Genetically Engineered Antibody Molecules and Their Application

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INTRODUCTION

Antibodies have long been recognized for their remarkable specificity. Indeed, the classical studies by Landsteiner demonstrated that antibodies are able to distinguish between *ortho, meta,* and *para* forms of the same haptenic group.[1] Antibodies have therefore seemed ideal candidates for the so-called magic bullet, which could with exquisite specificity identify and destroy an undesirable substance or cell. Antibodies also seem to be a potential means to deliver a drug to a defined target, provided that an antibody specific for the unique chemical differences inherent in that target can be produced.

Originally, the source of antibodies was antisera, which by their nature are limited in quantity and heterogeneous in quality. After the development of hybridomas,[2] a potentially unlimited quantity of antibodies with precisely defined specificities became available. A more recent source of antibodies has been genetically engineered antibodies produced by transfectomas.[3-6] With transfectomas, one is not limited by the specificity and isotype that happens to result following fusion to a normal spleen cell; both of these can be changed by the appropriate genetic manipulation. Transfectomas can be used to isotype switch both within[7] and between species.

Chimeric antibodies in which the specificity derived from a mouse myeloma or hybridoma is joined to human constant region have been produced.[4,8,9] These molecules in part overcome the species limitations inherent in monoclonal antibodies. It has become relatively easy to produce mouse monoclonals of a desired specificity; it has proven much more difficult to produce human monoclonals with the desired properties. Human monoclonals would be preferable for many applications, especially *in vivo* diagnosis and therapy.

The ability to express antibody molecules following gene transfection provides additional advantages. Using *in vitro* mutagenesis, it is possible to make alterations in the variable region of the molecule that alter the binding specificity. It is also possible to express antigen binding domains covalently associated with non-Ig sequences.[10] Such molecules may be useful in delivering protein drugs, such as some toxins to specific cells. In addition, it is possible to make covalent changes in the constant region that facilitate drug delivery; these might include changes in size, effector function, and changes to facilitate the covalent attachment of a drug without altering the binding affinity of the antibody.

Vectors for Transfection

Gene transfection is an inefficient process, with usually $<10^{-4}$ of treated cells going on to become stably transfected. Therefore, there must be a method to select the rare transfected cells from the many non-transfected cells.

The most commonly used vectors are based on those developed by Berg and co-workers.[11–13] These have several essential features. Firstly, they contain a plasmid origin of replication and a marker selectible in prokaryotes. This makes it relatively easy to obtain large quantities of DNA and facilitates their genetic manipulation. Secondly, they contain a marker expressible and selectible in eukaryotes. This consists of a eukaryotic transcription unit with an SV40 promoter, splice, and poly A addition site. Into this eukaryotic transcription unit is placed a dominant selectible marker derived from prokaryotes. One of these, *neo,* derived from the Tn5 transposon, encodes a phosphotransferase that inactivates the kanamycin-like antibiotic G418, an inhibitor of protein synthesis. A second selectible gene, *gpt,* (xanthine-guanine phosphoribosyltransferase) provides resistance to mycophenolic acid, an inhibitor of purine biosynthesis. It is important that both of these are dominant selectible markers so they can be used with cell lines that have not been drug marked; in addition, they select against entirely different biochemical pathways, hence they can be used simultaneously to generate double drug resistance lines. This is important when selecting for the expression of two different transfected genes (see below).

Production of a functional antