4000 rotor) and the supernatant is filtered first through a 0.45 μ membrane and then through a 0.22 μ membrane. The medium is stored at 4°C until it can be processed.

Two liters of conditioned medium is concentrated to 220 ml in a spiral ultrafiltration unit (Amicon, Corp., Cherry Hill Drive, Danvers, MA 01923) that is equipped with an S1Y30 (YM30) Diaflo cartridge. The concentrate is diluted with 400 ml of protein A binding buffer (3M NaCl, 1.5M glycine pH 8.9) and again concentrated to 200 ml. The concentrate is treated in batch with 0.5 ml Fast Flow Protein A Sepharose 4 (Pharmacia, Inc., 800 Centennial Avenue, Piscataway, NJ 08854) using a raised stir bar to agitate the mixture. After an overnight incubation at 4°C, the resin is collected by centrifugation (5 minutes, 50 g), washed twice with 20 volumes of protein A binding buffer (using centrifugation to recover the resin), and transferred to a column for subsequent treatment. The column is washed four times

with 0.5 ml of protein A binding buffer, two times with 0.25 ml of PBS, and the IgG is eluted with Pierce IgG elution buffer (Pierce Chemical Co., Rockford, IL. 61105 Cat No. 21004Y or 21009Y). 180 µl fractions are collected, which are neutralized with 20 µl of 1M HEPES pH 7.5. Fractions are analyzed for absorbance at 280 nm and by SDS-PAGE. The gel is stained with Coomassie blue. Peak fractions are pooled. 100 µl (14 ml/ml) is diluted with 100 µl of PBS and subjected to gel filtration on a Superose 6 FPLC column (Pharmacia, Inc., 800 Centennial Avenue, Piscataway, NJ 08854) in PBS. The column is run at 20 ml/hour and 1.5 minute fractions are collected. Peak column fractions are pooled, aliquoted, frozen on dry ice, and stored at -70°C. SDS-polyacrylamide gel profile of the final product is obtained under reducing and non-reducing conditions. In some cases when the sample is analyzed under non-reducing conditions, about 10% of the product is not an intact antibody. Studies in these cases indicate that this product is a heavy-light chain dimer. This has been previously recognized as a problem with IgG4 antibodies.

Example 7

Determination of Relative Binding Affinities of Humanized Recombinant Anti-VLA4 Antibodies

Recombinant antibodies according to the present invention are purified, as described in Example 6, and are assayed to determine their specificity for VLA4 and their binding affinity or potency. In particular, the potency of a recombinant anti-VLA4 antibody was assessed by calculating the half-maximal binding constant (reported as ng/ml or μg/ml of purified antibody) using two different assays described as follows.

1. Inhibition of VLA4-dependent adhesion to VCAM1

The critical function of an anti-VLA4 antibody is defined by the ability to inhibit the VCAM1/VLA4 adhesion pathway. It has been previously shown (Lobb et al., 1991a, [81]) that purified recombinant soluble VCAM1 (rsVCAM1) can be immobilized on plastic and is a functional adhesion molecule. Immobilized rsVCAM1 binds VLA4-expressing cells such as the human B cell line Ramos, and this binding can be inhibited by MAbs to VCAM1, such as 4B9 or MAbs to VLA4, such as HP1/2. This assay provides a reproducible method to assess the potency of any humanized recombinant antibody. Briefly, the antibody solution is diluted, and the serial antibody dilutions are incubated with Ramos cells, which are then incubated with rsVCAM1-coated plates. The Ramos cells are fluorescently labelled as described by Lobb, 1991b [82], and binding assessed by fluorescence in 96 well cluster plates according to the following protocol.

Recombinant soluble VCAM1 was prepared and purified essentially as described by Lobb et al., 1991a [81]. Soluble VCAM is diluted to 10 μg/ml in 0.05 M $NaHCO_3$, (15mM $NaHCO_3$, 35mM Na_2CO_3) pH 9.2. Then 50 μl/well is added into a Linbro Titertek polystyrene 96 well plate, flat bottom, Flow Labs catalog #76-231-05. The plate is incubated at 4°C overnight.

Following this incubation, the contents of the wells are removed by inverting and blotting the plate. To the empty wells, 100 µl/well of 1% of BSA in PBS, 0.02% NaN_3 is added for 1 hour or longer at room temperature. If the plate is not to be used immediately, it can be blocked and stored for one week at 4°C. BSA is added to some wells to assess non-specific binding.

For binding quantitation, VLA4 presenting cells, preferably Ramos cells, should be prelabelled. The cells may be radiolabelled or fluorescently labelled. For radiolabelling, prelabelling of the cells may be done overnight using ^{3}H-thymidine (0.5 µCi/ml). Alternatively, and preferably, the cells are preincubated with BCECF-AM (chemical name: 2',7'-bis-(2-carboxyethyl)-5(and -6) carboxyfluorescein, acetoxymethyl ester, Molecular Probes Inc., Eugene, Oregon, catalog #B-1150). For this method, cells are suspended to 5 x 10^6/ml, 2 µM BCECF-AM is added and the mixture is incubated for 30 minutes at 37°C. Following either method, the cells are washed with RPMI, 2% FBS, pH 7.4. RPMI with 1% FBS may also be used.

For the binding study, 2-4 x 10^6 cells/ml in RPMI, 2% FBS are resuspended, then 50 µl of labelled cells are added per well for 10 minutes of binding at room temperature.

After the 10 minute incubation, the contents of the wells are removed by inversion and the plates washed 1-2 times gently with RPMI, 2% FBS. When examined under a light microscope, BSA blank wells should have very few cells bound. A brief inverted spin may be included to remove cells not firmly attached and the plates may be washed again 1-2 times.

For the BCECF-AM method, 100 µl of 1% NP40 is added to each well to solubilize the cells and then the plate is read on a fluorescence plate scanner. (If the radiolabelling method is used, 100 µl of 0.1% NaOH is

added to each well and then the contents of each well are transferred to scintillation vials containing cocktail).

A volume of 50 µl of labelled cells should be counted to obtain a total known value added to each well. Then the 50 µl of labelled cells are added to either a well containing only 100 µl of 1% NP40 or to a scintillation vial depending on the method used.

For antibody blocking studies, 100 µl/well of murine HP1/2 MAb (anti-VLA4) typically at 10 µg/ml in RPMI, 2% FBS are added to the rsVCAM1 coated plates and incubated for 30 minutes at room temperature prior to cell binding as described above. MAb HP1/2 (anti-VLA4) or any recombinant humanized anti-VLA4 antibody prepared as described herein must be preincubated with labelled cells for 30 minutes at room temperature prior to the cell binding. Concentrations of the antibodies preincubated will vary, but generally concentrations were in the range of about 1 µg/ml.

In these adhesion assays, murine HP1/2 inhibits Ramos cell binding completely at about 40 ng/ml, and half maximally at about 15 ng/ml (10 µM). The results of adhesion assays as represented by the calculated half-maximal binding constants using humanized recombinant anti-VLA4 antibodies made according to the present invention are shown in Table III. The number (n) of experiments performed for each value is indicated for the recombinant humanized antibodies. As discussed below, these results generally compare well with the results obtained with the FACS binding assay.

The potency of recombinant Stage 0, Stage 1, Stage 2 and Stage 3 antibodies having the VK1 (DQL) light chain that had been purified from stably transfected YB2/0 cell lines was measured in the adhesion assay, as shown in Table III. The results showed that there was no inhibition detected in concentrations up to 1 µg/ml (1000 ng/ml) with the Stage 0-B and 0-C humanized antibodies.

The results with the recombinant Stage 3 antibodies STAW and AS having the improved VK2 (SVMDY) light chain showed that the AS/SVMDY antibody was at least equipotent and perhaps more potent than the murine HP1/2 antibody. Certain Stage 2 and Stage 3 constructs showed potencies of about 20% to about 100% of the potency of the murine HP1/2 antibody.

2. FACS Assays

The binding of humanized recombinant antibodies to the cell surface can be assessed directly by fluorescence activated cell sorter (FACS) analysis, using fluorescently labelled antibodies. This is a standard technique that also provides half-maximal binding information following dose response measurements. The FACS methods are described in Lobb et al., 1991b [82].

Briefly, 25 μl cells (4 x 10^6/ml in FACS buffer (PBS 2% FBS, 0.1% NaN_3) on ice are added to 5 μl of 5 μg/ml FITC or phycoerythrin (PE) conjugated antibody in FACS buffer, and incubated in V-bottomed microtiter wells on ice for 30 minutes. To the wells, 125 μl of FACS buffer is added, the plates are centrifuged at 350 x g for 5 minutes, and the supernatant is shaken off. To each well is added 125 μl FACS buffer, then the cells are transferred to 12 x 75 mm Falcon polystyrene tubes and resuspended to a final volume of 250 μl in FACS buffer. The mixture is analyzed on a Becton Dickinson FACStar. The results of the FACS assays as represented by the calculated half-maximal binding constructs using humanized recombinant anti-VLA4 antibodies made according to the present invention are shown in Table III and the number (n) of experiments performed for each value is indicated for the humanized antibodies. Table III also shows the potency calculated from the combined adhesion and FACS assays. Murine HP1/2 binds half-maximally to Ramos cells at 15 ng/ml. The AS/SVMDY humanized antibody binds half-maximally to Ramos cells at 12 ng/ml. Thus,

the two assays (i.e., adhesion and FACS assays) show an excellent correlation for the murine antibody and the humanized AS/SVMDY antibody.

TABLE III

SUMMARY OF HALF-MAXIMAL BINDING CONSTANTS FOR HUMANIZED RECOMBINANT ANTI-VLA4 ANTIBODIES

Antibody	Adhesion Assay	FACS Assay	Combination
Murine HP1/2	15 ng/ml	15 ng/ml	15 ng/ml
Stage 0 (Humanized heavy chain)	>1000 ng/ml (n=3)	-	-
Stage 1 (Humanized heavy chain)	228 ng/ml (n=6)	-	228 ng/ml (n=6)
Stage 2 (Ser mutant)	56 ng/ml (n=14)	47 ng/ml (n=6)	60 ng/ml (n=20)
Stage 3			
(STAW)	30 ng/ml (n=3)	33 ng/ml (n=3)	32 ng/ml (n=6)
(KAITAS)	85 ng/ml (n=2)	100 ng/ml (n=1)	90 ng/ml (n=3)
(SSE)	100 ng/ml (n=2)	40 ng/ml (n=1)	80 ng/ml (n=3)
(KRS)	50 ng/ml (n=2)	70 ng/ml (n=1)	57 ng/ml (n=3)
(AS)	28 ng/ml (n=2)	14 ng/ml (n=2)	21 ng/ml (n=4)
Constructs with improved light chain			
STAW/SVMDY	25 ng/ml (n=4)	35 ng/ml (n=3)	29 ng/ml (n=7)
AS/SVMDY	12 ng/ml (n=2)	12 ng/ml (n=2)	12 ng/ml (n=4)

Deposits

The following plasmids in E. coli K12 J221 (Iq) cells were deposited under the Budapest Treaty with American Type Culture Collection (ATCC), Rockville, Maryland (USA) on October 30, 1992. The deposits are identified as follows:

Plasmid	Accession No.
pBAG 184 (V_H-STAW)	69110
pBAG 183 (V_H-KAITAS)	69109
pBAG 185 (V_H-KRS)	69111
pBAG 207 (V_H-SSE)	69116
pBAG 195 (V_H-AS)	69113
pBAG 190 (VK1-DQL)	69112
pBAG 198 (VK2-SVMDY)	69115
pBAG 197 (VK3-DQMDY)	69114

In addition, an NSO cell line producing humanized recombinant anti-VLA4 antibody was deposited under the Budapest Treaty with American Type Culture Collection (ATCC), Rockville, Maryland (USA) on November 3, 1992. The deposit was given ATCC accession no. CRL 11175.

Sequences

The following is a summary of the sequences set forth in the Sequence Listing:

SEQ ID NO:1 DNA sequence of CG1FOR primer

SEQ ID NO:2 DNA sequence of CK2FOR primer

SEQ ID NO:3 DNA sequence of VH1BACK primer

SEQ ID NO:4 DNA sequence of VH5BACK primer

SEQ ID NO:5 DNA sequence of HP1/2 heavy chain variable region

SEQ ID NO:6 Amino acid sequence of HP1/2 heavy chain variable region

SEQ ID NO:7 DNA sequence of VK1BACK primer

SEQ ID NO:8	DNA sequence of VK7BACK primer
SEQ ID NO:9	DNA sequence of HP1/2 light chain variable region
SEQ ID NO:10	Amino acid sequence of HP1/2 light chain variable region
SEQ ID NO:11	DNA sequence of VH1FOR primer
SEQ ID NO:12	DNA sequence of VK3BACK primer
SEQ ID NO:13	DNA sequence of VK1FOR primer
SEQ ID NO:14	DNA sequence of VH insert in M13VHPCR1
SEQ ID NO:15	Amino acid sequence of VH insert in M13VHPCR1
SEQ ID NO:16	DNA sequence of VK insert in M13VKPCR2
SEQ ID NO:17	Amino acid sequence of VK insert in M13VKPCR2
SEQ ID NO:18	DNA sequence of OLIGO598
SEQ ID NO:19	DNA sequence of OLIGO599
SEQ ID NO:20	DNA sequence of OLIGO600
SEQ ID NO:21	DNA sequence of OLIGO605
SEQ ID NO:22	DNA sequence of OLIGO606
SEQ ID NO:23	DNA sequence of OLIGO607
SEQ ID NO:24	DNA sequence of OLIGO10
SEQ ID NO:25	DNA sequence of OLIGO385
SEQ ID NO:26	DNA sequence of OLIGO11
SEQ ID NO:27	DNA sequence of OLIGO391
SEQ ID NO:28	DNA sequence of Stage 1 heavy chain variable region
SEQ ID NO:29	Amino acid sequence of Stage 1 heavy chain variable region
SEQ ID NO:30	DNA sequence of VK1 (DQL) light chain variable region

SEQ ID NO:31 Amino acid sequence of VK1 (DQL) light chain variable region

SEQ ID NO:32 DNA sequence of Stage 2 heavy chain variable region

SEQ ID NO:33 Amino acid sequence of Stage 2 heavy chain variable region

SEQ ID NO:34 DNA sequence of OLIGO684

SEQ ID NO:35 DNA sequence of OLIGO683

SEQ ID NO:36 DNA sequence of OLIGO713

SEQ ID NO:37 DNA sequence of OLIGO716

SEQ ID NO:38 DNA sequence of STAW heavy chain variable region

SEQ ID NO:39 Amino acid sequence of STAW heavy chain variable region

SEQ ID NO:40 DNA sequence of OLIGO706

SEQ ID NO:41 DNA sequence of OLIGO707

SEQ ID NO:42 DNA sequence of KAITAS heavy chain variable region

SEQ ID NO:43 Amino acid sequence of KAITAS heavy chain variable region

SEQ ID NO:44 DNA sequence of OLIGO768

SEQ ID NO:45 DNA sequence of OLIGO769

SEQ ID NO:46 DNA sequence of SSE heavy chain variable region

SEQ ID NO:47 Amino acid sequence of SSE heavy chain variable region

SEQ ID NO:48 DNA sequence of OLIGO704

SEQ ID NO:49 DNA sequence of OLIGO705

SEQ ID NO:50 DNA sequence of KRS heavy chain variable region

SEQ ID NO:51 Amino acid sequence of KRS heavy chain variable region

SEQ ID NO:52 DNA sequence of OLIGO745

SEQ ID NO:53 DNA sequence of OLIGO746

SEQ ID NO:54 DNA sequence of AS heavy chain variable region

SEQ ID NO:55 Amino acid sequence of AS heavy chain variable region

SEQ ID NO:56 DNA sequence of OLIGO915

SEQ ID NO:57 DNA sequence of OLIGO917

SEQ ID NO:58 DNA sequence of OLIGO918

SEQ ID NO:59 DNA sequence of OLIOG919

SEQ ID NO:60 DNA sequence of OLIGO697

SEQ ID NO:61 DNA sequence of OLIGO698

SEQ ID NO:62 DNA sequence of VK2 (SVMDY) light chain variable region

SEQ ID NO:63 Amino acid sequence of VK2 (SVMDY) light chain variable region

SEQ ID NO:64 DNA sequence of OLIGO803

SEQ ID NO:65 DNA sequence of OLIGO804

SEQ ID NO:66 DNA sequence of VK3 (DQMDY) light chain variable region

SEQ ID NO:67 Amino acid sequence of VK3 (DQMDY) light chain variable region

SEQ ID NO:68 DNA sequence of PDLN heavy chain variable region

SEQ ID NO:69 Amino acid sequence of PDLN heavy chain variable region

SEQ ID NO:70 DNA sequence of PDLN light chain variable region

SEQ ID NO:71 Amino acid sequence of PDLN light chain variable region

SEQ ID NO:72 DNA sequence of Oligo 370-119

SEQ ID NO:73 DNA sequence of Oligo 370-120

SEQ ID NO:74 DNA sequence of Oligo 370-121

SEQ ID NO:75 DNA sequence of Oligo 370-122

SEQ ID NO:76 DNA sequence of Oligo 370-123

SEQ ID NO:77 DNA sequence of Oligo 370-124

SEQ ID NO:78 DNA sequence of Oligo 370-125

SEQ ID NO:79 DNA sequence of Oligo 370-126

SEQ ID NO:80 DNA sequence of Oligo 370-127

SEQ ID NO:81 DNA sequence of Oligo 370-128

SEQ ID NO:82 DNA sequence of Oligo 370-129

SEQ ID NO:83 DNA sequence of Oligo 370-130

SEQ ID NO:84 DNA sequence of Oligo 370-131

SEQ ID NO:85 DNA sequence of Oligo 370-132

SEQ ID NO:86 DNA sequence of Oligo 370-133

SEQ ID NO:87 DNA sequence of Oligo 370-134

SEQ ID NO:88 DNA sequence of Oligo 370-135

SEQ ID NO:89 DNA sequence of Oligo 370-136

SEQ ID NO:90 DNA sequence of Oligo 370-137

SEQ ID NO:91 DNA sequence of Oligo 370-138

SEQ ID NO:92 DNA sequence of Oligo 370-139

SEQ ID NO:93 DNA sequence of Oligo 370-140

SEQ ID NO:94 DNA sequence of Oligo 370-141

SEQ ID NO:95 DNA sequence of Oligo 370-142

SEQ ID NO:96 DNA sequence of VK1-DQL primer 370-247

SEQ ID NO:97 DNA sequence of VK1-DQL primer 370-210

SEQ ID NO:98 DNA sequence of VK2-SVMDY primer 370-269

SEQ ID NO:99 DNA sequence of VK3-DQMDY primer 370-268

While we have hereinbefore described a number of embodiments of this invention, it is apparent that our basic embodiments can be altered to provide other

embodiments that utilize the compositions and processes of this invention. Therefore, it will be appreciated that the scope of this invention includes all alternative embodiments and variations which are defined in the foregoing specification and by the claims appended hereto; and the invention is not to be limited by the specific embodiments that have been presented herein by way of example.

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[81] Lobb et al., 1991a, "Expression and Functional Characterization of a Soluble Form of Vascular Cell Adhesion Molecule 1", Biochem. Biophys. Res. Comm. 178: 1498-1504

[82] Lobb et al., 1991b, "Expression and Functional Characterization of a Soluble Form of Endothelial-Leukocyte Adhesion Molecule 1", J. Immunol. 147: 124-129

Each of the above-listed references is hereby incorporated by reference in its entirety.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Lobb, Roy R.; Carr, Frank J.; Tempest, Philip R.

(ii) TITLE OF INVENTION: Recombinant Anti-VLA4 Antibody Molecules

(iii) NUMBER OF SEQUENCES: 99

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Allegretti & Witcoff, Ltd.
(B) STREET: 10 South Wacker Drive, Suite 3000
(C) CITY: Chicago
(D) STATE: IL
(E) COUNTRY: US
(F) ZIP: 60606

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: McNicholas, Janet M.
(B) REGISTRATION NUMBER: 32,918
(C) REFERENCE/DOCKET NUMBER: 92,445/D012 US

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 312-715-1000
(B) TELEFAX: 312-715-1234

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "CG1FOR PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGAAGCTTAG ACAGATGGGG GTGTCGTTTT G 31

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "CK2FOR PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGAAGCTTGA AGATGGATAC AGTTGGTGCA GC 32

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "VH1BACK PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AGGTSMARCT GCAGSAGTCW GG 22

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "VK5BACK PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TTGAATTCGG TGCCAGARCW SAHATYGTKA TG 32

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 360 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "pBAG159 insert: HP1/2 heavy chain variable region; 1, 3 & 6 = E, Q & E in HP1/2"

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..360

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
GTC AAA CTG CAG CAG TCT GGG GCA GAG CTT GTG AAG CCA GGG GCC TCA        48
Val Lys Leu Gln Gln Ser Gly Ala Glu Leu Val Lys Pro Gly Ala Ser
  2               6                  11                  16

GTC AAG TTG TCC TGC ACA GCT TCT GGC TTC AAC ATT AAA GAC ACC TAT        96
Val Lys Leu Ser Cys Thr Ala Ser Gly Phe Asn Ile Lys Asp Thr Tyr
             21                  26                  31

ATG CAC TGG GTG AAG CAG AGG CCT GAA CAG GGC CTG GAG TGG ATT GGA       144
Met His Trp Val Lys Gln Arg Pro Glu Gln Gly Leu Glu Trp Ile Gly
         36                  41                  46

AGG ATT GAT CCT GCG AGT GGC GAT ACT AAA TAT GAC CCG AAG TTC CAG       192
Arg Ile Asp Pro Ala Ser Gly Asp Thr Lys Tyr Asp Pro Lys Phe Gln
     51                  56                  61

GTC AAG GCC ACT ATT ACA GCG GAC ACG TCC TCC AAC ACA GCC TGG CTG       240
Val Lys Ala Thr Ile Thr Ala Asp Thr Ser Ser Asn Thr Ala Trp Leu
 66                  71                  76                  81
```

```
CAG CTC AGC AGC CTG ACA TCT GAG GAC ACT GCC GTC TAC TAC TGT GCA        288
Gln Leu Ser Ser Leu Thr Ser Glu Asp Thr Ala Val Tyr Tyr Cys Ala
                 86                  91                  96

GAC GGA ATG TGG GTA TCA ACG GGA TAT GCT CTG GAC TTC TGG GGC CAA        336
Asp Gly Met Trp Val Ser Thr Gly Tyr Ala Leu Asp Phe Trp Gly Gln
            101                 106                 111

GGG ACC ACG GTC ACC GTC TCC TCA                                       360
Gly Thr Thr Val Thr Val Ser Ser
        116                 121
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 120 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Val Lys Leu Gln Gln Ser Gly Ala Glu Leu Val Lys Pro Gly Ala Ser
  2                 6                  11                  16

Val Lys Leu Ser Cys Thr Ala Ser Gly Phe Asn Ile Lys Asp Thr Tyr
             21                  26                  31

Met His Trp Val Lys Gln Arg Pro Glu Gln Gly Leu Glu Trp Ile Gly
         36                  41                  46

Arg Ile Asp Pro Ala Ser Gly Asp Thr Lys Tyr Asp Pro Lys Phe Gln
     51                  56                  61

Val Lys Ala Thr Ile Thr Ala Asp Thr Ser Ser Asn Thr Ala Trp Leu
 66                  71                  76                  81

Gln Leu Ser Ser Leu Thr Ser Glu Asp Thr Ala Val Tyr Tyr Cys Ala
                 86                  91                  96

Asp Gly Met Trp Val Ser Thr Gly Tyr Ala Leu Asp Phe Trp Gly Gln
             101                 106                 111

Gly Thr Thr Val Thr Val Ser Ser
         116                 121
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "VK1BACK PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GACATTCAGC TGACCCAGTC TCCA 24

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "VK7BACK PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TTGAATTCGG AGTTGATGGG AACATTGTAA TG 32

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 318 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..318
(D) OTHER INFORMATION: /product= "HP1/2 light chain variable region"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1

(D) OTHER INFORMATION: /note= "pBAG172 insert: HP1/2 light chain variable region"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
AGT ATT GTG ATG ACC CAG ACT CCC AAA TTC CTG CTT GTT TCA GCA GGA        48
Ser Ile Val Met Thr Gln Thr Pro Lys Phe Leu Leu Val Ser Ala Gly
 1               5                  10                  15

GAC AGG GTT ACC ATA ACC TGC AAG GCC AGT CAG AGT GTG ACT AAT GAT        96
Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Ser Val Thr Asn Asp
            20                  25                  30

GTA GCT TGG TAC CAA CAG AAG CCA GGG CAG TCT CCT AAA CTG CTG ATA       144
Val Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile
        35                  40                  45

TAT TAT GCA TCC AAT CGC TAC ACT GGA GTC CCT GAT CGC TTC ACT GGC       192
Tyr Tyr Ala Ser Asn Arg Tyr Thr Gly Val Pro Asp Arg Phe Thr Gly
    50                  55                  60

AGT GGA TAT GGG ACG GAT TTC ACT TTC ACC ATC AGC ACT GTG CAG GCT       240
Ser Gly Tyr Gly Thr Asp Phe Thr Phe Thr Ile Ser Thr Val Gln Ala
65                  70                  75                  80

GAA GAC CTG GCA GTT TAT TTC TGT CAG CAG GAT TAT AGC TCT CCG TAC       288
Glu Asp Leu Ala Val Tyr Phe Cys Gln Gln Asp Tyr Ser Ser Pro Tyr
                85                  90                  95

ACG TTC GGA GGG GGG ACC AAG CTG GAG ATC                              318
Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile
            100                 105
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 106 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Ser Ile Val Met Thr Gln Thr Pro Lys Phe Leu Leu Val Ser Ala Gly
 1               5                  10                  15

Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Ser Val Thr Asn Asp
            20                  25                  30

Val Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile
        35                  40                  45
```

Tyr Tyr Ala Ser Asn Arg Tyr Thr Gly Val Pro Asp Arg Phe Thr Gly
50 55 60

Ser Gly Tyr Gly Thr Asp Phe Thr Phe Thr Ile Ser Thr Val Gln Ala
65 70 75 80

Glu Asp Leu Ala Val Tyr Phe Cys Gln Gln Asp Tyr Ser Ser Pro Tyr
85 90 95

Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile
100 105

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "VH1FOR PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TGAGGAGACG GTGACCGTGG TCCCTTGGCC CCAG 34

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "VK3BACK PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GACATTCAGC TGACCCA 17

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "VK1FOR PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTTAGATCTC CAGCTTGGTC CC 22

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 823 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..621
(D) OTHER INFORMATION: /note= "VH insert in M13 VHPCR1"

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 261..621

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: join(122..167, 250..260)

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 261..621

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: join(122..167, 250..621)

(ix) FEATURE:
(A) NAME/KEY: TATA_signal
(B) LOCATION: 38..45

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 351..365
(D) OTHER INFORMATION: /note= "CDR1"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 408..458
(D) OTHER INFORMATION: /note= "CDR2"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 555..587
(D) OTHER INFORMATION: /note= "CDR3"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: (621^622)
(D) OTHER INFORMATION: /note= "splice to constant region"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
AAGCTTATGA ATATGCAAAT CCTCTGAATC TACATGGTAA ATATAGGTTT GTCTATACCA      60

CAAACAGAAA AACATGAGAT CACAGTTCTC TCTACAGTTA CTGAGCACAC AGGACCTCAC     120

C ATG GGA TGG AGC TGT ATC ATC CTC TTC TTG GTA GCA ACA GCT ACA  G      167
  Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr
  -19             -15                 -10                 -5

GTAAGGGGCT CACAGTAGCA GGCTTGAGGT CTGGACATAT ATATGGGTGA CAATGACATC     227

CACTTTGCCT TTCTCTCCAC AG  GT GTC CAC TCC CAG GTC CAA CTG CAG GAG      278
                         Gly Val His Ser Gln Val Gln Leu Gln Glu
                              -3          1               5

AGC GGT CCA GGT CTT GTG AGA CCT AGC CAG ACC CTG AGC CTG ACC TGC      326
Ser Gly Pro Gly Leu Val Arg Pro Ser Gln Thr Leu Ser Leu Thr Cys
            10                  15                  20

ACC GTG TCT GGC AGC ACC TTC AGC AGC TAC TGG ATG CAC TGG GTG AGA      374
Thr Val Ser Gly Ser Thr Phe Ser Ser Tyr Trp Met His Trp Val Arg
        25                  30                  35

CAG CCA CCT GGA CGA GGT CTT GAG TGG ATT GGA AGG ATT GAT CCT AAT      422
Gln Pro Pro Gly Arg Gly Leu Glu Trp Ile Gly Arg Ile Asp Pro Asn
    40                  45                  50

AGT GGT GGT ACT AAG TAC AAT GAG AAG TTC AAG AGC AGA GTG ACA ATG      470
Ser Gly Gly Thr Lys Tyr Asn Glu Lys Phe Lys Ser Arg Val Thr Met
55                  60                  65                  70
```

```
CTG GTA GAC ACC AGC AAG AAC CAG TTC AGC CTG AGA CTC AGC AGC GTG      518
Leu Val Asp Thr Ser Lys Asn Gln Phe Ser Leu Arg Leu Ser Ser Val
                75                  80                  85

ACA GCC GCC GAC ACC GCG GTC TAT TAT TGT GCA AGA TAC GAT TAC TAC      566
Thr Ala Ala Asp Thr Ala Val Tyr Tyr Cys Ala Arg Tyr Asp Tyr Tyr
            90                  95                  100

GGT AGT AGC TAC TTT GAC TAC TGG GGC CAA GGG ACC ACG GTC ACC GTC      614
Gly Ser Ser Tyr Phe Asp Tyr Trp Gly Gln Gly Thr Thr Val Thr Val
        105                 110                 115

TCC TCA G                                                            621
Ser Ser
    120
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 139 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly
-19             -15                 -10                 -5

Val His Ser Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Arg
            1               5                   10

Pro Ser Gln Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Ser Thr Phe
     15                  20                  25

Ser Ser Tyr Trp Met His Trp Val Arg Gln Pro Pro Gly Arg Gly Leu
 30                  35                  40                  45

Glu Trp Ile Gly Arg Ile Asp Pro Asn Ser Gly Gly Thr Lys Tyr Asn
                50                  55                  60

Glu Lys Phe Lys Ser Arg Val Thr Met Leu Val Asp Thr Ser Lys Asn
            65                  70                  75

Gln Phe Ser Leu Arg Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val
        80                  85                  90

Tyr Tyr Cys Ala Arg Tyr Asp Tyr Tyr Gly Ser Ser Tyr Phe Asp Tyr
     95                  100                 105
```

Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser
110 115 120

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 594 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..632
(D) OTHER INFORMATION: /note= "VK insert in M13 VKPCR2"

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 273..594

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: join(134..179, 262..272)

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 273..594

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: join(134..179, 262..594)

(ix) FEATURE:
(A) NAME/KEY: TATA_signal
(B) LOCATION: 50..57

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 342..374
(D) OTHER INFORMATION: /note= "CDR1"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 420..440
(D) OTHER INFORMATION: /note= "CDR2"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 537..563
(D) OTHER INFORMATION: /note= "CDR3"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: (594^595)
(D) OTHER INFORMATION: /note= "splice to constant region"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
CTCTTAAACT TCAAGCTTAT GAATATGCAA ATCCTCTGAA TCTACATGGT AAATATAGGT      60

TTGTCTATAC CACAAACAGA AAAACATGAG ATCACAGTTC TCTCTACAGT TACTGAGCAC     120

ACAGGACCTC ACC ATG GGA TGG AGC TGT ATC ATC CTC TTC TTG GTA GCA         169
               Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala
               -19             -15                 -10

ACA GCT ACA  G GTAAGGGGCT CACAGTAGCA GGCTTGAGGT CTGGACATAT            219
Thr Ala Thr
        -5

ATATGGGTGA CAATGACATC CACTTTGCCT TTCTCTCCAC AG  GT GTC CAC TCC         272
                                               Gly Val His Ser
                                                    -3

GAC ATC CAG CTG ACC CAG AGC CCA AGC AGC CTG AGC GCC AGC GTG GGT        320
Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
  1               5                  10                  15

GAC AGA GTG ACC ATC ACC TGT AGA GCC AGC GGT AAC ATC CAC AAC TAC        368
Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gly Asn Ile His Asn Tyr
            20                  25                  30

CTG GCT TGG TAC CAG CAG AAG CCA GGT AAG GCT CCA AAG CTG CTG ATC        416
Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
        35                  40                  45

TAC TAC ACC ACC ACC CTG GCT GAC GGT GTG CCA AGC AGA TTC AGC GGT        464
Tyr Tyr Thr Thr Thr Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

AGC GGT AGC GGT ACC GAC TTC ACC TTC ACC ATC AGC AGC CTC CAG CCA        512
Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

GAG GAC ATC GCC ACC TAC TAC TGC CAG CAC TTC TGG AGC ACC CCA AGG        560
Glu Asp Ile Ala Thr Tyr Tyr Cys Gln His Phe Trp Ser Thr Pro Arg
                85                  90                  95

ACG TTC GGC CAA GGG ACC AAG GTG GAA ATC AAA C                         594
Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
            100                 105
```

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 126 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

```
Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly
-19             -15                 -10                  -5

Val His Ser Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala
              1               5                  10

Ser Val Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gly Asn Ile
     15                  20                  25

His Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
 30                  35                  40                  45

Leu Leu Ile Tyr Tyr Thr Thr Thr Leu Ala Asp Gly Val Pro Ser Arg
                50                  55                  60

Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser
            65                  70                  75

Leu Gln Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Gln His Phe Trp Ser
         80                  85                  90

Thr Pro Arg Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
     95                  100                 105
```

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequence of 598 oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TGTCTCACCC AGTGCATATA GGTGTCTTTA ATGTTGAAGC CAGACACGCT GCAG 54

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 71 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequence of 599 oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CAGCATTGTC ACTCTGACCT GGAACTTCGG GTCATATTTA GTATCGCCAC TCGCAGGATC 60

AATCCTTCCA A 71

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 70 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequence of 600 oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GGTCCCTTGG CCCCAGAAGT CCAGAGCATA TCCCGTTGAT ACCCACATTC CGTCTGCACA 60

ATAATAGACC 70

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequence of 605 oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TCCCTTGGCC GAACGTGTAC GGAGAGCTAT AATCCTGCTG GCAGTAGTAG G 51

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequence of 606 oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

ATCTGCTTGG GCACACCAGT GTAGCGATTG GATGCATAGT AGATCAGCAG CT 52

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 61 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequence of 607 oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TCTGCTGGTA CCAAGCTACA TCATTAGTCA CACTCTGACT GGCCTTACAG GTGATGGTCA 60

C 61

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequence OLIGO 10 oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GTAAAACGAC GGCCAGT 17

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequence of OLIGO 385 oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GCGGGCCTCT TCGCTATTACGC 22

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequence of OLIGO 11 oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

AACAGCTATG ACCATG 16

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequence of OLIGO 391 oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CTCTCTCAGG GCCAGGCGGT GA 22

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 429 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 1..57

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 58..429

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..429

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "pMDR1019 insert: Stage 1 heavy chain variable region "

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

```
ATG GAC TGG ACC TGG AGG GTC TTC TGC TTG CTG GCT GTA GCA CCA GGT        48
Met Asp Trp Thr Trp Arg Val Phe Cys Leu Leu Ala Val Ala Pro Gly
-19             -15                 -10                 -5

GCC CAC TCC CAG GTC CAA CTG CAG GAG AGC GGT CCA GGT CTT GTG AGA        96
Ala His Ser Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Arg
              1               5                  10

CCT AGC CAG ACC CTG AGC CTG ACC TGC ACC GTG TCT GGC TTC AAC ATT       144
Pro Ser Gln Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Phe Asn Ile
     15                  20                  25

AAA GAC ACC TAT ATG CAC TGG GTG AGA CAG CCA CCT GGA CGA GGT CTT       192
Lys Asp Thr Tyr Met His Trp Val Arg Gln Pro Pro Gly Arg Gly Leu
 30                  35                  40                  45

GAG TGG ATT GGA AGG ATT GAT CCT GCG AGT GGC GAT ACT AAA TAT GAC       240
Glu Trp Ile Gly Arg Ile Asp Pro Ala Ser Gly Asp Thr Lys Tyr Asp
                 50                  55                  60

CCG AAG TTC CAG GTC AGA GTG ACA ATG CTG GTA GAC ACC AGC AAG AAC       288
Pro Lys Phe Gln Val Arg Val Thr Met Leu Val Asp Thr Ser Lys Asn
             65                  70                  75

CAG TTC AGC CTG AGA CTC AGC AGC GTG ACA GCC GCC GAC ACC GCG GTC       336
Gln Phe Ser Leu Arg Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val
         80                  85                  90

TAT TAT TGT GCA GAC GGA ATG TGG GTA TCA ACG GGA TAT GCT CTG GAC       384
Tyr Tyr Cys Ala Asp Gly Met Trp Val Ser Thr Gly Tyr Ala Leu Asp
     95                  100                 105

TTC TGG GGC CAA GGG ACC ACG GTC ACC GTC TCC TCA GGT GAG TCC           429
Phe Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Glu Ser
110                 115                 120
```

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 143 amino acids

(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

```
Met Asp Trp Thr Trp Arg Val Phe Cys Leu Leu Ala Val Ala Pro Gly
-19             -15                 -10                  -5

Ala His Ser Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Arg
               1               5                  10

Pro Ser Gln Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Phe Asn Ile
     15                  20                  25

Lys Asp Thr Tyr Met His Trp Val Arg Gln Pro Pro Gly Arg Gly Leu
 30                  35                  40                  45

Glu Trp Ile Gly Arg Ile Asp Pro Ala Ser Gly Asp Thr Lys Tyr Asp
                50                  55                  60

Pro Lys Phe Gln Val Arg Val Thr Met Leu Val Asp Thr Ser Lys Asn
            65                  70                  75

Gln Phe Ser Leu Arg Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val
         80                  85                  90

Tyr Tyr Cys Ala Asp Gly Met Trp Val Ser Thr Gly Tyr Ala Leu Asp
     95                  100                 105

Phe Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Glu Ser
110                 115                 120
```

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 386 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 1..57

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 58..386

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..386

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "pBAG190 insert: VK1 (DQL) light chain variable region"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

```
ATG GGT TGG TCC TGC ATC ATC CTG TTC CTG GTT GCT ACC GCT ACC GGT        48
Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly
-19             -15                 -10                 -5

GTT CAC TCC GAC ATC CAG CTG ACC CAG AGC CCA AGC AGC CTG AGC GCC        96
Val His Ser Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala
              1                5                  10

AGC GTG GGT GAC AGA GTG ACC ATC ACC TGT AAG GCC AGT CAG AGT GTG       144
Ser Val Gly Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Ser Val
     15                  20                  25

ACT AAT GAT GTA GCT TGG TAC CAG CAG AAG CCA GGT AAG GCT CCA AAG       192
Thr Asn Asp Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
 30                  35                  40                  45

CTG CTG ATC TAC TAT GCA TCC AAT CGC TAC ACT GGT GTG CCA AGC AGA       240
Leu Leu Ile Tyr Tyr Ala Ser Asn Arg Tyr Thr Gly Val Pro Ser Arg
                 50                  55                  60

TTC AGC GGT AGC GGT AGC GGT ACC GAC TTC ACC TTC ACC ATC AGC AGC       288
Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser
             65                  70                  75

CTC CAG CCA GAG GAC ATC GCC ACC TAC TAC TGC CAG CAG GAT TAT AGC       336
Leu Gln Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Asp Tyr Ser
         80                  85                  90

TCT CCG TAC ACG TTC GGC CAA GGG ACC AAG GTG GAA ATC AAA CGT AAG TG    386
Ser Pro Tyr Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Lys
     95                  100                 105
```

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 128 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

```
Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly
-19             -15                 -10                  -5

Val His Ser Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala
              1                 5                  10

Ser Val Gly Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Ser Val
     15                  20                  25

Thr Asn Asp Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
 30                  35                  40                  45

Leu Leu Ile Tyr Tyr Ala Ser Asn Arg Tyr Thr Gly Val Pro Ser Arg
                 50                  55                  60

Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser
             65                  70                  75

Leu Gln Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Asp Tyr Ser
         80                  85                  90

Ser Pro Tyr Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Lys
     95                  100                 105
```

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 429 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 1..57

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 58..429

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..429

(ix) FEATURE:
(A) NAME/KEY: misc_feature

(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "pMDR1028 insert: Stage 2 heavy chain variable region"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

```
ATG GAC TGG ACC TGG AGG GTC TTC TGC TTG CTG GCT GTA GCA CCA GGT      48
Met Asp Trp Thr Trp Arg Val Phe Cys Leu Leu Ala Val Ala Pro Gly
-19             -15                 -10                 -5

GCC CAC TCC CAG GTC CAA CTG CAG GAG AGC GGT CCA GGT CTT GTG AGA      96
Ala His Ser Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Arg
               1                   5                  10

CCT AGC CAG ACC CTG AGC CTG ACC TGC ACC GTG TCT GGC TTC AAC ATT     144
Pro Ser Gln Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Phe Asn Ile
     15                  20                  25

AAA GAC ACC TAT ATG CAC TGG GTG AGA CAG CCA CCT GGA CGA GGT CTT     192
Lys Asp Thr Tyr Met His Trp Val Arg Gln Pro Pro Gly Arg Gly Leu
 30                  35                  40                  45

GAG TGG ATT GGA AGG ATT GAT CCT GCG AGT GGC GAT ACT AAA TAT GAC     240
Glu Trp Ile Gly Arg Ile Asp Pro Ala Ser Gly Asp Thr Lys Tyr Asp
                 50                  55                  60

CCG AAG TTC CAG GTC AGA GTG ACA ATG CTG GTA GAC ACC AGC AGC AAC     288
Pro Lys Phe Gln Val Arg Val Thr Met Leu Val Asp Thr Ser Ser Asn
             65                  70                  75

CAG TTC AGC CTG AGA CTC AGC AGC GTG ACA GCC GCC GAC ACC GCG GTC     336
Gln Phe Ser Leu Arg Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val
         80                  85                  90

TAT TAT TGT GCA GAC GGA ATG TGG GTA TCA ACG GGA TAT GCT CTG GAC     384
Tyr Tyr Cys Ala Asp Gly Met Trp Val Ser Thr Gly Tyr Ala Leu Asp
     95                  100                 105

TTC TGG GGC CAA GGG ACC ACG GTC ACC GTC TCC TCA GGT GAG TCC         429
Phe Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Glu Ser
110                 115                 120
```

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 143 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

```
Met Asp Trp Thr Trp Arg Val Phe Cys Leu Leu Ala Val Ala Pro Gly
-19             -15                 -10                  -5

Ala His Ser Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Arg
              1                5                  10

Pro Ser Gln Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Phe Asn Ile
     15                  20                  25

Lys Asp Thr Tyr Met His Trp Val Arg Gln Pro Pro Gly Arg Gly Leu
 30                  35                  40                  45

Glu Trp Ile Gly Arg Ile Asp Pro Ala Ser Gly Asp Thr Lys Tyr Asp
                 50                  55                  60

Pro Lys Phe Gln Val Arg Val Thr Met Leu Val Asp Thr Ser Ser Asn
             65                  70                  75

Gln Phe Ser Leu Arg Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val
         80                  85                  90

Tyr Tyr Cys Ala Asp Gly Met Trp Val Ser Thr Gly Tyr Ala Leu Asp
     95                  100                 105

Phe Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Glu Ser
110                 115                 120
```

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequence of 684 oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

AGACACCAGC AGCAACCAGT TCAG 24

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequence of 683 oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

TGAACTGGTT GCTGCTGGTG TCTA 24

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequence of 713 oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

ACCAGCAGCA ACACAGCCTG GCTGAGACTC AGCAGCG 37

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequence of 716 oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GCTGAGTCTC AGCCAGGCTG TGTTGCTGCT GGTGTCGA 38

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 429 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 1..57

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 58..429

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..429

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "pBAG184 insert: STAW heavy chain variable region"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

```
ATG GAC TGG ACC TGG AGG GTC TTC TGC TTG CTG GCT GTA GCA CCA GGT        48
Met Asp Trp Thr Trp Arg Val Phe Cys Leu Leu Ala Val Ala Pro Gly
-19             -15                 -10                 -5

GCC CAC TCC CAG GTC CAA CTG CAG GAG AGC GGT CCA GGT CTT GTG AGA        96
Ala His Ser Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Arg
             1               5                  10

CCT AGC CAG ACC CTG AGC CTG ACC TGC ACC GTG TCT GGC TTC AAC ATT       144
Pro Ser Gln Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Phe Asn Ile
     15                  20                  25

AAA GAC ACC TAT ATG CAC TGG GTG AGA CAG CCA CCT GGA CGA GGT CTT       192
Lys Asp Thr Tyr Met His Trp Val Arg Gln Pro Pro Gly Arg Gly Leu
 30                  35                  40                  45
```

```
GAG TGG ATT GGA AGG ATT GAT CCT GCG AGT GGC GAT ACT AAA TAT GAC      240
Glu Trp Ile Gly Arg Ile Asp Pro Ala Ser Gly Asp Thr Lys Tyr Asp
                50                  55                  60

CCG AAG TTC CAG GTC AGA GTG ACA ATG CTG GTA GAC ACC AGC AGC AAC      288
Pro Lys Phe Gln Val Arg Val Thr Met Leu Val Asp Thr Ser Ser Asn
            65                  70                  75

ACA GCC TGG CTG AGA CTC AGC AGC GTG ACA GCC GCC GAC ACC GCG GTC      336
Thr Ala Trp Leu Arg Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val
        80                  85                  90

TAT TAT TGT GCA GAC GGA ATG TGG GTA TCA ACG GGA TAT GCT CTG GAC      384
Tyr Tyr Cys Ala Asp Gly Met Trp Val Ser Thr Gly Tyr Ala Leu Asp
    95                  100                 105

TTC TGG GGC CAA GGG ACC ACG GTC ACC GTC TCC TCA GGT GAG TCC          429
Phe Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Glu Ser
110                 115                 120
```

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 143 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

```
Met Asp Trp Thr Trp Arg Val Phe Cys Leu Leu Ala Val Ala Pro Gly
-19                 -15                 -10                  -5

Ala His Ser Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Arg
                 1                   5                  10

Pro Ser Gln Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Phe Asn Ile
     15                  20                  25

Lys Asp Thr Tyr Met His Trp Val Arg Gln Pro Pro Gly Arg Gly Leu
 30                  35                  40                  45

Glu Trp Ile Gly Arg Ile Asp Pro Ala Ser Gly Asp Thr Lys Tyr Asp
                50                  55                  60

Pro Lys Phe Gln Val Arg Val Thr Met Leu Val Asp Thr Ser Ser Asn
            65                  70                  75

Thr Ala Trp Leu Arg Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val
        80                  85                  90
```

Tyr Tyr Cys Ala Asp Gly Met Trp Val Ser Thr Gly Tyr Ala Leu Asp
95 100 105

Phe Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Glu Ser
110 115 120

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequence of 706 oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

AGTTCCAGGT CAAAGCGACA ATTACGGCAG ACACCAGCAA 40

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequence of 707 oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

CTTGCTGGTG TCTGCCGTAA TTGTCGCTTT GACCTGGAAC 40

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 429 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 1..57

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 58..429

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..429

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "pBAG183 insert: KAITAS heavy chain variable region"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

```
ATG GAC TGG ACC TGG AGG GTC TTC TGC TTG CTG GCT GTA GCA CCA GGT        48
Met Asp Trp Thr Trp Arg Val Phe Cys Leu Leu Ala Val Ala Pro Gly
-19             -15                 -10                 -5

GCC CAC TCC CAG GTC CAA CTG CAG GAG AGC GGT CCA GGT CTT GTG AGA        96
Ala His Ser Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Arg
               1                5                  10

CCT AGC CAG ACC CTG AGC CTG ACC TGC ACC GTG TCT GGC TTC AAC ATT       144
Pro Ser Gln Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Phe Asn Ile
     15                  20                  25

AAA GAC ACC TAT ATG CAC TGG GTG AGA CAG CCA CCT GGA CGA GGT CTT       192
Lys Asp Thr Tyr Met His Trp Val Arg Gln Pro Pro Gly Arg Gly Leu
 30                  35                  40                  45

GAG TGG ATT GGA AGG ATT GAT CCT GCG AGT GGC GAT ACT AAA TAT GAC       240
Glu Trp Ile Gly Arg Ile Asp Pro Ala Ser Gly Asp Thr Lys Tyr Asp
                50                  55                  60

CCG AAG TTC CAG GTC AAA GCG ACA ATT ACG GCA GAC ACC AGC AGC AAC       288
Pro Lys Phe Gln Val Lys Ala Thr Ile Thr Ala Asp Thr Ser Ser Asn
            65                  70                  75

CAG TTC AGC CTG AGA CTC AGC AGC GTG ACA GCC GCC GAC ACC GCG GTC       336
Gln Phe Ser Leu Arg Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val
        80                  85                  90
```

```
TAT TAT TGT GCA GAC GGA ATG TGG GTA TCA ACG GGA TAT GCT CTG GAC      384
Tyr Tyr Cys Ala Asp Gly Met Trp Val Ser Thr Gly Tyr Ala Leu Asp
     95                  100                 105

TTC TGG GGC CAA GGG ACC ACG GTC ACC GTC TCC TCA GGT GAG TCC      429
Phe Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Glu Ser
110                 115                 120
```

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 143 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

```
Met Asp Trp Thr Trp Arg Val Phe Cys Leu Leu Ala Val Ala Pro Gly
-19             -15                 -10                  -5

Ala His Ser Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Arg
               1                   5                  10

Pro Ser Gln Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Phe Asn Ile
     15                  20                  25

Lys Asp Thr Tyr Met His Trp Val Arg Gln Pro Pro Gly Arg Gly Leu
 30                  35                  40                  45

Glu Trp Ile Gly Arg Ile Asp Pro Ala Ser Gly Asp Thr Lys Tyr Asp
                 50                  55                  60

Pro Lys Phe Gln Val Lys Ala Thr Ile Thr Ala Asp Thr Ser Ser Asn
             65                  70                  75

Gln Phe Ser Leu Arg Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val
         80                  85                  90

Tyr Tyr Cys Ala Asp Gly Met Trp Val Ser Thr Gly Tyr Ala Leu Asp
     95                  100                 105

Phe Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Glu Ser
110                 115                 120
```

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequence of 768 oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

CTCAGCAGCG TGACATCTGA GGACACCGCG GTCTAT 36

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequence of 769 oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

ATAGACCGCG GTGTCCTCAG ATGTCACGCT GCTGAG 36

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 372 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..372
(D) OTHER INFORMATION: /note= "pBAG207 insert: SSE heavy chain variable region"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide

(B) LOCATION: 1..372

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..372

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

```
CAG GTC CAA CTG CAG GAG AGC GGT CCA GGT CTT GTG AGA CCT AGC CAG        48
Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Arg Pro Ser Gln
 1               5                  10                  15

ACC CTG AGC CTG ACC TGC ACC GTG TCT GGC TTC AAC ATT AAA GAC ACC        96
Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Phe Asn Ile Lys Asp Thr
            20                  25                  30

TAT ATG CAC TGG GTG AGA CAG CCA CCT GGA CGA GGT CTT GAG TGG ATT       144
Tyr Met His Trp Val Arg Gln Pro Pro Gly Arg Gly Leu Glu Trp Ile
        35                  40                  45

GGA AGG ATT GAT CCT GCG AGT GGC GAT ACT AAA TAT GAC CCG AAG TTC       192
Gly Arg Ile Asp Pro Ala Ser Gly Asp Thr Lys Tyr Asp Pro Lys Phe
    50                  55                  60

CAG GTC AGA GTG ACA ATG CTG GTA GAC ACC AGC AGC AAC CAG TTC AGC       240
Gln Val Arg Val Thr Met Leu Val Asp Thr Ser Ser Asn Gln Phe Ser
65                  70                  75                  80

CTG AGA CTC AGC AGC GTG ACA TCT GAG GAC ACC GCG GTC TAT TAT TGT       288
Leu Arg Leu Ser Ser Val Thr Ser Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

GCA GAC GGA ATG TGG GTA TCA ACG GGA TAT GCT CTG GAC TTC TGG GGC       336
Ala Asp Gly Met Trp Val Ser Thr Gly Tyr Ala Leu Asp Phe Trp Gly
            100                 105                 110

CAA GGG ACC ACG GTC ACC GTC TCC TCA GGT GAG TCC                       372
Gln Gly Thr Thr Val Thr Val Ser Ser Gly Glu Ser
        115                 120
```

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 124 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

```
Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Arg Pro Ser Gln
 1               5                  10                  15

Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Phe Asn Ile Lys Asp Thr
            20                  25                  30

Tyr Met His Trp Val Arg Gln Pro Pro Gly Arg Gly Leu Glu Trp Ile
        35                  40                  45

Gly Arg Ile Asp Pro Ala Ser Gly Asp Thr Lys Tyr Asp Pro Lys Phe
    50                  55                  60

Gln Val Arg Val Thr Met Leu Val Asp Thr Ser Ser Asn Gln Phe Ser
65                  70                  75                  80

Leu Arg Leu Ser Ser Val Thr Ser Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Asp Gly Met Trp Val Ser Thr Gly Tyr Ala Leu Asp Phe Trp Gly
            100                 105                 110

Gln Gly Thr Thr Val Thr Val Ser Ser Gly Glu Ser
        115                 120
```

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequence of 704 oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

```
TGCACTGGGT GAAACAGCGA CCTGGACGAG G                              31
```

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequence of 705 oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

CCTCGTCCAG GTCGCTGTTT CACCCAGTGC A 31

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 429 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 1..57

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 58..429

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..429

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "pBAG185 insert: KRS heavy chain variable region"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

```
ATG GAC TGG ACC TGG AGG GTC TTC TGC TTG CTG GCT GTA GCA CCA GGT        48
Met Asp Trp Thr Trp Arg Val Phe Cys Leu Leu Ala Val Ala Pro Gly
-19             -15                 -10                  -5

GCC CAC TCC CAG GTC CAA CTG CAG GAG AGC GGT CCA GGT CTT GTG AGA        96
Ala His Ser Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Arg
              1               5                  10
```

```
CCT AGC CAG ACC CTG AGC CTG ACC TGC ACC GTG TCT GGC TTC AAC ATT        144
Pro Ser Gln Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Phe Asn Ile
     15                  20                  25

AAA GAC ACC TAT ATG CAC TGG GTG AAA CAG CGA CCT GGA CGA GGT CTT        192
Lys Asp Thr Tyr Met His Trp Val Lys Gln Arg Pro Gly Arg Gly Leu
 30                  35                  40                  45

GAG TGG ATT GGA AGG ATT GAT CCT GCG AGT GGC GAT ACT AAA TAT GAC        240
Glu Trp Ile Gly Arg Ile Asp Pro Ala Ser Gly Asp Thr Lys Tyr Asp
                 50                  55                  60

CCG AAG TTC CAG GTC AGA GTG ACA ATG CTG GTA GAC ACC AGC AGC AAC        288
Pro Lys Phe Gln Val Arg Val Thr Met Leu Val Asp Thr Ser Ser Asn
             65                  70                  75

CAG TTC AGC CTG AGA CTC AGC AGC GTG ACA GCC GCC GAC ACC GCG GTC        336
Gln Phe Ser Leu Arg Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val
         80                  85                  90

TAT TAT TGT GCA GAC GGA ATG TGG GTA TCA ACG GGA TAT GCT CTG GAC        384
Tyr Tyr Cys Ala Asp Gly Met Trp Val Ser Thr Gly Tyr Ala Leu Asp
     95                  100                 105

TTC TGG GGC CAA GGG ACC ACG GTC ACC GTC TCC TCA GGT GAG TCC            429
Phe Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Glu Ser
110                 115                 120
```

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 143 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

```
Met Asp Trp Thr Trp Arg Val Phe Cys Leu Leu Ala Val Ala Pro Gly
-19             -15                 -10                 -5

Ala His Ser Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Arg
              1                   5                  10

Pro Ser Gln Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Phe Asn Ile
     15                  20                  25

Lys Asp Thr Tyr Met His Trp Val Lys Gln Arg Pro Gly Arg Gly Leu
 30                  35                  40                  45
```

```
Glu Trp Ile Gly Arg Ile Asp Pro Ala Ser Gly Asp Thr Lys Tyr Asp
                50                  55                  60

Pro Lys Phe Gln Val Arg Val Thr Met Leu Val Asp Thr Ser Ser Asn
            65                  70                  75

Gln Phe Ser Leu Arg Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val
        80                  85                  90

Tyr Tyr Cys Ala Asp Gly Met Trp Val Ser Thr Gly Tyr Ala Leu Asp
    95                  100                 105

Phe Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Glu Ser
110                 115                 120
```

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequence of 745 oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

TGACCTGCAC CGCGTCTGGC TTCAAC 26

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequence of 746 oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

TTGAAGCCAG ACGCGGTGCA GGTCAG 26

(2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 429 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 1..57

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 58..429

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..429

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "pBAG195 insert: AS heavy chain variable region"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

```
ATG GAC TGG ACC TGG AGG GTC TTC TGC TTG CTG GCT GTA GCA CCA GGT        48
Met Asp Trp Thr Trp Arg Val Phe Cys Leu Leu Ala Val Ala Pro Gly
-19             -15                 -10                 -5

GCC CAC TCC CAG GTC CAA CTG CAG GAG AGC GGT CCA GGT CTT GTG AGA        96
Ala His Ser Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Arg
              1               5                  10

CCT AGC CAG ACC CTG AGC CTG ACC TGC ACC GCG TCT GGC TTC AAC ATT       144
Pro Ser Gln Thr Leu Ser Leu Thr Cys Thr Ala Ser Gly Phe Asn Ile
     15                  20                  25

AAA GAC ACC TAT ATG CAC TGG GTG AGA CAG CCA CCT GGA CGA GGT CTT       192
Lys Asp Thr Tyr Met His Trp Val Arg Gln Pro Pro Gly Arg Gly Leu
 30                  35                  40                  45

GAG TGG ATT GGA AGG ATT GAT CCT GCG AGT GGC GAT ACT AAA TAT GAC       240
Glu Trp Ile Gly Arg Ile Asp Pro Ala Ser Gly Asp Thr Lys Tyr Asp
                 50                  55                  60
```

```
CCG AAG TTC CAG GTC AGA GTG ACA ATG CTG GTA GAC ACC AGC AGC AAC      288
Pro Lys Phe Gln Val Arg Val Thr Met Leu Val Asp Thr Ser Ser Asn
            65                  70                  75

CAG TTC AGC CTG AGA CTC AGC AGC GTG ACA GCC GCC GAC ACC GCG GTC      336
Gln Phe Ser Leu Arg Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val
        80                  85                  90

TAT TAT TGT GCA GAC GGA ATG TGG GTA TCA ACG GGA TAT GCT CTG GAC      384
Tyr Tyr Cys Ala Asp Gly Met Trp Val Ser Thr Gly Tyr Ala Leu Asp
    95                  100                 105

TTC TGG GGC CAA GGG ACC ACG GTC ACC GTC TCC TCA GGT GAG TCC          429
Phe Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Glu Ser
110                 115                 120
```

(2) INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 143 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

```
Met Asp Trp Thr Trp Arg Val Phe Cys Leu Leu Ala Val Ala Pro Gly
-19             -15                 -10                 -5

Ala His Ser Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Arg
                1               5                   10

Pro Ser Gln Thr Leu Ser Leu Thr Cys Thr Ala Ser Gly Phe Asn Ile
    15                  20                  25

Lys Asp Thr Tyr Met His Trp Val Arg Gln Pro Pro Gly Arg Gly Leu
30                  35                  40                  45

Glu Trp Ile Gly Arg Ile Asp Pro Ala Ser Gly Asp Thr Lys Tyr Asp
                50                  55                  60

Pro Lys Phe Gln Val Arg Val Thr Met Leu Val Asp Thr Ser Ser Asn
            65                  70                  75

Gln Phe Ser Leu Arg Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val
        80                  85                  90

Tyr Tyr Cys Ala Asp Gly Met Trp Val Ser Thr Gly Tyr Ala Leu Asp
    95                  100                 105
```

Phe Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Glu Ser
110 115 120

(2) INFORMATION FOR SEQ ID NO:56:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequence of 915 oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

TATTATTGTG CAAGAGGAAT GTGGGTATC 29

(2) INFORMATION FOR SEQ ID NO:57:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequence of 917 oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

ATACCCACAT TCCTCTTGCA CAATAATAG 29

(2) INFORMATION FOR SEQ ID NO:58:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequence of 918 oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

CTGCACCGTG TCTGGCTTCA CCTTCAGCGA CACCTATATG C 41

(2) INFORMATION FOR SEQ ID NO:59:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequence of 919 oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

GCATATAGGT GTCGCTGAAG GTGAAGCCAG ACACGGTGCA G 41

(2) INFORMATION FOR SEQ ID NO:60:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequence of 697 oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

GGTGTCCACT CCAGCATCGT GATGACCCAG A 41

(2) INFORMATION FOR SEQ ID NO:61:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequence of 698 oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

TCTGGGTCAT CACGATGCTG GAGTGGACAC C 41

(2) INFORMATION FOR SEQ ID NO:62:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 386 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 1..57

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 58..386

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..386

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "pBAG198 insert: VK2 (SVMDY) light chain variable region"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

ATG GGT TGG TCC TGC ATC ATC CTG TTC CTG GTT GCT ACC GCT ACC GGT 48
Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly
-19 -15 -10 -5

```
GTC CAC TCC AGC ATC GTG ATG ACC CAG AGC CCA AGC AGC CTG AGC GCC          96
Val His Ser Ser Ile Val Met Thr Gln Ser Pro Ser Ser Leu Ser Ala
                1               5                  10

AGC GTG GGT GAC AGA GTG ACC ATC ACC TGT AAG GCC AGT CAG AGT GTG         144
Ser Val Gly Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Ser Val
     15                  20                  25

ACT AAT GAT GTA GCT TGG TAC CAG CAG AAG CCA GGT AAG GCT CCA AAG         192
Thr Asn Asp Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
 30                  35                  40                  45

CTG CTG ATC TAC TAT GCA TCC AAT CGC TAC ACT GGT GTG CCA GAT AGA         240
Leu Leu Ile Tyr Tyr Ala Ser Asn Arg Tyr Thr Gly Val Pro Asp Arg
                50                  55                  60

TTC AGC GGT AGC GGT TAT GGT ACC GAC TTC ACC TTC ACC ATC AGC AGC         288
Phe Ser Gly Ser Gly Tyr Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser
            65                  70                  75

CTC CAG CCA GAG GAC ATC GCC ACC TAC TAC TGC CAG CAG GAT TAT AGC         336
Leu Gln Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Asp Tyr Ser
        80                  85                  90

TCT CCG TAC ACG TTC GGC CAA GGG ACC AAG GTG GAA ATC AAA CGT AAG TG      386
Ser Pro Tyr Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Lys
    95                  100                 105
```

(2) INFORMATION FOR SEQ ID NO:63:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 128 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

```
Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly
-19             -15                 -10                 -5

Val His Ser Ser Ile Val Met Thr Gln Ser Pro Ser Ser Leu Ser Ala
                1               5                  10

Ser Val Gly Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Ser Val
     15                  20                  25

Thr Asn Asp Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
 30                  35                  40                  45
```

Leu Leu Ile Tyr Tyr Ala Ser Asn Arg Tyr Thr Gly Val Pro Asp Arg
50 55 60

Phe Ser Gly Ser Gly Tyr Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser
65 70 75

Leu Gln Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Asp Tyr Ser
80 85 90

Ser Pro Tyr Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Lys
95 100 105

(2) INFORMATION FOR SEQ ID NO:64:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequence of 803 oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

GGTGTCCACT CCGACATCCA GATGACCCAG AG 32

(2) INFORMATION FOR SEQ ID NO:65:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequence of 804 oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

CTCTGGGTCA TCTGGATGTC GGAGTGGACA CC 32

(2) INFORMATION FOR SEQ ID NO:66:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 386 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 1..57

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 58..386

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..386

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "pBAG197 insert: VK3 (DQMDY) light chain variable region"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

```
ATG GGT TGG TCC TGC ATC ATC CTG TTC CTG GTT GCT ACC GCT ACC GGT        48
Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly
-19             -15                 -10                 -5

GTC CAC TCC GAC ATC CAG ATG ACC CAG AGC CCA AGC AGC CTG AGC GCC        96
Val His Ser Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala
               1                 5                  10

AGC GTG GGT GAC AGA GTG ACC ATC ACC TGT AAG GCC AGT CAG AGT GTG       144
Ser Val Gly Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Ser Val
     15                  20                  25

ACT AAT GAT GTA GCT TGG TAC CAG CAG AAG CCA GGT AAG GCT CCA AAG       192
Thr Asn Asp Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
 30                  35                  40                  45

CTG CTG ATC TAC TAT GCA TCC AAT CGC TAC ACT GGT GTG CCA GAT AGA       240
Leu Leu Ile Tyr Tyr Ala Ser Asn Arg Tyr Thr Gly Val Pro Asp Arg
                50                  55                  60
```

```
TTC AGC GGT AGC GGT TAT GGT ACC GAC TTC ACC TTC ACC ATC AGC AGC        288
Phe Ser Gly Ser Gly Tyr Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser
            65                  70                  75

CTC CAG CCA GAG GAC ATC GCC ACC TAC TAC TGC CAG CAG GAT TAT AGC        336
Leu Gln Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Asp Tyr Ser
        80                  85                  90

TCT CCG TAC ACG TTC GGC CAA GGG ACC AAG GTG GAA ATC AAA CGT AAG TG     386
Ser Pro Tyr Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Lys
    95                  100                 105
```

(2) INFORMATION FOR SEQ ID NO:67:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 128 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

```
Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly
-19             -15                 -10                 -5

Val His Ser Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala
              1                   5                  10

Ser Val Gly Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Ser Val
     15                  20                  25

Thr Asn Asp Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
 30                  35                  40                  45

Leu Leu Ile Tyr Tyr Ala Ser Asn Arg Tyr Thr Gly Val Pro Asp Arg
                 50                  55                  60

Phe Ser Gly Ser Gly Tyr Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser
             65                  70                  75

Leu Gln Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Asp Tyr Ser
         80                  85                  90

Ser Pro Tyr Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Lys
     95                  100                 105
```

(2) INFORMATION FOR SEQ ID NO:68:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 429 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..429
(D) OTHER INFORMATION: /note= "pMDR1023 insert: PDLN heavy chain variable region"

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 1..57

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 58..429

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..429

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

```
ATG GAC TGG ACC TGG AGG GTC TTC TGC TTG CTG GCT GTA GCA CCA GGT        48
Met Asp Trp Thr Trp Arg Val Phe Cys Leu Leu Ala Val Ala Pro Gly
-19             -15                 -10                 -5

GCC CAC TCC CAG GTC CAA CTG CAG GAG TCC GGT GCT GAA GTT GTT AAA        96
Ala His Ser Gln Val Gln Leu Gln Glu Ser Gly Ala Glu Val Val Lys
               1                   5                  10

CCG GGT TCC TCC GTT AAA CTG TCC TGC AAA GCT TCC GGT TTC AAC ATC       144
Pro Gly Ser Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Phe Asn Ile
     15                  20                  25

AAA GAC ACC TAC ATG CAC TGG GTT AAA CAG CGT CCG GGT CAG GGT CTG       192
Lys Asp Thr Tyr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu
 30                  35                  40                  45

GAA TGG ATC GGT CGT ATC GAC CCG GCT TCC GGT GAC ACC AAA TAC GAC       240
Glu Trp Ile Gly Arg Ile Asp Pro Ala Ser Gly Asp Thr Lys Tyr Asp
                 50                  55                  60

CCG AAA TTC CAG GTT AAA GCT ACC ATC ACC GCT GAC GAA TCC ACC TCC       288
Pro Lys Phe Gln Val Lys Ala Thr Ile Thr Ala Asp Glu Ser Thr Ser
             65                  70                  75

ACC GCT TAC CTG GAA CTG TCC TCC CTG CGT TCC GAA GAC ACC GCT GTT       336
Thr Ala Tyr Leu Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val
         80                  85                  90
```

```
TAC TAC TGC GCT GAC GGT ATG TGG GTT TCC ACC GGT TAC GCT CTG GAC      384
Tyr Tyr Cys Ala Asp Gly Met Trp Val Ser Thr Gly Tyr Ala Leu Asp
     95                  100                 105

TTC TGG GGT CAG GGT ACC ACG GTC ACC GTC TCC TCA GGT GAG TCC      429
Phe Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Glu Ser
110                 115                 120
```

(2) INFORMATION FOR SEQ ID NO:69:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 143 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

```
Met Asp Trp Thr Trp Arg Val Phe Cys Leu Leu Ala Val Ala Pro Gly
-19             -15                 -10                  -5

Ala His Ser Gln Val Gln Leu Gln Glu Ser Gly Ala Glu Val Val Lys
              1               5                  10

Pro Gly Ser Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Phe Asn Ile
     15                  20                  25

Lys Asp Thr Tyr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu
 30                  35                  40                  45

Glu Trp Ile Gly Arg Ile Asp Pro Ala Ser Gly Asp Thr Lys Tyr Asp
                 50                  55                  60

Pro Lys Phe Gln Val Lys Ala Thr Ile Thr Ala Asp Glu Ser Thr Ser
             65                  70                  75

Thr Ala Tyr Leu Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val
         80                  85                  90

Tyr Tyr Cys Ala Asp Gly Met Trp Val Ser Thr Gly Tyr Ala Leu Asp
     95                  100                 105

Phe Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Glu Ser
110                 115                 120
```

(2) INFORMATION FOR SEQ ID NO:70:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 383 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..376
(D) OTHER INFORMATION: /note= "pMDR1025 insert: PDLN light chain variable region"

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 1..57

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 58..376

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..376

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

```
ATG GGT TGG TCC TGC ATC ATC CTG TTC CTG GTT GCT ACC GCT ACC GGT        48
Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly
-19             -15                 -10                 -5

GTT CAC TCC ATC GTT ATG ACC CAG TCC CCG GAC TCC CTG GCT GTT TCC        96
Val His Ser Ile Val Met Thr Gln Ser Pro Asp Ser Leu Ala Val Ser
               1               5                  10

CTG GGT GAA CGT GTT ACC ATC AAC TGC AAA GCT TCC CAG TCC GTT ACC       144
Leu Gly Glu Arg Val Thr Ile Asn Cys Lys Ala Ser Gln Ser Val Thr
     15                  20                  25

AAC GAC GTT GCT TGG TAC CAG CAG AAA CCG GGT CAG TCC CCG AAA CTG       192
Asn Asp Val Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Leu
 30                  35                  40                  45

CTG ATC TAC TAC GCT TCC AAC CGT TAC ACC GGT GTT CCG GAC CGT TTC       240
Leu Ile Tyr Tyr Ala Ser Asn Arg Tyr Thr Gly Val Pro Asp Arg Phe
                50                  55                  60

TCC GGT TCC GGT TAC GGT ACC GAC TTC ACC TTC ACC ATC TCC TCC GTT       288
Ser Gly Ser Gly Tyr Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Val
            65                  70                  75

CAG GCT GAA GAC GTT GCT GTT TAC TAC TGC CAG CAG GAC TAC TCC TCC       336
Gln Ala Glu Asp Val Ala Val Tyr Tyr Cys Gln Gln Asp Tyr Ser Ser
        80                  85                  90
```

```
CCG TAC ACC TTC GGT GGT GGT ACC AAA CTG GAG ATC TAA GGA TCC TC        383
Pro Tyr Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile  *
 95                  100                 105
```

(2) INFORMATION FOR SEQ ID NO:71:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 124 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

```
Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly
-19             -15                 -10                  -5

Val His Ser Ile Val Met Thr Gln Ser Pro Asp Ser Leu Ala Val Ser
              1               5                  10

Leu Gly Glu Arg Val Thr Ile Asn Cys Lys Ala Ser Gln Ser Val Thr
     15                  20                  25

Asn Asp Val Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Leu
 30                  35                  40                  45

Leu Ile Tyr Tyr Ala Ser Asn Arg Tyr Thr Gly Val Pro Asp Arg Phe
                 50                  55                  60

Ser Gly Ser Gly Tyr Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Val
             65                  70                  75

Gln Ala Glu Asp Val Ala Val Tyr Tyr Cys Gln Gln Asp Tyr Ser Ser
         80                  85                  90

Pro Tyr Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile  *
 95                  100                 105
```

(2) INFORMATION FOR SEQ ID NO:72:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1

(D) OTHER INFORMATION: /note= "Oligo 370-119 corresponding to 58-117 VH-PDLN"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

CAGGTTCAGC TGCAGGAGTC CGGTGCTGAA GTTGTTAAAC CGGGTTCCTC CGTTAAACTG 60

(2) INFORMATION FOR SEQ ID NO:73:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Oligo 370-120 corresponds to 118-177 VH-PDLN"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

TCCTGCAAAG CTTCCGGTTT CAACATCAAA GACACCTACA TGCACTGGGT TAAACAGCGT 60

(2) INFORMATION FOR SEQ ID NO:74:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Oligo 370-121 corresponds to 178-237 VH-PDLN"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:74:

CCGGGTCAGG GTCTGGAATG GATCGGTCGT ATCGACCCGG CTTCCGGTGA CACCAAATAC 60

(2) INFORMATION FOR SEQ ID NO:75:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Oligo 370-122 corresponds to 238-303 VH-PDLN"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:75:

GACCCGAAAT TCCAGGTTAA AGCTACCATC ACCGCTGACG AATCCACCTC CACCGCTTAC 60

CTGGAA 66

(2) INFORMATION FOR SEQ ID NO:76:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 63 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Oligo 370-123 corresponds to 304-366 VH-PDLN"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:

CTGTCCTCCC TGCGTTCCGA AGACACCGCT GTTTACTACT GCGCTGACGG TATGTGGGTT 60

TCC 63

(2) INFORMATION FOR SEQ ID NO:77:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1

(D) OTHER INFORMATION: /note= "Oligo 370-124 corresponds to 367-420 VH-PDLN"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:77:

ACCGGTTACG CTCTGGACTT CTGGGGTCAG GGTACCACGG TCACCGTTTC CTCC 54

(2) INFORMATION FOR SEQ ID NO:78:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 63 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Oligo 370-125 corresponds to reverse VH-PDLN 420-358"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:78:

GGAGGAAACG GTGACCGTGG TACCCTGACC CCAGAAGTCC AGAGCGTAAC CGGTGGAAAC 60

CCA 63

(2) INFORMATION FOR SEQ ID NO:79:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Oligo 370-126 corresponds to reverse VH-PDLN 357-311"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:79:

CATACCGTCA GCGCAGTAGT AAACAGCGGT GTCTTCGGAA CGCAGGG 47

(2) INFORMATION FOR SEQ ID NO:80:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 67 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Oligo 370-127 corresponds to reverse VH-PDLN 310-244"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:80:

AGGACAGTTC CAGGTAAGCG GTGGAGGTGG ATTCGTCAGC GGTGATGGTA GCTTTAACCT 60

GGAATTT 67

(2) INFORMATION FOR SEQ ID NO:81:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Oligo 370-128 corresponds to reverse VH-PDLN 243-186"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:

CGGGTCGTAT TTGGTGTCAC CGGAAGCCGG GTCGATACGA CCGATCCATT CCAGACCCTG 60

(2) INFORMATION FOR SEQ ID NO:82:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Oligo 370-129 corresponds to reverse VH-PDLN 185-124"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:82:

ACCCGGACGC TGTTTAACCC AGTGCATGTA GGTGTCTTTG ATGTTGAAAC CGGAAGCTTT 60

(2) INFORMATION FOR SEQ ID NO:83:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Oligo 370-130 corresponds to reverse VH-PDLN 123-58"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:83:

GCAGGACAGT TTAACGGAGG AACCCGGTTT AACAACTTCA GCACCGGACT CCTGCAGCTG 60

AACCTG 66

(2) INFORMATION FOR SEQ ID NO:84:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature

(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Oligo 370-131 corresponds to 1-58 VK-PDLN"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:84:

AGCTTACCAT GGGTTGGTCC TGCATCATCC TGTTCCTGGT TGCTACCGCT ACCGGTGTTC 60

ACTCCA 66

(2) INFORMATION FOR SEQ ID NO:85:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Oligo 370-132 corresponds to 59-124 VK-PDLN"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:85:

TCGTTATGAC CCAGTCCCCG GACTCCCTGG CTGTTTCCCT GGGTGAACGT GTTACCATCA 60

ACTGCA 66

(2) INFORMATION FOR SEQ ID NO:86:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Oligo 370-133 corresponds to 125-190 VK-PDLN"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:86:

AAGCTTCCCA GTCCGTTACC AACGACGTTG CTTGGTACCA GCAGAAACCG GGTCAGTCCC 60

CGAAAC 66

(2) INFORMATION FOR SEQ ID NO:87:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Oligo 370-134 corresponds to 191-256 VK-PDLN"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:87:

TGCTGATCTA CTACGCTTCC AACCGTTACA CCGGTGTTCC GGACCGTTTC TCCGGTTCCG 60

GTTACG 66

(2) INFORMATION FOR SEQ ID NO:88:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Oligo 370-135 corresponds to 257-322 VK-PDLN"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:88:

GTACCGACTT CACCTTCACC ATCTCCTCCG TTCAGGCTGA AGACGTTGCT GTTTACTACT 60

GCCAGC 66

(2) INFORMATION FOR SEQ ID NO:89:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Oligo 370-136 corresponds to 323-376 VK-PDLN"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:89:

AGGACTACTC CTCCCCGTAC ACCTTCGGTG GTGGTACCAA ACTGGAGATC TAAG 54

(2) INFORMATION FOR SEQ ID NO:90:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 63 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Oligo 370-137 corresponds to reverse VK-PDLN 380-318"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:90:

GATCCTTAGA TCTCCAGTTT GGTACCACCA CCGAAGGTGT ACGGGGAGGA GTAGTCCTGC 60

TGG 63

(2) INFORMATION FOR SEQ ID NO:91:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Oligo 370-138 corresponds to reverse VK-PDLN 317-252"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:91:

CAGTAGTAAA CAGCAACGTC TTCAGCCTGA ACGGAGGAGA TGGTGAAGGT GAAGTCGGTA 60

CCGTAA 66

(2) INFORMATION FOR SEQ ID NO:92:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Oligo 370-139 corresponds to reverse VK-PDLN 251-186"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:92:

CCGGAACCGG AGAAACGGTC CGGAACACCG GTGTAACGGT TGGAAGCGTA GTAGATCAGC 60

AGTTTC 66

(2) INFORMATION FOR SEQ ID NO:93:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature

(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Oligo 370-140 corresponds to reverse VK-PDLN 185-120"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:93:

GGGGACTGAC CCGGTTTCTG CTGGTACCAA GCAACGTCGT TGGTAACGGA CTGGGAAGCT 60

TTGCAG 66

(2) INFORMATION FOR SEQ ID NO:94:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Oligo 370-141 corresponds to reverse VK-PDLN 119-54"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:94:

TTGATGGTAA CACGTTCACC CAGGGAAACA GCCAGGGAGT CCGGGGACTG GGTCATAACG 60

ATGGAG 66

(2) INFORMATION FOR SEQ ID NO:95:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Oligo 370-142 corresponds to reverse VK-PDLN 53-1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:95:

TGAACACCGG TAGCGGTAGC AACCAGGAAC AGGATGATGC AGGACCAACC CATGGTA 57

(2) INFORMATION FOR SEQ ID NO:96:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequence of VK1-DQL primer 307-247"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:96:

ACCGCTACCG GTGTTCACTC CGACATCCAG CTGACCCAGA GCCCAAGCAG C 51

(2) INFORMATION FOR SEQ ID NO:97:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 56 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequecne of VK1-DQL primer 370-210"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:97:

CTGAGGATCC AGAAAGTGCA CTTACGTTTG ATTTCCACCT TGGTCCCTTG GCCGAA 56

(2) INFORMATION FOR SEQ ID NO:98:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequence of VK2-SVMDY primer 370-269"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:98:

CTCTCCACCG GTGTCCACTC CAGCATCGTG ATGACCCAGA GCCCAAGCAG C 51

(2) INFORMATION FOR SEQ ID NO:99:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "DNA sequence of VK3-DQMDY primer 370-268"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:99:

CTCTCCACCG GTGTCCACTC CGACATCCAG ATGACCCAGA GCCCAAGCAG C 51

WHAT IS CLAIMED IS:

1. A recombinant antibody molecule comprising antigen binding regions derived from the heavy or light chain variable regions of an anti-VLA4 antibody.

2. A humanized recombinant antibody molecule having specificity for VLA4 and having an antigen binding site wherein at least one of the complementarity determining regions (CDR) of the variable regions are derived from a non-human anti-VLA4 antibody.

3. A humanized recombinant heavy chain according to claim 2 comprising non-human CDRs at positions 31-35 (CDR1), 50-65 (CDR2) and 95-102 (CDR3) (Kabat numbering).

4. A humanized recombinant heavy chain according to claim 3 comprising non-human residues at framework positions 27-30 (Kabat numbering).

5. A humanized recombinant heavy chain according to claim 4 comprising additional non-human residues at framework position 75 (Kabat numbering).

6. A humanized recombinant heavy chain according to claim 5 comprising additional non-human residues at framework position(s) 77-79 or 66-67 and 69-71 or 84-85 or 38 and 40 or 24.

7. A humanized recombinant light chain according to claim 2 comprising non-human CDRs at positions 24-34 (CDR1), 50-56 (CDR2) and 89-97 (CDR3).

8. A humanized recombinant light chain according to claim 7 comprising non-human residues at framework positions 60 and 67.

9. A humanized recombinant antibody molecule comprising at least one antibody heavy chain according to claim 3 and at least one antibody light chain according to claim 7.

10. A humanized recombinant antibody molecule according to claim 7 wherein the non-human CDRs are derived from the HP1/2 murine monoclonal antibody.

11. DNA encoding an antibody heavy chain according to claim 3.

12. DNA encoding an antibody light chain according to claim 7.

13. DNA encoding an antibody molecule according to claim 10.

14. A vector comprising DNA according to claim 11.

15. A vector comprising DNA according to claim 12.

16. A vector comprising DNA according to claim 13.

17. An expression vector comprising DNA encoding an antibody heavy chain according to claim 3 in operative combination with DNA encoding an antibody light chain according to claim 7.

18. An expression vector comprising DNA encoding an antibody molecule according to claim 10.

19. Host cells transformed with a vector according to claim 14 and a vector according to claim 15.

20. Host cells transformed with a vector according to claim 16.

21. A process for the production of a humanized recombinant anti-VLA4 antibody comprising:

(a) producing an expression vector comprising an operon having a DNA sequence encoding an antibody heavy or light chain wherein at least one of the CDRs of the variable domain are derived from a non-human anti-VLA4 antibody and the remaining immunoglobulin-derived parts of the antibody chain are derived from a human immunoglobulin;

(b) producing an expression vector comprising an operon having a DNA sequence encoding a complementary antibody light or heavy chain wherein at least one of the CDRs of the variable domain are derived from a non-human anti-VLA4 antibody and the remaining immunoglobulin-derived parts of the antibody chain are derived from a human immunoglobulin;

(c) transfecting a host cell with each vector; and

(d) culturing the transfected cell line to produce the humanized recombinant anti-VLA4 antibody molecule.

22. A process according to claim 21 wherein the DNA sequence encoding the heavy chain and the light chain comprise the same vector.

23. A therapeutic composition comprising an antibody molecule, or a fragment thereof, according to claim 1 in combination with a pharmaceutically acceptable diluent, excipient or carrier.

24. A diagnostic composition comprising an antibody molecule, or a fragment thereof, according to claim 1 in a detectably labelled form.

25. A method of treatment comprising administering an effective therapeutic amount of an antibody according to claim 1 to a human or animal subject.

26. A method for treating inflammation resulting from a response of a specific defense system in a mammalian subject which comprises providing to a subject in need of such treatment an amount of an anti-inflammatory agent sufficient to suppress the inflammation, wherein the anti-inflammatory agent is an antibody according to claim 1.

27. A humanized recombinant anti-VLA4 antibody molecule having the characteristics of an antibody which comprises a humanized heavy chain comprising a variable heavy chain region selected from the group consisting of V_H - STAW (SEQ ID NO:39), V_H - KAITAS (SEQ ID NO:43), V_H - SSE (SEQ ID NO:47), V_H - KRS (SEQ ID NO:51), and V_H - AS (SEQ ID NO: 55), in combination with a humanized light chain comprising a light chain variable region selected from the group consisting of VK - DQL (SEQ ID NO: 31), VK2 - SVMDY (SEQ ID NO: 63), and VK3 - DQMDY (SEQ ID NO: 67).

28. DNA encoding the humanized heavy chain and the humanized light chain according to claim 27.

29. A vector comprising DNA according to claim 28.

30. An expression vector comprising DNA encoding an antibody molecule according to claim 27.

31. Host cells transformed with a vector according to claim 29.

32. Host cells transformed with a vector according to claim 30.

33. Host cells according to claim 32 that are ATCC CRL 11175.

34. A humanized recombinant anti-VLA4 antibody molecule having a potency from about 20% to about 100% of the potency of an antibody which comprises a humanized heavy chain comprising a variable heavy chain region of V_H - AS (SEQ ID NO: 55), in combination with a humanized light chain comprising a light chain variable region of VK2 - SVMDY (SEQ ID NO: 63).

35. A therapeutic composition comprising an antibody molecule, or a fragment thereof, according to claim 27 or 34 in combination with a pharmaceutically acceptable diluent, excipient or carrier.

36. A diagnostic composition comprising an antibody molecule, or a fragment thereof, according to claim 27 or 34 in a detectably labelled form.

37. A method of treatment comprising administering an effective amount of an antibody according to claim 27 or 34 to a human or animal subject.

38. A method for treating inflammation resulting from a response of a specific defense system in a mammalian subject which comprises providing to a subject in need of such treatment an amount of an anti-inflammatory agent sufficient to suppress the inflammation, wherein the anti-inflammatory agent is an antibody according to claim 27 or 34.

39. A humanized recombinant anti-VLA4 antibody molecule that is the antibody produced by ATCC CRL 11175 or an antibody having the characteristics of the antibody produced by ATCC CRL 11175.

40. A humanized recombinant anti-VLA4 antibody molecule that has a potency from about 20% to about 100% of the potency of the antibody produced by ATCC CRL 11175.

41. A humanized recombinant anti-VLA4 antibody molecule that has a potency from about 20% to about 100% of the potency of the antibody produced by the murine monoclonal antibody HP1/2.

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(54) Title: INTERCELLULAR ADHESION MEDIATORS

(57) Abstract

Novel selectin ligands have been identified and various uses for the ligands and antibodies reactive thereto are provided, including targeted delivery of liposome formulations.

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INTERCELLULAR ADHESION MEDIATORS

FIELD OF THE INVENTION

The present invention relates to compositions and methods for reducing or controlling inflammation and for treating inflammatory disease processes and other pathological conditions mediated by intercellular adhesion.

BACKGROUND OF THE INVENTION

Vascular endothelial cells and blood platelets play key roles in a number of biological responses by selectively binding certain cells, for instance phagocytic leukocytes, in the blood stream. For example, endothelial cells preferentially bind monocytes and granulocytes prior to their migration through the blood vessel wall and into surrounding tissue in an inflammatory response. Certain inflammation-triggering compounds are known to act directly on the vascular endothelium to promote the adhesion of leukocytes to vessel walls, which cells then move through the walls and into areas of injury or infection. Cellular adhesion to vascular endothelium is also thought to be involved in tumor metastasis. Circulating cancer cells apparently take advantage of the body's normal inflammatory mechanisms and bind to areas of blood vessel walls where the endothelium is activated.

Blood platelets are also involved in similar responses. Platelets are known to become activated during the initiation of hemostasis and undergo major morphological, biochemical, and functional changes (e.g., rapid granule exocytosis, or degranulation), in which the platelet alpha granule membrane becomes fused with the external plasma membrane. As a result, new cell surface proteins become expressed that confer on the activated platelet new functions, such as the ability to bind both other activated platelets and other cells. Activated platelets are recruited into growing thrombi or are cleared rapidly from the blood circulation.

Activated platelets are known to bind to phagocytic leukocytes, including monocytes and neutrophils. Examples of pathological and other biological processes which are thought to be mediated by this process include atherosclerosis, blood clotting and inflammation.

Recent work has revealed that specialized cell surface receptors on endothelial cells and platelets, designated endothelial leukocyte adhesion molecule-1 (ELAM-1) and granule membrane protein-140 (GMP-140), respectively, are involved in the recognition of various circulating cells by the endothelium and platelets. These receptors are surface glycoproteins with a lectin-like domain, a region with homology to epidermal growth factor, and a region with homology to complement regulatory proteins (see, Bevilacqua et al., Science 243:1160 (1989), which is incorporated herein by reference). For example, ELAM-1 has been shown to mediate endothelial leukocyte adhesion, which is the first step in many inflammatory responses. Specifically, ELAM-1 binds human neutrophils, monocytes, eosinophils, certain T-lymphocytes (N. Graber et al., J. Immunol., 145:819 (1990)), NK cells, and the promyelocytic cell line HL-60.

The term "selectin" has been suggested for a general class of receptors, which includes ELAM-1 and GMP-140, because of their lectin-like domain and the selective nature of their adhesive functions. These cell surface receptors are expressed on a variety of cells. GMP-140 (also known as PADGEM) is present on the surface of platelets and endothelial cells, where it mediates platelet-leukocyte and endothelium-leukocyte interactions. Another member of the selectin class is the MEL-14 antigen and its human analog LAM-1 which are cell surface receptors of lymphocytes, and act as lymph node homing receptors. The exact nature of the ligand recognized by selectin receptors, however, has remained largely unknown.

Various other methods have been previously developed to block the action of selectins and thus inhibit cellular adhesion. For instance, the use of monoclonal antibodies directed to ELAM-1 has been proposed as a method to inhibit endothelial-leukocyte adhesion as a treatment for pathological

responses, such as inflammation. Endothelial interleukin-8 has also been shown to be an inhibitor of leukocyte-endothelial interactions.

With the elucidation of the ligand-receptor interaction it will be possible to develop highly specific, efficient inhibitors of selectin-mediated cellular adhesion which would be useful in therapeutic regimens. The ligand(s) could also be used to target other pharmaceutical compounds, such as anti-inflammatory agents or anti-oxidants, to the sites of injury. To date, however, insufficient understanding of the interaction of the ligand(s) and receptor molecules on the respective cells has hindered these efforts. The present invention fulfills these and other related needs.

SUMMARY OF THE INVENTION

Novel compositions which selectively bind a selectin cell surface receptor and which have at least one oligosaccharide moiety are provided by the present invention. The compositions inhibit intercellular adhesion mediated by the selectin cell surface receptor and thereby are capable, for example, of inhibiting inflammatory and other pathological responses associated with cellular adhesion. Generally, the composition comprises sialic acid and fucose, a sulfate, or a phosphate. In related embodiments the composition that binds the selectin may be a glycoprotein, a glycolipid, or an oligosaccharide.

In one aspect, pharmaceutical compositions are provided. The pharmaceutical compositions can be, for example, liposomes which comprise a ligand oligosaccharide moiety capable of selectively binding a selectin receptor and a pharmaceutically acceptable carrier. The liposome containing the ligand may also serve as a targeting vehicle for a conventional chemotherapeutic agent, which agent is contained within the liposome and delivered to targeted cells which express a selectin receptor. Typically the chemotherapeutic agent is an anti-inflammatory agent or an anti-oxidant. Using the ligands described herein to target chemical agents encapsulated within liposomes is a convenient and effective

method for reducing therapeutic levels of a drug and minimizing side effects.

In other aspects, the invention comprises methods of inhibiting intercellular adhesion in a patient for a disease process such as inflammation or reperfusion injury by administering to the patient a therapeutically effective dose of a compound comprising a moiety capable of binding a selectin cell surface receptor. The cell surface receptor, such as ELAM-1 or GMP-140, may be expressed on vascular endothelial cells or platelets. The inflammatory process may be, for example, rheumatoid arthritis. The compound which is administered may have an oligosaccharide moiety having the chemical formula: NeuAcα2,3Galβ1,4(Fucα1,3)GlcNAcβ1-R_1; wherein R_1 is an amino acid, oligopeptide, lipid, or oligosaccharide.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates the ability of cells which express SLX (LEC 11) to bind to IL-1β activated endothelial cells compared to those cells which express non-sialylated Le^x (CHO-K1 and LEC 12).

Fig. 2 illustrates the ability of monoclonal antibodies specific for SLX to block selectin-mediated binding of HL-60 cells at 37°C (Fig. 2A) and 4°C (Fig. 2B) compared to monoclonal antibodies which do not bind SLX determinants.

Fig. 3 illustrates the effects of incubating LEC 11 (Fig. 3A) and LEC 12 (Fig. 3B) cells with SLX and non-SLX specific monoclonal antibodies on binding to activated endothelial cells.

Fig. 4 illustrates the results obtained by treating HL-60, LEC11 and LEC12 cells with sialidase before binding to activated endothelial cells.

Fig. 5 compares the ability of liposomes which contain glycolipids with SLX, Le^x, or similar carbohydrate structures to inhibit the binding of HL-60 cells to activated endothelial cells.

Fig. 6 compares the inhibition of GMP-140 mediated platelet adhesion by monoclonal antibodies specific for SLX and Le^x determinants.

Fig. 7 compares the ability of liposomes which contain glycolipids with SLX, Le^x, or similar carbohydrate structures to inhibit the binding of HL-60 cells to activated platelets.

Fig. 8 compares the ability of liposomes which contain glycolipids with SLX, Le^x, or similar carbohydrate structures to inhibit the binding of PMNs to activated platelets.

Fig. 9 shows inhibition of GMP-140 mediated adehsion by glycolipids with the terminal sialic acid either NeuAc or NeuGc.

DESCRIPTION OF THE PREFERRED EMBODIMENT

Compositions and methods are provided for inhibiting inflammatory and other disease responses mediated by cellular adhesion. The present invention also provides compounds (e.g., glycoconjugates and monoclonal antibodies) which have the ability to block or inhibit the adhesion of the cells mediated by selectin cell surface receptors. Methods for preparing and screening for such compounds are also provided. In addition, diagnostic and therapeutic uses for the compounds are provided.

A basis of the present invention is the discovery of a carbohydrate moiety recognized by selectin cell surface receptors. As discussed above, selectins, also known as the "LEC-CAM" family of cell adhesion molecules, are unique glycoproteins expressed on the surface of a variety of cells. For instance, ELAM-1 is inducibly expressed on vascular endothelial cells (Bevilacqua et al., supra and Hession et al., Proc. Nat'l. Acad. Sci., 87:1673-1677 (1990), both of which are incorporated herein by reference). This receptor has been demonstrated to be induced by inflammatory cytokines such as interleukin Iβ (IL-Iβ) and tumor necrosis factor α (TNFα), as well as bacterial endotoxin (lipopolysaccharide) (see, Bevilacqua et al., Proc. Natl. Acad. Sci., 84:9238-9242 (1987) which is incorporated herein by reference). These compounds

act directly on endothelial cells in vitro to substantially augment polymorphonuclear leukocyte (neutrophil), and monocyte adhesion (Bevilacqua et al., Proc. Natl. Acad. Sci., supra).

As discussed above, GMP-140 is a membrane glycoprotein of platelet and endothelial secretory granules (Geng et al., Nature, 343, 757-760 (1990) which is incorporated herein by reference). Activated platelets which express GMP-140 on their surface are known to bind to monocytes and neutrophils (Jungi et al., Blood 67:629-636 (1986)), and also to monocyte-like cell lines, e.g., HL60 and U937 (Jungi et al., supra; Silverstein et al., J. Clin. Invest. 79:867-874 (1987)), all of which are incorporated herein by reference. GMP-140 is an alpha granule membrane protein of molecular weight 140,000 that is expressed on the surface of activated platelets upon platelet stimulation and granule secretion (Hsu-Lin et al., J. Biol. Chem. 259:9121-9126 (1984); Stenberg et al., J. Cell Biol. 101:880-886 (1985); Berman et al., J. Clin. Invest. 78:130-137 (1986)). It is also found in megakaryocytes (Beckstead et al., Blood 67:285-293 (1986)), and in endothelial cells (McEver et al., Blood 70:355a (1987)) within the Weibel-Palade bodies (Bonfanti et al., Blood 73:1109-1112 (1989)). Furie et al. U.S. Patent No. 4,783,330, describe monoclonal antibodies reactive with GPM-140. All of the foregoing references are incorporated herein by reference.

A third selectin receptor is the lymphocyte homing receptor, MEL-14 antigen or LAM-1 (Gallatin et al., Nature 304:30-34 (1983); Siegellman et al., Science, 243:1165-1172 (1989); Rosen, Cell Biology, 1:913-919 (1989); and Lasky et al. Cell 56:1045-1055 (1989) all of which are incorporated herein by reference). In addition to lymphocyte homing, MEL-14 antigen/LAM-1 is believed to function early in neutrophil binding to the endothelium.

The structure and function of selectin receptors has been elucidated by cloning and expression of full length cDNA encoding each of the above receptors (see, e.g., Bevilacqua et al., Science, supra, (ELAM-1), Geng et al., supra, (GMP 140), and Lasky et al., supra, (MEL-14 antigen)). The extracellular portion of selectins can be divided into three segments based

on homologies to previously described proteins. The N-terminal region (about 120 amino acids) is related to the C-type mammalian lectin protein family as described by Drickamer, J. Biol. Chem., 263: 9557-9560 (1988) (which is incorporated herein by reference) that includes low affinity IgE receptor CD23. A polypeptide segment follows, which has a sequence that is related to proteins containing the epidermal growth factor (EGF) motif. Lastly, after the EGF domain are one or more tandem repetitive motifs of about 60 amino acids each, related to those found in a family of complement regulatory proteins.

Since selectin receptors comprise a lectin-like domain, the specificity of the molecules is likely to be based on protein-carbohydrate interactions. Evidence provided here indicates that a sialylated, fucosylated N-acetyllactosamine unit of the Lewis X antigen, designated here as SLX, is a moiety recognized by the lectin region of the selectin receptor. In particular, the evidence shows recognition of this moiety by both ELAM-1 and GMP-140. Compounds of the present invention comprise this fucosylated, sialylated N-acetyllactosamine unit in a variety of configurations.

The nomenclature used to describe the oligosaccharide moieties of the present invention follows the conventional nomenclature. Standard abbreviations for individual monosaccharides are used. For instance, 2-N-acetylglucosamine is represented by GlcNAc, fucose is Fuc, galactose is Gal, and glucose is Glc. Two sialic acids which may be present on the oligosaccharides of the present invention are 5-N-acetylneuraminic acid (NeuAc) and 5-N-glycolylneuraminic acid (NeuGc). Unless otherwise indicated, all sugars except fucose (L-isomer) are D-isomers in the cyclic pyranose configuration. The two anomers of the cyclic forms are represented by α and β.

The monosaccharides are generally linked by glycosidic bonds to form oligo- and polysaccharides. The orientation of the bond with respect to the plane of the rings is indicated by α and β. The particular carbon atoms that form the bond between the two monosaccharides are also noted. Thus, a β glycosidic bond between C-1 of galactose and C-4 of glucose is represented by Galβ1,4Glc. For the D-sugars (e.g., D-

GlcNAc, D-Gal, and D-NeuAc) the designation α means the hydroxyl attached to C-1 (C-2 in NeuAc) is below the plane of the ring and β is above the ring. In the case of L-fucose, the α designation means the hydroxyl is above the ring and β means it is below.

Having identified SLX as a carbohydrate ligand that mediates leukocyte-endothelial and leukocyte-platelet cell adhesion, compounds comprising SLX or its mimetics can be purified or synthesized de novo. Once obtained, such compounds can be used for a variety of purposes, including, for example, competitive inhibition of the binding of SLX-bearing cells to cells which express the selectin receptors. By binding of the compounds of the invention to a cell surface selectin, interaction of the selectin with the native SLX ligand on migrating cells will be prevented, interfering with normal and pathological binding of leukocytes and other cells to the endothelium or platelets. Thus, compounds which contain one or more SLX-R units or mimetics can serve as effective inhibitors of, for instance, inflammation, atherosclerosis, clotting and other endothelial or platelet-mediated pathologies.

Compounds containing SLX can be obtained from the cell surface glycoproteins or glycolipids from a number of cells. For instance, the SLX antigen is present on N-linked carbohydrate groups of the cell surface glycoproteins of LEC11 cells, a glycosylation mutant of CHO cells. LEC11 expresses this unique glycopeptide which contains a terminal structure bearing both sialic acid and fucose in the SLX sequence:

NeuAcα2,3Galβ1,4GlcNAcβ1-R
|α1,3
Fuc

(SLX-R)

where R is:

2Man Fuc
|α1,6 ±|α1,6
Manβ1,4GlcNAcβ1,4GlcNAcβ1,Asn
|α1,4
(±SLX)β1,2Man

(See, Stanley et al., J. Biol. Chem., 263:11374 (1988), which is incorporated herein by reference.) Using the procedure described below, it was demonstrated that the LEC11 mutant bound to activated human vascular endothelial cells. Neither wild type CHO cells nor other related glycosylation mutant CHO cell lines without the particular glycosylation pattern (SLX) showed the same level of binding.

In the SLX moiety expressed by LEC11 cells, the sialic acid is in the form of NeuAc. The sialic acid may be in other forms, such as NeuGc, without significantly affecting binding. For instance, SLX isolated from bovine erythrocytes comprises NeuGc. As demonstrated in Example IX, below, the affinity for selectin receptors is the same for both forms. Thus, the term "SLX" as used herein refers to the minmal tetrasaccharide unit shown above in which the terminal sialic acid is NeuAc, NeuGc or other equivalent forms of sialic acid. Structures illustrated herein which show the sialic acid residue as NeuAc are understood to include these other forms, in particular NeuGc.

Naturally occuring variations on the basic SLX moiety are also recognized by selectin receptors. For instance, evidence provided in Example VIII, below, shows that an oligosaccharide moiety, termed SY2 (also known as the VIM antigen), having the structure
NeuGcα2,3Galβ1,4GlcNAcβ1,3Galβ1,4(Fucα1,3)GlcNAcβ1,3Galβ1,4Glcβ

binds selectin receptors as well as SLX. The SY2 moiety comprises two sialylated N-acetyllactosamine units, one of which is SLX. Thus, oligosaccharides recognized by selectin receptors may comprise a number of the sialylated N-acetyllactosamine units, at least one of which is fucosylated (see, Teimeyer et al., Proc. Natl. Acad. Sci. (USA) 88:1138-1142 (1991), which is incorporated herein by reference.

Sources that can be used to obtain the SLX unit include any cell which naturally expresses the moiety on glycolipid or glycoprotein carbohydrate groups. Thus, polymorphonuclear neutrophils, lymphocytes, tumor cells or HL-60 cells have been used to purify this unit. Other cells which bind to activated vascular endothelium can also be used to isolate the ligand (see, Symington et al., J. Immunol. 134:2498-2506 (1985), Mizoguchi et al., J. Biol. Chem. 259:11949-11957 (1984), Mizoguchi et al., J. Biol. Chem. 259:11943-11948 (1984), Paietta et al., Cancer Res. 48:28-287 (1988), all of which are incorporated herein by reference).

Compounds containing SLX or its mimetics can be prepared from natural sources using methods well known in the art for isolating surface glycoproteins, glycopeptides, oligosaccharides and glycolipids from cells (See, e.g., Gerard, "Purification of glycoproteins" and Thomas et al., "Purification of membrane proteins," both in Guide to Protein Purification, Vol. 182, Methods in Enzymology (Deutscher ed., 1990), which is incorporated herein by reference). For example, LEC11 cells can be used to obtain glycoprotein or glycolipid which contains the SLX unit using, for instance, the method described in Stanley et al., supra. Briefly, in one method LEC11 cells are infected with vesicular stomatitis virus. The structural carbohydrate alterations exhibited by LEC11 are then expressed on the N-linked biantennary carbohydrates of the G glycoprotein of the virus. The virus is purified by equilibrium gradient centrifugation, and glycopeptides are purified using proteinase digestion as described by Stanley et al.

Several approaches are used to isolate a selectin-binding moiety from HL-60, HT-29, colo 205, neutrophils, and

other cell lines which contain a ligand recognized by selectins. Since the ligand is generally expressed on the cell surface of these cell types, one approach consists of isolating a plasma membrane fraction enriched in the ligand. Once plasma membranes have been isolated, the ligands may be isolated and subsequently identified using monoclonal antibodies, particularly those which are reactive with the SLX oligosaccharide structure, such as monoclonal antibodies FH6, SNH3 and CSLEX-1.

To characterize a selectin ligand, release of the oligosaccharide from the glycopeptide is generally the first step in the structural analysis of the oligosaccharide chain. This is accomplished by chemical cleavage of the protein-carbohydrate linkage, or by specifically releasing the oligosaccharide with endoglycosidases. In most cases, different procedures may be used to establish the correct conditions for an individual glycoprotein. Asparagine-linked oligosaccharides are released by hydrazinolysis, endoglycosidases, vigorous alkaline hydrolysis, and trifluoroacetolysis. O-linked carbohydrate units are released by alkaline β-elimination. The oligosaccharides are separated from the glycopeptides by gel filtration. The resulting oligosaccharides are then separated from each other using a combination of gel filtration, HPLC, thin layer chromatography, and ion exchange chromatography. The isolated oligosaccharides are then fully analyzed. Complete structural analysis of the purified oligosaccharide units requires the determination of the monosaccharide units, their ring form, configuration (D or L), anomeric linkage (α or β), the positions of the linkages between the sugars and their sequence. In addition, the position of any substituent groups are established. Methylation analysis is used to determine the positions of the glycosidic linkages between the monosaccharides. The anomeric configuration of the sugar residues can be addressed using 500-MHz ^{1}H NMR spectroscopy. The conditions and methods used to perform a complete structural carbohydrate analysis are described generally in Beeley, Laboratory Techniques in Biochemistry and Molecular Biology, eds. Burdon and

Knippenberg, Elsevier, Amsterdam (1985), incorporated herein by reference.

The state of the art techniques to fully characterize the sugars of an oligosaccharide include the use of several analytical techniques such as FAB-MS (fast atom bombardment-mass spectrometry), HPAE (high pH anion exchange chromatography) and ^{1}H-NMR. These techniques are complementary. Recent examples of how these techniques are used to fully characterize the structure of an oligosaccharide can be found in the analysis by Spellman et al., J. Biol. Chem. 264:14100 (1989), and Stanley et al., supra. Other methods include positive ion fast atom bombardment mass spectroscopy (FAB-MS) and methylation analysis by gas chromatography - electron impact mass spectroscopy (GC/EI-MS) (see, EPO Application No. 89305153.2, which is incorporated herein by reference).

One approach to characterizing the selectin ligand on glycolipids consists of disrupting the cells using organic solvents, isolating the glycolipids, and identifying those glycolipids reactive with monoclonal antibodies to SLX, such as FH6, SNH3, SNH4, CSLEX-1, or VIM-2, for example, and then determining the structure of the oligosaccharide chains. To obtain glycolipids and gangliosides which contain SLX, standard methods for glycolipid preparation can be used (see, e.g., Ledeen et al., J. Neurochem. 21:829 (1973), which is incorporated herein by reference). For example, glycolipids are extracted from HL-60, HT-29, PMNs, human leukocytes, and other cell lines expressing the selectin ligand by methods generally known to those skilled in the arts (see, e.g., Symington et al., J. Immunol. 134:2498 (1985) and Macher and Beckstead, Leukemia Res. 14:119-130 (1990)). Cells are grown in suspension and are harvested by centrifugation. Glycolipids are extracted from the cell pellet by chloroform/methanol 2:1 and isopropyl alcohol/hexane/water 55:25:20 as described by Kannagi et al., J. Biol. Chem. 257:14865 (1982). The resulting extracts are partitioned by a chloroform/methanol/water (3:2:1) Folch partition. The resulting upper phase of the extraction contains gangliosides and the lower phase contains glycolipids.

The upper phase containing gangliosides (glycosphingolipids that contain at least one sialic acid moiety) are isolated and separated into neutral and acidic fractions using DEAE-Sephadex chromatography as described in detail by Ledeen and Yu, Methods Enzymol. 83:139 (1982). The resulting gangliosides are pooled, lyophilized, and dissolved in chloroform/methanol (2:1). The lower phase of the Folch partition contains glycolipids. These are isolated and separated on preparative thin-layer chromatography using chloroform/methanol/water (60:35:8) as the solvent system as described by Symington.

To identify those gangliosides and glycolipids which contain the selectin ligand, immunochemical glycolipid analysis is performed according to the procedure of Magnani et al., Anal. Biochem. 109:399 (1980). Briefly, the ganglioside pool described above is chromatographed by thin layer chromatography. The thin layer plate is then incubated with ^{125}I labeled FH6, or other monoclonal antibody which binds specifically to SLX. Following incubation with the labeled antibody, the plate is exposed to radiographic detection film and developed. Black spots on the X-ray film correspond to gangliosides that bind to the monoclonal antibody, and those gangliosides are recovered by scraping the corresponding areas of the silica plate and eluting the gangliosides with chloroform/methanol/water. Glycolipids are also dried and resuspended in chloroform and developed in a similar thin layer system and probed with the radiolabeled antibody. Structural analysis of oligosaccharides derived from glycolipids is performed essentially as described for glycoproteins.

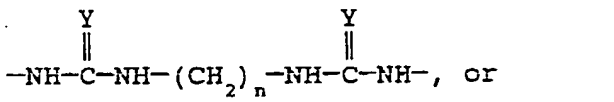
Oligosaccharides comprising the SLX unit can be prepared from glycoproteins by methods well known in the art (see, e.g., Gerard, supra, at pp. 537-539). Typically, N-glycosidase F (N-glycanase) is used to cleave N-linked oligosaccharides while O-linked groups are cleaved with endo-N-acetylgalactosaminidase.

Synthetic compounds containing SLX or its mimetics attached to a variety of moieties can be prepared depending on the particular use desired. For example, SLX can be converted

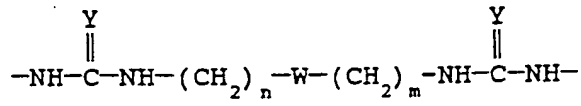
to a ganglioside by linking a ceramide moiety to the C-1 of the reducing terminal GlcNAc unit. SLX structures can also be linked to a wide variety of other moieties such as variously substituted amino groups, heterocyclic compounds, ether linkages with branched or unbranched carbon chains, and ether linkages with aryl or alkylaryl moieties. The SLX unit may also be bound to various amino acids, amino acid mimetics, oligopeptides or proteins.

The term "alkyl" as used herein means a branched or unbranched saturated or unsaturated hydrocarbon chain, including lower alkyls of 1-7 carbons such as methyl, ethyl, n-propyl, butyl, n-hexyl, and the like, cycloalkyls (3-7 carbons), cycloalkylmethyls (4-8 carbons), and arylalkyls. The term "aryl" refers to a radical derived from an aromatic hydrocarbon by the removal of one atom, e.g., phenyl from benzene. The aromatic hydrocarbon may have more than one unsaturated carbon ring, e.g., naphthyl. "Heterocyclic compounds" refers to ring compounds having three or more atoms in which at least one of the atoms is other than carbon (e.g., N, O, S, Se, P, or As). Examples of such compounds include furans, pyrimidines, purines, pyrazines and the like.

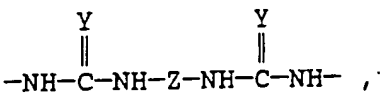
For the synthesis of polyvalent forms of SLX, monomeric units containing SLX can be joined to form molecules having one to about four or more SLX moieties. An example of such a polyvalent form is one in which the oligosaccharide units are linked by the following moieties:



, or



wherein, n and m are the same or different and are integers from 2 to 12; Y is O or S; and W is O, S, or NH; or



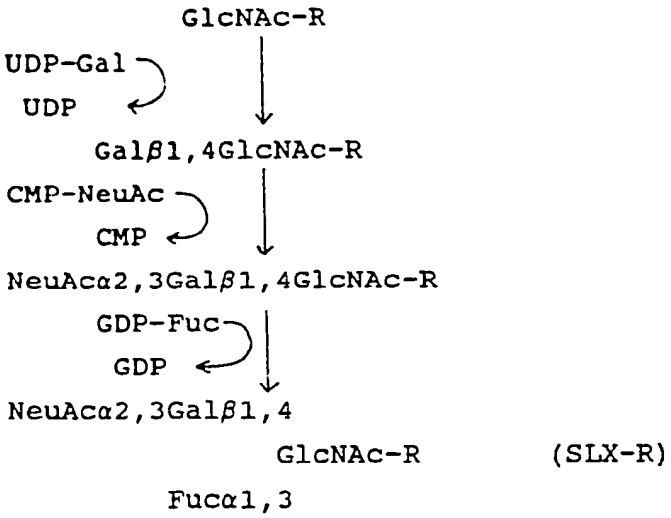
wherein, Z is a 5- to 14-membered ring and the substituents on the ring are in a cis- or trans-relationship, and the substituents are in a 1,2 to 1,(p/2)+1 arrangement, where p is the size of the ring. If the ring is a heterocyclic ring (e.g., one comprising nitrogen atoms) the oligosaccharide moieties are preferably linked to the nitrogen atoms on the ring. Examples of heterocyclic compounds that are suitable for this purpose include piperazine and homopiperazine.

Alternatively, polyvalent forms of SLX or its mimetics can be created by attaching the desired moiety to preformed carrier moieties with multiple sites of attachment. Examples include attachment of SLX to the amino groups of lysine and lysine-containing peptides, proteins, glycoproteins or the asparagine side-chain of such compounds.

One method of preparing polyvalent forms of SLX is by addition of desired monosaccharide residues to polysaccharides. For instance, the conversion of a polysaccharide which contains the linear core structure of SLX into a polyvalent SLX containing polysaccharide is achieved by enzymatic fucosylation. Native polysaccharide type Ia obtained from Group B Streptococcus is preferably used. The entire 200,000 dalton polysaccharide can be used for this purpose as well as fragemnts thereof. Thus, polysaccharides having a molecular weight between about 5,000 and about 300,000 can be used. A molecular weight between about 25,000 and about 100,000 is preferred. Any number of side chains on the polysaccharide type 1a may be fucosylated for the polysaccharide to have activity. Typically, between about 5 and about 200 side chains are fucosylated, preferably between about 50 and about 150 are fucosylated.

The synthesis of the SLX moiety can be accomplished using chemical, enzymatic, or combined chemical and enzymatic strategies. (see, e.g., EPO Publication No. 319,253, which is incorporated herein by reference.) In a preferred method (Scheme I below), a compound containing one or more N-acetylglucosamine units (GlcNAc-R) can be reacted sequentially

with a galactosyltransferase (N-acetylglucosamine β1,4 galactosyltransferase (E.C. 2.4.1.90)), a sialytransferase (Galβ1,4GlcNAc α2,3 sialyltransferase (E.C. 2.4.99.6) or Galβ1,3GalNAc α2,3 sialytransferase (E.C. 2.4.99.4) and a fucosyltransferase (N-acetylglucosaminide α1,3 fucosyltransferase (E.C. 2.4.1.152)) to yield the final SLX-containing structures. In this case, R may be a carrier moiety or activatable intermediate that will allow attachment to a suitable carrier moiety. Each enzymatic reaction uses the appropriate nucleotide sugar as a donor substrate to generate the following intermediates in the synthesis of SLX. The glycosyl transfer reactions may optimally be carried out with added alkaline phosphatase (e.g., from calf intestine, CIAP) to consume the nucleoside phosphate byproduct which may inhibit the reaction.



Scheme I

The general conditions for preparative enzymatic synthesis of carbohydrate groups analogous to SLX are known (see, e.g., Toone et al., Tetrahedron 45:5365-5422 (1989); Wong et al., J. Am. Chem. Soc. 47:5416-5418 (1982); Unverzagt et al., J. Am. Chem. Soc. 112:9308-9309 (1990); Prieels et al., J. Biol. Chem. 256:10456-10463 (1981), all of which are incorporated herein by reference). Each of the key enzymatic reactions has been demonstrated (Beyer et al., Adv. Enzymol.

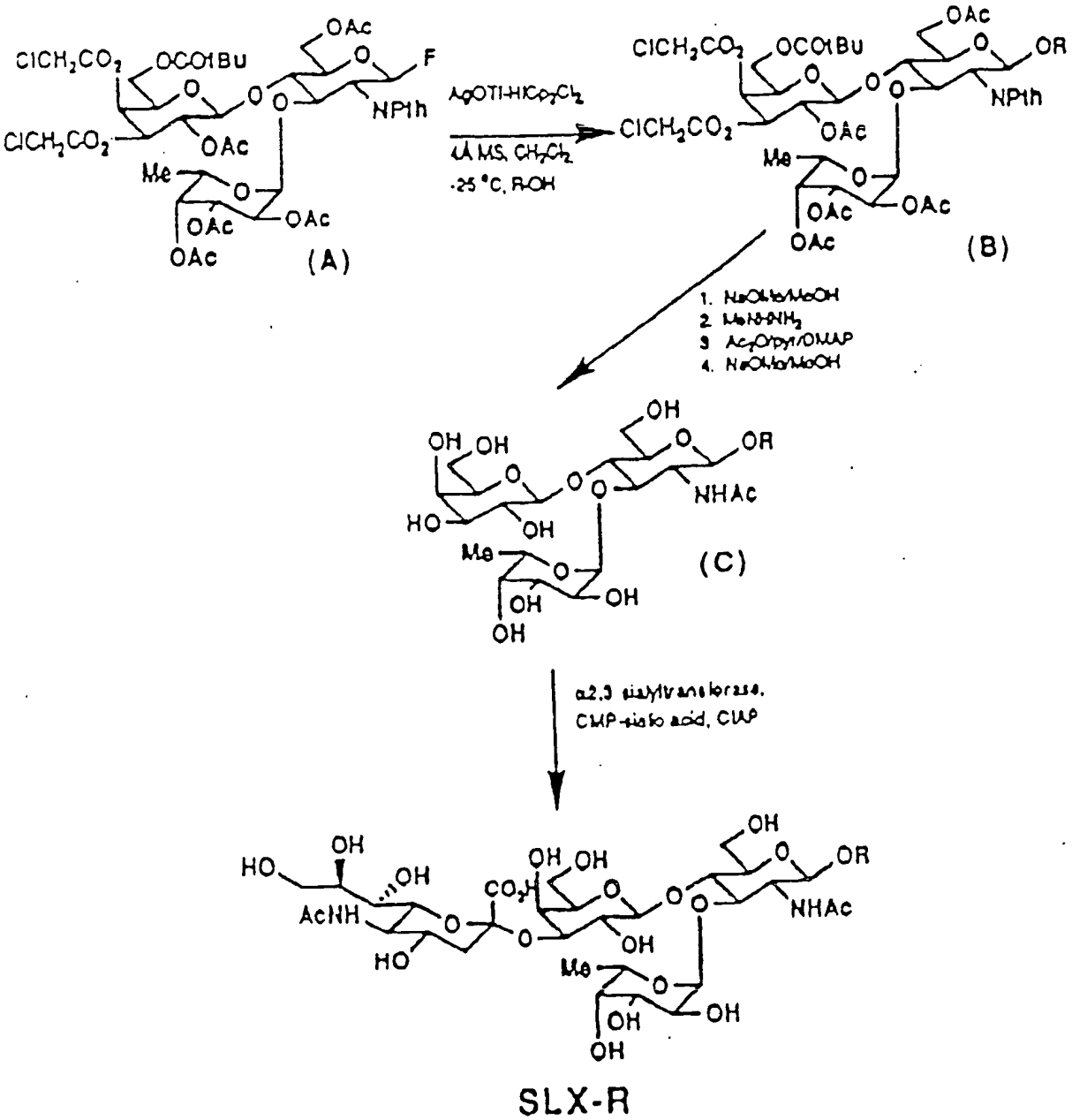
52:23-176 (1981); Toone et al., supra; and Howard et al., J. Biol. Chem. 262:16830-16837 (1981); all of which are incorporated herein by reference). For preparative reactions, the galactosyltransferase and the sialyltransferase(s) are purified from natural sources (Beyer et al., supra, and Weinstein et al., J. Biol. Chem. 257:13835-13844 (1982), which are incorporated herein by reference). Fucosyltransferases may also be identified from natural sources, as generally described in Crawley and Hindsgaul, Carbohyd. Res. 193:249-256 (1989), incorporated by reference herein. The cDNAs of the galactosyltransferase and a sialyltransferase have been cloned (Paulson and Colley, J. Biol.Chem. 264:17615-17618 (1989), which is incorporated herein by reference), allowing the production of soluble recombinant enzymes for large-scale preparative synthesis (Colley et al., J. Biol. Chem. 264:17619-17622 (1989)).

To obtain sufficient amounts of fucosyltransferase for large-scale reaction, the enzyme can be cloned and expressed as a recombinant soluble enzyme by someone with ordinary skill in the art. As a preferred method RNA can be subtracted from the wild type CHO cells and LEC11 cells as described by Chirgwin et al., Biochemistry 18:5214-5299 (1979), and the poly A+ RNA isolated by chromatography on oligo(dT)-cellulose. Next, cDNA from the LEC-11 cells can be prepared as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed. (1989), Cold Spring Harbor Press, New York, which is incorporated herein by reference. The cDNA can be subtracted using the method of Davis (Handbook of Experimental Immunology, Vol. 2, pp. 1-13 (1986)) using excess poly A+ RNA from wild type CHO cells, which do not express the desired fucosyltransferase, but otherwise have most of the mRNA species of LEC11 cells. A cDNA library can then be constructed in the CDM8 expression vector using the subtracted cDNA (Seed, Nature 329:840-842 (1987)). Clones expressing the fucosyltransferase can be isolated using the expression cloning method described by Larsen et al., Proc. Natl. Acad. Sci. 86:8227-8231 (1989), employing transfection of COS-1 cells and screening for cells expressing the SLX antigen with the FH6

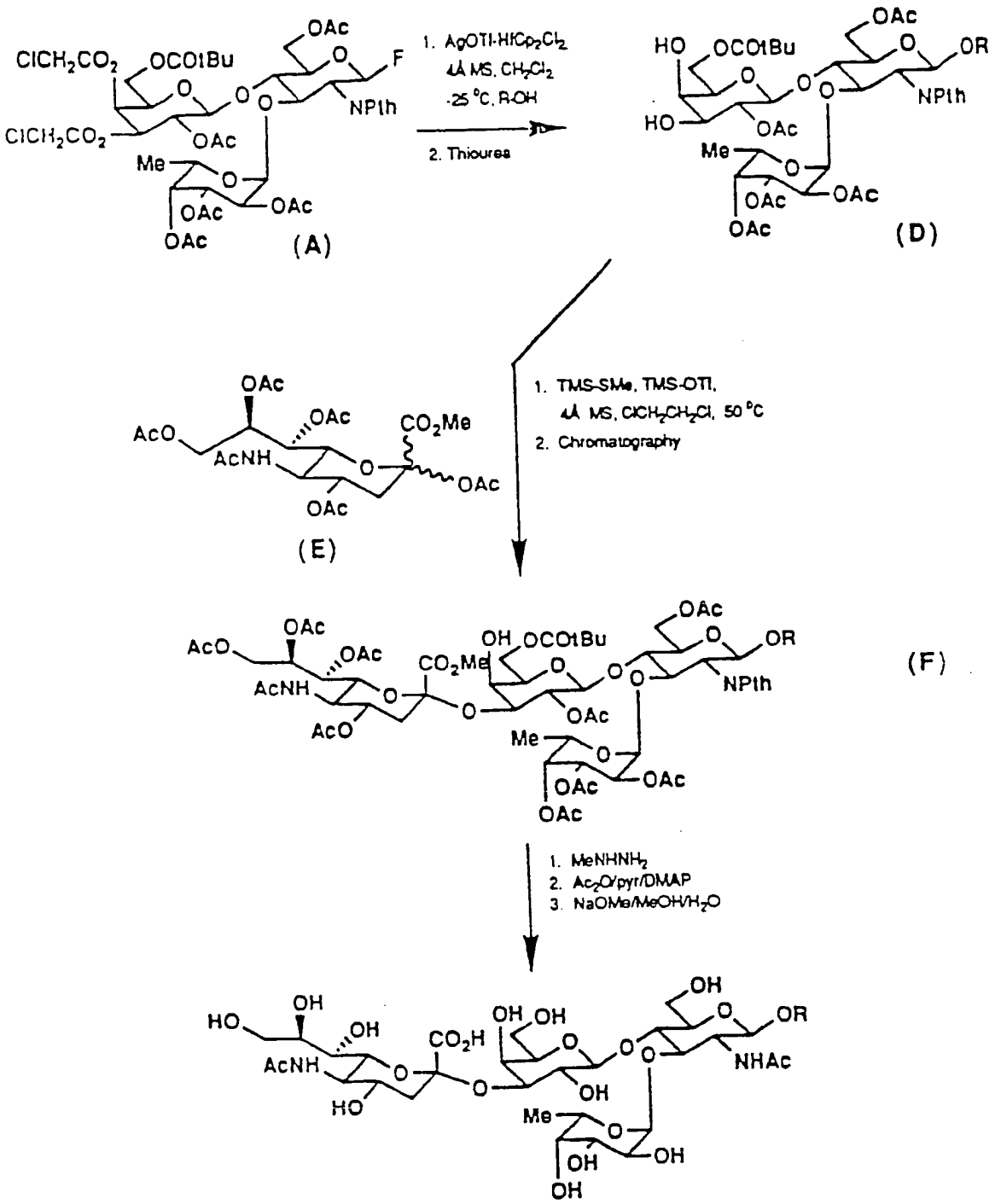
antibody or other antibody with specificity for the SLX antigen. The full-length clone of the fucosyltransferase can then be used to produce a soluble recombinant enzyme as taught by Colley et al., supra.

Another source of SLX is α_1-acid glycoprotein, which is a plasma glycoprotein, the carbhydrate moities of which can be fucosylated to produce SLX (see, Alpha,-Acid glycoprotein: Genetics, Biochemistry, Physiological Functions, and Pharmacology, Bauman et al. ed. (Wiley 1989), and Walz, et al. Science 250:1132-1135 (1990), both of which are incorporated herein by reference).

Although enzymatic or combined chemical and enzymatic synthesis of SLX compounds are preferred, chemical synthesis is also possible, as shown in Schemes II and IIa below.



Scheme II


1. AgOTf-HfCp2Cl2
4Å MS, CH2Cl2
-25 °C, R-OH
2. Thiourea
(A)
(D)
1. TMS-SMe, TMS-OTf,
4Å MS, ClCH2CH2Cl, 50 °C
2. Chromatography
(E)
(F)
1. MeNHNH2
2. Ac2O/pyr/DMAP
3. NaOMe/MeOH/H2O
SLX-R

Scheme IIa

Component pieces of the SLX structure have been synthesized. Nicolaou, et al., (J. Amer. Chem. Soc. 112:3693 (1990)) have published the total synthesis of the tumor-associated Le^x family of glycosphingolipids. Therein is described the synthesis of the protected trisaccharide Galβ1,4(Fucα1,3)GlcNAc (A). Reaction of this intermediate with an appropriate glycosyl acceptor (e.g., an alcohol moiety) results in compound (B). Selective deprotection and acetylation of the glucosamine moiety are carried out essentially as described in Nicolaou, et al. to afford compound (C). Reaction of (C) with a sialyltransferase as described above furnishes the desired product SLX-R, although this may be produced in relatively low yield using Scheme II.

Modified fucosides may be included in the synthetic schemes to provide for SLX analogues which vary in this moiety. For example, α-D-arabinosyl glycosides may be synthesized following known procedures, Nicolaou et al., J.Amer.Chem.Soc. 112:3693-3695 (1990) through the use of tri-O-benzyl arabinosyl halides. Other C-5 aryl or alkyl substituted arabinosyl moieties may be synthesized, Danishefsky et al., J.Amer.Chem.Soc. 107:1274 (1985), Danishefsky, Aldrichimica Acta. 19:59-68 (1986) and references therein, and introduced into the disaccharide in the same manner. All of these references are incorporated herein by reference.

According to alternative Scheme IIa, the trisaccharide (A) is partially deprotected to furnish (D), which is subsequently reacted with the peracetylated sialic acid methyl ester (E) following a procedure described by Kameyama et al., XV Intl. Carbohyd. Symp., Abst. No. A096, (1990), and Carbihydrate Res., 209:c1-c4 (1991) (which are incorporated herein by reference), yielding (F) after chromatographic purification. Treatment of (F) sequentially with methylhydrazine, N-acetylation, O-deacetylation and ester hydrolysis furnishes SLX-R.

Preferred examples of R for scheme II and IIa include alkyl (straight chain, branched, saturated, mono- and poly-unsaturated); serine (D or L); serine containing peptides; di- and tri-alkanolamines (e.g. $[HO(CH_2)_n]_2NH$, $[HO(CH_2)_n]_3N$; wherein

n = C_2-C_{20} as straight chain, branched, unsaturated, mono- and poly-unsaturated). R can also be aryl, substituted aryl (e.g., Me, OH, I; alone or in combination including ^{125}I), alkylaryl, arylalkyl or other moiety, as the skilled artisan would include for the desired use. The introduction of iodine into phenolic compounds such as tyrosine is known in the art. Radical groups containing phenols are useful for the introduction of ^{125}I radioisotope, yielding compounds which are useful in diagnosis.

The SLX ligand as disclosed here may also be used to assay for the presence of compounds which are capable of inhibiting intercellular adhesion mediated by selectins. A number of methods can be used to assay the biological activity of test compounds for the ability to inhibit the selectin-mediated response. Ideally, the assays of the present invention allow large scale in vitro or in vivo screening of a variety of compounds.

The agent or test compound to be screened will typically be a synthetic or naturally-produced biomolecule, such as a peptide, polypeptide, protein (e.g., monoclonal antibody), carbohydrate (e.g., oligosaccharide), glycoconjugate, nucleic acid, and the like. The compounds are synthetically produced using, for instance, the methods for synthesizing oligosaccharides described above (see, also, Khadem, Carbohydrate Chemistry (Academic Press, San Diego,CA, 1988), which is incorporated herein by reference). Methods for synthesizing polypeptides of defined composition are well known in the art (see, Atherton et al. Solid Phase Peptide Synthesis (IRL Press, Oxford, 1989) which is incorporated herein by reference). If the synthetic test compounds are polymeric (e.g., polypeptides or polysaccharides) they are preferably altered in a systematic way to identify the sequence of monomers which have the desired effect (see, e.g., U.S. Patent No. 4,833,092, which is incorporated herein by reference). Test compounds may also be isolated from any natural source, such as animal, plant, fungal, or bacterial cells in accordance with standard procedures as described above. Potentially useful monoclonal antibodies can be prepared according to standard methods described in more detail, below.

The assays of the present invention are particularly useful in identifying compounds which act as antagonists or agonists of a ligand molecule. Antagonists are compounds which reverse the physiological effect of a ligand or exclude binding of the ligand to the receptor. An antagonist usually competes directly or indirectly with the ligand for the receptor binding site and, thus, reduces the proportion of ligand molecules bound to the receptor. Typically, an antagonist will be the topographical equivalent of the natural ligand and will compete directly with the ligand for the binding site on the selectin. Such a compound is referred to here as a "mimetic." An SLX mimetic is a molecule that conformationally and functionally serves as substitute for an SLX moiety in that it is recognized by a selectin receptor. Alternatively, if the ligand and the test compound can bind the receptor simultaneously, the compound may act non-competitively. A non-competitive inhibitor acts by decreasing or inhibiting the subsequent physiological effects of receptor-ligand interactions rather than by diminishing the proportion of ligand molecules bound to the receptor. Finally, the assays of the present invention can be used to identify synthetic or naturally occurring agonists, that is, compounds which bind the receptor and initiate a physiological response similar to that of the natural ligand.

Numerous direct and indirect methods for in vitro screening of inhibitors of ligand-receptor interactions are available and known to those skilled in the art. For instance, the ability to inhibit adhesion of SLX-bearing cells to cells expressing a particular selectin can be determined. As discussed above, selectin receptor genes have been cloned, thus the genes can be inserted and expressed in a wide variety of cells, such as COS cells, CHO cells and the like. In addition, cells which do not normally express SLX are capable of being transformed with one or more glycosyltransferase genes which confer on the transformed cells the ability to synthesize the ligand. (see, e.g., Lowe et al., Cell 63:475-484 (1990), which is incorporated herein by reference.) Typically, the test compound or agent is incubated with labelled SLX-bearing cells and activated endothelial cells immobilized on a solid surface.

Inhibition of cellular adhesion is then determined by detecting label bound to the surface after appropriate washes. In an exemplified assay described below, promyelocytic HL-60 cells and activated human endothelial cells or activated platelets are used.

Since a ligand specific for selectin receptors has now been identified, isolated ligand molecules can also be used in the assays. The terms "isolated selectin-binding agent" or "isolated SLX moiety" as used herein refer to a selectin binding or SLX-bearing compound that is in other than its native state, e.g., not associated with the cell membrane of a cell that normally expresses the ligand. Thus, an isolated SLX moiety may be a component of an isolated molecule, such as an oligosaccharide or a glycoconjugate. The isolated molecule may be synthesized or prepared from the membranes of SLX-bearing cells. Alternatively, the isolated selectin-binding agent or SLX moiety may be associated with a liposome or attached to a solid surface before use in the assay. Methods for preparing SLX-bearing liposomes and for immobilizing various biomolecules are extensively discussed below.

Typically, the in vitro assays of the present invention are competition assays which detect the ability of a test compound to competitively inhibit binding of a compound known to bind either the receptor or the ligand. Inhibition of binding between SLX and a selectin receptor is usually tested. Inhibition of other binding interactions are also suitable, for instance, inhibition of the binding between a monoclonal antibody (e.g., FH6) and SLX or between an SLX mimetic and a selectin inhibitor can be used. Numerous types of competitive assays are known (see, e.g., U.S. Patents No. 3,376,110, 4,016,043, and Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Publications, N.Y. (1988), which are incorporated herein by reference).

The assays of the present invention are also suitable for measuring binding of a test compound to one component alone rather than using a competition assay. For instance, immunoglobulins can be used to identify compounds that contain the SLX moiety. Standard procedures for monoclonal antibody

assays, such as ELISA, may be used (see, Harlow and Lane, supra). When assaying for glycolipids comprising the SLX antigen, the reactivity of the monoclonal antibody with the antigen can be assayed by TLC immunostaining by the method originally described in Magnani et al., Anal. Biochem. 109:399-402 (1980) or by solid-phase radioimmunoassay as described by Kanagi et al., Cancer Res. 43:4997-5005 (1983); which are incorporated herein by reference. Glycoproteins can be assayed by standard immunoblotting procedures as described in Harlow and Lane, supra. Sandwich assay formats are also suitable (see, e.g., U.S. Patent Nos. 4,642,285; 4,299,916; and 4,391,904; and Harlow and Lane, supra all of which are incorporated herein by reference). Typically, compounds which have been identified in a binding assay will be further tested to determine their ability to inhibit receptor-ligand interactions.

Other assay formats involve the detection of the presence or absence of various physiological changes in either ligand-bearing or selectin-bearing cells that result from the interaction. Examples of suitable assays include the measurement of changes in transcription activity induced by binding (see, e.g., EPO Publication No. 3712820), the detection of various cell mediated extra-cellular effects (see, e.g., PCT Publication No. 90/00503), and the detection of changes in the membrane potential of individual cells (see, e.g., U.S. Patent No. 4,343,782), all of which are incorporated herein by reference. Alternatively, conformational changes in isolated receptors or ligands can be detected; see, e.g., U.S. Patent No. 4,859,609, which is incorporated herein by reference.

Any component of the assay, including the ligand, the receptor, or the test compound, may be bound to a solid surface. Many methods for immobilizing biomolecules on solid surfaces are known in the art. For instance, the solid surface may be a membrane (e.g., nitrocellulose), a microtiter dish (e.g., PVC or polystyrene) or a bead. The desired component may be covalently bound or noncovalently attached through unspecific bonding.

A wide variety of organic and inorganic polymers, both natural and synthetic may be employed as the material for the solid surface. Illustrative polymers include polyethylene, polypropylene, poly(4-methylbutene), polystyrene, polymethacrylate, poly(ethylene terephthalate), rayon, nylon, poly(vinyl butyrate), silicones, polyformaldehyde, cellulose, cellulose acetate, nitrocellulose, etc. Other materials which may be employed, include paper, glasses, ceramics, metals, metalloids, semiconductive materials, cermets or the like. In addition are included substances that form gels, such as proteins, e.g., gelatins, lipopolysaccharides, silicates, agarose and polyacrylamides or polymers which form several aqueous phases, such as dextrans, polyalkylene glycols (alkylene of 2 to 3 carbon atoms) or surfactants e.g. amphiphilic compounds, such as phospholipids, long chain (12-24 carbon atoms) alkyl ammonium salts and the like. Where the solid surface is porous, various pore sizes may be employed depending upon the nature of the system.

In preparing the surface, a plurality of different materials may be employed, particularly as laminates, to obtain various properties. For example, protein coatings, such as gelatin can be employed to avoid non-specific binding, simplify covalent conjugation, enhance signal detection or the like.

If covalent bonding between a compound and the surface is desired, the surface will usually be polyfunctional or be capable of being polyfunctionalized. Functional groups which may be present on the surface and used for linking can include carboxylic acids, aldehydes, amino groups, cyano groups, ethylenic groups, hydroxyl groups, mercapto groups and the like. The manner of linking a wide variety of compounds to various surfaces is well known and is amply illustrated in the literature. See for example Immobilized Enzymes, Ichiro Chibata, Halsted Press, New York, 1978, and Cuatrecasas, J. Biol. Chem. 245 3059 (1970) which is incorporated herein by reference.

In addition to covalent bonding, various methods for noncovalently binding an assay component can be used. Noncovalent binding is typically nonspecific absorption of a

compound to the surface. Typically, the surface is blocked with a second compound to prevent nonspecific binding of labelled assay components. Alternatively, the surface is designed such that it nonspecifically binds one component but does not significantly bind another. For example, a surface bearing a lectin such as Concanavalin A will bind a carbohydrate containing compound but not a labelled protein that lacks glycosylation. Various solid surfaces for use in noncovalent attachment of assay components are reviewed in U.S. Patent Nos. 4,447,576 and 4,254,082, which are incorporated herein by reference.

Many assay formats employ labelled assay components such as SLX ligands, SLX mimetics, immunoglobulins, receptors, or test compounds. The label may be coupled directly or indirectly to the desired component of the assay according to methods well known in the art. A wide variety of labels may be used. The component may be labelled by any one of several methods. The most common method of detection is the use of autoradiography with ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P labelled compounds or the like. The choice of radioactive isotope depends on research preferences due to ease of synthesis, varying stability, and half lives of the selected isotopes. Other non-radioactive labels include ligands which bind to labelled antibodies, fluorophores, chemiluminescent agents, enzymes, and antibodies which can serve as specific binding pair members for a labelled ligand. The choice of label depends on sensitivity required, ease of conjugation with the compound, stability requirements, and available instrumentation.

Non-radioactive labels are often attached by indirect means. Generally, a ligand molecule (e.g., biotin) is covalently bound to the molecule. The ligand then binds to an anti-ligand (e.g., streptavidin) molecule which is either inherently detectable or covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound. Ligands and anti-ligands may be varied widely. Where a ligand has a natural anti-ligand, for example, biotin, thyroxine, and cortisol, it can be used in conjunction with the labelled, naturally occurring anti-

ligands. Alternatively, any haptenic or antigenic compound can be used in combination with an antibody.

The molecules can also be conjugated directly to signal generating compounds, e.g., by conjugation with an enzyme or fluorophore. Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases, esterases and glycosidases, or oxidoreductases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, etc. Chemiluminescent compounds include luciferin, and 2,3-dihydrophthalazinediones, e.g., luminol. For a review of various signal producing systems which may be used, see, U.S. Patent No. 4,391,904, which is incorporated herein by reference.

As discussed above, in addition to various inhibitor compounds which comprise an accessible SLX unit or SLX mimetic, the present invention also provides monoclonal antibodies capable of inhibiting intercellular adhesion mediated by selectins as well as methods for identifying such antibodies. The monoclonal antibodies bind a selectin ligand or receptor and block cellular adhesion. Thus, the multitude of techniques available to those skilled in the art for production and manipulation of various immunoglobulin molecules can be applied to inhibit intercellular adhesion.

As used herein, the term "immunoglobulin" refers to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. The immunoglobulins may exist in a variety of forms besides antibodies, including for example, Fv, Fab, and $F(ab)_2$, as well as in single chains (e.g., Huston et al., Proc. Nat. Acad. Sci. U.S.A. 85:5879-5883 (1988) and Bird et al., Science 242:423-426 (1988), which are incorporated herein by reference). (see, generally, Hood et al., Immunology, 2nd ed., Benjamin, N.Y. (1984), and Hunkapiller and Hood, Nature 323:15-16 (1986), which are incorporated herein by reference.)

Antibodies which bind the SLX antigen may be produced by a variety of means. The production of non-human monoclonal antibodies, e.g., murine, lagomorpha, equine, etc., is well known and may be accomplished by, for example, immunizing the animal with the SLX antigen or a preparation containing a glycoprotein or glycolipid comprising the antigen. Antibody-producing cells obtained from the immunized animals are immortalized and screened, or screened first for the production of antibody which inhibits the interaction of the viral surface protein with the receptor molecule and then immortalized. For a discussion of general procedures of monoclonal antibody production see Harlow and Lane, Antibodies, A Laboratory Manual (1988), supra.

The generation of human monoclonal antibodies to a human antigen (in the case of an SLX unit isolated from human tissue) may be difficult with conventional techniques. Thus, it may be desirable to transfer the antigen binding regions of the non-human antibodies, e.g., the $F(ab')_2$ or hypervariable regions, to human constant regions (Fc) or framework regions by recombinant DNA techniques to produce substantially human molecules. Such methods are generally known in the art and are described in, for example, U.S. 4,816,397, EP publications 173,494 and 239,400, which are incorporated herein by reference. Alternatively, one may isolate DNA sequences which encode a human monoclonal antibody or portions thereof that specifically bind to the human SLX by screening a DNA library from human B cells according to the general protocol outlined by Huse et al., Science 246:1275-1281 (1989), incorporated herein by reference, and then cloning and amplifying the sequences which encode the antibody (or binding fragment) of the desired specificity.

A number of currently available monoclonal antibodies can be used according to the present invention to inhibit intercellular adhesion mediated by selectins. For instance, CSLEX-1 (see, Campbell et al., J. Biol. Chem. 259:11208-11214 (1984)), VIM-2, which recognizes a sequence slightly different from SLX (see, Macher et al., supra), FH6 (described in U.S. Patent No. 4,904,596) (all references are incorporated herein

by reference) or SH_3 and SH_4 generated by Dr. S. Hakomori of the Biomembrane Institute in Seattle, Washington.

The compounds of the present invention, including immunoglobulins, can be used in preparing pharmaceutical formulations as discussed below. If the compound is an oligosaccharide or glycoconjugate, the SLX or SLX-mimetic moiety can be presented in a variety of forms, but should be able to effectively bind to a selectin receptor, such as ELAM-1, GMP-140, or MEL-14 antigen and thereby inhibit intercellular adhesion.

The pharmaceutical compositions of the present invention can be used to block or inhibit cellular adhesion associated with a number of disorders. For instance, a number of inflammatory disorders are associated with selectins expressed on vascular endothelial cells and platelets. The term "inflammation" is used here to refer to reactions of both the specific and non-specific defense systems. A specific defense system reaction is a specific immune system reaction to an antigen. Example of specific defense system reactions include antibody response to antigens, such as viruses, and delayed-type hypersensitivity. A non-specific defense system reaction is an inflammatory response mediated by leukocytes generally incapable of immunological memory. Such cells include macrophages, eosinophils and neutrophils. Examples of non-specific reactions include the immediate swelling after a bee sting, and the collection of PMN leukocytes at sites of bacterial infection (e.g., pulmonary infiltrates in bacterial pneumonias and pus formation in abscesses).

Other treatable disorders include, e.g., rheumatoid arthritis, post-ischemic leukocyte-mediated tissue damage (reperfusion injury), frost-bite injury or shock, acute leukocyte-mediated lung injury (e.g., adult respiratory distress syndrome), asthma, traumatic shock, septic shock, nephritis, and acute and chronic inflammation, including atopic dermatitis, psoriasis, and inflammatory bowel disease. Various platelet-mediated pathologies such as atherosclerosis and clotting can also be treated. In addition, tumor metastasis can be inhibited or prevented by inhibiting the adhesion of

circulating cancer cells. Examples include carcinoma of the colon and melanoma.

By way of example, reperfusion injury is particularly amenable to treatment by compositions of the present invention. Compositions which inhibit a GMP-140 selectin-ligand interaction may be particularly useful for treating or preventing reperfusion injury. The present invention may be used prophylactically prior to heart surgery to enhance post-surgical recovery.

Because GMP-140 is stored in Weibel-Palade bodies of platelets and endothelial cells and is released upon activation by thrombin to mediate adhesion of neutrophils and monocytes, inhibitors of the GMP-140 -ligand interaction may be especially useful in minimizing tissue damage which often accompanies thrombotic disorders. For instance, such inhibitors may be of therapeutic value in patients who have recently experienced stroke, myocardial infarctions, deep vein thrombosis, pulmonary embolism, etc. The compounds are especially useful in pre-thrombolytic therapy.

Compositions of the invention find particular use in treating the secondary effects of septic shock or disseminated intravascular coagulation (DIC). Leukocyte emigration into tissues during septic shock or DIC often results in pathological tissue destruction. Furthermore, these patients may have widespread microcirculatory thrombi and diffuse inflammation. The therapeutic compositions provided herein inhibit leukocyte emigration at these sites and mitigates tissue damage.

The inhibitors of selectin-ligand interaction also are useful in treating traumatic shock and acute tissue injury associated therewith. Because the selectins play a role in recruitment of leukocytes to the sites of injury, particularly ELAM-1 in cases of acute injury and inflammation, inhibitors thereof may be administered locally or systemically to control tissue damage associated with such injuries. Moreover, because of the specificity of such inhibitors for sites of inflammation, e.g., where ELAM-1 receptors are expressed, these compositions will be more effective and less likely to cause

complications when compared to traditional anti-inflammatory agents.

Thus, the present invention also provides pharmaceutical compositions which can be used in treating the aforementioned conditions. The pharmaceutical compositions are comprised of biomolecules or other compounds which comprise an SLX unit, antibodies which bind to SLX, or other compounds which inhibit the interaction between the SLX ligand and selectin receptors, together with pharmaceutically effective carriers. A biomolecule of the present invention may be a peptide, polypeptide, protein (e.g., an immunoglobulin), carbohydrate (e.g., oligosaccharide or polysaccharide), glycoconjugate (e.g., glycolipid or glycoprotein), nucleic acid, and the like. The pharmaceutical compositions are suitable for use in a variety of drug delivery systems. For a brief review of present methods for drug delivery, see, Langer, Science 249:1527-1533 (1990), which is incorporated herein by reference.

In light of the complexity of the inflammatory response in mammals, one of skill will readily recognize that the pharmaceutical compositions of the present invention may comprise SLX bearing compounds in admixture with other compounds known to interfere with the function of other cellular adhesion molecules. For instance, members of the integrin family of adhesion molecules are thought to play a role in the extravasation of leukocytes at points of infection. For a review of intercellular adhesion receptors, including selectin receptors, and their role immune function, see Springer, Nature 346:425-434 (1990), which is incorporated herein by reference. In addition, successful treatment using the pharmaceutical compositions of the present invention may also be determined by the state of development of the condition to be treated. Since different adhesion molecules may be up or down regulated in response to a variety of factors during the course of the disease or condition, one of skill will recognize that different pharmaceutical compositions may be required for treatment of different inflammatory states.

In one embodiment, the SLX ligand of the pharmaceutical composition can be used to target conventional anti-inflammatory drugs or other agents to specific sites of tissue injury. By using a selectin-binding oligosaccharide moiety such as an SLX ligand or SLX mimetic to target a drug to a selectin receptor on, e.g., a vascular endothelial cell, such drugs can achieve higher concentrations at sites of injury. Side effects from the conventional anti-inflammatory chemotherapeutic agents can be substantially alleviated by the lower dosages, the localization of the agent at the injury sites and/or the encapsulation of the agent prior to delivery.

The targeting component, i.e., the SLX ligand or an SLX mimetic which binds to a desired selectin, can be directly or indirectly coupled to the chemotherapeutic agent. The coupling, which may be performed by means, generally known in the art, should not substantially inhibit the ability of the ligand to bind the receptor nor should it substantially reduce the activity of the chemotherapeutic agent. A variety of chemotherapeutics can be coupled for targeting. For example, anti-inflammatory agents which may be coupled include SLX-bearing compounds of the present invention, immunomodulators, platelet activating factor (PAF) antagonists, cyclooxygenase inhibitors, lipoxygenase inhibitors, and leukotriene antagonists. Some preferred moieties include cyclosporin A, indomethacin, naproxen, FK-506, mycophenolic acid, etc. Similarly, anti-oxidants, e.g., superoxide dismutase, are useful in treating reperfusion injury when targeted by a SLX ligand or mimetic. Likewise, anticancer agents can be targeted by coupling the SLX ligand or mimetic to the chemotherapeutic agent. Examples of agents which may be coupled include daunomycin, doxorubicin, vinblastine, bleomycin, etc.

The selectin receptor targeting may also be accomplished via amphipaths, or dual character molecules (polar:nonpolar) which exist as aggregates in aqueous solution. Amphipaths include nonpolar lipids, polar lipids, mono- and diglycerides, sulfatides, lysolecithin, phospholipids, saponin, bile acids and salts. These molecules can exist as emulsions and foams, micelles, insoluble monolayers, liquid crystals,

phospholipid dispersions and lamellar layers. These are generically referred to herein as liposomes. In these preparations the drug to be delivered is incorporated as part of a liposome in conjunction with a SLX ligand or mimetic which binds to the selectin receptor. Thus, liposomes filled with a desired chemotherapeutic agent can be directed to a site of tissue injury by the selectin-SLX ligand interaction. When the liposomes are brought into proximity of the affected cells, they deliver the selected therapeutic compositions.

The liposomes of the present invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of, e.g., liposome size and stability of the liposomes in the bloodstream.

Typically, the major lipid component in the liposomes is phosphatidylcholine. Phosphatidylcholines having a variety of acyl chain groups of varying chain length and degree of saturation are available or may be isolated or synthesized by well-known techniques. In general, less saturated phosphatidylcholines are more easily sized, particularly when the liposomes must be sized below about 0.3 microns, for purposes of filter sterilization. Methods used in sizing and filter-sterilizing liposomes are discussed below. The acyl chain composition of phospholipid may also affect the stability of liposomes in the blood. One preferred phosphatidylcholine is partially hydrogenated egg phosphatidylcholine.

Targeting of liposomes using a variety of targeting agents (e.g., ligands, receptors and monoclonal antibodies) is well known in the art. (see, e.g., U.S. Patent Nos. 4,957,773 and 4,603,044, both of which are incorporated herein by reference). Glycoproteins and glycolipids of a variety of molecular weights can be used as targeting agents. Typically, glycoproteins having a molecular weight less than about 300,000 daltons, preferably between about 40,000 and about 250,000 are used, more preferably between about 75,000 and about 150,000. Glycolipids of molecular weight of less than about 10,000 daltons, preferably between about 600 and about 4,000 are used.

Standard methods for coupling targeting agents to liposomes can be used. These methods generally involve incorporation into liposomes of lipid components, such as phosphatidylethanolamine, which can be activated for attachment of targeting agents, or derivatized lipophilic compounds, such as lipid derivatized bleomycin.. Antibody targeted liposomes can be constructed using, for instance, liposomes which incorporate protein A (see, Renneisen, et al., J. Biol. Chem., 265:16337-16342 (1990) and Leonetti et al., Proc. Natl. Acad. Sci. (USA) 87:2448-2451 (1990), both of which are incorporated herein by reference).

Targeting mechanisms generally require that the targeting agents be positioned on the surface of the liposome in such a manner that the target agents are available for interaction with the selectin receptor. The liposome is typically fashioned in such a way that a connector portion is first incorporated into the membrane at the time of forming the membrane. The connector portion must have a lipophilic portion which is firmly embedded and anchored in the membrane. It must also have a hydrophilic portion which is chemically available on the aqueous surface of the liposome. The hydrophilic portion is selected so that it will be chemically suitable to form a stable chemical bond with the targeting agent which is added later. Therefore, the connector molecule must have both a lipophilic anchor and a hydrophilic reactive group suitable for reacting with the target agent and holding the target agent in its correct position, extended out from the liposome's surface. In some cases it is possible to attach the target agent to the connector molecule directly, but in most instances it is more suitable to use a third molecule to act as a chemical bridge, thus linking the connector molecule which is in the membrane with the target agent which is extended, three dimensionally, off of the vesicle surface.

Liposome charge is an important determinant in liposome clearance from the blood, with negatively charged liposomes being taken up more rapidly by the reticuloendothelial system (Juliano, Biochem. Biophys. Res. Commun. 63:651 (1975)) and thus having shorter half-lives in

the bloodstream. Liposomes with prolonged circulation half-lives are typically desirable for therapeutic and diagnostic uses. Liposomes which can be maintained from 8, 12, or up to 24 hours in the bloodstream provide sustained release of the selectin-ligand inhibitors of the invention, or may facilitate targeting of the inhibitors (which may be labelled to provide for in vivo diagnostic imaging) to a desired site before being removed by the reticuloendothelial system.

Typically, the liposomes are prepared with about 5-15 mole percent negatively charged phospholipids, such as phosphatidylglycerol, phosphatidylserine or phosphatidylinositol. Added negatively charged phospholipids, such as phosphatidylglycerol, also serves to prevent spontaneous liposome aggregating, and thus minimize the risk of undersized liposomal aggregate formation. Membrane-rigidifying agents, such as sphingomyelin or a saturated neutral phospholipid, at a concentration of at least about 50 mole percent, and 5-15 mole percent of monosialylganglioside, may provide increased circulation of the liposome preparation in the bloodstream, as generally described in U.S. Pat. No. 4, 837,028, incorporated herein by reference.

Additionally, the liposome suspension may include lipid-protective agents which protect lipids and drug components against free-radical and lipid-peroxidative damages on storage. Lipophilic free-radical quenchers, such as alphatocopherol and water-soluble iron-specific chelators, such as ferrioxianine, are preferred.

A variety of methods are available for preparing liposomes, as described in, e.g., Szoka et al., Ann. Rev. Biophys. Bioeng. 9:467 (1980), U.S. Pat. Nos. 4, 235,871, 4,501,728 and 4,837,028, incorporated herein by reference. One method produces multilamellar vesicles of heterogeneous sizes. In this method, the vesicle forming lipids are dissolved in a suitable organic solvent or solvent system and dried under vacuum or an inert gas to form a thin lipid film. If desired, the film may be redissolved in a suitable solvent, such as tertiary butanol, and then lyophilized to form a more homogeneous lipid mixture which is in a more easily hydrated

powder-like form. This film is covered with an aqueous solution of the targeted drug and the targeting component and allowed to hydrate, typically over a 15-60 minute period with agitation. The size distribution of the resulting multilamellar vesicles can be shifted toward smaller sizes by hydrating the lipids under more vigorous agitation conditions or by adding solubilizing detergents such as deoxycholate.

The hydration medium contains the targeted drug at a concentration which is desired in the interior volume of the liposomes in the final liposome suspension. Typically the drug solution contains between 10-100 mg/ml in a buffered saline. The concentration of the targeting SLX molecule or mimetic which binds a selectin is generally between about 0.1 - 20 mg/ml.

Following liposome preparation, the liposomes may be sized to achieve a desired size range and relatively narrow distribution of liposome sizes. One preferred size range is about 0.2-0.4 microns, which allows the liposome suspension to be sterilized by filtration through a conventional filter, typically a 0.22 micron filter. The filter sterilization method can be carried out on a high through-put basis if the liposomes have been sized down to about 0.2-0.4 microns.

Several techniques are available for sizing liposomes to a desired size. One sizing method is described in U.S. Pat. No. 4,737,323, incorporated herein by reference. Sonicating a liposome suspension either by bath or probe sonication produces a progressive size reduction down to small unilamellar vesicles less than about 0.05 microns in size. Homogenization is another method which relies on shearing energy to fragment large liposomes into smaller ones. In a typical homogenization procedure, multilamellar vesicles are recirculated through a standard emulsion homogenizer until selected liposome sizes, typically between about 0.1 and 0.5 microns, are observed. In both methods, the particle size distribution can be monitored by conventional laser-beam particle size discrimination.

Extrusion of liposome through a small-pore polycarbonate membrane or an asymmetric ceramic membrane is also an effective method for reducing liposome sizes to a

relatively well-defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired liposome size distribution is achieved. The liposomes may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in liposome size.

Even under the most efficient encapsulation methods, the initial sized liposome suspension may contain up to 50% or more drug and targeting agent in free (non-encapsulated) form. Therefore, to maximize the advantages of liposomal targeted drug, it is important to remove free drug and targeting agent from the final injectable suspension.

Several methods are available for removing non-entrapped compound from a liposome suspension. In one method, the liposomes in the suspension are pelleted by high-speed centrifugation leaving free compound and very small liposomes in the supernatant. Another method involves concentrating the suspension by ultrafiltration, then resuspending the concentrated liposomes in a drug-free replacement medium. Alternatively, gel filtration can be used to separate large liposome particles from solute molecules.

Following treatment to remove free drug and/or targeting agent, the liposome suspension is brought to a desired concentration for use in intravenous administration. This may involve resuspending the liposomes in a suitable volume of injection medium, where the liposomes have been concentrated, for example by centrifugation or ultrafiltration, or concentrating the suspension, where the drug removal step has increased total suspension volume. The suspension is then sterilized by filtration as described above. The liposome-ligand preparation may be administered parenterally or locally in a dose which varies according to, e.g., the manner of administration, the drug being delivered, the particular disease being treated, etc.

For pharmaceutical compositions which comprise the SLX ligand, and/or SLX mimetics which bind to selectin receptors, the dose of the compound will vary according to, e.g., the particular compound, the manner of administration, the particular disease being treated and its severity, the

overall health and condition of the patient, and the judgment of the prescribing physician. For example, for the treatment of reperfusion injury, the dose is in the range of about 50 μg to 2,000 mg/day for a 70 kg patient. Ideally, therapeutic administration should begin as soon as possible after the myocardial infarction or other injury. The pharmaceutical compositions are intended for parenteral, topical, oral or local administration, such as by aerosol or transdermally, for prophylactic and/or therapeutic treatment. The pharmaceutical compositions can be administered in a variety of unit dosage forms depending upon the method of administration. For example, unit dosage forms suitable for oral administration include powder, tablets, pills, capsules and dragees.

Preferably, the pharmaceutical compositions are administered intravenously. Thus, this invention provides compositions for intravenous administration which comprise a solution of the compound dissolved or suspended in a pharmaceutically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, e.g., water, buffered water, 0.4% saline, and the like. These compositions may be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

The concentration of SLX ligand or mimetic, which may be combined with other SLX ligands or mimetics to form a "cocktail" for increased efficacy in the pharmaceutical formulation, can vary widely, i.e., from less than about 0.05%, usually at or at least about 1% to as much as 10 to 30% by weight and will be selected primarily by fluid volumes,

viscosities, etc., in accordance with the particular mode of administration selected. The cocktail may also comprise a monoclonal antibody which binds to selectin receptor, e.g., a monoclonal antibody to ELAM-1 or GMP-140, combined with the SLX ligand, a ligand mimetic or a monoclonal antibody to the ligand, so as to effectively inhibit the ligand-receptor interaction. As described above, the cocktail components may be delivered via liposome preparations.

Thus, a typical pharmaceutical composition for intravenous infusion could be made up to contain 250 ml of sterile Ringer's solution, and 25 mg of the compound. Actual methods for preparing parenterally administrable compounds will be known or apparent to those skilled in the art and are described in more detail in for example, Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Company, Easton, PA (1985), which is incorporated herein by reference.

For solid compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient, that is, one or more SLX ligands or mimetics of the invention, preferably about 20% (see, Remington's, supra).

For aerosol administration, the compounds are preferably supplied in finely divided form along with a surfactant and propellant. Typical percentages of SLX oligosaccharide ligands or mimetics are 0.05% - 30% by weight, preferably 1% - 10%. The surfactant must, of course, be nontoxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride such as, for

example, ethylene glycol, glycerol, erythritol, arabitol, mannitol, sorbitol, the hexitol anhydrides derived from sorbitol, and the polyoxyethylene and polyoxypropylene derivatives of these esters. Mixed esters, such as mixed or natural glycerides may be employed. The surfactant may constitute 0.1%-20% by weight of the composition, preferably 0.25-5%. The balance of the composition is ordinarily propellant. Liquefied propellants are typically gases at ambient conditions, and are condensed under pressure. Among suitable liquefied propellants are the lower alkanes containing up to 5 carbons, such as butane and propane; and preferably fluorinated or fluorochlorinated alkanes. Mixtures of the above may also be employed. In producing the aerosol, a container equipped with a suitable valve is filled with the appropriate propellant, containing the finely divided compounds and surfactant. The ingredients are thus maintained at an elevated pressure until released by action of the valve.

The compositions containing the compounds can be administered for prophylactic and/or therapeutic treatments. In therapeutic applications, compositions are administered to a patient already suffering from a disease, as described above, in an amount sufficient to cure or at least partially arrest the symptoms of the disease and its complications. An amount adequate to accomplish this is defined as "therapeutically effective dose." Amounts effective for this use will depend on the severity of the disease and the weight and general state of the patient, but generally range from about 0.5 mg to about 2,000 mg of SLX oligosaccharide or SLX mimetic per day for a 70 kg patient, with dosages of from about 5 mg to about 200 mg of the compounds per day being more commonly used.

In prophylactic applications, compositions containing the compounds of the invention are administered to a patient susceptible to or otherwise at risk of a particular disease. Such an amount is defined to be a "prophylactically effective dose." In this use, the precise amounts again depend on the patient's state of health and weight, but generally range from about 0.5 mg to about 1,000 mg per 70 kilogram patient, more

commonly from about 5 mg to about 200 mg per 70 kg of body weight.

Single or multiple administrations of the compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical formulations should provide a quantity of SLX oligosaccharide or SLX mimetic of this invention sufficient to effectively treat the patient.

The compounds may also find use as diagnostic reagents. For example, labeled compounds can be used to locate areas of inflammation or tumor metastasis in a patient suspected of having an inflammation. For this use, the compounds can be labeled with ^{125}I, ^{14}C, or tritium.

The following examples are offered by way of illustration, not by way of limitation.

EXAMPLE I

Isolation of α1,3-fucosyltransferase I from Golgi Apparatus

LEC11, HL-60, HT-29, certain adenocarcinomas (colo 205 cells in particular), and polymorphonuclear leukocytes (PMN, neutrophils) contain a very specific α1,3-fucosyltransferase I, which is able to transfer fucose from GDP-fucose to the sialylated substrates NeuAcα2,3Galβ1,4GlcNAc or NeuGcα2,3Galβ1,4GlcNAc.

It is well known in the art that fucose is transferred to oligosaccharide chains in the lumen of the Golgi apparatus via specific fucosyltransferases, reviewed in Schacter and Roseman, in "The Biochemistry of Glycoproteins and Proteoglycans", W. Lennarz, ed., Plenum Press, New York, pp. 85-160 (1980), which is incorporated herein by reference. Since the subcellular localization of the fucosyltransferases is in the Golgi apparatus, the first step in the isolation of these enzymes is to isolate a Golgi apparatus fraction from a cell line which expresses this novel and specific α1,3-fucosyltransferase.

Golgi apparatus-derived vesicle fractions are prepared by a modification of the procedure described by Balch et al., Cell, 39:405 (1984) which is incorporated herein by

reference. The LEC11, HL-60, HT-29, PMN, colo 205 or other cell lines containing the α1,3-fucosyltransferase I are grown in suspension to a density of approximately 5 X 10^5 cells/ml. Cells are harvested from the suspension culture by centrifugation at 2,000 X g. The resulting cell pellet from a 12 liter suspension (6 X 10^9 cells) is resuspended in 3 volumes (packed cell volume) of ice-cold 0.25M sucrose (w/v) solution containing Tris-Cl (10mM), pH 7.0, heat inactivated fetal calf serum (7%), and Aprotinin (100 μg/ml, Sigma Chemical, Co. St. Louis, Mo.).

The cells are disrupted (approximately 60 strokes) with a tight fitting Wheaton glass dounce homogenizer using the A pestle. The homogenate is centrifuged for 5 min. at 500 X g in a table-top clinical centrifuge. Lipid and insoluble material remaining at the top of the solution in the centrifuge tube is discarded. The cloudy supernatant is transferred to a clean tube, and the sucrose concentration of the supernatant fraction is then adjusted to 40% (w/v) sucrose in Tris-Cl (20 mM), pH 7.0, with the aid of a refractometer. Five milliliters of this suspension is transferred to an ultracentrifuge tube and is layered sequentially with 2.5 ml of 35% (w/v) sucrose in Tris-Cl (10 mM, pH 7.0) and 2.0 ml of 29% (W/v) sucrose in 10 mM Tris-Cl buffer. The gradient is centrifuged for 1 hr. at 110,000 X g in a SW-41 rotor (Beckman) at 5°C. Golgi apparatus enriched vesicles are collected from the 29% to 35% sucrose interphase. Other subcellular fractions are found at other interphases in the gradient; e.g., vesicles derived from the rough and smooth endoplasmic reticulum band below the Golgi derived vesicles, etc. The band removed from the 29% to 35% interphase is analyzed for the presence and amount of sialyltransferase activity.

The enzyme sialytransferase is only known to be found within Golgi apparatus-derived vesicles and is used by those trained in the art as a marker to assess the authenticity of the band collected from the 29-35% interphase. Sialyltransferase assays are performed using asialofetuin as the acceptor as described by Briles et al., J. Biol. Chem., 252:1107 (1977). A good Golgi apparatus derived vesicle

preparation from LEC cells typically has a sialyltransferase-specific activity of 3.0 nmole/mg protein/hr.

The resulting Golgi apparatus preparation is then used as a source of the α1,3-fucosyltransferase I used in the enzymatic synthesis described above.

EXAMPLE II

Demonstration of Intercellular Adhesion by Cells Expressing SLX

The ability of LEC11 cells (which express SLX) to bind to activated endothelial cells expressing ELAM-1 was compared to that of CHO cells and another glycosylation mutant, LEC12, which expresses the structure Le^x, a non-sialylated form of SLX.

MATERIALS

Passage 5 human umbilical vein endothelial cells (HUVEC) (Clonetics) which had been grown on a gelatin coated 48 well assay plate were used as the source of endothelial cells. Cells were stimulated with IL-1β (Genzyme) at 30 μg/ml. Cells were stimulated for exactly 4 hrs. HL-60 cells provided by American Type Culture Collection (ATCC No. CCL 240) were used as the source of control ligand bearing cells. These were harvested from bulk culture in RPMI 1640 (Gibco) containing Penicillin (100 units/ml)/ Streptomycin (100 Mcg/ml)(Irvine Scientific), L-Glutamine (2mM) (Irvine Scientific) and 10% Fetal Bovine Serum (Hazleton) (hereafter referred to as CRPMI). LEC11, LEC12 and CHO-K1 were provided by Dr. P. Stanley. They were grown in suspension culture in complete alpha MEM containing ribonucleotides and deoxyribonucleotides (Gibco), Penicillin (100 units/ml)/ Streptomycin (100 μg/ml)(Irvine Scientific), L-Glutamine (2mM) (Irvine Scientific) and 10% Fetal Bovine Serum (Hazelton).

PROCEDURE

1. HL-60, LEC11, LEC12 and CHO-K1 cells were harvested and washed in CRPMI. A viable cell count was made using trypan blue. 3 x 10^6 cells of each type were pelleted in

a 10 ml test tube and 300 μl of ^{51}Cr (450 μCi) (New England Nuclear) was added to each pellet. The tubes were allowed to incubate 1 hour at 37°C with gentle agitation.

2. Labeled cells were washed 3X in medium and resuspended to 2 x 10^5/ 400 μl (6ml). The tubes were then placed in a 4°C ice bath.

3. After 4 hours incubation with IL-1β the assay plate containing activated HUVEC was removed from the incubator and chilled for 15 minutes by placing the plate in a 4°C ice bath.

4. When the temperature in both samples had equilibrated, the medium was removed from the assay wells with a pasteur pipette a few wells at a time.

5. Labeled cells were added to the wells in 400 μl volumes equal to 2 x 10^5 cells/well. Three 400 μl aliquots of each cell suspension were placed in glass tubes for determination of input CPMs.

6. The plate was incubated in the ice water bath for 30 minutes.

7. Unbound cells were removed from the wells of the assay plate by systematic resuspension using a pasteur pipette followed by addition and removal of 0.7 ml of medium.

8. All of the medium was removed from the wells and a solution of 0.125 M Tris, 2% SDS and 10% glycerin was added (0.3 ml). The plate was allowed to stand for 30 minutes and then 0.5 ml of dH_2O was added to each well.

9. The fluid in each well was resuspended with a P1000 pipette and transferred to a glass test tube. The P1000 tip was ejected into the tube.

10. The tubes, including those containing the input CPM samples were counted in a gamma counter.

11. CPMs bound in each well were divided by the input CPMs for each sample to determine the % bound. The mean and standard deviation of triplicate assay points were plotted.

The results obtained in this experiment, shown in Fig. 1, indicate that cells expressing SLX have the ability to bind effectively to activated vascular endothelial cells expressing ELAM-1. These data show that LEC11 cells which express high levels of the unique carbohydrate SLX bind exceptionally well to IL-1β activated HUVEC, while LEC12 and CHO-K1 which lack significant quantities of this carbohydrate are poor binders of the activated HUVEC. This conclusion is further supported by the observation that this binding occurs at 4°C, a characteristic of ELAM-1 mediated binding.

EXAMPLE III

Inhibition of Intercellular Adhesion by Monoclonal Antibodies Specific for SLX.

Two sets of experiments are described which confirm that the ligand on neutrophils for ELAM-1 contains an oligosaccharide where the terminal sugars are NeuAcα2,3Galβ1,4(Fuc α1,3)GlcNac(SLX).

These experiments are performed by assaying the ability of monoclonal antibodies specific for sialylated Le^x and for the unsialylated form, Le^x, to block the ELAM-1 mediated adhesion of HL-60 cells to IL-1β stimulated HUVEC.

A. Monoclonal Antibody Panel 1

Materials: Passage 3 HUVEC from cultures initiated for the present experiments were used as described above. Two sets of triplicate wells were left unstimulated as controls. Four triplicates were stimulated with IL-1β (Genzyme) at 10 μg/ml and 4 at 20 μg/ml. Cells were stimulated for exactly 4 hours. HL-60 cells obtained from the American Type Culture Collection were used as the source of ligand bearing cells. These were harvested from bulk culture in RPMI-1640 (Gibco)

containing penicillin (100 units/ml), streptomycin (100 μg/ml) (Irvine Scientific), L-Glutamine (2mM) (Irvine Scientific) and 10% Fetal Bovine Serum (Hazleton) (hereafter referred to as cRPMI).

Monoclonal antibody preparations included SNH3 (IgM) at about 20 μg/ml and SH1 (IgG3) at about 10 μg/ml. The specificity of SNH3 is for SLX, while SH1 recognizes the unsialylated structure.

Procedure:

1. HL-60 cells were harvested and washed in CRPMI. A viable cell count was made using trypan blue. 3 x 10^6 cells were placed in each of 2, 10 ml test tubes and 300 μl of ^{51}Cr (450 μCi) (New England Nuclear) was added to each tube. The tubes were allowed to incubate 1 hour at 37°C with gentle agitation.

2. The antibodies were supplied as hybridoma culture supernatants and contained 0.01% NaN3 and 0.05% thimerosal. To remove these preservatives, 5 ml. of each antibody was dialysed against 3 changes of 500 ml each of outdated tissue culture medium over 72 hours.

3. Antibodies were collected from dialysis and 3.5 ml of each was placed in 10 ml tubes. The remainder was retained for use in an ELISA assay for HL-60 binding. 7 ml of RPMI 1640 5% FCS was placed in a 4th tube for use as a control.

4. Labeled HL-60 cells were washed 3X in CRPMI and pooled into one tube. They were then centrifuged and resuspended to 1 ml in medium.

5. 200 μl of cell suspension was added to each of the antibody containing tubes and 400 μl to the control tube. Tubes were incubated 20 min. at 37°C with gentle agitation.

6. The stimulated HUVEC assay plate was removed from the incubator and the medium was removed from the wells with a pasteur pipette, a few wells at a time.

7. 0.5 ml of cell suspension was added to each of triplicate wells. Control cells were plated on unstimulated and stimulated HUVEC at both IL-1β concentrations. Test cells were added to stimulated wells only.

8. 0.5 ml aliquots of each cell suspension were added to glass tubes to be used to determine the input CPMs.

9. The assay plate was returned to the incubator (5% CO_2, 37°C) for 30 min.

10. An aliquot of each cell suspension was mixed with an equal volume of trypan blue and the cells were examined microscopically for viability. The results were: Control = 98%, SH1 = 92%, and SNH3 = 99%.

11. Unbound cells were removed from the wells of the assay plate by systematic resuspension using a pasteur pipette followed by addition and removal of 0.7 ml of medium.

12. All of the medium was removed from the wells and a solution of 0.125 M Tris, 2% SDS (Bio-Rad) and 10% glycerin (Fisher) was added (0.3 ml). The plates were allowed to stand for 30 min. and then 0.6 ml of dH_2O was added to each well.

13. The fluid in each well was resuspended with a P1000 pipette and transferred to a glass test tube. The P1000 tip was ejected into the tube.

14. The tubes, including those containing the input counts per minute (CPM) samples were counted in a gamma counter.

15. CPMs bound in each well were divided by the input CPMs for each sample to determine the % bound. The mean and standard deviation of triplicate assay points were plotted.

Replicates were judged to be best in the experiment in which high IL-1β was used to induce the endothelial cells.

The results showed that the monoclonal antibody SNH3 blocked the binding of HL-60 cells to IL-1β stimulated HUVEC via the ELAM-1 receptor. The control antibody SH1 which does not bind the SLX determinant did not block binding of HL-60 cells to ELAM-1. This suggests that the terminal sialic acid in the ligand is necessary for binding to ELAM-1.

B. Monoclonal Antibody Panel 2

Materials: Passage 3 HUVEC which had been grown on gelatin coated 48 well assay plates (Costar) were used as the source of endothelial cells. The plates were prepared as previously described above. Two sets of triplicate wells were left unstimulated as controls. Seven triplicates on each plate were stimulated with IL-1β at 30 μg/ml in 0.5 ml of EGM-UV. Cells were stimulated for exactly 4 hrs. HL-60 cells (ATCC) were used as the source of ligand bearing cells. These were harvested from bulk culture in CRPMI. Fresh hybridoma supernatants containing monoclonal antibodies included: FH6 (IgM) a lower affinity mAb; SNH-3 (IgM) (20 μg/ml); SH-1 (IgG_3) (10 μg/ml); FH-2 (IgM) a Le^x reactive mAb; SNH-4 (IgG3) a high affinity antibody; and CSLEX-1 (IgM) (provided by Dr. P. Terasaki, UCLA as purified immunoglobulin at 2.8 mg/ml, diluted to 9 μg/ml in Dulbecco's Modified Eagles Medium (DMEM) containing 5% FCS for use in this assay). The specificities of the antibodies were as follows: FH6, SNH-4, SNH3 and CSLEX-1 were specific for SLX; FH2 and SH1 were specific for the unsialylated Le^x.

Procedure:

1. HL-60 cells were harvested and washed in CRPMI. A viable cell count was made using trypan blue. 3×10^6 cells were placed in each of 2, 10 ml test tubes and 300 μl of ^{51}Cr (450 μCi) (New England Nuclear) was added to each tube. The tubes were allowed to incubate 1 hour at 37°C with gentle agitation.

2. Labeled HL-60 cells were washed 3X in DMEM containing 5% FCS (hereafter referred to as cDMEM) and pooled into one tube. They were then centrifuged and resuspended to 4 x 10^6 cells per ml in the same medium.

3. 3.2 ml of each monoclonal antibody culture supernatant, and 3.2 ml purified CSLEX-1 (29 μg), were added to separate test tubes; a control tube received 6.4 ml of medium.

4. 200 μl of cell suspension (equal to about 8 x 10^5 cells) was added to tubes containing the monoclonal antibodies and 400 μl to the control tube. Tubes were then incubated 20 min. at 37°C with gentle agitation.

5. The stimulated HUVEC assay plate was removed from the incubator and the wells were washed one time with cDMEM and the medium was removed from the wells with a pasteur pipette, a few wells at a time.

6. 0.4 ml of cell suspension was added to each well of one of the two plates. Control cells were plated on unstimulated and stimulated HUVEC. Antibody treated cells were added to stimulated wells only.

7. 0.4 ml aliquots of each cell suspension were added to glass tubes to be used to determine the input CPMs.

8. The assay plate was incubated at 37°C for 30 min.

9. The remainder of each cell suspension and the assay plate were placed in an ice bath to chill for 20 min.

10. The cell suspensions were plated on the chilled plate as for the 37°C plate above. This plate was incubated for 30 min. at 40°C.

The remaining steps of the assay were performed as described in steps 11-15 of Section A above, except that in step 12 the plates were allowed to stand for 15 min. rather than 30 min.

The results, shown in Fig. 2A, indicate that the monoclonal antibodies SNH-3, FH6, SNH-4 and CSLEX-1, all specific for SLX, significantly blocked the binding of HL-60 cells to IL-1β stimulated HUVEC via the ELAM-1 receptor when incubated at 37°C. The monoclonal antibodies specific for Le^x (FH2 and SH1) were not effective inhibitors. Thus, the ligand for ELAM-1 contains the sialylated Le^x antigen or a similar structure found in cell surface glycoproteins or glycolipids.

When incubated at 4°C (Fig. 2B), antibodies FH6 and SNH-3 (both IgM's) enhanced binding. In these tests there appeared to be significant agglutination of the HL-60 cells in the wells, which may account for this observation.

C. Monoclonal Antibodies Block Adhesion of LEC11 Cells to Cells which Express ELAM-1

In this set of experiments the ability of monoclonal antibodies specific for SLX and for the unsialylated form, Le^x, to block the ELAM-1 mediated adhesion of LEC11 cells (which express SLX) and LEC12 cells (which express Le^x) to IL-1β stimulated HUVEC.

Materials: Passage 4 HUVEC served as the source of endothelial cells. The plates were prepared as previously described. Two sets of triplicate wells were left unstimulated as controls. 7 triplicates on each plate were stimulated with IL-1β at 30 μg/ml in a 0.5 ml volume of EGM-UV. Cells were stimulated for exactly 4 hrs. LEC11 and LEC12 cells, described generally in Stanley et al., J. Biol. Chem., 263:11374 (1988), supra, were provided by Dr. P. Stanley. They were grown in suspension culture in complete alpha MEM containing ribonucleotides and deoxyribonucleotides (Gibco), penicillin (100 units/ml)/streptomycin (100 μg/ml) (Irvine Scientific), L-Glutamine (2mM) (Irvine Scientific) and 10% FBS (Hazelton). The monoclonal antibodies used in these experiments are

described in Section B, above. They included: FH6, SNH-3, SH-1, FH-2, SNH-4 and CSLEX-1.

Procedure:

1. LEC11 and LEC12 cells were harvested and washed in CRPMI. A viable cell count was made using trypan blue. 3 x 10^6 cells of each cell line were placed in each of 2, 10 ml test tubes and 300 µl of ^{51}Cr (450 µCi) (New England Nuclear) was added to each tube. The tubes were allowed to incubate 1 hr. at 37°C with gentle agitation.

2. The radiolabeled cells were washed X3 in cDMEM and pooled into one tube. They were then centrifuged and resuspended to 4 x 10^6 cells per ml in the same medium.

3. 1.6 ml of each monoclonal antibody supernatant, and 1.6 ml purified CSLEX-1 (15 µg), were added to separate test tubes; control tubes received 3.2 ml medium.

4. 200 µl of cell suspension equal to 4 x 10^5 LEC11 or LEC12 cells were added to tubes containing the monoclonal antibodies and 400 µl to the control tube. Tubes were incubated 20 min. at 37°C with gentle agitation.

5. The stimulated HUVEC assay plate was removed from the incubator and the wells were washed one time with cDMEM and the medium was removed from the wells with a pasteur pipette, a few wells at a time.

6. The cell suspensions and the assay plate were placed in an ice bath to chill for 20 min.

7. 0.4 ml of cell suspension was added to each well of the previously described assay plate. Control cells were plated on unstimulated and stimulated HUVEC. Antibody treated cells were added to stimulated wells only. Each assay was done in triplicate.

8. 0.4 ml aliquots of each cell suspension were added to glass tubes to be used to determine the input CPMs.

9. The assay plate was incubated for 30 min. at 4°C.

The remaining steps of the assay were performed as described in steps 11-15 of Section A, above, except that in step 12 the plates were allowed to stand for 15 min.

The results shown, in Figs. 3A and 3B, indicate that the monoclonal antibodies SNH-3, FH6, SNH-4 and CSLEX-1 (all specific for SLX) significantly blocked the binding of LEC11 cells to IL-1β stimulated HUVEC via the ELAM-1 receptor. LEC12 cells, which do not express the SLX epitope, did not bind the activated endothelium. The monoclonal antibodies specific for Le^x (FH2 and SH1) caused minor inhibition of LEC11 binding.

Further confirmation that SLX is a primary ligand for ELAM-1 receptor was provided by removing sialic acid from LEC 11 and HL-60 cells. In these experiments the treatment of LEC 11 and HL-60 cells prior to adhesion assays with Clostridium perfringens neuraminidase (sialidase), 1.6 U/ml (Type X, Sigma Chem. Co.) for 90 min. at 37°C during ^{51}Cr-labelling. The results, shown in Fig. 4, confirm that sialidase substantially reduced the adhesion of LEC 11 and HL-60 cells by 70-85% to activated endothelial cells.

EXAMPLE IV

Liposomes of Glycosphingolipids Block Binding of SLX Cells to Activated Endothelial Cells

This Example describes the preparation of liposomes which contain various biosynthetically produced glycosphingolipids on which the terminal carbohydrate units are either SLX, Le^x, or similar but not identical compounds. The ability of the liposomes which contain SLX or SLX mimetics to block the binding of SLX-expressing HL-60 cells and LEC11 cells to endothelial cells which have been stimulated to express ELAM-1 by treatment with IL-1β is shown.

Materials: The glycosphingolipids used in this experiment are shown in Table I; they were obtained from the Biomembrane Institute, Seattle, WA, and were either purified or biosynthetically produced and characterized by NMR and mass spectrometry, as generally described in Hakomori, S. I., et al., J. Biol. Chem., 259:4672 (1984), and Fukushi Y., et al., J. Biol. Chem. 259:10511 (1984), incorporated by referenced herein. S-diLe^x (SLX) was synthesized enzymatically by adding fucosyl residues using a colo 205 cell line as enzyme source and SH as substrate. Nonsialylated diLe^x was similarly synthesized using nLc6 as substrate and the cell line NCI H-69. See Holmes et al., J. Biol. Chem. 260:7619 (1985), incorporated by reference herein. SPG and SH were purified form bovine red blood cells, and nLc6 was produced by chemical removal of the terminal sialosyl residue from SH.

Table 1. Glycolipids tested for liposome inhibition of ELAM-1 mediated cell adhesion

Generic	IUPAC	Structure
nLc_6	nLc_6	Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer
diLe^x	III^{3}V^{3}Fuc$_2$nLc$_6$	Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer 3 3 ↑ ↑ Fucα1 Fucα1
SPG	IV^{3}NeuAcnLc$_4$	NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer
SH	VI^{3}NeuAcnLc$_6$	NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer
S-diLe^x	III^{3}V^{3}Fuc$_2$VI^{3}NeuAcnLc$_6$	NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer 3 3 ↑ ↑ Fucα1 Fucα1

Liposomes containing the glycosphingolipids were formed as follows: 100 μg of glycolipid was added to 300 μg phosphatidylcholine (Sigma, egg yolk) and 500 μg cholesterol (Sigma) in chloroform-methanol (2:1) and the whole solution evaporated to dryness by N_2 in 15 ml screwcap tubes.

Passage 3 HUVEC, which had been grown on to confluence on a gelatin coated 48 well assay plate (Costar) were used as the source of endothelial cells. The plates were prepared as previously described. Two sets of triplicate wells were left unstimulated as controls. 14 triplicates were stimulated with IL-1β at 30 μg/ml in a 0.5 ml volume of EGM-UV. Cells were stimulated for exactly 4 hrs. HL-60 cells and LEC11 cells were cultured as described above.

Procedure:

1. One 48 well Costar cluster dish containing HUVEC grown to confluence on gelatin was removed from the incubator and the medium in each well was removed with a pasteur pipette and replaced either with 0.5 ml fresh EGM-UV medium or with the same medium containing 30 μg/ml IL-1β, and the plate then returned to the incubator for 4 hrs.

2. HL-60 cells and LEC11 cells were harvested and washed in CRPMI. A viable cell count was made using trypan blue. 6×10^6 cells of each cell type were radiolabeled as follows: 3×10^6 cells of each type were placed in each of 2, 10 ml test tubes and 300 μl of ^{51}Cr (450 μCi) (New England Nuclear) was added to each tube. The tubes were allowed to incubate 1 hr. at 37°C with gentle agitation.

3. Radiolabeled HL-60 and LEC11 cells were washed 3X in CRPMI and pooled into one tube. They were then centrifuged and resuspended to 2×10^6 cells per ml in the same medium.

4. The stimulated HUVEC assay plate was removed from the incubator and the wells were washed two times with RPMI 1640 containing 5 mg/ml bovine serum albumin (BSA).

5. Liposomes were prepared as follows: The evaporated pellets were dissolved in 100 μl of absolute ethanol and sonicated for 2 min. Two ml of PBS was added slowly to the tubes over two minutes while continuing to sonicate. This stock was diluted 1:10 in RPMI 1640 medium just prior to use and 50 μl of a stock solution of BSA at 100 mg/ml was added to each 1 ml of diluted liposomes to make a final concentration of 5 mg/ml BSA.

6. The medium was removed from the wells of the assay plate with a pasteur pipette, a few wells at a time, and 0.3 ml of liposome suspension was added to each of 6 IL-1β stimulated assay wells. Control wells received the liposome buffer containing ethanol, RPMI 1640 and BSA at the same concentrations as in the liposome containing wells. Control buffer was plated on unstimulated and stimulated HUVEC. Liposome containing samples were added to stimulated wells only.

7. The plates were incubated for 40 min. at 37°C and then 50 μl of ^{51}Cr labeled HL-60 or LEC11 cells were added to the assay wells. Each cell line was assayed in triplicate on each liposome preparation. The final concentration of cells was 10^5/350 μl/well. Three aliquots of 50 μl of each cell suspension were added to glass tubes to be used to determine the input CPMs, and the assay plate incubated at 37°C for 30 min.

8. Unbound cells were removed from the wells of the assay plates by systematic resuspension using a pasteur pipette followed by addition and removal of 0.7 ml of medium. All of the medium was removed from the wells and a solution of 0.125 M Tris, 2% SDS and 10% glycerin was added (0.3 ml). The plates were allowed to stand for 15 min. and then 0.5 ml of dH_2O was added to each well.

9. The fluid in each well was resuspended and transferred to a glass test tube. The pipette tip was ejected into the tube. The tubes, including those containing the input CPM samples, were counted in a gamma counter. CPMs bound in each well were divided by the input CPMs for each sample to determine the % bound. The means and standard error of triplicate assay points were plotted.

As shown in Fig. 5, liposomes containing selected glycolipids having terminal sequences which contained SLX (S-diLe^x, Table 1) dramatically inhibited adhesion of HL-60 cells to activated endothelial cells at 4°C. Liposomes containing glycolipids with Le^x (di-Le^x) or other related carbohydrate structures (Table 1) exhibited minimal inhibition that was not dependent on the structure of the carbohydrate group. Similar results were obtained with LEC 11 cell adhesion. When the experiments were performed at 37°C, HL-60 cell adhesion was reduced by liposomes containing glycolipids with the SLX structure (S-diLe^x, 70%), and also to a lesser extent by liposomes containing Le^x (di-Le^x, 40%) suggesting that Le^x may also interact with ELAM-1, but with a lower affinity. These experiments show that biosynthetically produced SLX or similar SLX mimetic compounds when formulated into liposome compositions can serve as therapeutic compounds for, e.g., the reduction of leukocyte infiltration into inflammatory sites.

Jurkat cells bind to IL-1 activated endothelial cells predominantly through the V-CAM (endothelial cell) - VLA-4 (Jurkat cell) adhesion pair (Wayner et al., J. Cell Biol. 109:1321), in contrast to the adhesion of HL-60 and LEC 11 cells to activated endothelial cells through the ELAM-1 receptor. Jurkat cell adhesion was not inhibited by liposomes which contained SLX, but was completely inhibited by monoclonal antibody to the α subunit of the integrin molecule VLA-4. This result demonstrates that SLX liposome inhibition of HL-60 and LEC 11 cells is not a stearic effect attributable to binding of liposomes to endothelial cells, but supports the conclusion that SLX liposomes inhibit the adhesion through a direct competition with the ligand binding site of ELAM-1.

EXAMPLE V

Antibodies to SLX Inhibit GMP-140 Mediated Binding on Activated Human Platelets

In this Example the ability of monoclonal antibodies specific for SLX and for the unsialylated Le^x to block the GMP-140 mediated adhesion of HL-60 cells to activated human platelets was determined.

Materials: HL-60 cells are described above and were used as the source of ligand bearing cells. Jurkat cells were used as the non-ligand bearing control. Monoclonal antibodies SH-1, FH-2, SNH-4, and CSLEX-1 are also described above.

Procedure:

1. Blood was drawn from a normal human donor into a syringe containing ACD anticoagulant (dextrose, 2.0 g; sodium citrate 2.49 g; and citric acid 1.25 g; to 100 ml with dH_2O) at a ratio of 6 parts blood to 1 part anticoagulant.

Platelets were isolated by differential centrifugation as follows: Blood was centrifuged at 800 rpm (approx. 90 x g) for 15 min. at room temp. The supernatant was collected and centrifuged at 1200 rpm (approximately 400 x g) for 6 min. The supernatant was removed and centrifuged at 2000 rpm (1200 x g) for 10 min. to pellet the platelets. The platelet button was washed 2 times with Tyrode-HEPES buffer, pH 6.5 (NaCl 8.0 g; KCl 0.2 g; $NaH_2PO_4 \cdot H_2O$ 0.057 g; $MgCl_2 \cdot 6H_2O$ 0.184 g; $NaHCO_3$ 0.1 g; Dextrose, 1.0 g; and HEPES, 2.383 g; bring to 1 L with DI water, adjust to pH 6.5 with 1N NaOH) followed by one wash in PBS. Platelets were suspended to a concentration of 10^8/ml in PBS.

2. Approximately 20 min. before the platelets were finally resuspended, 48 well plates were coated with 0.1% gelatin and incubated to 15 min. at 37°C. Excess gelatin was removed by pipette immediately fore the addition of the platelet suspension. Platelets were activated by the addition of 0.25 units of thrombin/ml (Sigma T-6759) of platelet

suspension. Platelets were allowed to stand at room temperature for 20 min.

3. To prepare bound, activated platelets, 300 µl of the platelet suspension was added to each well of the gel coated plate. The plate was incubated at 37°C to 15 min., then spun at 800 rpm (90 xg) for 2 min. The unbound platelets were removed by washing the plate 3 times with PBS.

4. Since platelets possess highly reactive Fc receptors, to prevent uptake of any aggregated IgG from the antibody preparation, the platelet Fc receptors were blocked as follows: Purified mouse IgG W6/32 (IgG_{2a}) at 27 mg/ml was aggregated by heating at 63°C for 5 min. 300 µl of the heated preparation at 20 µg/ml in PBS was added to each well of the platelet-coated plate. The plate was incubated at 37°C for 15 min. then washed with PBS.

5. HL-60 and Jurkat cells were harvested and washed in CRPMI. A viable cell count was made using trypan blue, 3 x 10^6 cells of each type were placed in each of 2, 10 ml test tubes and 300 µl of ^{51}Cr (450 µCi) (New England Nuclear) was added to each tube. The tubes were incubated for 1 hr. at 37°C with gentle agitation.

6. Radiolabeled cells were washed 3X in CRPMI and pooled into one tube. They were then centrifuged and resuspended to 4 x 10^6 cells per ml in the same medium.

7. 1.6 ml of each monoclonal antibody culture supernatant, and 1.6 ml of purified CSLEX-1 (15 µg) were added to separate test tubes; control tubes received 1.6 ml of medium.

8. 100 µl of the labeled HL-60 of Jurkat cell suspension (containing 4 x 10^5 cells) was added to each of the tubes which contained monoclonal antibody. They were incubated for 20 min. at 37°C with gentle agitation. Following this

incubation period, 0.3 ml of each cell suspension (containing 7.5×10^4 cells) was added to each well of the previously described assay plate containing bound activated platelets. Each assay was done in triplicate.

9. The assay plate was centrifuged at 90 xg for 2 min. and then incubated for 5 min. at room temp. Unbound cells were removed from the wells of the assay plate by inverting the plate into a radioactive waste receptacle and blotting the plate on towels. The wells were washed X3 by carefully adding 300 µl PBS to each well and inverting and blotting the plate. All of the medium was removed from the wells and 0.3 ml of a solution of 0.125 M Tris, 2% SDS and 10% glycerin was added. The plates were allowed to stand for 15 min. and then 0.6 ml of dH_2O was added to each well.

10. The fluid in each well was resuspended with a pipette and transferred to a glass test tube. The tip was ejected into the tube. The tubes, including those containing the input samples, were counted in a gamma counter. CPMs bound in each well were divided by the input CPMs for each sample to determine the % bound. Input CPMs were determined by counting a 0.3 ml aliquot of each cell suspension described in step 8.

The results, shown in Fig. 6, indicate that the monoclonal antibodies SNH-4 and CSLEX-1 specific for SLX blocked the binding of HL-60 cells to GMP-140 on activated platelets. The monoclonal antibodies specific for Le^x (FH2 and SH-1) also blocked this binding but to a lesser extent. This Example suggests that both SLX and Le^x may be ligands for GMP-140, but that the SLX structure may be of a higher affinity for GMP-140 than the Le^x structure.

EXAMPLE VI

Liposomes of Glycosphingolipids Block Binding of SLX Cells to Activated Platelets

This Example demonstrates the ability of the liposomes which contain SLX or SLX mimetics to block the binding of SLX-expressing HL-60 cells and PMNs to platelets

which have been stimulated to express GMP-140 by treatment with Thrombin. The assays generally followed the protocol described in Larsen et al., Cell 63: 467-474 (1990), which is incorporated herein by reference.

Materials:

Glycosphingolipids were prepared as described in Example IV. The platelets were prepared as described in Example V, except that blocking of Fc receptors was not performed. HL60 cells were prepared as described above.

PMNs were prepared from 50 ml of whole blood drawn from volunteer donors into heparinized vacutainer tubes, which were inverted to mix the blood. All steps were performed at 22-24 degrees C. Each 25 ml of blood was layered over 15 ml of Mono-Poly Resolving Medium (Flow Labs). The tubes were centrifuged at 800xg for 25 min followed by 1300xg for a further 25 min. The PMN layer was removed and placed in a clean 50cc centrifuge tube. Thirty ml of Hanks Balanced Salt Solution (Gibco) containing 20mM HEPES (Gibco) and 0.2% glucose (Fisher) was added to each tube, which were then centrifuged at 1900xg for 3 min. The PMNs were washed 3X in the same buffer by centrifugation at 1900xg for 3 min. PMNs were counted using a hemacytometer and resuspended to 2 $x10^6$/ml and held at room temperature until use.

Procedure

1. 20 ul of preparation of activated platelets were placed in each of 28 1.5 ml eppendorf tubes (14 duplicate samples).

2. 20 ul of the diluted liposomes at 10 ug, 5 ug or 2 ug, or of the control buffers, were added to the appropriate tube of each duplicate.

3. The platelets were incubated with the liposome preparations for 20 min. at room temp.

4. Neutrophils or HL-60 cells at 2 x 10^6 cells/ml were each added to one set of liposome treated platelets. 20 ul of cell suspension were added to each tube.

5. The tubes were mixed and allowed to stand at room temperature for 20 min. Then they were applied to a

hemacytometer and the cells were scored as positive (2 or more platelets attached/cell) or negative (less than 2 platelets attached/cell).

As shown in Fig. 7, liposomes containing selected glycolipids having terminal sequences which contained SLX (S-diLE^X, Table 1) dramatically inhibited adhesion of HL-60 cells to activated platelets. Liposomes containing glycolipids with Le^X (di-Le^X) or other related carbohydrate structures (Table 1) exhibited minimal inhibition that was not dependent on the structure of the carbohydrate group. Similar results were obtained with PMN cell adhesion (Fig. 8). These experiments show that biosynthetically produced SLX or similar SLX mimetic compounds when formulated into liposomes compositions can serve as therapeutic compounds for, e.g., the reduction of leukocyte binding to platelets in inflammatory sites.

EXAMPLE VII

Hexasaccharide SLX blocks binding of Neutrophils to platelets

In this example the ability of a minimal tetra-saccharide SLX to inhibit GMP-140 adhesion was compared to that of a hexasaccharide SLX. Briefly, platelets and neutrophils were isolated by the methods described above. Platelets were activated with thrombin and then incubated with dilutions of various oligosaccharides. Neutrophils were added and the effect of the saccharides on the adhesion of neutrophils to activated platelets was determined. The oligosaccharides used were as follows: SLX(hexa), NeuAcα2,3Galβ1,4 (Fucα1,3) GlcNacβ1,3 Galβ1,4Glc-O-$CH_2CH_2SiMe_3$ (the generous gift of Professor Hasegawa, Gifu University, Japan) and SLX(tetra), NeuAcα2,3Galβ1,4 (Fucα1,3)GlcNAc.

Procedure

1. Platelets were isolated as described above and were activated ($2x10^8$/ml) by incubation for 20 min at room temperature with thrombin at a final concentration of 0.25U/ml.

2. Neutrophils were isolated by layering heparinized blood over Mono-Poly Resolving Medium (Ficoll-Hypaque-Flow Laboratories), followed by centrifugation for 25 min at 2000rpm and then, a further 25 min at 2500rpm as described above.

3. For the assay, 20 μl of the platelet suspension ($2x10^8$/ml) was placed in an Eppendorf centrifuge tube. An equal volume of the oligosaccharide preparations at concentrations from 500 μg/ml to 2.0 μg/ml, or of glycolipid-liposome preparations (prepared as described, above), at concentrations from 2 μg/ml to 0.25 μg/ml, was added and the tubes were allowed to stand at room temperature for 20 min. Twenty μl of the neutrophil preparation ($2x10^6$/ml) was then added and the tubes were allowed to stand for a further 20 min at room temperature.

4. Adhesion of activated platelets to the neutrophils was assessed microscopically. One hundred neutrophils were evaluated. They were scored as positive if 2 or more platelets were attached and negative if less than 2 platelets were bound. The percent of cells with 2 or more bound platelets was calculated.

The results of two identical experiments are shown in Table 2.

TABLE 2

OLIGOSACCHARIDE	AMOUNT REQUIRED FOR 50% INHIBITION (μM)	
	EXPERIMENT 1	EXPERIMENT 2
SLX (hexa)	4.0	2.2
SLX (tetra)	69.0	54.0
Le^x	78.0	43.0

As indicated in Table 2 above, approximately 20 times more of the SLX-tetra saccharide is required for 50% inhibition of GMP-140 mediated binding of neutrophils to thrombin activated platelets than of the SLX-hexa saccharide. The amount of the tetra-saccharide required is approximately that needed for a similar degree of inhibition when the non-sialylated Le^x was used. These results suggest that the 5-6 sugar SLX moiety, especially including the GlcNAcβ1,3Gal structure constitutes a portion of the ligand for GMP-140 necessary for binding.

EXAMPLE VIII

Blocking adhesion using variant SLX structures

This example describes experiments testing various glycolipid structures on liposomes. In particular, SY2, a sialylated polysaccharide in which the fucose instead of being attached to the ultimate GlcNAc as in SLX, is attached to the penultimate GLcNAc was tested. Platelets and neutrophils were isolated by the methods described above. Platelets were activated with thrombin and then incubated with dilutions of various glycolipids embedded in liposomes prepared as described above. Neutrophils were added and the effect of the glycolipids on the adhesion of neutrophils to activated platelets was determined.

Structures of the various glycolipids examined are as follows: SDiY2, NeuGcα2,3Galβ1,4(Fucα1,3)GlcNacβ1,3Galβ1,4(Fucα1,3)GlcNAcβ1,3Galβ1,4Glcβ1,1Cer; SLX, NeuGcα2,3Galβ1,4(Fucα1,3)GlcNAcβ1,3Galβ1,4Glcβ1,1Cer; SY2, NeuGcα2,3Galβ1,4GlcNAcβ1,3Galβ1,4(Fucα1,3)GlcNAcβ1,3Galβ1,4Glcβ1,1Cer; SH, NeuGcα2,3Galβ1,4GlcNAcβ1,3Galβ1,4GlcNAcβ1,3Galβ1,4Glcβ1,1Cer; SPG, NeuGcα2,3Galβ1,4GlcNAcβ1,3Galβ1,4Glcβ1,1Cer.

The results of two identical experiments are shown in Table 3.

Table 3

GLYCOLIPID	AMOUNT REQUIRED FOR 50% INHIBITION (μM)
SY2 (Exp 1)	0.325
SY2 (Exp 2)	0.345
SLX (hexa)	0.30
SdiY2	0.36
SPG	No Inh.
SH	No Inh.

These results show that SY2 inhibited GMP-140 mediated adhesion of neutrophils to thrombin activated platelets equally as well as did SLX and SDiY2.

EXAMPLE IX

Blocking adhesion using further variants of SLX

The example demonstrates that the affinity of sialylated Le^x(SLX) for GMP-140 is the same whether the terminal sialic acid is in the form N-Acetyl neuraminate (NeuAc) or N-Glycol neuraminate (NeuGc). All materials were prepared as described above. Platelets and neutrophils were isolated by the methods described. Platelets were activated with thrombin and then incubated with dilutions of various glycolipids contained in liposomes. Neutrophils were added and the effect of the glycolipids on the adhesion of neutrophils to activated platelets was determined.

The results of an experiment in which synthetic SLX(NeuAc) and a preparation of SLX prepared by enzymatic fucosylation of of sialylparagloboside purified from bovine erythrocytes SLX(NeuGc), were directly compared are shown in Table 4.

Table 4

GLYCOLIPID	SOURCE	AMOUNT REQUIRED FOR 50% INHIBITION (μM)
SLX (NeuGc)	(Bovine Erythrocytes)	0.74
SLX (NeuAc)	(Synthetic)	0.67

These results show that SLX-hexasaccharide inhibited GMP-140 mediated adhesion of neutrophils to thrombin activated platelets equally well whether the sialic acid was NeuAc or NeuGc. This result indicates that either the N-acetyl or N-glycollyl derivative of sialic acid would also allow recognition of SLX by ELAM-1.

Various glycolipids were also tested in the same assay. The results are presented in Figure 9Structures of the glycolipids tested are as follows: SLX(hexa), NeuGcα2,3Galβ1,4(Fucα1,3)GlcNacβ1,3Galβ1,44Glcβ1,1Ceramide; α2,3 SLX cer, NeuAcα2,3Galβ1,4(Fucα1,3)GlcNAcβ1,3 Galβ1,4Glcβ1,1Ceramide; α2,6 SLX cer, NeuAcα2,6Galβ1,4 (fucα1,3)GlcNacβ1,3Galβ1,4Glcβ1,1Ceramide; SH, NeuGcα2,3Galβ1,4 GlcNAcβ1,3Galβ1,4GlcNAcβ1,3Galβ1,4Glcβ1,1Ceramide.

EXAMPLE X

Blocking adhesion using synthetic SLX

This example demonstrates that synthetic SLX binds ELAM-1 and inhibits neutrophil adhesion to activated endothelium. This example also shows that the linkage of the sialic acid affects binding to ELAM-1.

Two synthetic compounds were prepared. One comprised sialic acid in an α2.3 linkage, as in naturally occurring SLX. The second comprised sialic acid in an α2,6 linkage, to examine the importance of the nature of the linkage to receptor binding.

Liposomes were prepared by adding 12 μl of absolute ETOH to each tube, warming briefly in a 50°C water bath and sonicating for 2 min. 238 μl of warm phosphate buffered saline (PBS) was added slowly to each tube while sonicating and sonication was continued for a further 10 min. The final concentration of stock liposomes was 400 μg glycolipids/ml in 5% ETOH/PBS.

Procedure

1. HUVECs, PMNs, and liposomes were prepared as described above.

2. The stimulated HUVEC assay plate was removed from the incubator and the wells were washed two times with RPMI 1640 containing 5 mg/ml bovine serum albumin (BSA).

3. Liposomes stocks were diluted in the HBSS/BSA buffer to make solutions equal to: 40 μg/ml, 30 μg/ml, 15 μg/ml, 7.5 μg/ml, 3.75 μg/ml and 1.87 μg/ml. Similar dilutions were prepared from a control stock consisting of PBS-5% ETOH.

4. The medium was removed from the wells of the assay plate with a pasteur pipette, a few wells at a time.

5. 0.05 ml of each liposome suspension was added to duplicate wells on the stimulated assay plate. Control wells received the liposome buffer containing ethanol HBSS and BSA at the same concentrations as in the liposome containing wells. Control buffer was plated on unstimulated and stimulated HUVEC. Liposome containing samples were added to stimulated wells only.

6. The plates were incubated for 40 min at 37°C and then 50 μl of PMNs were added to the assay wells. The final concentration of cells was $5x10^5$ well in 100 μl.

7. The assay plate was returned to the incubator (5% CO_2, 37 °C) for 8 min.

8. Unbound cells were removed from the wells of the assay plates by systematic resuspension using a p200 multichannel pipette followed by addition and removal of 0.2 ml of medium.

9. All of the medium was removed from the wells and 50 μl of solubilization buffer was added. This consisted of citrate buffer (24.3 ml of 0.1 M Citric acid, 10.5 g/500 ml + 25.7 ml of 0.2 M dibasic sodium phosphate, 14.2 g/500 ml and SQ H_2O to 100 ml) containing 0.1% NO-40 detergent.

10. The plate was incubated on a rotary shaker for 10 min and then 0.05 ml of OPDA solution [8 mg o-phenylene-diamine, Sigma cat# P-1526, 8 μl of 30% H_2O_2 and 10 ml of citrate

buffer (as above)] was added to each well. The reaction was allowed to develop for 15 min and then 25 µl of 4N H_2SO_4 was added to each well to stop the reaction.

11. A reagent bulk was prepared by mixing 100 µl volumes of the solubilization buffer and the OPDA solution with 50 µl of 4N H_2SO_4.

12. 100 µl of supernatant was removed from each of 2 wells and transferred to a flexible ELISA assay plate (Falcon). The plate scanned spectrophotometrically at 492 nM within 30 min.

The results of the two experiments are presented in Table 5, below.

Table 5

	Concentration	(2,6) SLex Mean	(2,3)SLex Mean
1	20 µg/ml	0.663	0.156
2	15 µg/ml	0.636	0.270
3	7.5 µg/ml	0.602	0.359
4	3.75 µg/ml	0.655	0.483
5	1.87 µg/ml	0.690	0.580
6	.47 µg/ml	0.695	0.642
7	0 µg/ml	0.710	0.716

	Concentration	Control +IL-1B Mean	Control -IL-1B Mean
1	20 µg/ml	0.657	0.010
2	15 µg/ml	0.740	0.010
3	7.5 µg/ml	0.658	0.013
4	3.75 µg/ml	0.698	0.009
5	1.87 µg/ml	0.725	0.014
6	.47 µg/ml	0.782	0.018
7	0 µg/ml	0.708	0.016

These results show that liposomes containing synthetic α(2,3)SialylLe^x but not α(2,6)SialylLe^x inhibit neutrophil adhesion to activated endothelium in an ELAM-1 dependent binding assay. Thus, the α2,3 linkage of the sialic acid appears to be necessary for recognition by ELAM-1. In addition, the results show that a synthetically produced oligosaccharide, α(2,3)SialylLe^x, binds to ELAM-1 and blocks binding of neutrophils to activated endothelium. This compound or derivatives of this compound therefore constitute potential anti-inflammatory drug candidates.

EXAMPLE XI

Treatment of HL60 Cells with Endo-β-Galactosidase

This example describes experiments to determine whether the internal β-galactose-backbone sugar linkage of sialylated Le^x of HL60 cells was susceptible to cleavage by Endo-β-Galactosidase, an enzyme known to cleave an internal β-galactose linkage in polylactosaminyl structures, but not β-gal when GlaNAc is attached to mannose (core-type structures).

Procedure:

Platelets were isolated and activated with thrombin by the methods described above. Cultured HL60 cells were treated with endo-β-galactosidase as described below and the effect of enzyme treatment on the GMP-140 mediated adhesion of HL60 cells to activate platelets was determined.

Enzyme treatment of the HL60 cells was carried out as follows: 12.4 x10^6 cells were washed twice with Hanks Balanced Salt Solution containing 20mM HEPES and 0.2% glucose, followed by a single wash step in normal saline. The endo-β-galactosidase (0.1 Unit, ICN Chemicals, Inc., Irvine, CA) was dissolved in 200μl normal saline and 200μl sodium acetate buffer, pH 6.01. 200μl (containing 0.05U of enzyme) was added to 3 x 10^6 HL60 cells, and 200μl of the acetate buffer was added to a similar number of cells to be used as the buffer control. Both tubes were incubated at 37°C for 60 min. with

gentle shaking. The tubes were then cooled in ice and the cells were washed three times in HBSS containing HEPES and glucose and were then counted and suspended to $2x10^6$/ml.

For the assay, 20µl of Tyrode-HEPES buffer, pH 7.2 was placed in an Eppendorf tube. The same volume of activated platelets ($2x10^8$/ml) and HL60 cells ($2x10^6$/ml) was added and, after mixing, the tubes were allowed to stand at room temperature for 20 min. Adhesion of platelets to the HL60 cells was assessed microscopically as described earlier for adhesion of activated platelets to neutrophils.

The results of these experiments indicated that treatment of HL60 cells with Endo-β-Galactosidase inhibited their ability to bind to thrombin activated platelets by 87.5%. Thus, the minimal SLX-containing tetrasaccharide ligand for GMP-140 is probably attached to a lactose or polylactosaminyl structure rather than a mannose.

EXAMPLE XII

Fucosylated Polysaccharide blocks binding of Neutrophils to Platelets

In this example the ability of a fucosylated polysaccharide to inhibit GMP-140 mediated adhesion was compared to that of the non-fucosylated polysaccharide, a hexasaccharide SLX and Le^x. Briefly, platelets and neutrophils were isolated by the methods described above. Platelets were activated with thrombin and then incubated with dilutions of various oligosaccharides. Neutrophils were added and the effect of the saccharides on the adhesion of neutrophils to activated platelets was determined. The oligosaccharides used were as follows: Native polysaccharide and its fucosylated derivative (the preparation of both is described, below); SLX hexasaccharide, LNF III (Le^x) and LNF I (the structures are described above).

The conversion of a polysaccharide which contains the linear core structure of SLX into a polyvalent SLX containing polysaccharide was achieved by enzymatic fucosylation. The native polysaccharide type Ia was obtained from Group B Streptococcus as described by Jennings et al., Biochem. 22 1258-1263 (1983) which is incorporated herein by reference.

The appropriate bacterial strains are deposited with the American Type Culture Collection and have Deposit Nos. 12400, 31574, 12401, and 31575.

To prepare the fucosylated polysaccharide, the native type Ia polysaccharide 1 mg. was dissolved in a mixture of 6 μL of 1 M manganese chloride, guanosine 5'-diphosphate β-L-fucose with a radiolabelled tracer (specific activity 1.82 x 10^6 cpm/μmol), 0.9 μmoles in water 90 μL and water 137 μL. To this was added 100 μL solution of 3/4 fucosyl transferase isolated from human milk as previously described by Prieels et al., J.Biol.Chem. 256 10456-10463 (1981) which is incorporated herein by reference.

The reaction mixture of concentrated against a membrane (100K cut off) several times with water and the retentate lymphysized to give a powder. Resuspension and counting of label indicated approximately one half (i.e., about 100) of the available acceptor side chains had been fucosylated.

Procedure:

Platelets were isolated as described above and were activated ($2x10^8$/ml) by incubation for 20 min at room temperature with thrombin at a final concentration of 0.25U/ml.

Neutrophils were isolated by layering heparinized blood over Mono-Poly Resolving Medium (Ficoll-Hypaque, Flow Laboratories), followed by centrifugation for 25 min at 2000rpm and then, a further 25 min at 2500rpm as described above.

For the assay, 20 μl of the platelet suspension ($2x10^8$/ml) was placed in an Eppendorf centrifuge tube. An equal volume of the oligosaccharide preparations at concentrations from 500μg/ml to 2.0μg/ml was added and the tubes were allowed to stand at room temperature for 20 min. Twenty μl of the neutrophil preparation ($2x10^6$/ml) was then added and the tubes were allowed to stand for a further 20 min at room temperature.

Adhesion of activated platelets to the neutrophils was assessed microscopically. One hundred neutrophils were evaluated. They were scored as positive if 2 or more platelets were attached and negative if less than 2 platelets were bound.

The percent of cells with 2 or more bound platelets was calculated.

As shown in Table 6, the fucosylated polysaccharide very efficiently inhibited GMP-140 mediated binding of neutrophils to thrombin activated platelets- 50% inhibition was achieved with less than 1μg/ml. This compared to 20μg/ml which was required of the native polysaccharide and 8μg/ml of the SLX hexasaccharide for a similar degree of inhibition.

TABLE 6

OLIGOSACCHARIDE	AMOUNT REQUIRED FOR 50% INHIBITION (μg/ml)
Native Polysaccharide	20
Fucosylated Polysaccharide	<1
SLX Hexasaccharide	8
LNF III (Le^x)	35
LNF I	No Inhibition

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a compound having a selectin-binding oligosaccharide moiety.

2. A composition of claim 1, wherein the oligosaccharide moiety contains fucose and sialic acid.

3. A composition of claim 1, wherein the oligosaccharide moiety is R_1—Galβ1,4(Fucα1,3)GlcNAcβ1—R_2, wherein

R_1 is selected from the group consisting of NeuAcα2,3, NeuGcα2,3, NeuAcα2,3Galβ1,4GlcNAcβ1,3, and NeuGcα2,3Galβ1,4GlcNAcβ1,3; and

R_2 is selected from the group consisting of 1,3βGal, 1,2αMan, and 1,6αGalNAc.

4. A composition of claim 1, wherein the oligosaccharide moiety is on a polysaccharide.

5. A composition of claim 4 wherein the polysaccharide is a fucosylated polysaccharide type Ia of Group B streptococcus.

6. A composition of claim 5 wherein the polysaccharide has molecular weight between about 5,000 and 300,000 daltons.

7. A composition of claim 5 wherein the polysaccharide comprises between about 5 and about 200 fucosylated side chains.

8. A composition of claim 7 wherein the polysaccharide comprises between about 50 and about 150 fucosylated side chains.

9. A composition of claim 1 wherein the compound is a glycoprotein or a glycolipid.

10. A composition of claim 1, wherein selectin-binding moiety binds a selectin receptor expressed on a vascular endothelial cell or a platelet.

11. A composition of claim 1, wherein the cell surface receptor is ELAM-1 or GMP-140.

12. A pharmaceutical composition which comprises a pharmaceutically acceptable carrier and a liposome having a compound which comprises a selectin-binding oligosaccharide moiety.

13. A composition of claim 12, wherein the liposome encapsulates an anti-inflammatory chemotherapeutic agent.

14. A composition of claim 12, wherein the anti-inflammatory agent is cyclosporin A.

15. A composition of claim 12, wherein the oligosaccharide moiety comprises a fucose and a sialic acid residue.

16. A composition of claim 12, wherein the oligosaccharide moiety is R_1—Galβ1,4(Fucα1,3)GlcNAcβ1—R_2, wherein

R_1 is selected from the group consisting of NeuAcα2,3, NeuGcα2,3, NeuAcα2,3Galβ1,4GlcNAcβ1,3, and NeuGcα2,3Galβ1,4GlcNAcβ1,3; and

R_2 is selected from the group consisting of 1,3βGal, 1,2αMan, and 1,6αGalNAc.

17. A composition of claim 12 wherein the compound is a glycoprotein.

18. A composition of claim 12 wherein the glycoprotein has a molecular weight between 40,000 and about 250,000 daltons.

19. A composition of claim 1, wherein the compound is a glycolipid.

20. A composition of claim 14, wherein the glycolipid has a molecular weight between about 600 and about 4,000 daltons.

21. A composition of claim 12, wherein the compound is an oligosaccharide.

22. A composition of claim 12, wherein the selectin-binding moiety binds a selectin receptor expressed on a vascular endothelial cell or a platelet.

23. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a compound having an oligosaccharide moiety capable of selectively binding a selectin, the compound comprising:

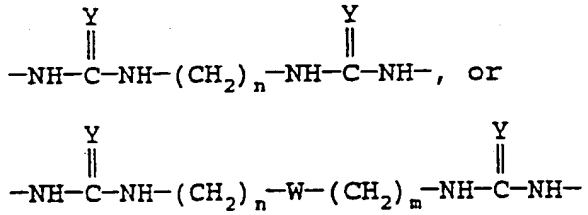
$$L_1-X_1$$

wherein

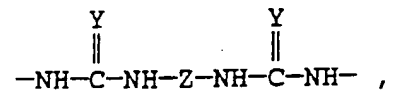
L_1 is the oligosaccharide moiety and is selected from the group consisting of SLX and SY2;

X_1 is selected from the group consisting of H, OH, NH_2, NHR_1, OR_1, OAryl, OAlkylAryl, OCeramide, R_1, Aryl, and AlkylAryl, wherein R_1 is a C_1-C_{20} alkyl.

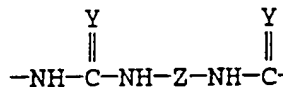
24. A compound of claim 23, wherein L is linked to



wherein, n and m are the same or different and are integers from 2 to 12; Y is O or S; and W is O, S, or NH; or to



wherein, n and m are integers from 2 to about 12;
Y is O or S; and
W is O, S, or NH, or to



wherein, Z is a 5- to 14-membered ring and the substituents on the ring are in a cis- or trans-relationship, and the substituents are in a 1,2 to 1,(p/2)+1 arrangement, where p is the size of the ring.

25. A composition comprising a heterocyclic compound having two nitrogen atoms and two oligosaccharide moieties of claim 23, each moiety being linked to a nitrogen atom.

26. A composition of claim 25, wherein the heterocyclic compound is a six or seven membered ring, selected from the group of piperazine or homopiperazine.

27. A composition comprising an amino acid linked to the oligosaccharide moiety of claim 23.

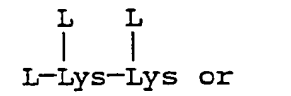
28. A composition of claim 27, wherein the amino acid is lysine, homolysine, ornithine, diaminobutyric acid, asparagine or diaminopropionic acid.

29. A composition of claim 28, wherein the amino acid is incorporated in an oligopeptide.

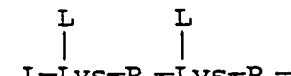
30. A composition of claim 29, wherein the oligopeptide comprises lysine, homolysine, ornithine, diaminobutyric acid, asparagine or diaminopropionic acid.

31. A composition of claim 30, wherein the oligopeptide further comprises an alanine, tyrosine or radioiodinated tyrosine.

32. A composition of claim 31, wherein the oligopeptide comprises, in a direction from the N-terminus to the C-terminus,



or



wherein R_1 and R_2 are any amino acid residue.

33. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and an immunoglobulin capable of selectively binding an oligosaccharide ligand recognized by a selectin cell surface receptor.

34. A composition of claim 33 wherein the ligand comprises the sequence NeuAcα2,3Galβ1,4(α1,3Fuc)GlcNAcβ1.

35. A composition of claim 33, wherein the oligosaccharide ligand is expressed by a leukocyte.

36. A composition of claim 33, wherein the selectin is expressed by a vascular endothelial cell or a platelet.

37. A composition of claim 33, wherein the selectin is ELAM-1.

38. A composition of claim 33, wherein the immunoglobulin is CSLEX-1, FH6, SNH_3, SNH_4 or VIM-2.

39. A composition of claim 33, wherein the composition is in unit dosage form.

40. A pharmaceutical composition for inhibiting selectin-mediated intercellular adhesion which comprises a compound capable of selectively binding a selectin receptor.

41. A composition of claim 40, wherein the compound comprises an oligosaccharide moiety having a fucose and a sialic acid residue.

42. A composition of claim 41, wherein the oligosaccharide moiety is SLX or SY2.

43. A composition of claim 40 wherein the compound is an immunoglobulin.

44. A composition of claim 40, wherein the cell surface receptor is expressed on vascular endothelial cells or platelets.

45. A composition of claim 40, wherein the cell surface receptor is ELAM-1 or GMP-140.

46. A composition of claim 40 wherein the selectin-mediated intercellular adhesion is associated with an inflammatory disease response.

47. A composition of claim 46, wherein the inflammatory disease process is reperfusion injury, asthma, psoriasis, septic shock or nephritis.

48. A method for inhibiting selectin-mediated intercellular adhesion in a patient, the method comprising administering a therapeutically effective dose of the pharmaceutical composition of claims 1, 12, 23, 33.

49. A method of claim 48, wherein the intercellular adhesion is associated with an inflammatory condition.

50. A method of claim 49, wherein the inflammatory condition is reperfusion injury, asthma, psoriasis, septic shock, or nephritis.

51. A method of inhibiting intercellular adhesion mediated by a selectin cell surface receptor in a patient, the method comprising administering to the patient a therapeutically effective dose of a compound having at least one oligosaccharide moiety capable of selectively binding the cell surface receptor.

52. A method of claim 51, wherein the oligosaccharide moiety comprises a fucose and a sialic acid residue.

53. A method of claim 51, wherein the oligosaccharide moiety is R_1—Galβ1,4(Fucα1,3)GlcNAcβ1—R_2, wherein

R_1 is selected from the group consisting of NeuAcα2,3, NeuGcα2,3, NeuAcα2,3Galβ1,4GlcNAcβ1,3, and NeuGcα2,3Galβ1,4GlcNAcβ1,3; and

R_2 is selected from the group consisting of 1,3βGal, 1,2αMan, and 1,6αGalNAc.

54. A method of claim 51, wherein the cell surface receptor is ELAM-1 or GMP-140.

55. A method of claim 51 wherein the oligosaccharide moiety is on a liposome.

56. A method of claim 51 wherein the oligosaccharide moiety is on a polysaccharide.

57. A method of claim 51 wherein the polysaccharide is a fucosylated polysaccharide type Ia of Group B streptococcus.

58. A method of claim 51, wherein the selectin mediates adhesion of a leukocyte, monocyte or neutrophil to the endothelial cell.

59. A method of claim 51, wherein the intercellular adhesion is associated with an inflammatory condition.

60. A method of claim 59, wherein the inflammatory condition is reperfusion injury, asthma, psoriasis, septic shock, nephritis, or traumatic shock.

61. A method of claim 51, wherein the intercellular adhesion is associated with metastasis.

62. A method of treating an inflammatory disease process mediated by a selectin cell surface receptor in a patient, the method comprising administering to the patient a therapeutically effective dose of a biomolecule having an oligosaccharide moiety capable of selectively binding the cell surface receptor.

63. A method of claim 62, wherein the oligosaccharide moiety contains sialic acid and fucose.

64. A method of claim 62, wherein the biomolecule has a chemical formula selected from the group consisting of NeuAcα2,3Galβ1,4(Fucα1,3)GlcNAcβ1-R_1, NeuGcα2,3Galβ1,4(Fucα1,3)GlcNAcβ1-R_1, and NeuGcα2,3Galβ1,4GlcNAcβ1,3Galβ1,4(Fucα1,3)GlcNAcβ1-R_1; wherein R_1 is selected from the group consisiting of an amino acid, an oligopeptide, a protein, a glycoprotein, and a polysaccharide.

65. A method of claim 62, wherein the cell surface receptor is ELAM-1 or GMP-140.

66. A method of assaying a test compound for the ability to inhibit selectin-mediated cellular adhesion, the method comprising the steps of:

contacting the test compound with a selectin receptor and an isolated selectin-binding agent; and

detecting the ability of the test compound to inhibit binding between the receptor and the agent.

67. A method of claim 66 wherein the agent comprises an SLX moiety, an SLX mimetic, or an immunoblogulin.

68. A method of claim 66 wherein the receptor is on an activated endothelial cell or a platelet.

69. A method of claim 66 wherein the receptor, the agent, or the test compound is labelled.

70. A method of claim 66 wherein the receptor or the agent are immobilized on a solid surface.

71. A method of claim 66 wherein the step of detecting the inhibition of binding is carried out by detecting a physiological change in a cell bearing the receptor.

72. A method of claim 66 wherein the test compound is an oligosaccharide or a glycoconjugate.

73. A method of claim 66 wherein the test compound comprises fucose and sialic acid.

74. A method of claim 73 wherein the test compound comprises an SLX moiety.

75. A method of claim 66 wherein the test compound is an immunoblogulin.

76. A method of assaying for the ability of an oligosaccharide moiety to selectively bind a selectin receptor, the method comprising contacting a test compound having the moiety with the receptor and determining the binding of the compound to the receptor.

77. A method of claim 76 wherein the test compound is labelled.

78. A method of claim 76 wherein the receptor is immobilized on a solid surface.

79. A method of claim 76 wherein the moiety comprises fucose and sialic acid.

80. A method of claim 76 wherein the step of contacting further comprises contacting the test compound with a selectin-binding agent and the step of determining binding is carried out by detecting the inhibition of binding between the receptor and the agent.

81. A method of claim 80 wherein the agent is an immunoblogulin.

82. A method of claim 80 wherein the agent comprises an SLX moiety.

83. A method of assaying a test compound for the ability to selectively bind an SLX moiety, the method comprising contacting the test compound with an isolated SLX moiety and determining the binding of the compound to the isolated SLX moiety.

84. A method of claim 83 wherein the isolated SLX moiety is immobilized on a solid surface.

85. A method of claim 83 wherein the test compound is labelled.

86. A method of claim 83 wherein the test compound is an immunoblogulin.

87. A method of claim 83 wherein the step of contacting further comprises contacting the test compound with an SLX-binding agent and the step of determining binding is carried out by detecting the inhibition of binding between the isolated SLX moiety and the SLX-binding agent.

88. A method of claim 87 wherein the SLX-binding agent is an immunoglobulin.

89. A method of claim 87 wherein the SLX-binding agent is a selectin receptor.

90. A pharmaceutical composition comprising a suitable carrier and a compound having a selectin-binding oligosaccharide moiety having the formula:

$$R_1 - Gal\beta 1,4(R_2)GlcNac\beta - R_3,$$

wherein, R_1 is NeuAcα2,3; NeuGcα2,3; NeuAcα2, 3Galβ1,4GlcNAcβ1,3;
or NeuGcα2,3Galβ1,4GlcNacβ1,3;
R_2 is L-Fucα1,3; D-Fucα1,3; Araα1,3; (R,S)-5-alkyl-Araα1,3 or (R,S)-5-aryl-Araα1,3;
R_3 is 1,3βGal; 1,2αMan; or 1,6αGalNAc.

91. A composition of claim 90, wherein the oligosaccharide moiety is on a glycoprotein or glycolipid.

92. A composition of claim 90, wherein the oligosaccharide moiety binds a selectin receptor expressed on a vascular endothelial cell or a platelet.

93. A composition of claim 90, wherein the cell surface receptor is ELAM-1 or GMP-140.

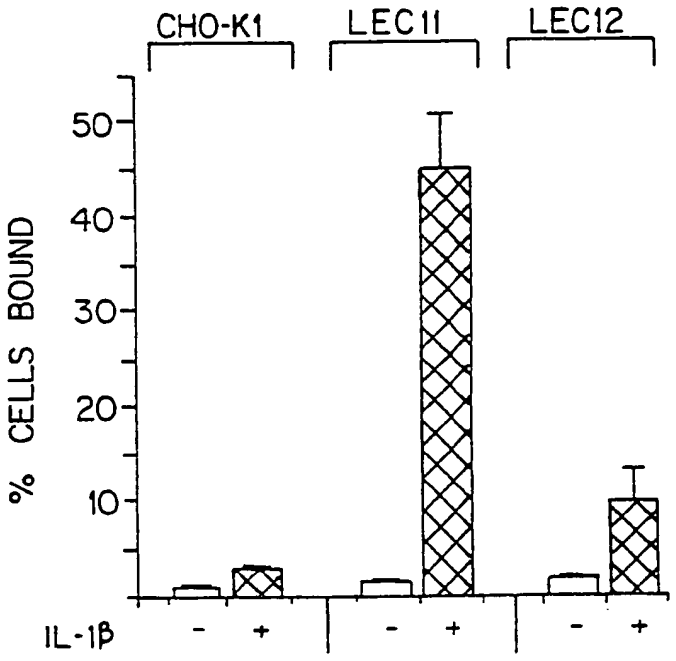


FIG. 1.

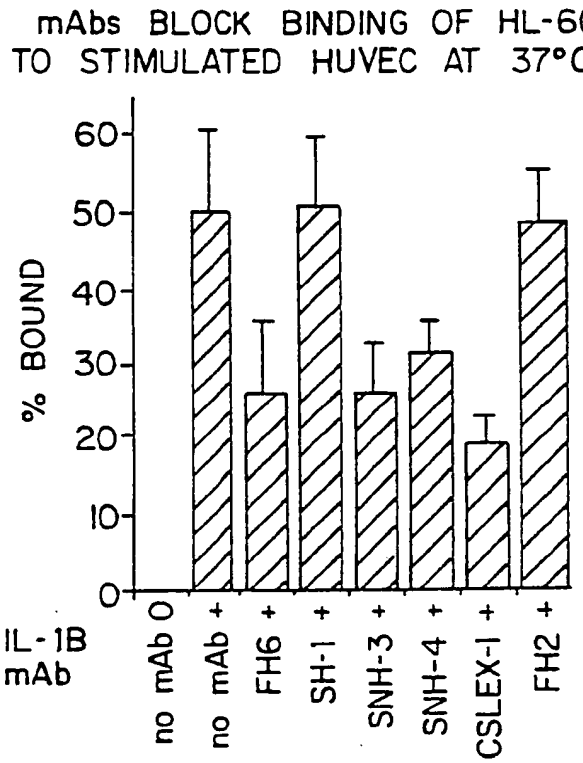


FIG. 2A.

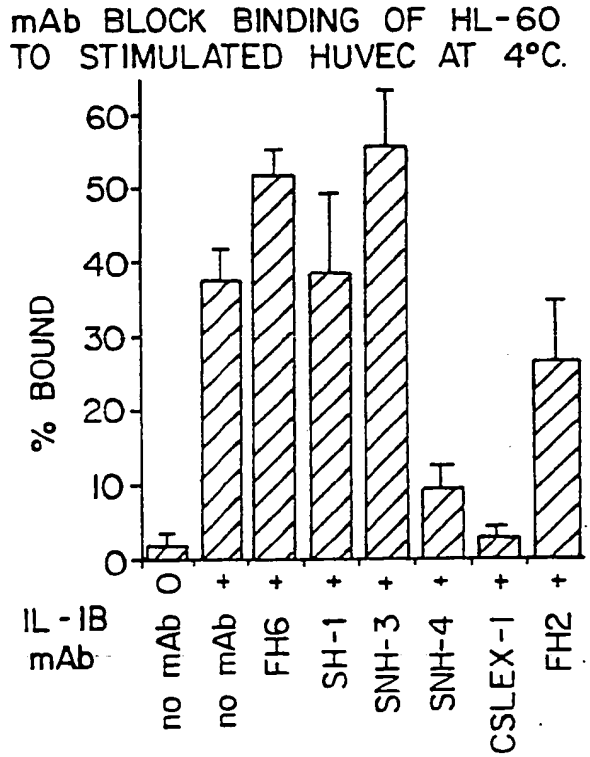


FIG. 2B.

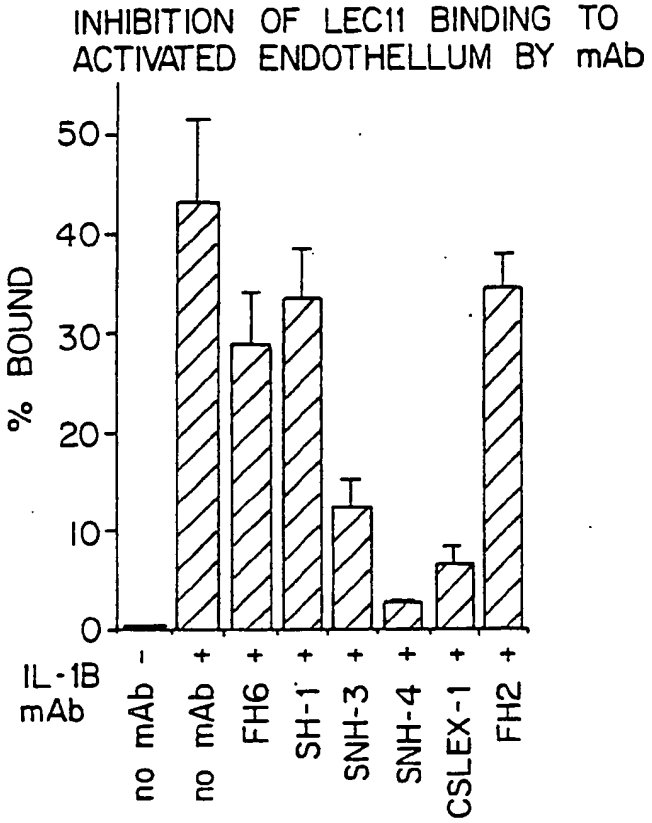


FIG. 3A.

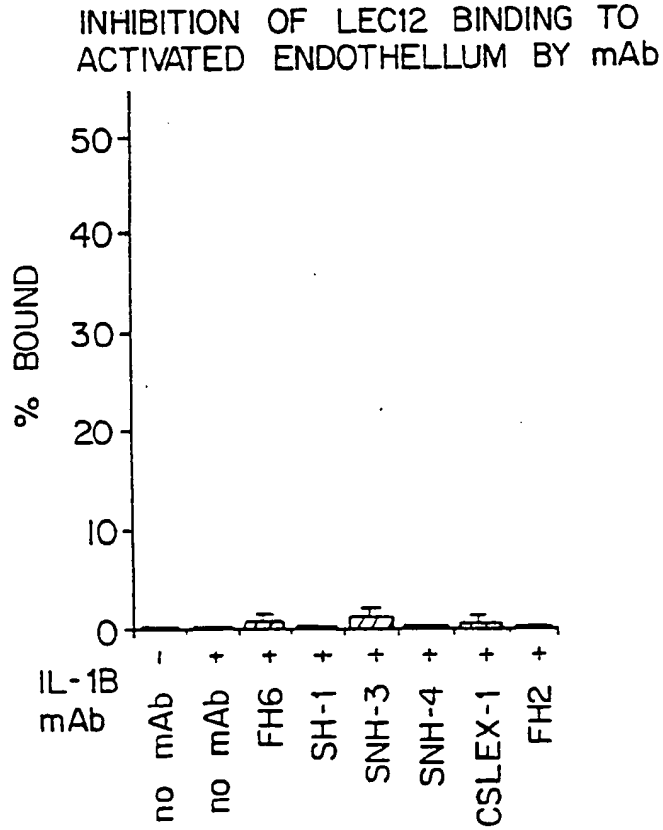


FIG. 3B.

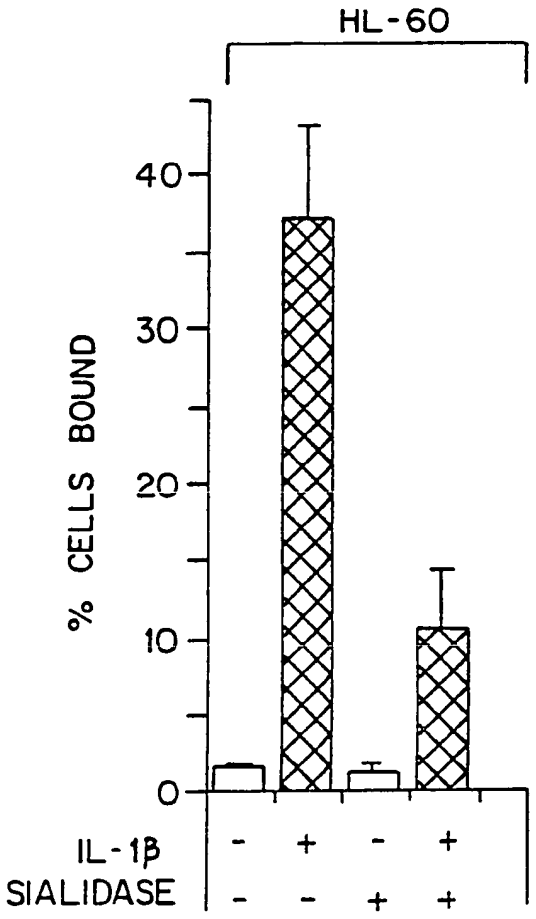


FIG. 4A.

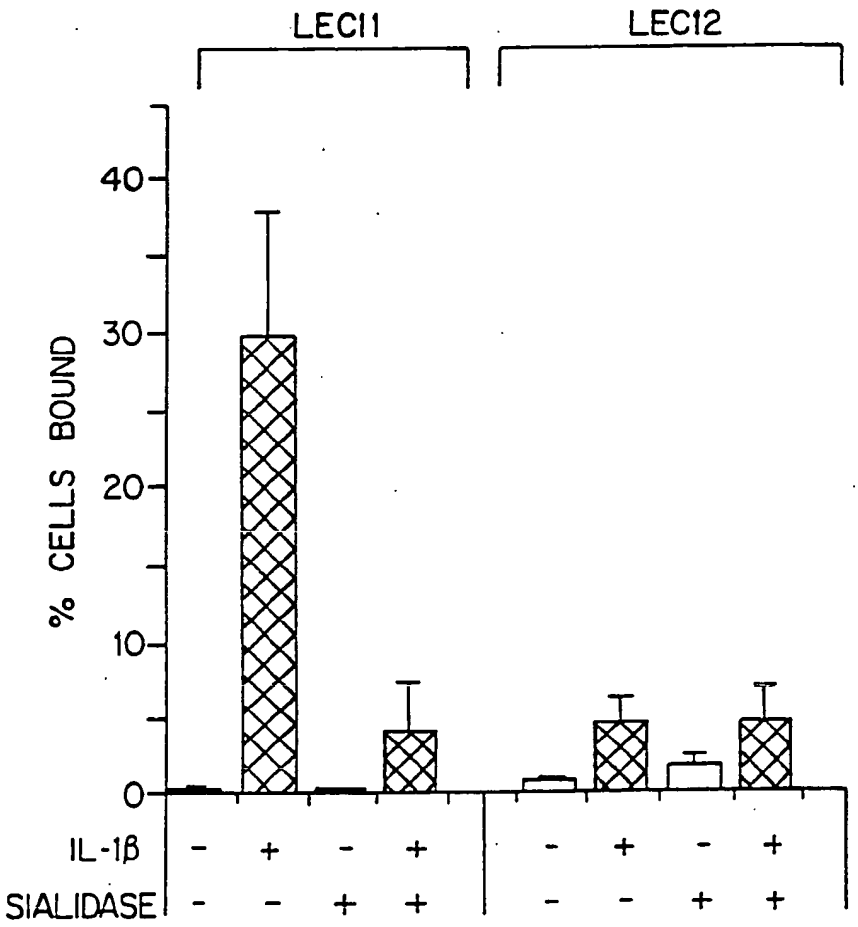


FIG. 4B.

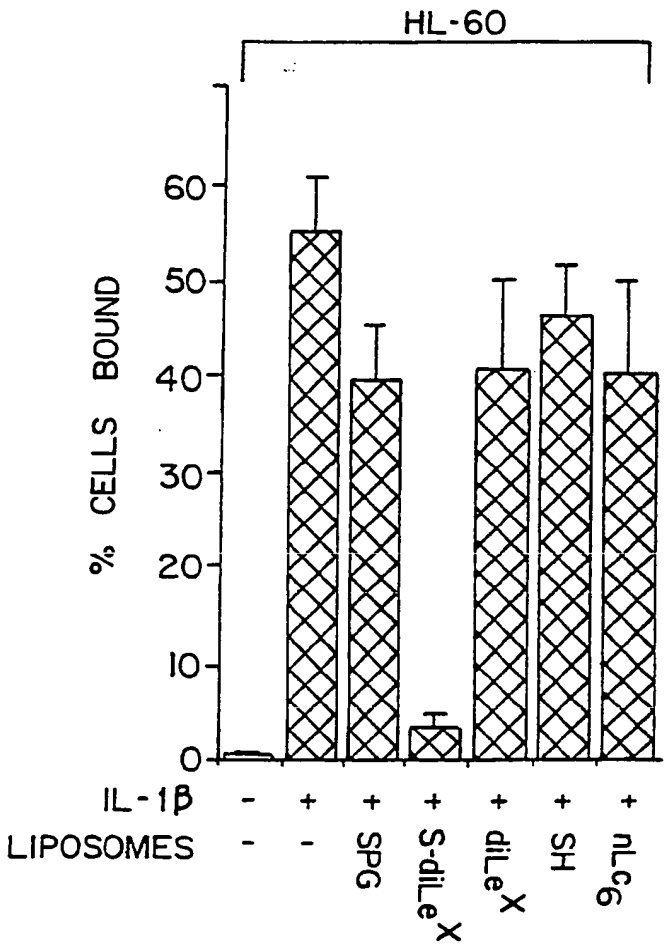


FIG. 5A.

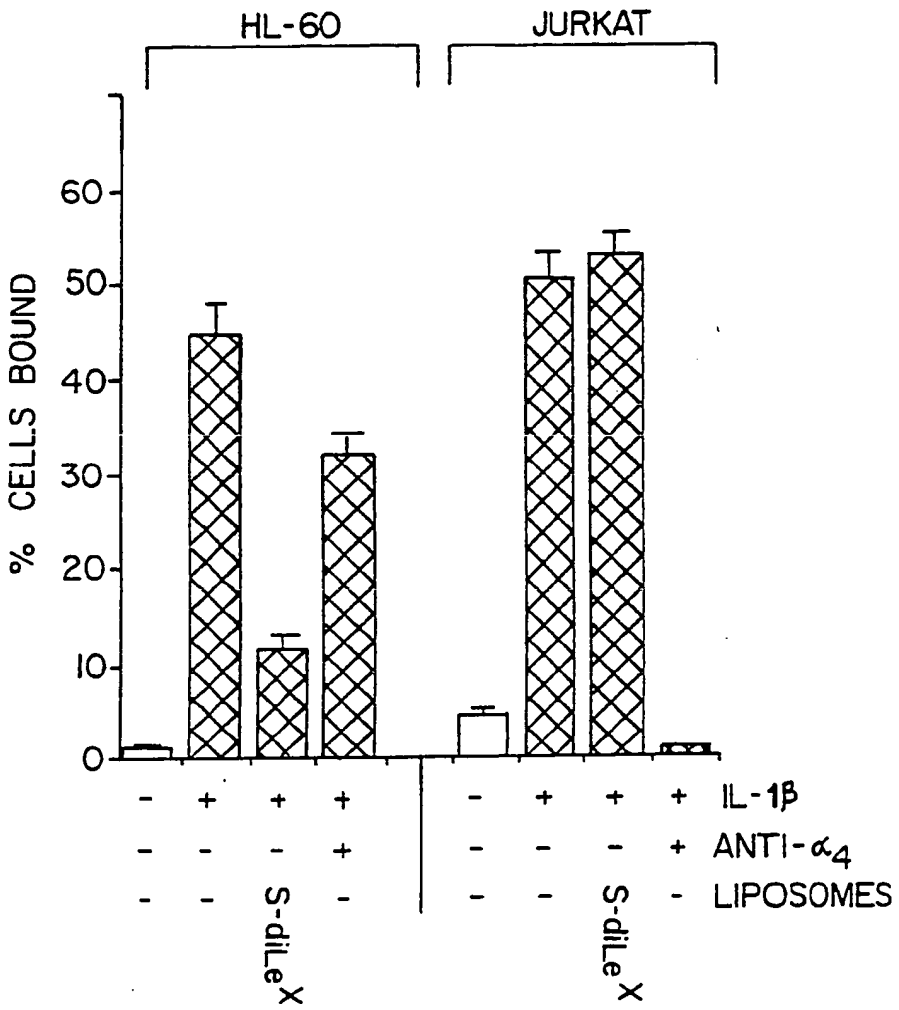


FIG. 5B.

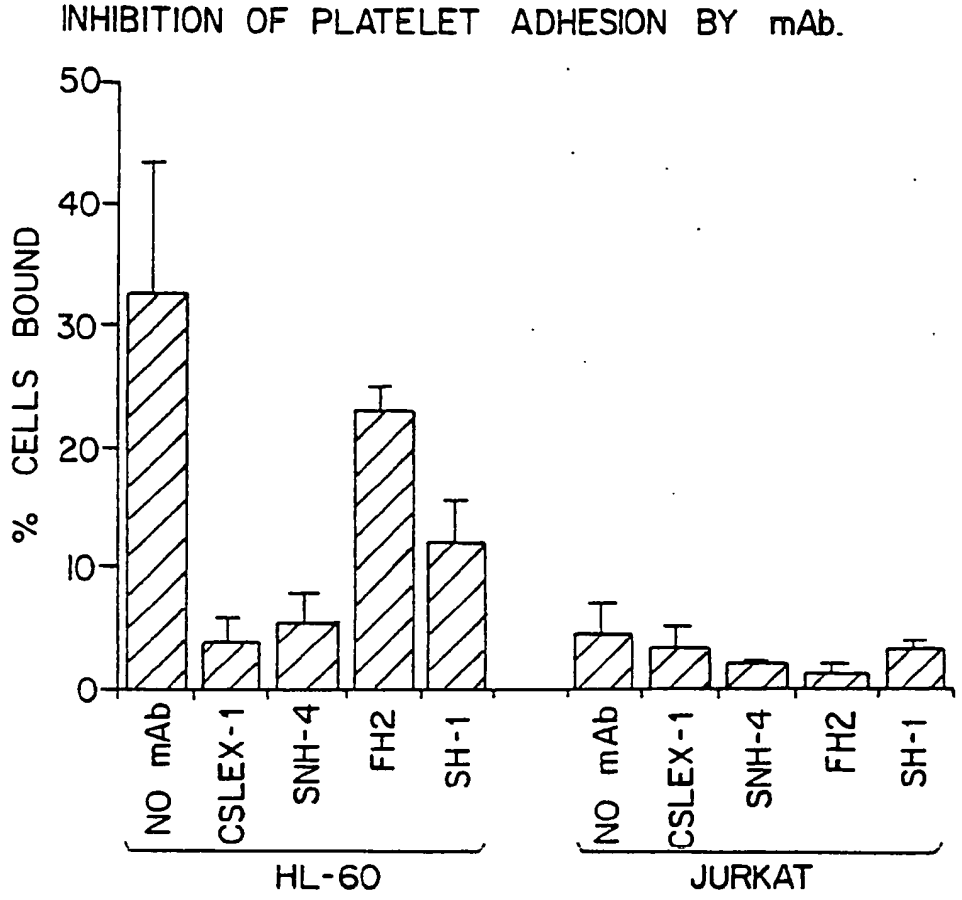


FIG. 6.

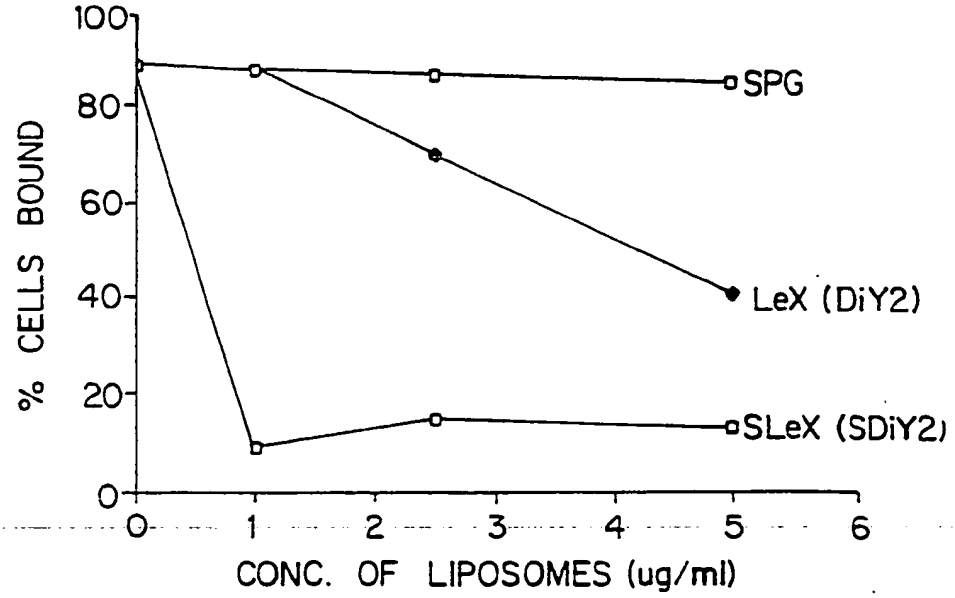


FIG. 7.

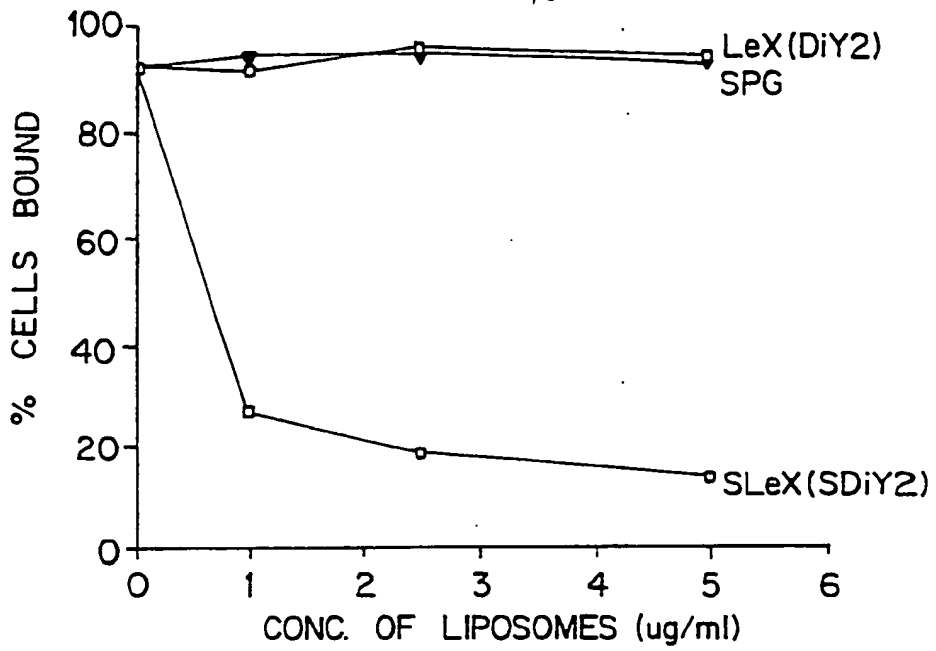


FIG. 8.

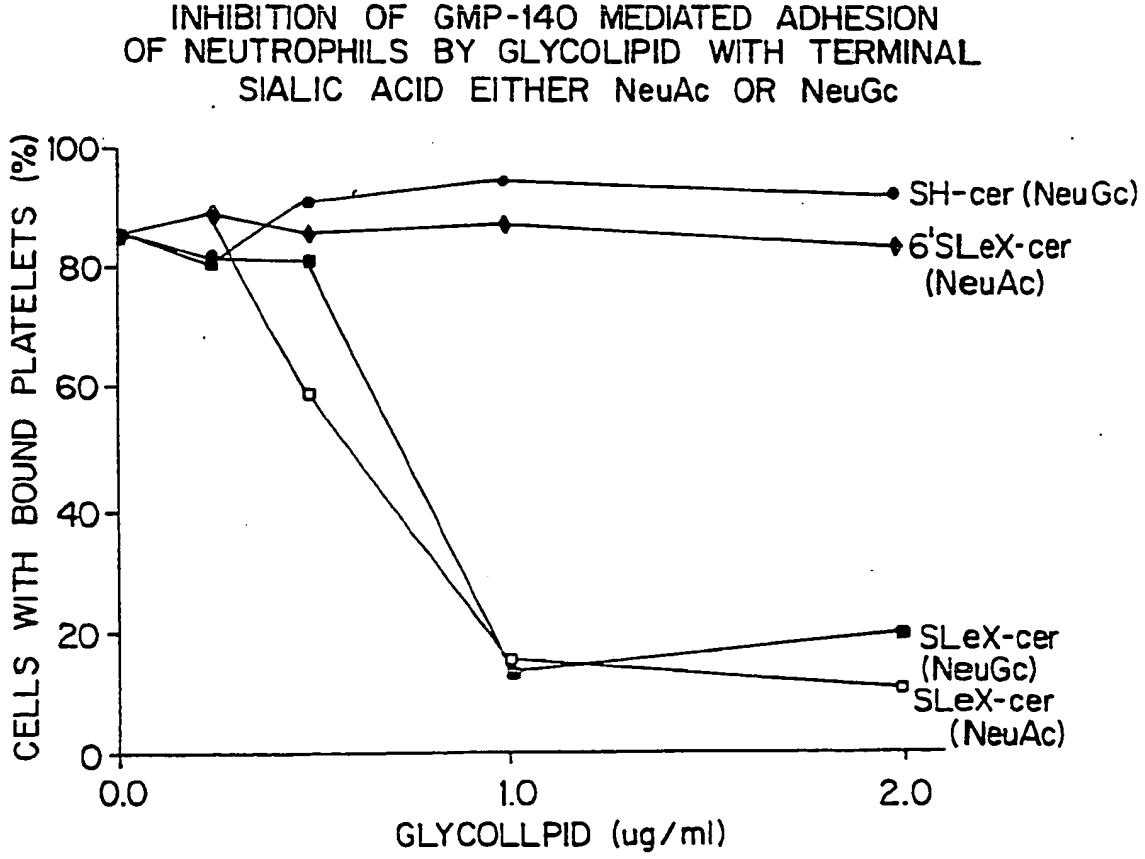


FIG. 9.

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/03592

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 31/70, 31/715, 39/00
U.S. CL.: 514/23,54; 536/1.1, 53, 123

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	514/23, 54; 536/1.1, 123, 53

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Chemical ABstracts Services: Selection or ELAM and polysaccharide(s) or oligosaccharide(s) or saccharide(s) or carbohydrate(s)

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
T	The Journal of Biological Chemistry, vol. 259, No. 7, issued 10 April 1984, S. Hakomori et al. "Novel Fucolipids Accumulating in Human Adenocarcinoma", pages 4672-4680, see entire document.	1-8,10-11 40-42, 44-54, 56-65,90 & 92-93
Y	The Journal of Biological Chemistry, vol. 259, no. 7, issued 10 April 1984, Y. Fukushi et al, "Novel Fucolipids Accumulating in Human Adenocarcinoma", pages 4681-4685, see entire document.	1-8,10-11 40-42, 44-54, 56-65, 90 & 92-93
Y	The Journal of Biological Chemistry, Vol. 259, no. 16, issued 25 August 1984, Y. Fukushi et al. "Novel Fucolipids Accumulating in Human Adenocarcinoma", pages 10511-10517, see entire document.	1-8,10-11 40-42, 44-54, 56-65, 90 & 92-93

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search: 21 September 1991

Date of Mailing of this International Search Report: 08 OCT 1991

International Searching Authority: ISA/US

Signature of Authorized Officer: Jasmine W Marie for Nancy S. Carson ebw

Form PCT/ISA/210 (second sheet) (Rev.11-87)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [2] not required to be searched by this Authority, namely:

2. ☐ Claim numbers .... , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

See attached sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

1-8, 10-11, 40-42, 44-54, 56-65, 90, 92 and 93

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	The Journal of Biological Chemistry. Vol. 260. no. 12. issued 25 June 1985. E.H. Holmes et al.. "Enzymatic Basis for the Accumulation of Glycolipids with X and Dimeric X Determinants in Human Lung Cancer Cells (NCI-H69)". pages 7619-7627, see entire document.	1-8,10-11.40-42. 44-54, 56-65.90 & 92-93
Y	The Journal of Biological Chemistry. Vol. 263. no. 23. issued 15 August 1988. P. Stanley et al. "The LEC11 Chinese Hamster Ovary Mutant Synthesizes N-linked Carbohydrates Containing Sialylated, Fucosylated Lactosamire Unites." pages 11374-11381, see entire document.	1-8,10-11.40-42. 44-54, 56-65.90 & 92-93
Y	Science. Vol. 243, issued 03 March 1989. M.P. Bevilacqua et al., "Endothelial Leukocyte Adhesion Molecule 1: An inducible Receptor for Neutrophils Related to Complement Regulation Proteins and Lectins". pages 1160-1165, see entire document.	1-8.10-11,40-42. 44-54. 56-65,90 & 92-93
Y,P	Science, Vol. 250, issued 23 November 1990. M.L. Phillips et al., "ELAM-1 mediates Cell Adhesion by Recognition of a Carbohydrate Ligand. Sialyl-Lex" pages 1130-1132, see entire document.	1-8,10-11,40-42. 44-54. 56-65.90 & 92-93
Y,P	Science, vol. 250, issued 23 November 1990. G. Walz et al., "Recognition by ELAM-1 of the Sialyl-Lex determinant on myeloid and tumor cells", pages 1132-1135, see entire document.	1-8,10-11,40-42. 44-54, 56-65.90 & 92-93

Form PCT/ISA/210 (extra sheet) (Rev.11-87)

Attachment to Form PCT/ISA/210 PartVI

Itemized summary of claims groupings

I. Claims 1-65 and 90-93, drawn to pharmaceutical compositions comprising compounds and a first method of using the compositions, classified in Class 424 subclass 85.8, Class 424 subclass 450, Class 514 subclass 8, Class 514 subclass 23, and Class 536 subclass 123.

There are independent and distinct species pertinent to the invention of Group I. The first named species, the moiety contains fucose and sialic acid and the compound is a polysaccharide (claims 4-8, 10, 11 and 90), will be searched to the extent that claims 1-65 and 90-93 embrace it. Note that a search of any other additional species within Group I requires payment of additional fees. The additional species are:

a)the compound is a glycopeptide or glycoprotein (claims 9, 27-32 and 91);

b)the compound is a glycolipid (claims 9, 19, 23, 24 and 91);

c)the composition comprises a liposome (claims 12-18 and 20-22);

d)the compound is heterocyclic (claims 25 and 26);

e)the composition comprises an immunoglobulin (claims 33-39).

II. Claims 66-75, drawn to a method of assaying a test compound for the ability to inhibit selectin-mediated cellular adhesion, classified in Class 424 subclass 85.8, Class 530 subclass 350 and Class 536 subclass 123.

There are independent and distinct species pertinent to the invention of Group II. The first named species, the agent comprises an SLX moiety, the receptor is on an activated endothelial cell, any of which may be labeled or immobilized (claims 66-70 and 72-75), will be searched to the extent that claims 66-75 embrace it upon payment of the requisite fee for Group II. Note that a search of any other additional species within Group II requires payment of additional fees. The additional species are:

f)the agent comprises an SLX mimetic (claim 67);

g)the agent comprises an immunoglobulin (claim 67);

h)the step of detecting is carried out by detecting physiological change in a cell (claim 71).

III. Claims 76-82, drawn to a method of assaying for the ability of an oligosaccharide moiety to selectively bind a selectin receptor, classified in Class 424 subclass 85.8, Class 536 subclass 123 and Class 530 subclass 350.

There are independent and distinct species pertinent to the invention of Group III. The first named species, the method

further comprises contacting the test compound with a selectin-binding agent wherein the agent is immunoglobulin (claims 80-81), will be searched to the extent that claims 76-82 embrace it upon payment of the requisite fee for Group III. Note that a search of any other additional species within Group III requires payment of additional fees. The additional species is

1)the agent is an SLX moiety (claim 82).

IV. Claims 83-89, drawn to a method of assaying a test compound for the ability to selectively bind an SLX moiety, classified in Class 536, subclass 123, Class 424 subclass 85.8 and Class 530 subclass 350.

There are independent and distinct species pertinent to the invention of Group IV. The first named species, the method further comprises contacting the test compound with an SLX-binding agent which is an immunoglobulin (claims 87-88), will be searched to the extent that claims 83-89 embrace it upon payment of the requisite fee for Group IV. Note that a search of any other additional species within Group IV requires payment of additional fees. The additional species is:

1)the SLX-binding agent is a selectin receptor (claim 89).

The inventions are distinct, each from the other because of the following reasons:

The process of Group I is materially distinct from the processes of Groups II, III and IV because the administration of a therapeutically effective dose is practiced with materially different process steps and has materially different purposes from the steps and purposes of testing compounds.

The inventions of Groups II, III and IV are distinct and independent each from the other as the claimed process steps are different, and the modes of detecting are different.

PCT Rules 13.1 and 13.2 do not provide for multiple distinct methods within a single general inventive concept.

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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60/082,581 20 April 1998 (20.04.98) US

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: GLYCOSYLATION ENGINEERING OF ANTIBODIES FOR IMPROVING ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY

(57) Abstract

The present invention relates to the field of glycosylation engineering of proteins. More particularly, the present invention is directed to the glycosylation engineering of proteins to provide proteins with improved therapeutic properties, e.g., antibodies, antibody fragments, or a fusion protein that includes a region equivalent to the Fc region of an immunoglobulin, with enhanced Fc-mediated cellular cytotoxicity.

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GLYCOSYLATION ENGINEERING OF ANTIBODIES FOR IMPROVING ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY

I. RELATION TO OTHER APPLICATIONS

This application claims priority to United States Provisional Application Serial No. 60/082,581, filed April 20, 1998, incorporated herein by reference in its entirety.

II. FIELD OF THE INVENTION

The present invention relates to the field of glycosylation engineering of proteins. More particularly, the present invention relates to glycosylation engineering to generate proteins with improved therapeutic properties, including antibodies with enhanced antibody-dependent cellular cytotoxicity.

III. BACKGROUND OF THE INVENTION

Glycoproteins mediate many essential functions in human beings, other eukaryotic organisms, and some prokaryotes, including catalysis, signalling, cell-cell communication, and molecular recognition and association. They make up the majority of non-cytosolic proteins in eukaryotic organisms. Lis and Sharon, 1993, *Eur. J. Biochem.* 218:1-27. Many glycoproteins have been exploited for therapeutic purposes, and during the last two decades, recombinant versions of naturally-occurring, secreted glycoproteins have been a major product of the biotechnology industry. Examples include erythropoietin (EPO), therapeutic monoclonal antibodies (therapeutic mAbs), tissue plasminogen activator (tPA), interferon-β, (IFN-β), granulocyte-macrophage colony stimulating factor (GM-CSF), and human chorionic gonadotrophin (hCH). Cumming *et al.*, 1991, *Glycobiology* 1:115-130.

The oligosaccharide component can significantly affect properties relevant to the efficacy of a therapeutic glycoprotein, including physical stability, resistance to protease attack, interactions with the immune system, pharmacokinetics, and specific biological activity. Such properties may depend not only on the presence or absence, but also on the specific structures, of oligosaccharides. Some generalizations between oligosaccharide structure and glycoprotein function can be made. For example, certain oligosaccharide structures mediate rapid clearance of the glycoprotein from the

bloodstream through interactions with specific carbohydrate binding proteins, while others can be bound by antibodies and trigger undesired immune reactions. Jenkins *et al.*, 1996, *Nature Biotechn.* 14:975-981.

Mammalian cells are the preferred hosts for production of therapeutic glycoproteins, due to their capability to glycosylate proteins in the most compatible form for human application. Cumming, 1991, *supra*; Jenkins *et al.*, 1996, *supra*. Bacteria very rarely glycosylate proteins, and like other types of common hosts, such as yeasts, filamentous fungi, insect and plant cells, yield glycosylation patterns associated with rapid clearance from the blood stream, undesirable immune interactions, and in some specific cases, reduced biological activity. Among mammalian cells, Chinese hamster ovary (CHO) cells have been most commonly used during the last two decades. In addition to giving suitable glycosylation patterns, these cells allow consistent generation of genetically stable, highly productive clonal cell lines. They can be cultured to high densities in simple bioreactors using serum-free media, and permit the development of safe and reproducible bioprocesses. Other commonly used animal cells include baby hamster kidney (BHK) cells, NS0- and SP2/0-mouse myeloma cells. More recently, production from transgenic animals has also been tested. Jenkins *et al.*, 1996, *supra*.

The glycosylation of recombinant therapeutic proteins produced in animal cells can be engineered by overexpression of glycosyl transferase genes in host cells. Bailey, 1991, *Science* 252:1668-1675. However, previous work in this field has only used constitutive expression of the glycoprotein-modifying glycosyl transferase genes, and little attention has been paid to the expression level.

IV. SUMMARY OF THE INVENTION

The present invention is directed, generally, to host cells and methods for the generation of proteins having an altered glycosylation pattern resulting in improved therapeutic values. In one specific embodiment, the invention is directed to host cells that have been engineered such that they are capable of expressing a preferred range of a glycoprotein-modifying glycosyl transferase activity which increases complex N-linked oligosaccharides carrying bisecting GlcNAc. In other embodiments, the present invention is directed to methods for the generation of modified glycoforms of glycoproteins, for example antibodies, including whole antibody molecules, antibody

fragments, or fusion proteins that include a region equivalent to the Fc region of an immunoglobulin, having an enhanced Fc-mediated cellular cytotoxicity, and glycoproteins so generated. The invention is based, in part, on the inventors' discovery that there is an optimal range of glycoprotein-modifying glycosyl transferase expression for the maximization of complex N-linked oligosaccharides carrying bisecting GlcNAc.

More specifically, the present invention is directed to a method for producing altered glycoforms of proteins having improved therapeutic values, *e.g.*, an antibody which has an enhanced antibody dependent cellular cytotoxicity (ADCC), in a host cell. The invention provides host cells which harbor a nucleic acid encoding the protein of interest, *e.g.*, an antibody, and at least one nucleic acid encoding a glycoprotein-modifying glycosyl transferase. Further, the present invention provides methods and protocols of culturing such host cells under conditions which permit the expression of said protein of interest, *e.g.*, the antibody having enhanced antibody dependent cellular cytotoxicity. Further, methods for isolating the so generated protein having an altered glycosylation pattern, *e.g.*, the antibody with enhanced antibody dependent cellular cytotoxicity, are described.

Furthermore, the present invention provides alternative glycoforms of proteins having improved therapeutic properties. The proteins of the invention include antibodies with an enhanced antibody-dependent cellular cytotoxicity (ADCC), which have been generated using the disclosed methods and host cells.

V. BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 depicts the representation of typical Fc-associated oligosaccharide structures.

FIGURE 2 depicts a Western blot analysis of tetracycline-regulated expression of GnT III in two different tTA-producing CHO clones. CHOt2 (lanes A and B) and CHOt17 (lanes C and D) cells were transfected with the pUDH10-3GnTIIIm expression vector and cultured for 36 h in the absence (lanes A and C) or presence of tetracycline, at a concentration of 400 ng/ml (lanes B and D). Cell lysates were then prepared for western blot analysis probing with an antibody (9E10), which recognizes specifically the c-myc tag added to GnT III at its carboxy-terminus.

FIGURE 3 depicts determination of the range of tetracycline concentrations where myc-tagged GnT III expression can be controlled. CHOt17 cells were transfected

with the pUDH10-3-GnTIIIm expression vector and then cultured for 48h in the presence of the indicated concentrations of tetracycline. GnT III levels in cell lysates from these cultures were compared using western blot analysis. GnT III was detected *via* the c-myc tag using 9E10 antibody.

FIGURES 4A through 4B depict screening of CHO clones for stable, tetracycline-regulated expression of GnT V (FIGURE 4A) or myc-tagged GnT III (FIGURE 4B) glycosyltransferases by western blot analysis. CHOt17 cells were co-transfected with a vector for expression of puromycin resistance (pPUR) and either pUHD10-3GnTV (FIGURE 4A) or pUDH10-3GnTIIIm (FIGURE 4B) and stable CHO clones were selected for resistance to puromycin (7.5 μ/ml), in the presence of tetracycline (2 μg/ml). Eight clones (1-8) for each glycosyltransferase were cultured for 48 h in the absence or presence (+) of tetracycline (2 μg/ml) and analysed by western blot using either an anti-GnT V antibody (FIGURE 4A) or an anti-myc (9E10) antibody (FIGURE 4B).

FIGURES 5A and 5B depict verification of activity of heterologous GnT V (FIGURE 5A) and Gn T III (FIGURE 5B) glycosyltransferaseas *in vivo* by lectin blot analysis. Cellular glycoproteins from various stable clones (numbered as in FIGURE 4), cultured in the absence or presence (+) of tetracycline (2 μg/ml), were resolved by SDS-PAGE, blotted to a membrane, and probed with either L-PHA (FIGURE 5A) or E-PHA (FIGURE 5B) lectins. These lectins bind with higher affinity to the oligosaccharide products of reactions catalyzed by GnT V and GnT III, respectively, than to the oligosaccharide substrates of these reactions. A molecular weight marker (MWM) was run in parallel. A comparison of lectin blots in FIGURES 5A and 5B indicates a broader range of substrates, among the endogenous CHO cell glycoproteins, for GnT III (FIGURE 5B) than for GnT V (FIGURE 5A).

FIGURES 6A through 6D depict inhibition of cell growth upon glycosyltransferase overexpression. CHO-tet-GnTIIIm cells were seeded to 5-10% confluency and cultured in the absence (FIGURES 6A and 6B) or presence (FIGURES 6C and 6D) of tetracycline. Cultures were photographed 45 (FIGURES 6A and 6C) and 85 (FIGURES 16B and 6D) hours after seeding.

FIGURE 7 depicts sequences of oligonucleotide primers used in PCRs for the construction of the chCE7 heavy chain gene. Forward and reverse primers are identified by the suffixes ".fwd" and ".rev", respectively. Overlaps between different

primers, necessary to carry out secondary PCR steps using the product of a primary PCR step as a template, are indicated. Restriction sites introduced, sequences annealing to the CE7 chimeric genomic DNA, and the synthetic leader sequence introduced, are also indicated.

FIGURE 8 depicts sequences of oligonucleotide primers used in PCRs for the construction of the chCE7 light chain gene. Forward and reverse primers are identified by the suffixes ".fwd" and ".rev" respectively. Overlaps between different primers, necessary to carry out secondary PCR steps using as a template the product of a primary PCR step, are indicated. Restriction sites introduced, sequences annealing to the CE7 chimeric genomic DNA, and the leader sequence introduced, are also indicated.

FIGURE 9 depicts MALDI/TOF-MS spectra of neutral oligosaccharide mixtures from chCE7 samples produced either by SP2/0 mouse myeloma cells (FIGURE 9A, oligosaccharides from 50 μg of CE7-SP2/0), or by CHO-tetGnTIII-chCE7 cell cultures differing in the concentration of tetracycline added to the media, and therefore expressing the GnT III gene at different levels. In decreasing order of tetracycline concentration, *i.e.*, increasing levels of GnT III gene expression, the latter samples are: CE7-2000t (FIGURE 9B, oligosaccharides from 37.5μg of antibody), CE7-60t (FIGURE 9C, oligosaccharides from 37.5 μg of antibody, CE7-30t (FIGURE 9D, oligosaccharides from 25 μg of antibody) and CE7-15t (FIGURE 9E, oligosaccharides from 10 μg of antibody).

FIGURE 10 depicts N-linked oligosaccharide biosynthetic pathways leading to bisected complex oligosaccharides *via* a GnT III-catalyzed reaction. M stands for Mannose; Gn, N-acetylglucosamine (GlcNAc); G, galactose; Gn^b, bisecting GlcNAc; f, fucose. The oligosaccharide nomenclature consists of enumerating the M, Gn, and G residues attached to the core oligosaccharide and indicating the presence of a bisecting GlcNAc by including a Gn^b. The oligosaccharide core is itself composed of 2 Gn residues and may or may not include a fucose. The major classes of oligosaccharides are shown inside dotted frames. Man I stands for Golgi mannosidase; GnT, GlcNAc transferase; and GalT, for galactosyltransferase. The mass associated with the major, sodium-associated oligosaccharide ion that is observed MALDI/TOF-MS analysis is shown beside each oligosaccharide. For oligosaccharides which can potentially be core-fucosylated, the masses associated with both fucosylated (+f) and non-fucosylated (-f) forms are shown.

FIGURE 11 depicts N-linked oligosaccharide biosynthetic pathway leading to bisected complex and bisected hybrid oligosaccharides *via* GnT III-catalyzed reactions. M stands for mannose; Gn N-acetylglucosamine (GlcNAc); G, galactose; Gn^b, bisecting GlcNAc; f, fucose. The oligosaccharide nomenclature consists of enumerating the M, Gn, and G residues attached to the common oligosaccharide and indicating the presence of bisecting GlcNAc by including a Gn^b. The oligosaccharide core is itself composed of 2 Gn residues and may or not include a fucose. The major classes of oligosaccharides are shown inside dotted frames. Man I stands for Golgi mannosidase; TnT, GlcNAc transferase; and GalT, for galactosyltransferase. The mass associated with major, sodium-associated oligosaccharide ion that is observed in MALDI/TOF-MS analysis is shown beside each oligosaccharide. For oligosaccharides which can potentially be core-fucosylated, the masses associated with both fucosylated (+f) and non -fucosylated (-f) forms are shown.

FIGURE 12 depicts ADCC activity of different chCE7 samples. Lysis of IMR-32 neuroblastoma cells by human lymphocytes (target:effector ratio of 1:19, 16 h incubation at 37 °C), mediated by different concentrations of chCE7 samples, was measured *via* retention of a fluorescent dye. The percentage of cytotoxicity is calculated relative to a total lysis control (by means of a detergent), after subtraction of the signal in the absence of antibody.

FIGURE 13 depicts the GnT III expression of different cultures of CHO-tet-GnTIII grown at different tetracycline concentrations used to produce distinct C2B8 antibody samples. Cell lysates from each culture grown at 2000ng/ml (Lane C) and 25ng/ml (Lane D) tetracycline concentrations were resolved by SDS-PAGE, blotted onto a membrane, and probed with 9E10 (*see supra)* and anti-mouse horseradish peroxidase as primary and secondary antibodies, respectively. Lane A depicts a negative control.

FIGURES 14A and 14B depict the specificity of antigen binding of the C2B8 anti-CD20 monoclonal antibody using an indirect immunofluorescence assay with cells in suspension. CD20 positive cells (SB cells; ATCC deposit no.ATCC CCL120) and CD20 negative cells (HSB cells; ATCC deposit no. ATCC CCL120.1), FIGURE 14A and 14B respectively, were utilized. Cells of each type were incubated with C2B8 antibody produced at 25ng/ml tetracycline as a primary antibody. Negative controls included HBSSB instead of primary antibody. An anti-human IgG Fc specific,

polyclonal, FITC conjugated antibody was used for all samples as a secondary antibody.

FIGURE 15 depicts the ADCC activity of different C2B8 antibody samples at different antibody concentrations (0.04-5μg/ml). Sample C2B8-nt represents the ADCC activity of the C2B8 antibody produced in a cell line without GnT III expression. Samples C2B8-2000t, C2B8-50t and C2B8-25t show the ADCC activity of three antibody samples produced at decreasing tetracycline concentrations (i.e., increasing GnT III expression).

VI. DEFINITIONS

Terms are used herein as generally used in the art, unless otherwise defined in the following:

As used herein, the term ***antibody*** is intended to include whole antibody molecules, antibody fragments, or fusion proteins that include a region equivalent to the Fc region of an immunoglobulin.

As used herein, the term ***glycoprotein-modifying glycosyl transferase*** refers to an enzyme that effects modification of the glycosylation pattern of a glycoprotein. Examples of glycoprotein-modifying glycosyl transferases include, but are not limited to glycosyl transferases such as GnT III, GnT V, GalT, and Man II.

As used herein, the term ***glycosylation engineering*** is considered to include any sort of change to the glycosylation pattern of a naturally occurring polypeptide or fragment thereof. Glycosylation engineering includes metabolic engineering of the glycosylation machinery of a cell, including genetic manipulations of the oligosaccharide synthesis pathways to achieve altered glycosylation of glycoproteins expressed in cells. Furthermore, glycosylation engineering includes the effects of mutations and cell environment on glycosylation.

As used herein, the term ***host cell*** covers any kind of cellular system which can be engineered to generate modified glycoforms of proteins, protein fragments, or peptides of interest, including antibodies and antibody fragments. Typically, the host cells have been manipulated to express optimized levels of at least one glycoprotein-modifying glycosyl transferase, including, but not limited to GnT III, GnT V, GalT, and Man II, and/or at least one glycosidase. Host cells include cultured cells, *e.g.*, mammalian cultured cells, such as CHO cells, BHK cells, NS0 cells, SP2/0 cells, or hybridoma cells, yeast cells, and insect cells, to name only few, but also cells comprised

within a transgenic animal or cultured tissue.

As used herein, the term *Fc-mediated cellular cytotoxicity* is intended to include antibody dependent cellular cytotoxicity (ADCC), and cellular cytotoxicity directed to those cells that have been engineered to express on their cell surface an Fc-region or equivalent region of an immunoglobin G, and cellular cytotoxicity mediated by a soluble fusion protein consisting of a target protein domain fused to the N-terminus of an Fc-region or equivalent region of an immunoglobulin G.

VII. DETAILED DESCRIPTION OF THE INVENTION

A. General Overview

The objective of the present invention is to provide glycoforms of proteins, in particular antibodies, including whole antibody molecules, antibody fragments, or fusion proteins that include a region equivalent to the Fc region of an immunoglobulin, to produce new variants of a therapeutic protein. The invention is based, in part, on the inventors' discovery that the glycosylation reaction network of a cell can be manipulated to maximize the proportion of certain glycoforms within the population, and that certain glycoforms have improved therapeutic characteristics. The invention is further based, in part, on the discovery of ways to identify glycoforms of proteins which have an improved therapeutic value, and how to generate them reproducibly. The invention is further based, in part, on the discovery that there is a preferred range of glycoprotein-modifying glycosyl transferase expression in the antibody-generating cell, for increasing complex N-linked oligosaccharides carrying bisecting GlcNAc.

As such, the present invention is directed, generally, to methods for the glycosylation engineering of proteins to alter and improve their therapeutic properties. More specifically, the present invention describes methods for producing in a host cell an antibody which has an altered glycosylation pattern resulting in an enhanced antibody dependent cellular cytotoxicity (ADCC). For the practice of the methods, the present invention provides host cells which harbor a nucleic acid encoding an antibody and at least one nucleic acid encoding a glycoprotein-modifying glycosyl transferase. Further, the present invention provides methods and protocols of culturing such host cells under conditions which permit the expression of the desired antibody having an altered glycosylation pattern resulting in an enhanced antibody dependent cellular

cytotoxicity. Further, methods for isolating the so generated antibody with enhanced antibody dependent cellular cytotoxicity are described.

In more specific embodiments of the invention, two monoclonal antibodies, namely the anti-neuroblastoma antibody chCE7, and the anti-CD20 antibody C2B8, have been used as model therapeutic glycoproteins, and the target glycoforms have been those carrying a special class of carbohydrate, namely bi-antennary complex N-linked oligosaccharides modified with bisecting N-acetylglucosamine (GlcNAc). In the model system provided by the invention, CHO cells are used as host cells, although many other cell systems may be contemplated as host cell system. The glycosyl transferase that adds a bisecting GlcNAc to various types of N-linked oligosaccharides, GlcNAc-transferase III (GnT III), is not normally produced by CHO cells. Stanley and Campell, 1984, *J. Biol. Chem.* 261:13370-13378.

To investigate the effects of GnT III overexpression experimentally, a CHO cell line with tetracycline-regulated overexpression of a rat GnT III cDNA was established. Using this experimental system, the inventors discovered that overexpression of GnT III to high levels led to growth inhibition and was toxic to the cells. Another CHO cell line with tetracycline-regulated overexpression of GnT V, which is a distinct glycosyl transferase, showed the same inhibitory effect, indicating that this may be a general feature of glycoprotein-modifying glycosyl transferase overexpression. The effect of the enzyme expression on the cell growth sets an upper limit to the level of glycoprotein-modifying glycosyl transferase overexpression and may therefore also limit the extent to which poorly accessible glycosylation sites can be modified by engineering of glycosylation pathways and patterns using unregulated expression vectors.

The production of a set of chCE7 mAb and C2B8 samples differing in their glycoform distributions by controlling GnT III expression in a range between basal and toxic levels are disclosed. Measurement of the ADCC activity of the chCE7 mAb samples showed an optimal range of GnT III expression for maximal chCE7 *in vitro* biological activity. The activity correlated with the level of Fc-associated bisected, complex oligosaccharides. Expression of GnT III within the practical range, *i.e.*, where no significant growth inhibition and toxicity are observed, led to an increase of the target bisected, complex structures for this set of chCE7 samples. The pattern of oligosaccharide peaks in MALDI/TOF-mass spectrometric analysis of chCE7 samples

produced at high levels of GnT III indicates that a significant proportion of potential GnT III substrates is diverted to bisected hybrid oligosaccharide by-products. Minimization of these by-products by further engineering of the pathway could therefore be valuable.

B. Identification And Generation Of Nucleic Acids Encoding A Protein For Which Modification Of The Glycosylation Pattern Is Desired

The present invention provides host cell systems suitable for the generation of altered glycoforms of any protein, protein fragment or peptide of interest, for which such an alteration in the glycosylation pattern is desired. The nucleic acids encoding such protein, protein fragment or peptide of interest may be obtained by methods generally known in the art. For example, the nucleic acid may be isolated from a cDNA library or genomic library. For a review of cloning strategies which may be used, *see*, *e.g.*, Maniatis, 1989, Molecular Cloning, A Laboratory Manual, Cold Springs Harbor Press, N.Y.; and Ausubel *et al.*, 1989, Current Protocols in Molecular Biology, (Green Publishing Associates and Wiley Interscience, N.Y.).

In an alternate embodiment of the invention, the coding sequence of the protein, protein fragment or peptide of interest may be synthesized in whole or in part, using chemical methods well known in the art. *See*, for example, Caruthers *et al.*, 1980, *Nuc. Acids Res. Symp. Ser.* 7:215-233; Crea and Horn, 1980, *Nuc. Acids Res. USA* 9:2331; Matteucci and Caruthers, 1980, *Tetrahedron Letters* 21:719; Chow and Kempe, 1981, *Nuc. Acids Res.* 9:2807-2817. Alternatively, the protein itself could be produced using chemical methods to synthesize its amino acid sequence in whole or in part. For example, peptides can be synthesized by solid phase techniques, cleaved from the resin, and purified by preparative high performance liquid chromatography. *E.g.*, *see* Creighton, 1983, Protein Structures And Molecular Principles, W.H. Freeman and Co., N.Y. pp. 50-60. The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (*e.g.*, the Edman degradation procedure; *see* Creighton, 1983, Proteins. Structures and Molecular Principles, W.H. Freeman and Co., N.Y., pp. 34-49).

In preferred embodiments, the invention provides methods for the generation and use of host cell systems for the production of glycoforms of antibodies or antibody

fragments or fusion proteins which include antibody fragments with enhanced antibody-dependent cellular cytotoxicity. Identification of target epitopes and generation of antibodies having potential therapeutic value, for which modification of the glycosylation pattern is desired, and isolation of their respective coding nucleic acid sequence is within the scope of the invention.

Various procedures known in the art may be used for the production of antibodies to target epitopes of interest. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments and fragments produced by an Fab expression library. Such antibodies may be useful, *e.g.*, as diagnostic or therapeutic agents. As therapeutic agents, neutralizing antibodies, *i.e.*, those which compete for binding with a ligand, substrate or adapter molecule, are of especially preferred interest.

For the production of antibodies, various host animals are immunized by injection with the target protein of interest including, but not limited to, rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*.

Monoclonal antibodies to the target of interest may be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique originally described by Kohler and Milstein, 1975, *Nature* 256:495-497, the human B-cell hybridoma technique (Kosbor *et al.*, 1983, *Immunology Today* 4:72; Cote *et al.*, 1983, *Proc. Natl. Acad. Sci. U.S.A.* 80:2026-2030) and the EBV-hybridoma technique (Cole *et al.*, 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In addition, techniques developed for the production of "chimeric antibodies" (Morrison *et al.*, 1984, *Proc. Natl. Acad. Sci. U.S.A.* 81:6851-6855; Neuberger *et al.*, 1984, *Nature* 312:604-608; Takeda *et al.*, 1985, *Nature* 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. Alternatively, techniques described for the production of single chain antibodies (U.S.

Patent No. 4,946,778) can be adapted to produce single chain antibodies having a desired specificity.

Antibody fragments which contain specific binding sites of the target protein of interest may be generated by known techniques. For example, such fragments include, but are not limited to, $F(ab')_2$ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries may be constructed (Huse *et al.*, 1989, *Science* 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity to the target protein of interest.

Once an antibody or antibody fragment has been identified for which modification in the glycosylation pattern are desired, the coding nucleic acid sequence is identified and isolated using techniques well known in the art. *See, supra.*

C. Generation Of Cell Lines For The Production Of Proteins With Altered Glycosylation Pattern

The present invention provides host cell expression systems for the generation of proteins having modified glycosylation patterns. In particular, the present invention provides host cell systems for the generation of glycoforms of proteins having an improved therapeutic value. Therefore, the invention provides host cell expression systems selected or engineered to increase the expression of a glycoprotein-modifying glycosyltransferase. Specifically, such host cell expression systems may be engineered to comprise a recombinant nucleic acid molecule encoding a glycoprotein-modifying glycosyltransferase, operatively linked to a constitutive or regulated promoter system. Alternatively, host cell expression systems may be employed that naturally produce, are induced to produce, and/or are selected to produce a glycoprotein-modifying glycosyltransferase.

In one specific embodiment, the present invention provides a host cell that has been engineered to express at least one nucleic acid encoding a glycoprotein-modifying glycosyl transferase. In one aspect, the host cell is transformed or transfected with a nucleic acid molecule comprising at least one gene encoding a glycoprotein-modifying glycosyl transferase. In an alternate aspect, the host cell has been engineered and/or selected in such way that an endogenous glycoprotein-

modifying glycosyl transferase is activated. For example, the host cell may be selected to carry a mutation triggering expression of an endogenous glycoprotein-modifying glycosyl transferase. This aspect is exemplified in one specific embodiment, where the host cell is a CHO lec10 mutant. Alternatively, the host cell may be engineered such that an endogenous glycoprotein-modifying glycosyl transferase is activated. In again another alternative, the host cell is engineered such that an endogenous glycoprotein-modifying glycosyl transferase has been activated by insertion of a regulated promoter element into the host cell chromosome. In a further alternative, the host cell has been engineered such that an endogenous glycoprotein-modifying glycosyl transferase.has been activated by insertion of a constitutive promoter element, a transposon, or a retroviral element into the host cell chromosome.

Generally, any type of cultured cell line can be used as a background to engineer the host cell lines of the present invention. In a preferred embodiment, CHO cells, BHK cells, NS0 cells, SP2/0 cells, or a hybridoma cell line is used as the background cell line to generate the engineered host cells of the invention.

The invention is contemplated to encompass engineered host cells expressing any type of glycoprotein-modifying glycosyl transferase as defined herein. However, in preferred embodiments, at least one glycoprotein-modifying glycosyl transferase expressed by the host cells of the invention is GnT III, or, alternatively, ß(1,4)-N-acetylglucosaminyltransferase V (GnT V). However, also other types of glycoprotein-modifying glycosyl transferase may be expressed in the host system, typically in addition to GnT III or GnT V, including β(1,4)-galactosyl transferase (GalT), and mannosidase II (Man II). In one embodiment of the invention, GnT III is coexpressed with GalT. In another embodiment of the invention, GnT III is coexpressed with Man II. In a further embodiment of the invention, GnT III is coexpressed with GalT and Man II. However, any other permutation of glycoprotein-modifying glycosyl transferases is within the scope of the invention. Further, expression of a glycosidase in the host cell system may be desired.

One or several nucleic acids encoding a glycoprotein-modifying glycosyl transferase may be expressed under the control of a constitutive promoter or, alternately, a regulated expression system. Suitable regulated expression systems include, but are not limited to, a tetracycline-regulated expression system, an ecdysone-inducible expression system, a lac-switch expression system, a glucocorticoid-inducible

expression system, a temperature-inducible promoter system, and a metallothionein metal-inducible expression system. If several different nucleic acids encoding glycoprotein-modifying glycosyl transferases are comprised within the host cell system, some of them may be expressed under the control of a constitutive promoter, while others are expressed under the control of a regulated promoter. The optimal expression levels will be different for each protein of interest, and will be determined using routine experimentation. Expression levels are determined by methods generally known in the art, including Western blot analysis using a glycosyl transferase specific antibody, Northern blot analysis using a glycosyl transferase specific nucleic acid probe, or measurement of enzymatic activity. Alternatively, a lectin may be employed which binds to biosynthetic products of the glycosyl transferase, for example, E_4-PHA lectin. In a further alternative, the nucleic acid may be operatively linked to a reporter gene; the expression levels of the glycoprotein-modifying glycosyl transferase are determined by measuring a signal correlated with the expression level of the reporter gene. The reporter gene may transcribed together with the nucleic acid(s) encoding said glycoprotein-modifying glycosyl transferase as a single mRNA molecule; their respective coding sequences may be linked either by an internal ribosome entry site (IRES) or by a cap-independent translation enhancer (CITE). The reporter gene may be translated together with at least one nucleic acid encoding said glycoprotein-modifying glycosyl transferase such that a single polypeptide chain is formed. The nucleic acid encoding the glycoprotein-modifying glycosyl transferase may be operatively linked to the reporter gene under the control of a single promoter, such that the nucleic acid encoding the glycoprotein-modifying glycosyl transferase and the reporter gene are transcribed into an RNA molecule which is alternatively spliced into two separate messenger RNA (mRNA) molecules; one of the resulting mRNAs is translated into said reporter protein, and the other is translated into said glycoprotein-modifying glycosyl transferase.

If several different nucleic acids encoding a glycoprotein-modifying glycosyl transferase are expressed, they may be arranged in such way that they are transcribed as one or as several mRNA molecules. If they are transcribed as a single mRNA molecule, their respective coding sequences may be linked either by an internal ribosome entry site (IRES) or by a cap-independent translation enhancer (CITE). They may be transcribed from a single promoter into an RNA molecule which is alternatively spliced

into several separate messenger RNA (mRNA) molecules, which then are each translated into their respective encoded glycoprotein-modifying glycosyl transferase.

In other embodiments, the present invention provides host cell expression systems for the generation of therapeutic proteins, for example antibodies, having an enhanced antibody-dependent cellular cytotoxicity, and cells which display the IgG Fc region on the surface to promote Fc-mediated cytotoxicity. Generally, the host cell expression systems have been engineered and/or selected to express nucleic acids encoding the protein for which the production of altered glycoforms is desired, along with at least one nucleic acid encoding a glycoprotein-modifying glycosyl transferase. In one embodiment, the host cell system is transfected with at least one gene encoding a glycoprotein-modifying glycosyl transferase. Typically, the transfected cells are selected to identify and isolate clones that stably express the glycoprotein-modifying glycosyl transferase. In another embodiment, the host cell has been selected for expression of endogenous glycosyl transferase. For example, cells may be selected carrying mutations which trigger expression of otherwise silent glycoprotein-modifying glycosyl transferases. For example, CHO cells are known to carry a silent GnT III gene that is active in certain mutants, *e.g.*, in the mutant Lec10. Furthermore, methods known in the art may be used to activate silent glycoprotein-modifying glycosyl transferase genes, including the insertion of a regulated or constitutive promoter, the use of transposons, retroviral elements, etc. Also the use of gene knockout technologies or the use of ribozyme methods may be used to tailor the host cell's glycosyl transferase and/or glycosidase expression levels, and is therefore within the scope of the invention.

Any type of cultured cell line can be used as background to engineer the host cell lines of the present invention. In a preferred embodiment, CHO cells, BHK cells, NS0 cells, SP2/0 cells. Typically, such cell lines are engineered to further comprise at least one transfected nucleic acid encoding a whole antibody molecule, an antibody fragment, or a fusion protein that includes a region equivalent to the Fc region of an immunoglobulin. In an alternative embodiment, a hybridoma cell line expressing a particular antibody of interest is used as background cell line to generate the engineered host cells of the invention.

Typically, at least one nucleic acid in the host cell system encodes GnT III, or, alternatively, GnT V. However, also other types of glycoprotein-modifying glycosyl transferase may be expressed in the host system, typically in addition to GnT III or GnT

V, including GalT, and Man II. In one embodimentof the invention, GnT III is coexpressed with GalT. In another embodiment of the invention, GnT III is coexpressed with Man II. In a further embodiment of the invention, GnT III is coexpressed with GalT and Man II. However, any other permutation of glycoprotein-modifying glycosyl transferases is within the scope of the invention. Further, expression of a glycosidase in the host cell system may be desired.

One or several nucleic acids encoding a glycoprotein-modifying glycosyl transferase may be expressed under the control of a constitutive promoter, or alternately, a regulated expression system. Suitable regulated expression systems include, but are not limited to, a tetracycline-regulated expression system, an ecdysone-inducible expression system, a lac-switch expression system, a glucocorticoid-inducible expression system, a temperature-inducible promoter system, and a metallothionein metal-inducible expression system. If several different nucleic acids encoding glycoprotein-modifying glycosyl transferases are comprised within the host cell system, some of them may be expressed under the control of a constitutive promoter, while others are expressed under the control of a regulated promoter. The optimal expression levels will be different for each protein of interest, and will be determined using routine experimentation. Expression levels are determined by methods generally known in the art, including Western blot analysis using a glycosyl transferase specific antibody, Northern blot analysis using a glycosyl transferase specific nucleic acid probe, or measurement of enzymatic activity. Alternatively, a lectin may be employed which binds to biosynthetic products of glycosyl transferase, for example, E_4-PHA lectin. In a further alternative, the nucleic acid may be operatively linked to a reporter gene; the expression levels of the glycoprotein-modifying glycosyl transferase are determined by measuring a signal correlated with the expression level of the reporter gene. The reporter gene may transcribed together with the nucleic acid(s) encoding said glycoprotein-modifying glycosyl transferase as a single mRNA molecule; their respective coding sequences may be linked either by an internal ribosome entry site (IRES) or by a cap-independent translation enhancer (CITE). The reporter gene may be translated together with at least one nucleic acid encoding said glycoprotein-modifying glycosyl transferase such that a single polypeptide chain is formed. The nucleic acid encoding the glycoprotein-modifying glycosyl transferase may be operatively linked to the reporter gene under the control of a single promoter, such that the nucleic acid

encoding the glycoprotein-modifying glycosyl transferase and the reporter gene are transcribed into an RNA molecule which is alternatively spliced into two separate messenger RNA (mRNA) molecules; one of the resulting mRNAs is translated into said reporter protein, and the other is translated into said glycoprotein-modifying glycosyl transferase.

If several different nucleic acids encoding a glycoprotein-modifying glycosyl transferase are expressed, they may be arranged in such way that they are transcribed as one or as several mRNA molecules. If they are transcribed as single mRNA molecule, their respective coding sequences may be linked either by an internal ribosome entry site (IRES) or by a cap-independent translation enhancer (CITE). They may be transcribed from a single promoter into an RNA molecule which is alternatively spliced into several separate messenger RNA (mRNA) molecules, which then are each translated into their respective encoded glycoprotein-modifying glycosyl transferase.

1. Expression Systems

Methods which are well known to those skilled in the art can be used to construct expression vectors containing the coding sequence of the protein of interest and the coding sequence of the glycoprotein-modifying glycosyl transferase and appropriate transcriptional/translational control signals. These methods include *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* recombination/genetic recombination. *See*, for example, the techniques described in Maniatis *et al.*, 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel *et al.*, 1989, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, N.Y.

A variety of host-expression vector systems may be utilized to express the coding sequence of the protein of interest and the coding sequence of the glycoprotein-modifying glycosyl transferase. Preferably, mammalian cells are used as host cell systems transfected with recombinant plasmid DNA or cosmid DNA expression vectors containing the coding sequence of the protein of interest and the coding sequence of the glycoprotein-modifying glycosyl transferase. Most preferably, CHO cells, BHK cells, NS0 cells, or SP2/0 cells, or alternatively, hybridoma cells are used as host cell systems. In alternate embodiments, other eukaryotic host cell systems may be contemplated, including, yeast cells transformed with recombinant yeast expression vectors containing

the coding sequence of the protein of interest and the coding sequence of the glycoprotein-modifying glycosyl transferase; insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing the coding sequence of the protein of interest and the coding sequence of the glycoprotein-modifying glycosyl transferase; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing the coding sequence of the protein of interest and the coding sequence of the glycoprotein-modifying glycosyl transferase; or animal cell systems infected with recombinant virus expression vectors (*e.g.*, adenovirus, vaccinia virus) including cell lines engineered to contain multiple copies of the DNA encoding the protein of interest and the coding sequence of the glycoprotein-modifying glycosyl transferase either stably amplified (CHO/dhfr) or unstably amplified in double-minute chromosomes (*e.g.*, murine cell lines).

For the methods of this invention, stable expression is generally preferred to transient expression because it typically achieves more reproducible results and also is more amenable to large scale production. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with the respective coding nucleic acids controlled by appropriate expression control elements (*e.g.*, promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows selection of cells which have stably integrated the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines.

A number of selection systems may be used, including, but not limited to, the herpes simplex virus thymidine kinase (Wigler *et al.*, 1977, *Cell* 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, *Proc. Natl. Acad. Sci. USA* 48:2026), and adenine phosphoribosyltransferase (Lowy *et al.*, 1980. *Cell* 22:817) genes, which can be employed in tk^-, $hgprt^-$ or $aprt^-$ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler *et al.*, 1980, *Natl. Acad. Sci. USA*

77:3567; O'Hare *et al.*, 1981, *Proc. Natl. Acad. Sci. USA* 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, *Proc. Natl. Acad. Sci. USA* 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin *et al.*, 1981, *J. Mol. Biol.* 150:1); and hygro, which confers resistance to hygromycin (Santerre *et al.*, 1984, *Gene* 30:147) genes. Recently, additional selectable genes have been described, namely trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman & Mulligan, 1988, *Proc. Natl. Acad. Sci. USA* 85:8047); the glutamine synthase system; and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue, 1987, *in*: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.).

2. Identification Of Transfectants Or Transformants That Express The Protein Having A Modified Glycosylation Pattern

The host cells which contain the coding sequence and which express the biologically active gene products may be identified by at least four general approaches; (a) DNA-DNA or DNA-RNA hybridization; (b) the presence or absence of "marker" gene functions; (c) assessing the level of transcription as measured by the expression of the respective mRNA transcripts in the host cell; and (d) detection of the gene product as measured by immunoassay or by its biological activity.

In the first approach, the presence of the coding sequence of the protein of interest and the coding sequence of the glycoprotein-modifying glycosyl transferase(s) inserted in the expression vector can be detected by DNA-DNA or DNA-RNA hybridization using probes comprising nucleotide sequences that are homologous to the respective coding sequences, respectively, or portions or derivatives thereof.

In the second approach, the recombinant expression vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (*e.g.*, thymidine kinase activity, resistance to antibiotics, resistance to methotrexate, transformation phenotype, occlusion body formation in baculovirus, etc.). For example, if the coding sequence of the protein of interest and the coding sequence of the glycoprotein-modifying glycosyl transferase are inserted within a marker gene sequence of the vector, recombinants containing the respective coding sequences can be

identified by the absence of the marker gene function. Alternatively, a marker gene can be placed in tandem with the coding sequences under the control of the same or different promoter used to control the expression of the coding sequences. Expression of the marker in response to induction or selection indicates expression of the coding sequence of the protein of interest and the coding sequence of the glycoprotein-modifying glycosyl transferase.

In the third approach, transcriptional activity for the coding region of the protein of interest and the coding sequence of the glycoprotein-modifying glycosyl transferase can be assessed by hybridization assays. For example, RNA can be isolated and analyzed by Northern blot using a probe homologous to the coding sequences of the protein of interest and the coding sequence of the glycoprotein-modifying glycosyl transferase or particular portions thereof. Alternatively, total nucleic acids of the host cell may be extracted and assayed for hybridization to such probes.

In the fourth approach, the expression of the protein products of the protein of interest and the coding sequence of the glycoprotein-modifying glycosyl transferase can be assessed immunologically, for example by Western blots, immunoassays such as radioimmuno-precipitation, enzyme-linked immunoassays and the like. The ultimate test of the success of the expression system, however, involves the detection of the biologically active gene products.

D. Generation And Use Of Proteins And Protein Fragments Having Altered Glycosylation Patterns

1. Generation And Use Of Antibodies Having Enhanced Antibody-Dependent Cellular Cytotoxicity

In preferred embodiments, the present invention provides glycoforms of antibodies and antibody fragments having an enhanced antibody-dependent cellular cytotoxicity.

Clinical trials of unconjugated monoclonal antibodies (mAbs) for the treatment of some types of cancer have recently yielded encouraging results. Dillman, 1997, *Cancer Biother. & Radiopharm.* 12:223-225; Deo *et al.*, 1997, *Immunology Today* 18:127. A chimeric, unconjugated IgG1 has been approved for low-grade or follicular B-cell non-Hodgkin's lymphoma (Dillman, 1997, *supra*), while another unconjugated mAb, a humanized IgG1 targeting solid breast tumors, has also been

showing promising results in phase III clinical trials. Deo *et al.*, 1997, *supra*. The antigens of these two mAbs are highly expressed in their respective tumor cells and the antibodies mediate potent tumor destruction by effector cells *in vitro* and *in vivo*. In contrast, many other unconjugated mAbs with fine tumor specificities cannot trigger effector functions of sufficient potency to be clinically useful. Frost *et al.*, 1997, *Cancer* 80:317-333; Surfus *et al.*, 1996, *J. Immunother.* 19:184-191. For some of these weaker mAbs, adjunct cytokine therapy is currently being tested. Addition of cytokines can stimulate antibody-dependent cellular cytotoxicity (ADCC) by increasing the activity and number of circulating lymphocytes. Frost *et al.*, 1997, *supra*; Surfus *et al.*, 1996, *supra*. ADCC, a lytic attack on antibody-targeted cells, is triggered upon binding of lymphocyte receptors to the constant region (Fc) of antibodies. Deo *et al.*, 1997, *supra*.

A different, but complementary, approach to increase ADCC activity of unconjugated IgG1s would be to engineer the Fc region of the antibody to increase its affinity for the lymphocyte receptors (FcγRs). Protein engineering studies have shown that FcγRs interact with the lower hinge region of the IgG CH2 domain. Lund *et al.*, 1996, *J. Immunol.* 157:4963-4969. However, FcγR binding also requires the presence of oligosaccharides covalently attached at the conserved Asn 297 in the CH2 region. Lund *et al.*, 1996, *supra*; Wright and Morrison, 1997, *Tibtech* 15:26-31, suggesting that either oligosaccharide and polypeptide both directly contribute to the interaction site or that the oligosaccharide is required to maintain an active CH2 polypeptide conformation. Modification of the oligosaccharide structure can therefore be explored as a means to increase the affinity of the interaction.

An IgG molecule carries two N-linked oligosaccharides in its Fc region, one on each heavy chain. As any glycoprotein, an antibody is produced as a population of glycoforms which share the same polypeptide backbone but have different oligosaccharides attached to the glycosylation sites. The oligosaccharides normally found in the Fc region of serum IgG are of complex bi-antennary type (Wormald *et al.*, 1997, *Biochemistry* 36:130-1380), with low level of terminal sialic acid and bisecting N-acetylglucosamine (GlcNAc), and a variable degree of terminal galactosylation and core fucosylation (FIGURE 1). Some studies suggest that the minimal carbohydrate structure required for FcγR binding lies within the oligosaccharide core. Lund *et al.*, 1996, *supra*. The removal of terminal galactoses results in approximately a two-fold

reduction in ADCC activity, indicating a role for these residues in FcγR receptor binding. Lund *et al.*, 1996, *supra*.

The mouse- or hamster-derived cell lines used in industry and academia for production of unconjugated therapeutic mAbs normally attach the required oligosaccharide determinants to Fc sites. IgGs expressed in these cell lines lack, however, the bisecting GlcNAc found in low amounts in serum IgGs. Lifely *et al.*, 1995, *Glycobiology* 318:813-822. In contrast, it was recently observed that a rat myeloma-produced, humanized IgG1 (CAMPATH-1H) carried a bisecting GlcNAc in some of its glycoforms. Lifely *et al.*, 1995, *supra*. The rat cell-derived antibody reached a similar *in vitro* ADCC activity as CAMPATH-1H antibodies produced in standard cell lines, but at significantly lower antibody concentrations.

The CAMPATH antigen is normally present at high levels on lymphoma cells, and this chimeric mAb has high ADCC activity in the absence of a bisecting GlcNAc. Lifely *et al.*, 1995, *supra*. Even though in the study of Lifely *et al.*, 1995, *supra*. the maximal *in vitro* ADCC activity was not increased by altering the glycosylation pattern, the fact that this level of activity was obtained at relatively low antibody concentrations for the antibody carrying bisected oligosaccharides suggests an important role for bisected oligosaccharides. An approach was developed to increase the ADCC activity of IgG1s with low basal activity levels by producing glycoforms of these antibodies carrying bisected oligosaccharides in the Fc region.

In the N-linked glycosylation pathway, a bisecting GlcNAc is added by the enzyme β(1,4)-N-acetylglucosaminyltransferase III (GnT III). Schachter, 1986, *Biochem. Cell Biol.* 64:163-181. Lifely *et al.*, 1995, *supra*, obtained different glycosylation patterns of the same antibody by producing the antibody in different cell lines with different but non-engineered glycosylation machineries, including a rat myeloma cell line that expressed GnT III at an endogenous, constant level. In contrast, we used a single antibody-producing CHO cell line, that was previously engineered to express, in an externally-regulated fashion, different levels of a cloned GnT III gene. This approach allowed us to establish for the first time a rigorous correlation between expression of GnT III and the ADCC activity of the modified antibody.

As demonstrated herein, *see*, Example 4, *infra*, C2B8 antibody modified according to the disclosed method had an about sixteen-fold higher ADCC activity than the standard, unmodified C2B8 antibody produced under identical cell culture and

purification conditions. Briefly, a C2B8 antibody sample expressed in CHO-tTA-C2B8 cells that do not have GnT III expression showed a cytotoxic activity of about 31% (at 1μg/ml antibody concentration), measured as *in vitro* lysis of SB cells (CD20+) by human lymphocytes. In contrast, C2B8 antibody derived from a CHO cell culture expressing GnT III at a basal, largely repressed level showed at 1μg/ml antibody concentration a 33% increase in ADCC activity against the control at the same antibody concentration. Moreover, increasing the expression of GnT III produced a large increase of almost 80% in the maximal ADCC activity (at 1μg/ml antibody concentration) compared to the control at the same antibody concentration. *See*, Example 4, *infra*.

Further antibodies of the invention having an enhanced antibody-dependent cellular cytotoxicity include, but are not limited to, anti-human neuroblastoma monoclonal antibody (chCE7) produced by the methods of the invention, a chimeric anti-human renal cell carcinoma monoclonal antibody (ch-G250) produced by the methods of the invention, a humanized anti-HER2 monoclonal antibody produced by the methods of the invention, a chimeric anti-human colon, lung, and breast carcinoma monoclonal antibody (ING-1) produced by the methods of the invention, a humanized anti-human 17-1A antigen monoclonal antibody (3622W94) produced by the methods of the invention, a humanized anti-human colorectal tumor antibody (A33) produced by the methods of the invention, an anti-human melanoma antibody (R24) directed against GD3 ganglioside produced by the methods of the invention, and a chimeric anti-human squamous-cell carcinoma monoclonal antibody (SF-25) produced by the methods of the invention. In addition, the invention is directed to antibody fragment and fusion proteins comprising a region that is equivalent to the Fc region of immunoglobulins. *See, infra*.

2. Generation And Use Fusion Proteins Comprising A Region Equivalent To An Fc Region Of An Immunoglobulin That Promote Fc-Mediated Cytotoxicity

As discussed above, the present invention relates to a method for enhancing the ADCC activity of therapeutic antibodies. This is achieved by engineering the glycosylation pattern of the Fc region of such antibodies, in particular by maximizing the proportion of antibody molecules carrying bisected complex oligosaccharides N-linked to the conserved glycosylation sites in their Fc regions. This

strategy can be applied to enhance Fc-mediated cellular cytotoxicity against undesirable cells mediated by any molecule carrying a region that is an equivalent to the Fc region of an immunoglobulin, not only by therapeutic antibodies, since the changes introduced by the engineering of glycosylation affect only the Fc region and therefore its interactions with the Fc receptors on the surface of effector cells involved in the ADCC mechanism. Fc-containing molecules to which the presently disclosed methods can be applied include, but are not limited to, (a) soluble fusion proteins made of a targeting protein domain fused to the N-terminus of an Fc-region (Chamov and Ashkenazi, 1996, *TIBTECH* 14: 52) and (b) plasma membrane-anchored fusion proteins made of a type II transmembrane domain that localizes to the plasma membrane fused to the N-terminus of an Fc region (Stabila, P.F., 1998, *Nature Biotech.* 16: 1357).

In the case of soluble fusion proteins (a) the targeting domain directs binding of the fusion protein to undesirable cells such as cancer cells, *i.e.*, in an analogous fashion to therapeutic antibodies. The application of presently disclosed method to enhance the Fc-mediated cellular cytotoxic activity mediated by these molecules would therefore be identical to the method applied to therapeutic antibodies. *See*, Example 2 of United States Provisional Application Serial Number 60/082,581, incorporated herein by reference.

In the case of membrane-anchored fusion proteins (b) the undesirable cells in the body have to express the gene encoding the fusion protein. This can be achieved either by gene therapy approaches, *i.e.*, by transfecting the cells *in vivo* with a plasmid or viral vector that directs expression of the fusion protein-encoding gene to undesirable cells, or by implantation in the body of cells genetically engineered to express the fusion protein on their surface. The later cells would normally be implanted in the body inside a polymer capsule (encapsulated cell therapy) where they cannot be destroyed by an Fc-mediated cellular cytotoxicity mechanism. However should the capsule device fail and the escaping cells become undesirable, then they can be eliminated by Fc-mediated cellular cytotoxicity. Stabila *et al.*, 1998, *Nature Biotech.* 16: 1357. In this case, the presently disclosed method would be applied either by incorporating into the gene therapy vector an additional gene expression cassette directing adequate or optimal expression levels of GnT III or by engineering the cells to be implanted to express adequate or optimal levels of GnT III. In both cases, the aim of the disclosed method is to increase or maximize the proportion of surface-displayed Fc regions carrying

bisected complex oligosaccharides.

The examples below explain the invention in more detail. The following preparations and examples are given to enable those skilled in the art to more clearly understand and to practice the present invention. The present invention, however, is not limited in scope by the exemplified embodiments, which are intended as illustrations of single aspects of the invention only, and methods which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

VIII. EXAMPLES

A. Example 1: Tetracycline-Regulated Overexpression Of Glycosyl Transferases In Chinese Hamster Ovary Cells

To establish a cell line in which the expression of GnT III could be externally-controlled, a tetracycline-regulated expression system was used. Gossen, M. and Bujard, H., 1992, *Proc. Nat. Acad. Sci.* USA, 89: 5547-5551. The amount of GnT III in these cells could be controlled simply by manipulating the concentration of tetracycline in the culture medium. Using this system, it was found that overexpression of GnT III to high levels led to growth inhibition and was toxic to the cells. Another CHO cell line with tetracycline-regulated overexpression of GnT V, a distinct glycoprotein-modifying glycosyl transferase, showed the same inhibitory effect, indicating that this may be a general feature of glycoprotein-modifying glycosyl transferase overexpression. This phenomenon has not been reported previously, probably due to the fact that inventigators generally have used constitutive promoters for related experiments. The growth effect sets an upper limit to the level of glycoprotein-modifying glycosyl transferase overexpression, and may thereby also limit the maximum extent of modification of poorly accessible glycosylation sites.

1. Materials And Methods

Establishment Of CHO Cells With Tetracycline-Regulated Expression Of Glycosyltransferases. In a first step, an intermediate CHO cell line

(CHO-tTA) was first generated that constitutively expresses a tetracycline-controlled transactivator (tTA) at a level for the adequate for the regulation system. Using Lipofectamine reagent (Gibco, Eggenfelden, Germany), CHO (DUKX) cells were co-transfected, with *pUHD15-1*, a vector for constitutive expression of the tTA gene (Gossen and Bujard, 1992, *Proc. Nat. Acad. Sci. USA*, 89: 5547-5551), and *pSV2Neo*, a vector for constitutive expression of a neomycin resistance gene (Clontech, Palo Alto, CA). Stable, drug-resistant clones were selected and screened for adequate levels of tTA expression *via* transient transfections with a tetracycline-regulated β-galactosidase expression vector, *pUHG16-3*. C-myc epitope-encoding DNA was added to the 3' end of the rat GnT III cDNA (Nishikawa *et al.*, 1992, *J. Biol. Chem.* 267:18199-18204) by PCR amplification. Nilsson *et al*, 1993, *J. Cell Biol.* 120:5-13. The product was sequenced and subcloned into *pUHD10-3*, a vector for tetracycline-regulated expression (Gossen and Bujard, *supra)* to generate the vector *pUHD10-3-GnT IIIm*. The human GnT V cDNA (Saito *et al.*, 1995, *Eur. J. Biochem.* 233:18-26), was directly subcloned into *pUHD10-3* to generate plasmid vector *pUHD10-3-GnT V*. CHO-tTA cells were co-transfected using a calcium phosphate transfection method (Jordan and Wurm, 1996, *Nucleic Acids Res.* 24:596-601), with *pPur*, a vector for constitutive expression of puromycin resistance (Clontech, Palo Alto, CA), and either the vector *pUHD10-3-GnT IIIm* or the vector *pUHD10-3-GnT V*. Puromycin resistant clones were selected in the presence of tetracycline, isolated and then analyzed for tetracycline-regulated expression of GnT III or GnT V *via* western blots analysis. *See, infra.*

Western And Lectin Blotting. For Western blot analysis of GnT III or GnT V, cell lysates were separated by SDS-PAGE and electroblotted to PVDF membranes (Millipore, Bedford, MA). GnT III was detected using the anti-c-myc monoclonal antibody 9E10 (Nilsson *et al.*, 1993, *J. Cell Biol.* 120:5-13) and GnT V using with an anti-GnT V rabbit polyclonal antibody (Chen *et al.*, 1995, *Glycoconjugate J.* 12:813-823). Anti-mouse or anti-rabbit IgG-horse radish peroxidase (Amersham, Arlington, IL) was used as secondary antibody. Bound secondary antibody was detected using an enhanced chemiluminescence kit (ECL kit, Amersham, Arlington, IL)

For lectin blot analysis of glycoproteins modified either by GnT III- or GnT V-catalyzed reactions, biotinylated E-PHA (Oxford Glycosciences, Oxford, United Kingdom) or L-PHA-digoxigenin (Boehringer Mannheim, Mannheim, Germany), respectively, were used. Merkle and Cummings, 1987. *Methods Enzymol.* 138:232-259.

2. Results And Discussion

Establishment Of CHO Cell Lines With Tetracycline-Regulated Overexpression Of Glycosyl Transferases. The strategy used for establishment of glycosyl transferase overexpressing cell lines consisted of first generating an intermediate CHO cell line constitutively expressing the tetracycline-controlled transactivator (tTA) at an adequate level for the system to work. Yin *et al.*, 1996, *Anal. Biochem.* 235:195-201. This level had to be high enough to activate high levels of transcription, in the absence of tetracycline, from the minimal promoter upstream of the glycosyl transferase genes. CHO cells were co-transfected with a vector for constitutive expression for tTA, driven by the human cytomegalovirus (hCMV) promoter/enhancer, and a vector for expression of a neomycin-resistance (Neo^R) gene. An excess of the tTA-expression vector was used and neomycin-resistant clones were isolated.

In mammalian cells, co-transfected DNA integrates adjacently at random locations within the chromosomes, and expression depends to a large extent on the site of integration and also on the number of copies of intact expression cassettes. A mixed population of clones with different expression levels of the transfected genes is generated. Yin *et al.*, 1996, *supra*. Selection for neomycin resistance merely selects for integration of an intact Neo^R expression cassette, while the use of an excess of the tTA-expression vector increases the probability of finding clones with good expression of tTA. The mixed population of clones has to be screened using a functional assay for tTA expression. Gossen and Bujard, 1992, *supra*; Yin *et al.*, 1996, *supra*. This was done by transfection of each clone with a second vector harboring a reporter gene, *lacZ*, under the control of the tet-promoter and screening for tetracycline-regulated (tet-regulated), transient expression (*i.e.*, one to three days after transfection) of ß-galactosidase activity. CHOt17, which showed the highest level of tet-regulated ß-galactosidase activity among twenty screened clones, was selected for further work.

CHOtl7 cells were tested for tet-regulated expression of GnT III by transfecting the cells with vector pUHDlO-3-GnT IIIm and comparing the relative levels of GnT III after incubation of the cells in the presence and absence of tetracycline for 36 h. GnT III levels were compared by western blot analysis, using a monoclonal antibody (9E10) which recognizes the c-myc peptide epitope tag at the carboxy-

terminus of GnT III. The tag had been introduced through a modification of the glycosyl transferase gene using PCR amplification. Various reports have demonstrated addition of peptide epitope tags to the carboxy-termini of glycosyl transferases, a group of enzymes sharing the same topology, without disruption of localization or activity. Nilsson *et al.*, 1993, *supra*; Rabouille *et al.*, 1995, *J. Cell Science* 108:1617-1627. FIGURE 2 shows that in clone CHOt17 GnT III accumulation is significantly higher in the absence than in the presence of tetracycline. An additional clone, CHOt2, which gave weaker activation of transcription in the b-galactosidase activity assay, was tested in parallel (FIGURE 2). GnT III and β-galactosidase expression levels follow the same pattern of tetracycline-regulation for both of these clones. The range of tetracycline concentrations where GnT III expression can be quantitatively controlled was found to be from 0 to 100 ng/ml (FIGURE 3). This result agrees with previous research using different cell lines and genes (Yin *et al.*, 1996, *supra*).

To generate a stable cell line with tet-regulated expression of GnT III, CHOt17 cells were co-transfected with vector pUHD10-3-GnT IIIm and vector, *pPUR*, for expression of a puromycin resistance gene. In parallel, CHOtl7 cells were co-transfected with pUHD10-3-GnT V and *pPUR* vectors to generate an analogous cell line for this other glycosyl transferase. A highly efficient calcium phosphate transfection method was used and the DNA was linearized at unique restriction sites outside the eucaryotic expression cassettes, to decrease the probability of disrupting these upon integration. By using a host in which the levels of tTA expressed had first been proven to be adequate, the probability of finding clones with high expression of the glycosyl transferases in the absence of tetracycline is increased.

Stable integrants were selected by puromycin resistance, keeping tetracycline in the medium throughout clone selection to maintain glycosyl transferase expression at basal levels. For each glycosyl transferase, sixteen puromycin resistant clones were grown in the presence and absence of tetracycline, and eight of each were analysed by western blot analysis (FIGURE 4). The majority of the clones showed good regulation of glycosyl transferase expression. One of the GnT III-expressing clones showed a relatively high basal level in the presence of tetracycline (FIGURE 4B, clone 3), which suggests integration of the expression cassete close to an endogenous CHO-cell enhancer; while two puromycin-resistant clones showed no expression of GnT III in the absence of tetracycline (FIGURE 4B, clones 6 and 8). Among the clones

showing good regulation of expression, different maximal levels of glycosyl transferase were observed. This may be due to variations in the site of integration or number of copies integrated. Activity of the glycosyl transferases was verified by E-PHA and L-PHA lectin binding to endogenous cellular glycoproteins derived from various clones grown in the presence and absence of tetracycline (FIGURE 5). Lectins are proteins which bind to specific oligosaccharide structures. E-PHA lectin binds to bisected oligosaccharides, the products of GnT III-catalyzed reactions, and L-PHA binds to tri- and tetra-antennary oligosaccharides produced by GnT V-catalyzed reactions (Merkle and Cummings, 1987, *Methods Enzymol.* 138:232-259). For each glycosyl transferase, a clone with high expression in the absence, but with undetectable expression in the presence of tetracycline (clone 6, FIGURE 4A, CHO-tet-GnT V, and clone 4, FIGURE 4B, CHO-tet-GnT IIIm) was selected for further work.

B. Example 2: Inhibition Of Cell Growth Effected By Glycosyl Transferase Overexpresseion

During screening of GnT III- and GnT V-expressing clones in the absence of tetracycline, *see*, Example 1, *supra*, approximately half of each set of clones showed a strong inhibition of growth. The extent of growth-inhibition varied among clones, and comparison with expression levels estimated from western blot analysis (FIGURE 4) suggested a correlation between the degree of growth-inhibition and glycosyl transferase overexpression. This correlation was firmly established by growing the final clones, CHO-tet-GnT IIIm and CHO-tet-GnT V, in different concentrations of tetracycline. A strong inhibition of growth was evident after two days of culture at low levels of tetracycline (FIGURE 6). Growth-inhibited cells displayed a small, rounded morphology instead of the typical extended shape of adherent CHO cells. After a few days, significant cell death was apparent from the morphology of the growth-inhibited cells.

Growth-inhibition due to glycosyl transferase overexpression has not hitherto been reported in the literature, probably due to the widespread use of constitutive promoters. Those clones giving constitutive expression of a glycosyl transferase at growth-inhibiting levels, would be lost during the selection procedure. This was avoided here by keeping tetracycline in the medium, *i.e.*, basal expression levels, throughout selection. Prior to selection, the frequency of clones capable of

expressing glycosyl transferases to growth-inhibiting levels using traditional mammalian vectors based on the constitutive hCMV promoter/enhancer would be expected to be lower. This is due to the fact that, for any given gene, the pUHD10-3 vector in CHO cell lines selected for high constitutive levels of tTA, gives significantly higher expression levels than constitutive hCMV promoter/enhancer-based vectors, as observed by others. Yin *et al.*, 1996, *supra*.

Inhibition of cell growth could be due to a direct effect of overexpression of membrane-anchored, Golgi-resident glycosyl transferases independent of their *in vivo* catalytic activity, *e.g.*, *via* misfolding in the endoplasmic reticulum (ER) causing saturation of elements which assist protein folding in the ER. This could possibly affect the folding and secretion of other essential cellular proteins. Alternatively, inhibition of growth could be related to increased *in vivo* activity of the glycosyl transferase leading to a change of the glycosylation pattern, in a function-disrupting fashion, of a set of endogenous glycoproteins necessary for growth under standard *in vitro* culture conditions.

Independent of the underlying mechanism, the growth-inhibition effect has two consequences for engineering the glycosylation of animal cells. First, it implies that cotransfection of constitutive glycosyl transferase expression vectors together with vectors for the target glycoprotein product is a poor strategy. Other ways of linking expression of these two classes of proteins, *e.g.*, through the use of multiple constitutive promoters of similar strength or use of multicistronic, constitutive expression vectors, should also be avoided. In these cases, clones with very high, constitutive expression of the target glycoprotein, a pre-requisite for an economical bioprocess, would also have high expression of the glycosyl transferase and would be eliminated during the selection process. Linked, inducible expression could also be problematic for industrial bioprocesses, since the viability of the growth-arrested cells would be compromised by the overexpression of the glycosyl transferase.

The second consequence is that it imposes an upper limit on glycosyl transferase overexpression for glycosylation engineering approaches. Clearly, the conversions of many glycosyl transferase-catalyzed reactions in the cell, at the endogenous levels of glycosyl transferases, are very high for several glycosylation sites. However, glycosylation sites where the oligosaccharides are somewhat inaccesible or are stabilized in unfavorable conformations for specific glycosyl tranferases also exist.

For example, it has been observed that addition of bisecting GlcNAc is more restricted to the oligosaccharides attached to the Fc region than to those located on the variable regions of human IgG antibodies. Savvidou *et al.*, 1984, *Biochemistry* 23:3736-3740. Glycosylation engineering of these restricted sites could be affected by such a limit on glycosyl transferase expression. Although this would imply aiming for an "unnatural" distribution of glycoforms, these could be of benefit for special therapeutic applications of glycoproteins.

C. Example 3: Engineering The Glycosylation Of An Anti-Human Neuroblastoma Antibody In Chinese Hamster Ovary Cells

In order to validate the concept of engineering a therapeutic antibody by modifying its glycosylation pattern, a chimeric anti-human neuroblastoma IgG1 (chCE7) was chosen which has insignificant ADCC activity when produced by SP2/0 recombinant mouse myeloma cells. ChCE7 recognizes a tumor-associated 190-kDa membrane glycoprotein and reacts strongly with all neuroblastoma tumors tested to date. It has a high affinity for its antigen ($K_d$of $10^{10}M^{-1}$) and, because of its high tumor-specificity, it is routinely used as a diagnostic tool in clinical pathology. Amstutz *et al.*, 1993. *Int. J. Cancer* 53:147-152. In recent studies, radiolabelled chCE7 has shown good tumor localization in human patients. Dürr, 1993, *Eur. J. Nucl. Med.* 20:858. The glycosylation pattern of chCE7, an anti-neuroblastoma therapeutic monoclonal antibody (mAb) was engineered in CHO cells with tetracycline-regulated expression of GnT III. A set of mAb samples differing in their glycoform distribution was produced by controlling GnT III expression in a range between basal and toxic levels, and their glycosylation profiles were analyzed by MALDI/TOF-MS of neutral oligosaccharides. Measurement of the ADCC activity of these samples showed an optimal range of GnT III expression for maximal chCE7 *in vitro* biological activity, and this activity correlated with the level of Fc-associated bisected, complex oligosaccharides.

1. Materials And Methods

Construction Of chCE7 Expression Vectors. Plasmid vectors 10CE7VH and 98CE7VL, for expression of heavy (IgG1) and light (kappa) chains, respectively, of anti-human neuroblastoma chimeric antibody chCE7, which contain chimeric genomic DNA including the mouse immunoglobulin

promoter/enhancer, mouse antibody variable regions, and human antibody constant regions (Amstutz *et al.*, 1993, *Int. J. Cancer* 53:147-152) were used as starting materials for the construction of the final expression vectors, pchCE7H and pchCE7L. Chimeric heavy and light chain chCE7 genes were reassambled and subcloned into the pcDNA3.1(+) vector. During reassembly, all introns were removed, the leader sequences were replaced with synthetic ones, Reff *et al.*, 1994, *Blood* 83:435-445, and unique restriction sites joining the variable and constant region sequences were introduced. Introns from the heavy constant region were removed by splicing with overlap-extension-PCR. Clackson *et al.*, 1991, General Applications of PCR to Gene Cloning and Manipulation, p. 187-214, *in*: McPherson *et al.* (ed.), PCR a Practical Approach, Oxford University Press, Oxford.

Production Of chCE7 In CHO Cells Expressing Different Levels Of GnT III. CHO-tet-GnT IIIm (*see, supra*) cells were co-transfected with vectors pchCE7H, pchCE7L, and pZeoSV2 (for Zeocin resistance, Invitrogen, Groningen, The Netherlands) using a calcium phosphate transfection method. Zeocin resistant clones were transferred to a 96-well cell culture plate and assayed for chimeric antibody expression using an ELISA assay specific for human IgG constant region. Lifely *et al*, 1995, *supra*. Four chCE7 antibody samples were derived from parallel cultures of a selected clone (CHO-tet-GnT IIIm-chCE7), grown in FMX-8 cell culture medium supplemented with 10% FCS; each culture containing a different level of tetracycline and therefore expressing GnT III at different levels. CHO-tet-GnT IIIm-chCE7 cells were expanded and preadapted to a different concentration of tetracycline during 7 days. The levels of tetracycline were 2000, 60, 30, and 15 ng/ml.

Purification Of chCE7 Antibody Samples. Antibody was purified from culture medium by Protein A affinity chromatography on a 1 ml HiTrap Protein A column (Pharmacia Biotech, Uppsala, Sweden), using linear pH gradient elution from 20 mM sodium phosphate, 20 mM sodium citrate, 500 mM sodium chloride, 0.01% Tween 20, 1M urea, pH 7.5 (buffer A) to buffer B (buffer A without sodium phosphate, pH 2.5). Affinity purified chCE7 samples were buffer exchanged to PBS on a 1 ml ResourceS cation exchange column (Pharmacia Biotech, Uppsala, Sweden). Final purity was judged to be higher than 95% from SDS-PAGE and Coomasie-Blue staining. The concentration of each sample was estimated from the absorbance at 280 nm.

Binding Of Antibodies To Neuroblastoma Cells. Binding affinity to human

neuroblastoma cells was estimated from displacement of ^{125}I-labeled chCE7 by the CHO-produced samples. Amstutz *et al*, 1993, *supra*.

Oligosaccharide Analysis By MALDI/TOF-MS. CE7-2000t, -60t, -30t, and -15t samples were treated with *A. urefaciens* sialidase (Oxford Glycosciences, Oxford, United Kingdom), following the manufacturer's instructions, to remove any sialic acid monosaccharide residues. The sialidase digests were then treated with peptide N-glycosidase F (PNGaseF, Oxford Glycosciences, Oxford, United Kingdom), following the manufacturer's instructions, to release the N-linked oligosaccharides. Protein, detergents, and salts were removed by passing the digests through microcolumns containing, from top to bottom, 20 ml of SepPak C18 reverse phase matrix (Waters, Milford, MA), 20 ml of Dowex AG 50W X8 cation exchange matrix (BioRad, Hercules, CA), and 20 ml of AG 4X4 anion exchange matrix (BioRad, Hercules, CA). The microcolumns were made by packing the matrices in a Gel Loader tip (Eppendorf, Basel, Switzerland) filled with ethanol, followed by an equilibration with water. Küster *et al.*, 1997, *Anal. Biochem.* 250:82-101. Flow through liquid and a 300 ml-water wash were pooled, filtered, evaporated to dryness at room temperature, and resuspended in 2 ml of deionized water. One microliter was applied to a MALDI-MS sample plate (Perseptive Biosystems, Farmingham, MA) and mixed with 1 ml of a 10 mg/ml dehydrobenzoic acid (DHB, Aldrich, Milwakee, Wisconsin) solution in acetonitrile. The samples were air dried and the resulting crystals were dissolved in 0.2 ml of ethanol and allowed to recrystallize by air drying. Harvey, 1993, *Rapid Mass. Spectrom.* 7:614-619. The oligosaccharide samples were then analyzed by matrix-assisted laser desorption ionization/time-of-flight-mass spectrometry (MALDI/TOF-MS) using an Elite Voyager 400 spectrometer (Perseptive Biosystems, Farmingham, MA), equipped with a delayed ion extraction MALDI-ion source, in positive ion and reflector modes, with an accelaration voltage of 20 kV. One hundred and twenty eight scans were averaged. Bisected biantennary complex oligosaccharide structures were assigned to five-HexNAc-associated peaks. Non-bisected tri-antennary N-linked oligosaccharides, the alternative five HexNAc-containing isomers, have never been found in the Fc region of IgGs and their syntheses are catalyzed by glycosyltransferases discrete from GnT III.

ADCC Activity Assay. Lysis of IMR-32 human neuroblastoma cells (target) by human lymphocytes (effector), at a target:effector ratio of 1:19, during a 16 h incubation at 37 °C in the presence of different concentrations of chCE7 samples, was

measured *via* retention of a fluorescent dye. Kolber *et al*, 1988, *J. Immunol. Methods* 108: 255-264. IMR-32 cells were labeled with the fluorescent dye Calcein AM for 20 min (final concentration 3.3 μM). The labeled cells (80'000 cells/well) were incubated for 1h with different concentrations of CE7 antibody. Then, monocyte depleted mononuclear cells were added (1'500'000 cells/well) and the cell mixture was incubated for 16 h at 37°C in a 5% $C0_2$ atmosphere. The supernatant was discarded and the cells were washed once with HBSS and lysed in Triton X-100 (0.1%). Retention of the fluorescent dye in IMR-32 cells was measured with a fluorometer (Perkin Elmer, Luminscence Spectrometer LS 50B, (Foster City, CA) and specific lysis was calculated relative to a total lysis control, resulting from exposure of the target to a detergent instead of exposure to antibody. The signal in the absence of antibody was set to 0% cytotoxicity. Each antibody concentration was analyzed by triplicate, and the assay was repeated three separate times.

2. Results And Discussion

Production Of chCE7 In CHO Cells Expressing Different Levels Of GnT III. ChCE7 heavy and light chain expression vectors were constructed incorporating the human cytomegalovirus (hCMV) promoter, the bovine growth hormone termination and polyadenylation sequences, and eliminating all heavy and light chain introns. This vector design was based on reports of reproducible high-level expression of recombinant IgG genes in CHO cells. Reff *et al.*, 1994, *supra*; Trill *et al.*, 1995, *Current Opinion Biotechnol.* 6:553-560. In addition, a unique restriction sites was introduced in each chain, at the junction between the variable and constant regions. These sites conserve the reading frame and do not change the amino acid sequence. They should enable simple exchange of the mouse variable regions, for the production of other mouse-human chimeric antibodies. Reff *et al.*, 1994, *supra*. DNA sequencing confirmed that the desired genes were appropriately assembled, and production of the chimeric antibody in transfected CHO cells was verified with a human Fc-ELISA assay.

CHO-tet-GnT IIIm-chCE7 cells, with stable, tetracycline-regulated expression of GnT III and stable, constitutive expression of chCE7, were established and scaled-up for production of a set of chCE7 samples. During scale-up, four parallel cultures derived from the same CHO clone were grown, each at a different level of tetracycline and therefore only differing in the level of expression of the GnT III gene.

This procedure eliminates any clonal effects from other variables affecting N-linked glycoform biosynthesis, permitting a rigorous correlation to be established between GnT III gene expression and biological activity of the glycosylated antibody. The tetracycline concentration ranged from 2000 ng/ml, *i.e.*, the basal level of GnT III expression, to 15 ng/ml, at which significant growth inhibition and toxicity due to glycosyl transferase overexpression was observed (*see, supra*). Indeed, only a small amount of antibody could be recovered from the latter culture. The second highest level of GnT III expression, using tetracycline at a concentration of 30 ng/ml, produced only a mild inhibition of growth. The purified antibody yield from this culture was approximately 70% that from the remaining two lower levels of GnT III gene overexpression.

The four antibody samples, CE7-2000t, -60t, -30t, and -15t, numbers denoting the associated concentration of tetracycline, were purified by affinity chromatography on Protein A and buffer exchanged to PBS using a cation exchange column. Purity was higher than 95% as judged from SDS-PAGE with Coomassie Blue staining. Binding assays to human neuroblastoma cells revealed high affinity to the cells and no significant differences in antigen binding among the different samples (estimated equilibrium dissociation constants varied between 2.0 and 2.7 x 10^{-10} M). This was as expected, since there are no potential N-linked glycosylation sites in the CE7 variable regions.

Oligosaccharide Distributions And Levels Of Bisected Complex Oligosaccharides Of Different chCE7 Samples. Oligosaccharide profiles were obtained by matrix-assisted laser desorption/ionization mass spectrometry on a time-of-flight instrument (MALDI/TOF-MS). Mixtures of neutral N-linked oligosaccharides derived from each of the four CHO-produced antibody samples and from a SP2/0 mouse myeloma-derived chCE7 (CE7-SP2/0) sample were analyzed using 2,5-dehydrobenzoic acid (2,5-DHB) as the matrix (FIGURE 9). Under these conditions, neutral oligosaccharides appear essentially as single [M + Na^+] ions, which are sometimes accompanied by smaller [M + K^+] ions, depending on the potassium content of the matrix. Bergweff *et al.*, 1995, *Glycoconjugate J.* 12:318-330.

This type of analysis yields both the relative proportions of neutral oligosaccharides of different mass, reflected by relative peak height, and the isobaric monosaccharide composition of each peak. Küster *et al.*, 1997, *supra*; Naven and

Harvey, 1996, *Rapid Commun. Mass Spectrom.* 10:1361-1366. Tentative structures are assigned to peaks based on the monosaccharide composition, knowledge of the biosynthetic pathway, and on previous structural data for oligosaccharides derived from the same glycoprotein produced by the same host, since the protein backbone and the cell type can have a strong influence on the oligosaccharide distribution. Field *et al.*, 1996, *Anal. Biochem.* 239:92-98. In the case of Fc-associated oligosaccharides, only bi-antennary complex oligosaccharides have been detected in IgGs present in human serum or produced by mammalian cell cultures under normal conditions. Wormald *et al.*, 1997, *Biochemistry* 36:1370-1380; Wright and Morrison, 1997, *Tibtech* 15:26-31. The pathway leading to these compounds is illustrated in FIGURE 10, including the mass of the [M + Na^+] ion corresponding to each oligosaccharide. High mannose oligosaccharides have also been detected on antibodies produced in the stationary and death phases of batch cell cultures. Yu Ip *et al.*, 1994, *Arch. Biochem. Biophys.* 308:387-399.

The two major peaks in the CE7-SP2/0 sample (FIGURE 9A) correspond to masses of fucosylated oligosaccharides with four N-acetylhexosamines (HexNAcs) containing either three (m/z 1486) or four (m/z 1648) hexoses. *See*, FIGURE 10, but note that the summarized notation for oligosaccharides in this figure does not count the two GlcNAcs of the core. This composition is consistent with core fucosylated, bi-antennary complex oligosaccharide structures carrying zero or one galactose residues, respectively, typical of Fc-associated oligosaccharides, and as previously observed in NMR analysis of Fc oligosaccharides derived from a chimeric IgG1 expressed in SP2/0 cells. Bergweff *et al.*, 1995, *supra*.

GnT III-catalyzed transfer of a bisecting GlcNAc to these bi-antennary compounds, which are the preferred GnT III acceptors, would lead to oligosaccharides with five HexNAcs (m/z 1689 and 1851, non- and mono-galactosylated, respectively, FIGURE 10), which are clearly absent in the CE7-SP2/0 sample. The latter peaks appear when chCE7 is expressed in CHO-tet-GnTIIIm cells. In the CHO-expressed antibodies the four HexNAc-containing peaks are also mainly fucosylated, although a small amount of non-fucosylated structures is evident from the peak at m/z 1339 (*see*, FIGURE 10). The level of galactosylation is also not very different between the CHO- and SP2/0-derived material. At the basal level of GnT III expression (CE7-2000t sample. FIGURE 9B), the molecules with five HexNAcs are present in a lower

proportion than those with four HexNAcs. A higher level of GnT III expression (CE7-60t sample, FIGURE 9C) led to a reversal of the proportions in favor of oligosaccharides with five HexNAcs. Based on this trend, bisected, bi-antennary complex oligosaccharide structures can be assigned to compounds with five HexNAcs in these samples. Tri-antennary N-linked oligosaccharides, the alternative five HexNAc-containing isomers, have never been found in the Fc region of IgGs and their syntheses are catalyzed by GlcNAc-transferases discrete from GnT III.

A further increase in GnT III expression (CE7-30t sample, FIGURE 9D) did not lead to any significant change in the levels of bisected complex oligosaccharides. Another peak (m/z 1543) containing five HexNAcs appears at low, but relatively constant levels in the CHO-GnTIII samples and corresponds in mass to a non-fucosylated, bisected-complex oligosaccharide mass (FIGURE 10). The smaller peaks at m/z 1705 and 1867, also correspond to five HexNAc-containing bi-antennary complex oligosaccharides. They can be assigned either to potassium adducts of the peaks at m/z 1689 and 1851 (mass difference of 16 Da with respect to sodium adducts) (Küster *et al.*, 1997, *supra*) or to mono- and bi-galactosylated, bisected complex oligosaccharides without fucose (FIGURE 10). Together, the bisected complex oligosaccharides amount to approximately 25% of the total in sample CE7-2000t and reach approximately 45 to 50% in samples CE7-60t and CE7-30t.

Additional information From The Oligosaccharide Profiles Of chCE7 Samples. Although the levels of bisected complex oligosaccharides were not higher in sample CE730t, increased overexpression of GnT III did continue to reduce, albeit to a small extent, the proportions of substrate bi-antennary complex oligosaccharide substrates. This was accompanied by moderate increases in two different, four HexNAc-containing peaks (m/z 1664 and 1810). The latter two peaks can correspond either to galactosylated bi-antennary complex oligosaccharides or to bisected hybrid compounds (FIGURE 11). A combination of both classes of structures is also possible. The relative increase in these peaks is consistent with the accumulation of bisected hybrid by-products of GnT III overexpression. Indeed, the sample produced at the highest level of GnT III overexpression, CE7-15t, showed a large increase in the peak at m/z 1664, a reduction in the peak at m/z 1810 and a concomitant reduction of complex bisected oligosaccharides to a level of approximately 25%. *See*, peaks with m/z 1689 and 1851 in FIGURE 9E and the corresponding structures in FIGURE 11. Higher

accumulation of non-fucosylated (m/z 1664) bisected hybrid by-products, instead of fucosylated ones (m/z 1810), would agree with the fact that oligosaccharides which are first modified by GnT III can no longer be biosynthetic substrates for core α1,6-fucosyltransferase. Schachter, 1986, *Biochem. Cell Biol.* 64:163-181.

The peak at m/z 1257 is present at a level of 10 -15% of the total in the CHO-derived samples and at a lower level in CE7-SP2/0 (FIGURE 9). It corresponds to five hexoses plus two HexNAcs. The only known N-linked oligosaccharide structure with this composition is a five mannose-containing compound of the high-mannose type. Another high mannose oligosaccharide, a six mannose one (m/z 1420), is also present at much lower levels. As mentioned above, such oligosaccharides have been detected in the Fc of IgGs expressed in the late phase of batch cell cultures. Yu Ip *et al.*, 1994, *supra*.

Antibody Dependent Cellular Cytotoxicity Of chCE7 Samples. ChCE7 shows some ADCC activity, measured as *in vitro* lysis of neuroblastoma cells by human lymphocytes, when expressed in CHO-tet-GnTIIIm cells with the minimum level of GnT III overexpression (FIGURE 12, sample CE7-2000t). Raising the level of GnT III produced a large increase in ADCC activity (FIGURE 12, sample CE7-60t). Further overexpression of GnT III was not accompanied by an additional increase in activity (FIGURE 12, sample CE7-30t), and the highest level of expression actually led to reduced ADCC (FIGURE 12, sample CE7-15t). Besides exhibiting the highest ADCC activities, both CE7-60t and CE7-30t samples show significant levels of cytotoxicity at very low antibody concentrations. These results show that there is an optimal range of GnT III overexpression in CHO cells for ADCC activity, and comparison with oligosaccharide profiles shows that activity correlates with the level of Fc-associated, bisected complex oligosaccharides.

Given the importance of bisected complex oligosaccharides for ADCC activity, it would be useful to engineer the pathway to further increase the proportion of these compounds. Overexpression of GnT III to levels approaching that used for sample CE7-30t is within the biotechnologically practical range where no significant toxicity and growth inhibiton are observed. At this level of expression, the non-galactosylated, non-bisected, bi-antennary complex oligosaccharides, *i.e.*, the preferred, potential GnT III substrates, are reduced to less than 10% of the total. *See*, m/z 1486 peak, FIGURE 9D. However, only 50% are converted to the desired bisected

biantennary complex structures. The rest are either diverted to bisected, hybrid oligosaccharide byproducts or consumed by the competing enzyme β1,4-galactosyltransferase, GalT (FIGURE 11).

Resolution of the bisected hybrid and the non-bisected, galactosylated complex oligosaccharide peaks by complementary structural analyses would determine how much each potential, undesired route is consuming. The growth of the m/z 1664 and 1810 peaks at high GnT III overexpression levels suggests that at least a fraction of these peaks corresponds to bisected hybrid oligosaccharides (FIGURE 11). In theory, a flux going to bisected hybrid compounds can be reduced by co-overexpression of enzymes earlier in the pathway such as mannosidase II together with GnT III. On the other hand, competition between GnT III and GalT for bisected complex oligosaccharide substrates could potentially be biased towards GnT III-catalyzed reactions, by increasing the intra-Golgi concentration of UDP-GlcNAc while overexpressing GnT III. GnT III transfers a GlcNAc from the co-substrate UDP-GlcNAc to the different oligosaccharides. Should the intra-Golgi concentration of UDP-GlcNAc co-substrate be sub-saturating for GnT III, then increasing it, either by manipulation of the culture medium composition or by genetic manipulation of sugar-nucleotide transport into the Golgi, could favor GnT III in a competition for oligosaccharides with GalT.

It remains to be determined whether the increase in ADCC activity results from the increase in both the galactosylated and non-galactosylated, bisected complex oligosaccharides, or only from one of these forms. *See*, peaks at m/z 1689 and 1851 in FIGURE 9. If it is found that galactosylated, bisected complex bi-antennary oligosaccharides are the optimal structures for increased ADCC activity, then maximizing the fraction of these compounds on the Fc region would require overexpression of both GnT III and GalT. Given the competitive scenario discussed previously, the expression levels of both genes would have to be carefully regulated. In addition, it would be valuable to try to re-distribute overexpressed GalT as much as possible towards the TGN instead of the trans-Golgi cisterna. The latter strategy may be realized by exchanging the transmembrane region-encoding sequences of GalT with those of α2,6-sialyltransferase (Chege and Pfeffer, 1990, *J. Cell. Biol.* 111:893-899).

D. Example 4: Engineering The Glycosylation Of The Anti-CD20 Monoclonal Antibody C2B8

C2B8 is an anti-human CD20 chimeric antibody, Reff, M.E. *et al*, 1994, *supra*. It recieved FDA approval in 1997 and is currently being used, under the comercial name of RituxanTM, for the treatment of Non-Hodgkin's lymphoma in the United States. It is derived from CHO cell culture and therefore should not carry bisected oligosaccharides. *See, supra*. In order to produce an improved version of this antibody, the method demonstrated previously for the chCE7 anti-neuroblastoma antibody was applied. *See, supra*. C2B8 antibody modified according to the disclosed method had a higher ADCC activity than the standard, unmodified C2B8 antibody produced under identical cell culture and purification conditions.

1. Material And Methods

Synthesis Of The Variable Light And Variable Heavy Chain Regions Of Chimeric Anti-CD20 Monoclonal Antibody (C2B8). The VH and VL genes of the C2B8 antibody were assembled synthetically using a set of overlapping single-stranded oligonucleotides (primers) in a one-step process using PCR, Kobayashi *et al*, 1997, *Biotechniques* 23: 500-503. The sequence data coding for mouse immunoglobulin light and heavy chain variable regions (VL and VH respectively) of the anti-CD20 antibody were obtained from a published international patent application (International Publication Number: WO 94/11026). The assembled DNA fragments were subcloned into pBluescriptIIKS(+) and sequenced by DNA cycle sequencing to verify that no mutations had been introduced.

Contruction Of Vectors For Expression Of Chimeric Anti-CD20 Monoclonal Antibody (C2B8). VH and VL coding regions of the C2B8 monoclonal antibody were subcloned in pchCE7H and pchCE7L respectively. In the subcloning, the sequences coding for the variable heavy and light chains of the anti-neuroblastoma CE7 (*see, supra)* were exchanged with the synthetically assembled variable heavy and variable light chain regions of C2B8.

Generation Of CHO-tet-GnTIIIm Cells Expressing C2B8 Antibody. The method for the generation of a CHO-tet-GntIIIm cell line expressing C2B8 antibody was exactly the same as for CHO-tet-GnTIIIm-CE7. *See. supra*. The clone chosen for further work was named CHO-tet-GnTIIIm-C2B8.

Generation Of CHO-tTA Expressing C2B8 Antibody. CHO-tTA is the parental cell line of CHO-tet-GnTIIIm. *See, supra.* The method for the generation of a CHO-tTA cell line expressing C2B8 antibody without GnT III expression was exactly the same as for CHO-tet-GnTIIIm-C2B8 and CHO-tet-GnTIIIm-chCE7. *See, supra.* The clone chosen for further work was named CHO-tTA-C2B8.

Production Of C2B8 Antibody Samples. Two C2B8 antibody samples were derived from parallel CHO-tet-GnTIIIm-C2B8 cultures; each culture containing different levels of tetracycline and therefore expected to express GnTIII at different levels. The levels of tetracycline were 2000, 50, and 25ng/ml. The C2B8 antibody samples derived from these cultures were designated as C2B8-2000t, C2B8-50t, and C2B8-25t, respectively. In parallel, one antibody sample (C2B8-nt) was made from a CHO-tTA-C2B8 culture, this cell line does not express GnT III. CHO-tTA-C2B8 cells were cultured without tetracycline.

Analysis Of GnT III Expression. For Western blot analysis of GnT III, cell lysates of each of the production cultures were resolved by SDS-PAGE and electroblotted to polyvinylidene difluoride membranes. Anti-c-myc monoclonal antibody 9E10 and anti-mouse IgG-horseradish peroxidase (Amersham, Arlington, IL) were used as primary and secondary antibodies respectively. Bound antibody was detected using an enhanced chemiluminiscence kit (Amersham, Arlington, IL).

Purification Of C2B8 Antibody Samples. Antibody samples were purified using the same procedure as for the chCE7 antibody samples. *See, supra.* The concentration was measured using a fluorescence based kit from Molecular Probes (Leiden, The Netherlands).

Verification Of Specific C2B8 Antigen Binding . The specificity of antigen binding of the C2B8 anti-CD20 monoclonal antibody was verified using an indirect immunofluorescence assay with cells in suspension. For this study, CD20 positive cells (SB cells; ATCC deposit no.ATCC CCL120) and CD20 negative cells (HSB cells; ATCC deposit no. ATCC CCL120.1) were utilized. Cells of each type were incubated with C2B8 antibody produced at 25ng/ml tetracycline, as a primary antibody. Negative controls included HBSSB instead of primary antibody. An anti-human IgG Fc specific, polyclonal, FITC conjugated antibody was used for all samples as a secondary antibody (SIGMA, St. Louis, MO). Cells were examined using a Leica (Bensheim, Germany) fluorescence microscope.

ADCC Activity Assay. Lysis of SB cells (CD20+ target cells; ATCC deposit no. ATCC CCL120) by human monocyte depleted peripheral blood mononuclear cells (effector cells) in the presence of different concentrations of C2B8 samples was performed basically following the same procedure described in Brunner *et al.*, 1968, *Immunology* 14:181-189. The ratio of effector cells to target cells was 100:1.

2. Results And Discussion

GnT III Is Expressed At Different Levels In Different Cell Lines And Cultures. The cells of the parallel CHO-tet-GnTIIIm-C2B8 cultures, each culture containing different levels of tetracycline (2000, 50, and 25ng/ml) and therefore expected to express GnTIII at different levels, were lysed and the cell lysates were resolved by SDS-PAGE and detected by Western blotting. The lysates of the culture grown at 25ng/ml tetracycline showed an intense band at the corresponding molecular weight of GnT III whereas cultures grown at 50 and at 2000ng/ml had much less expression of GnT III as shown in FIGURE 13.

Verification Of Specific C2B8 Antigen Binding. C2B8 samples produced from parallel cultures of cells expressing different levels of GnT III were purified from the culture supernatants by affinity chromatography and buffer exchanged to PBS on a cation exchange column. Purity was estimated to be higher than 95% from Coomassie Blue staining of an SDS-PAGE under reducing conditions. These antibody samples were derived from expression of antibody genes whose variable regions were synthesized by a PCR assembly method. Sequencing of the synthetic cDNA fragments revealed no differences to the original C2B8 variable region sequences previously published in an international patent application (International Publication Number WO 94/11026). Specific binding of the samples to human CD20, the target antigen of C2B8, was demonstrated by indirect immunofluorescence using a human lymphoblastoid cell line SB expressing CD20 on its surface and an HSB lymphoblastoid cell line lacking this antigen. Antibody sample C2B8-25t gave positive staining of SB cells (FIGURE 14A), but not of HSB cells under identical experimental conditions (see FIGURE 14B). An additional negative control consisted of SB cells incubated with PBS buffer instead of C2B8-25t antibody. It showed no staining at all.

In Vitro ADCC Activity Of C2B8 Samples. The antibody sample C2B8-nt expressed in CHO-tTA-C2B8 cells that do not have Gnt III expression (*see. supra*)

showed 31% cytotoxic activity (at 1μg/ml antibody concentration), measured as *in vitro* lysis of SB cells (CD20+) by human lymphocytes (FIGURE 15, sample C2B8-nt). C2B8-2000t antibody derived from a CHO-tet-GnTIII culture grown at 2000ng/ml of tetracycline (*i.e.*, at the basal level of cloned GnT III expression) showed at 1 μg/ml antibody concentration a 33% increase in ADCC activity with respect to the C2B8-nt sample at the same antibody concentration. Reducing the concentration of tetracycline to 25ng/ml (sample C2B8-25t), which significantly increased GnTIII expression, produced a large increase of almost 80% in the maximal ADCC activity (at 1 μg/ml antibody concentration) with respect to the C2B8-nt antibody sample at the same antibody concentration (FIGURE 15, sample C2B8-25t).

Besides exhibiting the highest ADCC activity, C2B8-25t showed significant levels of cytotoxicity at very low antibody concentrations. The C2B8-25t sample at 0.06μg/ml showed an ADCC activity similar to the maximal ADCC activity of C2B8-nt at 1μg/ml. This result showed that sample C2B8-25t, at a 16- fold lower antibody concentration, reached the same ADCC activity as C2B8-nt. This result indicates that the chimeric anti-CD20 antibody C2B8 produced in a cell line actively expressing GnT III was significantly more active than the same antibody produced in a cell line that did not express GnT III.

One advantage of this antibody using the methods of the invention is that (1) lower doses of antibody have to be injected to reach the same therapeutic effect, having a benefical impact in the economics of antibody production, or (2) that using the same dose of antibody a better therapeutic effect is obtained.

E. Example 5: Establishment Of CHO Cell Lines With Constitutive Expression Of Glycosyltransferase Genes At Optimal Levels Leading To Maximal ADCC Activity

In some applications of the method for enhancing the ADCC it may be desirable to use constitutive rather than regulated expression of GnT III on its own or together with other cloned glycosyltransferases and/or glycosidases. However, the inventors have demonstrated that ADCC activity of the modified antibody depends on the expression level of GnT III. *See, supra.* Therefore, it is important to select a clone with constitutive expression of GnT III alone or together with other glycosyltransferase and/or glycosidase genes at optimal or near optimal levels. The optimal levels of

expression of GnT III, either alone or together with other glycosyl transferases such as β(1,4)-galactosyl transferase (GalT), are first determined using cell lines with regulated expression of the glycosyl transferases. Stable clones with constitutive expression of GnT III and any other cloned glycosyltransferase are then screened for expression levels near the optimum.

1. Determination Of Near-optimal Expression Levels

Construction Of A Vector For Regulated GnT III Expression linked To GFP Expression. Each glycosyl transferase gene is linked, *via* an IRES sequence, to a reporter gene encoding a protein retained in the cell, *e.g.*, green fluorescent protein (GFP) or a plasma membrane protein tagged with a peptide that can be recognized by available antibodies. If more than one glycosyl transferase is being tested, a different marker is associated with each glycosyl transferase, *e.g.*, GnT III may be associated to GFP and GalT may be associated to blue fluorescent protein (BFP). An eucaryotic expression cassette consisting of the GnT III cDNA upstream of an IRES element upstream of the GFP cDNA is first assembled by standard subcloning and/or PCR steps. This cassette is then subcloned in the tetracycline regulated expression vector pUHD10-3 (*see, supra*), downstream of the tet-promoter and upstream of the termination and polyadenylation sequences resulting in vector pUHD10-3-GnTIII-GFP.

Establishment Of CHO Cells With Regulated GnTIII Expression Linked To GFP Expression And Constitutive chCE7 Antibody Expression. CHO-tTA cells (*see, supra*) expressing the tetracycline-responsive transactivator, are co-transfected with vector pUHD10-3-GnTIII-GFP and vector pPur for expression of a puromycin-resistance gene. *See, supra.* Puromycin resistant clones are selected in the presence of tetracycline. Individual clones are cultured by duplicate in the presence (2μg/ml) or absence of tetracycline. Six clones that show inhibition of growth in the absence of tetracycline, due to glycosyltransferase overexpression (*see, supra*), are selected and analyzed by fluorescence-activated cell sorting (FACS) for detection of the GFP-associated signal. A clone giving the highest induction ratio, defined as the ratio of fluorescence in the absence of tetracycline to fluorescence in the presence of tetracycline is chosen for further work and designated as CHO-tet-GnTIII-GFP. CHO-tet-GnTIII-GFP are transfected with expression vectors for antibody chCE7 and a clone with high constitutive expression of this antibody is selected CHO-tet-GnTIII-GFP-

chCE7. *See, supra.*

Production Of chCE7 Samples, Measurement Of ADCC Activity And Determination Of Optimal GnTIII Expression Levels. Parallel cultures of CHO-tet-GnTIII-GFP-chCE7 are grown at different levels of tetracycline, and therefore expressing GnTIII together with GFP at different levels. chCE7 antibody samples are purified from the culture supernatants by affinity chromatography. In parallel, the cells from each culture are analyzed by FACS to determine the mean level of GFP-associated fluorescence, which is correlated to the expression level of GnT III, of each culture. The *in vitro* ADCC activity of each chCE7 antibody sample is determined (*see, supra*) and the maximal *in vitro* ADCC activity of each sample is plotted against the mean fluorescence of the cells used to produce it.

2. Establishment Of A CHO Cell Line With Constitutive GnTIII expression At Near-optimal Levels

The GnTIII-IRES-GFP cassette (*see, supra*) is subcloned in a constitutive expression vector. CHO cells are stably co-transfected with this vector and a vector for puromycin resistance. Puromycin resistant cells are selected. This population of stably transfected cells is then sorted *via* FACS, and clones are selected which express the levels of reporter GFP gene near the within the range where optimal or near-optimal ADCC activity is achieved. *See, supra.* This final transfection step may be done either on CHO cells already stably expressing a therapeutic antibody or on empty CHO cells, *e.g.*, DUKX or DG44 dhfr- CHO cells. In the latter case, the clones obtained from the procedure described above will be transfected with therapeutic antibody-expression vectors in order to generate the final antibody-producing cell lines.

F. Example 6: Cell Surface Expression Of A Human IgG Fc Chimera With Optimized Glycosylation

Encapsulated cell therapy is currently being tested for a number of diseases. An encapsulated cell implant is designed to be surgically placed into the body to deliver a desired therapeutic substance directly where it is needed. However, if once implanted the encapsulated device has a mechanical failure, cells can escape and become undesirable. One way to destroy escaped, undesirable cells in the body is *via* an Fc-mediated cellular cytoxicity mechanism. For this purpose, the cells to be

encapsulated can be previously engineered to express a plasma membrane-anchored fusion protein made of a type II transmembrane domain that localizes to the plasma membrane fused to the N-terminus of an Fc region. Stabila, P.F., 1998, *supra.* Cells inside the capsule are protected against Fc-mediated cellular cytoxicity by the capsule, while escaped cells are accesible for destruction by lymphocytes which recognize the surface-displayed Fc regions, *i.e.*, *via* an Fc-mediated cellular cytoxicity mechanism. This example illustrates how this Fc-mediated cellular cytoxicity activity is enhanced by glycosylation engineering of the displayed Fc regions.

1. Establishment Of Cells Expressing The Fc Chimera On Their Surface And Expressing GnTIII

Cells to be implanted for a particular therapy, for example baby hamster kidney (BHK) cells, which already produce the surface-displayed Fc chimera and a secreted, therapeutic protein, are first stably transfected with a vector for constitutive expression of GnTIII linked *via* an IRES element to expression of GFP. *See, supra.* Stable transfectants are selected by means of a marker incorporated in the vector, *e.g.*, by means of a drug resistance marker and selected for survival in the presence of the drug.

2. Screening Of Cells Expressing Diffent Levels Of GnTIII And Measurement

Stable transfectants are analyzed by fluorescence-activated cell sorting (FACS) and a series of clones with different mean fluorescence levels are selected for further studies. Each selected clone is grown and reanalyzed by FACS to ensure stability of GFP, and therefore associated GnT III, expression.

3. Verification Of Different Levels Of Bisected Complex Oligosaccharides On The Displayed Fc Regions

Fc regions from three clones with different levels of GFP-associated fluorescence and from the original BHK cells not transfected with the GnTIII-IRES-GFP vector are solubilized from the membrane by means of a detergent and then purified by affinity chromatography. The oligosaccharides are then removed, purified and analyzed by MALDI-TOF/MS. *See, supra.* The resulting MALDI-TOF/MS profiles show that the Fc-regions of the modified, fluorescent clones carry

different proportions of bisected complex oligosaccharides. The MALDI profile from the unmodified cells does not show any peak associated to bisected oligosaccharides. The clone with carrying the highest levels of bisected complex oligosaccharides on the displayed Fc regions is chosen for further work.

4. ***In vitro* Fc-mediated Cellular Cytoxicity Activity Assay**

Two Fc-mediated cellular cytoxicity activity assays are then conducted in parallel. In one assay the target cells are derived from the clone selected above. In the parallel assay the target cells are the original cells to be encapsulated and which have not been modified to express GnTIII. The assay is conducted using the procedure described previously (*see, supra*) but in the absence of any additional antibody, since the target cells already display Fc regions. This experiment demonstrates that the Fc-mediated cellular cytoxicity activity against the cells expressing GnT III is higher than that against cells not expressing this glycosyltransferase.

All references cited within the body of the instant specification are hereby incorporated by reference in their entirety.

CLAIMS

What is claimed is:

1. A host cell engineered to express at least one nucleic acid encoding a glycoprotein-modifying glycosyl transferase at a regulated level.

2. The host cell of Claim 1, wherein a nucleic acid molecule comprising at least one gene encoding a glycoprotein-modifying glycosyl transferase has been introduced in said host cell.

3. The host cell of Claim 1, wherein said host cell has been engineered such that an endogenous glycoprotein-modifying glycosyl transferase is activated.

4. The host cell of Claim 2 or 3, wherein said host cell is a CHO cell, a BHK cell, a NS0 cell, a SP2/0 cell, or a hybridoma cell.

5. The host cell of Claim 3, wherein said endogenous glycoprotein-modifying glycosyl transferase has been activated by insertion of a regulated promoter element into the host cell chromosome.

6. The host cell of Claim 2 or 3, wherein said glycoprotein-modifying glycosyl transferase is GnT III, GnT V, Man II, or Gal T.

7. The host cell of Claim 2 or 3, wherein said host cell is engineered to express at least two different glycoprotein-modifying glycosyl transferases selected from the group consisting of GnT III, GnT V, Man II, and Gal T.

8. The host cell of Claim 7, wherein at least one gene encoding a glycoprotein-modifying glycosyl transferase is operably linked to a constitutive promoter element.

9. The host cell of Claim 2, wherein at least one gene encoding a

glycoprotein-modifying glycosyl transferase is operably linked to a regulated promoter element.

10. The host cell of Claim 5 or 9, wherein the regulated promoter element is a tetracycline-regulated promoter system, an ecdysone-inducible promoter system, a lac-switch promoter system, a glucocorticoid-inducible promoter system, a temperature-inducible promoter system, or a metallothionein metal-inducible promoter system.

11. A host cell engineered to express at least one nucleic acid molecule encoding a glycoprotein-modifying glycosyl transferase, wherein said host cell is capable of producing a protein having enhanced Fc-mediated cellular cytotoxicity.

12. The host cell of Claim 11, wherein said protein is a whole antibody molecule, an antibody fragment, or a fusion protein that includes a region equivalent to the Fc region of an immunoglobulin.

13. The host cell of Claim 12, wherein a nucleic acid molecule comprising at least one gene encoding a glycoprotein-modifying glycosyl transferase has been introduced into said host cell chromosome.

14. The host cell of Claim 12, wherein said host cell has been selected to carry a mutation triggering expression of an endogenous glycoprotein-modifying glycosyl transferase.

15. The host cell of Claim 14, wherein said host cell is the mutant lec10.

16. The host cell of Claim 12, wherein said host cell has been engineered such that an endogenous glycoprotein-modifying glycosyl transferase is activated.

17. The host cell of Claim 16, wherein said endogenous glycoprotein-modifying glycosyl transferase has been activated by insertion of a regulated promoter element into the host cell chromosome.

18. The host cell of Claim 16, wherein said endogenous glycoprotein-modifying glycosyl transferase has been activated by insertion of a constitutive promoter element, a transposon, or a retroviral element into the host cell chromosome.

19. The host cell of Claim 11 or 13, further comprising at least one transfected nucleic acid encoding an antibody molecule, an antibody fragment, or a fusion protein that includes a region equivalent to the Fc region of an immunoglobulin.

20. The host cell of Claim 13, wherein at least one gene encoding a glycoprotein-modifying glycosyl transferase is operably linked to a constitutive promoter element.

21. The host cell of Claim 13, wherein at least one gene encoding a glycoprotein-modifying glycosyl transferase is operably linked to a regulated promoter element.

22. The host cell of Claim 21, wherein the regulated promoter element is a tetracycline-regulated promoter system, an ecdysone-inducible promoter system, a lac-switch promoter system, a glucocorticoid-inducible promoter system, a temperature-inducible promoter system, or a metallothionein metal-inducible promoter system.

23. The host cell of Claim 11, wherein said host cell is a hybridoma cell.

24. The host cell of Claim 11, wherein said engineered host cell is an engineered CHO cell, an engineered BHK cell, an engineered NS0 cell, or an engineered SP2/0 cell.

25. The host cell of Claim 11, wherein said host cell comprises at least one transfected nucleic acid encoding a chimeric anti-CD20 monoclonal antibody (C2B8).

26. The host cell of Claim 11, wherein said host cell comprises at least one transfected nucleic acid encoding a chimeric anti-human neuroblastoma monoclonal

antibody (chCE7).

27. The host cell of Claim 11, wherein said host cell comprises at least one transfected nucleic acid encoding a chimeric anti-human renal cell carcinoma monoclonal antibody (ch-G250), a humanized anti-HER2 monoclonal antibody, a chimeric anti-human colon, lung, and breast carcinoma monoclonal antibody (ING-1), a humanized anti-human 17-1A antigen monoclonal antibody (3622W94), a humanized anti-human colorectal tumor antibody (A33), an anti-human melanoma antibody (R24) directed against GD3 ganglioside, or a chimeric anti-human squamous-cell carcinoma monoclonal antibody (SF-25).

28. The host cell of Claim 11, wherein at least one nucleic acid molecule encodes ß(1,4)-N-acetylglucosaminyltransferase III (GnT III).

29. The host cell of Claim 28, further comprising at least one nucleic acid encoding a ß(1,4)-galactosyl transferase (GalT).

30. The host cell of Claim 28, further comprising at least one nucleic acid encoding a mannosidase II (Man II).

31. The host cell of Claim 28, further comprising at least one nucleic acid encoding a β(1,4)-galactosyl transferase (GalT) and at least one nucleic acid encoding a mannosidase II (Man II).

32. A method for producing a protein compound having enhanced Fc-mediated cellular cytotoxicity in a host cell, comprising:

(a) providing a host cell engineered to express a glycoprotein-modifying glycosyl transferase at a regulated level, chosen to improve glycosylation of a protein compound of interest, wherein said host cell expresses at least one nucleic acid encoding an antibody, an antibody fragment, or a fusion protein that includes a region equivalent to the Fc region of an immunoglobulin;

(b) culturing said host cell under conditions which permit the production of said protein compound having enhanced Fc-mediated dependent cellular

cytotoxicity; and

(c) isolating said protein compound having enhanced Fc-mediated cellular cytotoxicity.

33. The method of Claim 32, wherein in step (a), said host cell comprises at least one nucleic acid encoding a whole antibody.

34. The method of Claim 32, wherein in step (a), said host cell comprises at least one nucleic acid encoding an antibody fragment.

35. The method of Claim 32, wherein in step (a), said host cell comprises at least one nucleic acid encoding a fusion protein comprising a region resembling a glycosylated Fc region of an immunoglobulin.

36. The method of Claim 32, wherein said host cell comprises at least one transfected nucleic acid encoding a chimeric anti-CD20 monoclonal antibody (C2B8).

37. The method of Claim 32, wherein said host cell comprises at least one transfected nucleic acid encoding a chimeric anti-human neuroblastoma monoclonal antibody (chCE7).

38. The method of Claim 32, wherein said host cell comprises at least one transfected nucleic acid encoding a chimeric anti-human renal cell carcinoma monoclonal antibody (ch-G250), a humanized anti-HER2 monoclonal antibody, a chimeric anti-human colon, lung, and breast carcinoma monoclonal antibody (ING-1), a humanized anti-human 17-1A antigen monoclonal antibody (3622W94), a humanized anti-human colorectal tumor antibody (A33), an anti-human melanoma antibody (R24) directed against GD3 ganglioside, or a chimeric anti-human squamous-cell carcinoma monoclonal antibody (SF-25).

39. The method of Claim 32, wherein with at least one gene encoding a glycoprotein-modifying glycosyl transferase has been introduced into said host cell.

40. The host cell of Claim 32, wherein said host cell has been selected to carry a mutation triggering expression of an endogenous glycoprotein-modifying glycosyl transferase.

41. The host cell of Claim 40, wherein said host cell is the mutant lec10.

42. The host cell of Claim 32, wherein said host cell has been engineered such that an endogenous glycoprotein-modifying glycosyl transferase is activated.

43. The method of Claim 32, wherein said glycosyl transferase is a ß(1,4)-N-acetylglucosaminyltransferase III (GnT III).

44. The method of Claim 43, wherein said GnT III is expressed using a constitutive promoter system.

45. The method of Claim 43, wherein said GnT III is expressed using a regulated promoter system.

46. The method of Claim 45, wherein said regulated promoter system is a tetracycline-regulated promoter system, an ecdysone-inducible promoter system, a lac-switch promoter system, a glucocorticoid-inducible promoter system, a temperature-inducible promoter system, or a metallothionein metal-inducible promoter system.

47 The method of Claim 32, wherein said glycosyl transferase is a β(1,4)-galactosyl transferase (GalT).

48. The method of Claim 47, wherein said GalT is expressed using a constitutive promoter system.

49. The method of Claim 47, wherein said GalT is expressed using a regulated promoter system.

50. The method of Claim 49, wherein said regulated promoter system is

a tetracycline-regulated promoter system, an ecdysone-inducible promoter system, a lac-switch promoter system, a glucocorticoid-inducible promoter system, a temperature-inducible promoter system, or a metallothionein metal-inducible promoter system.

51. The method of Claim 32, wherein said host cell is engineered to express a plurality of nucleic acids encoding a glycosyl transferase at a regulated level, chosen to improve glycosylation of a protein compound of interest, wherein at least one nucleic acid encodes GnT III and at least one nucleic acid encodes a β(1,4)-galactosyl transferase (GalT).

52. The host cell of Claim 51, wherein a nucleic acid molecule comprising at least one gene encoding a glycoprotein-modifying glycosyl transferase has been introduced into said host cell.

53. The host cell of Claim 51, wherein said host cell has been selected to carry a mutation triggering expression of at least one endogenous glycoprotein-modifying glycosyl transferase.

54. The host cell of Claim 51, 52, or 53, wherein said host cell has been engineered such that an endogenous glycoprotein-modifying glycosyl transferase is activated.

55. The method of Claim 32, wherein said host cell comprises a plurality of nucleic acids encoding a glycoprotein-modifying glycosyl transferase at a regulated level, chosen to improve glycosylation of a protein compound of interest, wherein at least one nucleic acid encodes GnT III and at least one nucleic acid encodes a mannosidase II (Man II).

56. The host cell of Claim 55, wherein a nucleic acid molecule comprising at least one gene encoding a glycoprotein-modifying glycosyl transferase has been introduced into said host cell.

57. The host cell of Claim 55, wherein said host cell has been selected to

carry a mutation triggering expression of at least one endogenous glycoprotein-modifying glycosyl transferase.

58. The host cell of Claim 55, 56, or 57, wherein said host cell has been engineered such that an endogenous glycoprotein-modifying glycosyl transferase is activated.

59. The method of Claim 32, wherein said host cell comprises a plurality of nucleic acids encoding a glycoprotein-modifying glycosyl transferase at a regulated level, chosen to improve glycosylation of a protein of interest, wherein at least one nucleic acid encodes GnT III, at least one nucleic acid encodes β(1,4)-galactosyl transferase (GalT), and at least one nucleic acid encodes mannosidase II (Man II).

60. The host cell of Claim 59, wherein a nucleic acid molecule comprising at least one gene encoding a glycoprotein-modifying glycosyl transferase has been introduced into said host cell.

61. The host cell of Claim 59, wherein said host cell has been selected to carry a mutation triggering expression of at least one endogenous glycoprotein-modifying glycosyl transferase.

62. The host cell of Claim 59, 60, or 61, wherein said host cell has been engineered such that an endogenous glycoprotein-modifying glycosyl transferase is activated.

63. The method of Claim 32, wherein the expression level of at least one glycoprotein-modifying glycosyl transferase has been selected to produce an antibody molecule, an antibody fragment, or a fusion protein that includes a region equivalent to the Fc region of an immunoglobulin having enhanced Fc-mediated cellular cytotoxicity at a higher level than the Fc-mediated cellular cytotoxicity obtained from a different expression level of the same glycosyl transferase gene.

64. The method of Claim 63, wherein said expression levels are

determined by Western blot analysis using a glycosyl transferase-specific antibody.

65. The method of Claim 63, wherein said expression levels are determined by Northern blot analysis using a glycosyl transferase-specific probe.

66. The method of Claim 63, wherein said expression levels are determined by measuring the enzymatic activity of glycosyl transferase.

67. The method of Claim 63, wherein said expression levels are determined using a lectin which binds to biosynthetic products of glycoprotein-modifying glycosyl transferase.

68. The method of Claim 67, wherein the lectin is E_4-PHA lectin.

69. The method of Claim 63, wherein said nucleic acid encoding said glycoprotein-modifying glycosyl transferase is operatively linked to a reporter gene, and wherein said expression levels of said glycosyl transferase are determined by measuring a signal correlated with the expression level of said reporter gene.

70. The method of Claim 69, wherein said reporter gene is transcribed together with at least one nucleic acid encoding said glycoprotein-modifying glycosyl transferase as a single RNA molecule and their respective coding sequences are linked either by an internal ribosome entry site (IRES) or by a cap-independent translation enhancer (CITE).

71. The method of Claim 69, wherein said reporter gene is translated together with at least one nucleic acid encoding said glycoprotein-modifying glycosyl transferase such that a single polypeptide chain is formed.

72. The method of Claim 63, wherein said nucleic acid encoding said glycoprotein-modifying glycosyl transferase is operatively linked to a reporter gene under the control of a single promoter, wherein said nucleic acid encoding said glycoprotein-modifying glycosyl transferase and said reporter gene are transcribed into

an RNA molecule which is alternatively spliced into two separate messenger RNA (mRNA) molecules, wherein one of the resulting mRNAs is translated into said reporter protein, and the other is translated into said glycoprotein-modifying glycosyl transferase.

73. The method of Claim 32, wherein said host cell further comprises a nucleic acid encoding a glycosidase.

74. An antibody having enhanced antibody dependent cellular cytotoxicity (ADCC) produced by the host cells of Claim 11.

75. A chimeric anti-CD20 monoclonal antibody (C2B8) having enhanced antibody dependent cellular cytotoxicity (ADCC) produced by the host cells of Claim 25.

76. A chimeric anti-human neuroblastoma monoclonal antibody (chCE7) having enhanced antibody dependent cellular cytotoxicity (ADCC) produced using the host cells of Claim 26.

77. A chimeric anti-human renal cell carcinoma monoclonal antibody (ch-G250) having enhanced antibody dependent cellular cytotoxicity (ADCC) produced using the host cells of Claim 27.

78. A humanized anti-HER2 monoclonal antibody having enhanced antibody dependent cellular cytotoxicity (ADCC) produced using the host cells of Claim 27.

79. A chimeric anti-human colon, lung, and breast carcinoma monoclonal antibody (ING-1) having enhanced antibody dependent cellular cytotoxicity (ADCC) produced using the host cells of Claim 27.

80. A humanized anti-human 17-1A antigen monoclonal antibody (3622W94) having enhanced antibody dependent cellular cytotoxicity (ADCC)

produced using the host cells of Claim 27.

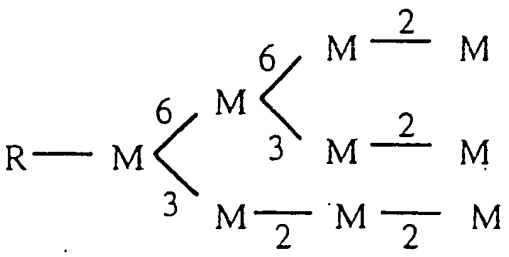
81. A chimeric anti-human squamous-cell carcinoma monoclonal antibody (SF-25) having enhanced antibody dependent cellular cytotoxicity (ADCC) produced using the host cells of Claim 27.

82. A humanized anti-human colorectal tumor antibody (A33), having enhanced antibody dependent cellular cytotoxicity (ADCC) produced using the host cells of Claim 27.

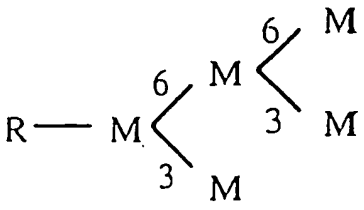
83. An anti-human melanoma antibody (R24) directed against GD3 ganglioside, having enhanced antibody dependent cellular cytotoxicity (ADCC) produced using the host cells of Claim 27.

84. An antibody fragment that includes a region equivalent to the Fc region of an immunoglobulin, having enhanced Fc-mediated cellular cytotoxicity produced using the host cells of Claim 11.

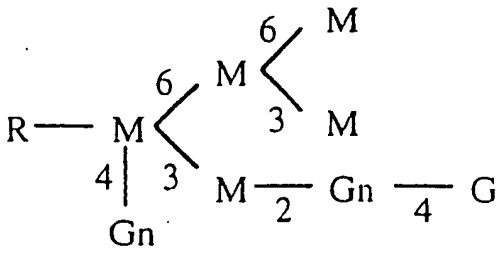
85. A fusion protein that includes a region equivalent to the Fc region of an immunoglobulin, having enhanced Fc-mediated cellular cytotoxicity produced using the host cells of Claim 11.



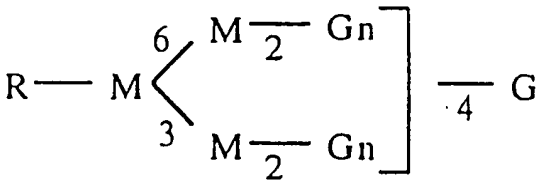
M_9. High-mannose



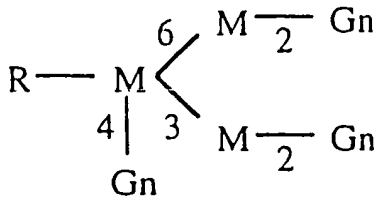
M_5. High-mannose



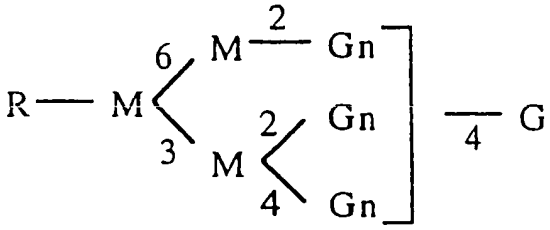
M_5GnGn^bG. Bisected hybrid



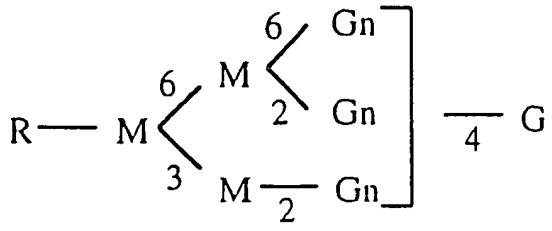
M_3Gn_2G. Bi-antennary complex



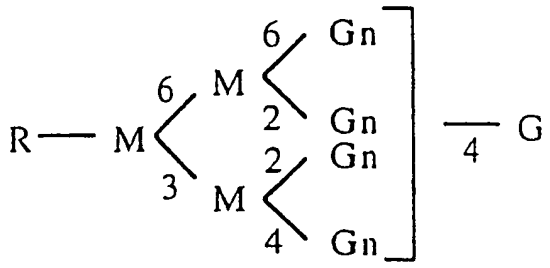
$M_3Gn_2Gn^b$. Bisected bi-antennary complex



M_3Gn_3G. Tri-antennary complex



$M_3Gn_3'G$. Tri'-antennary complex



M_3Gn_4G. Tetra-antennary complex

FIG.1

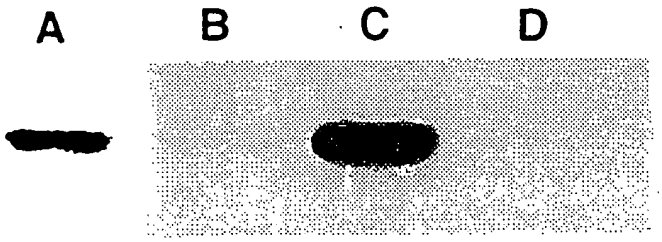


FIG.2

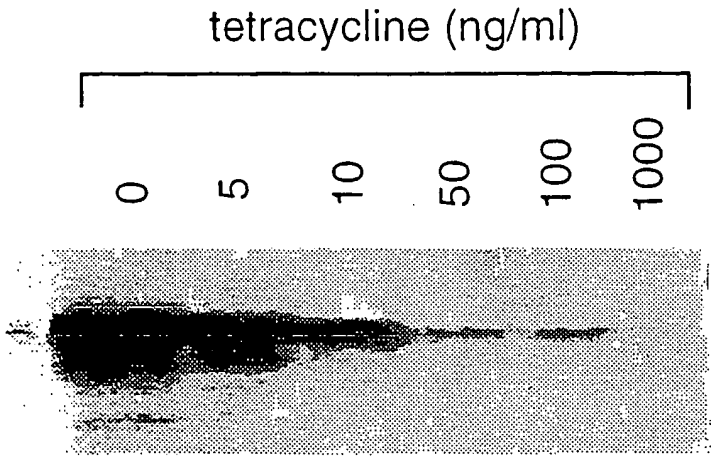


FIG.3

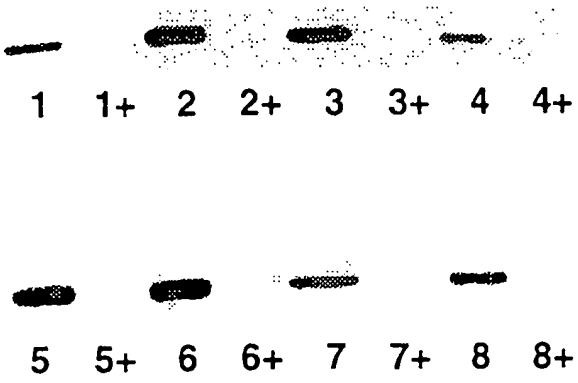


FIG.4A

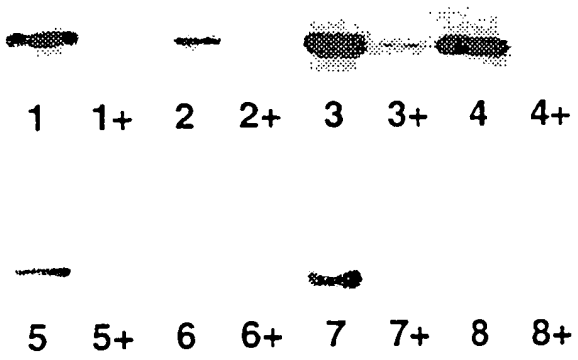


FIG.4B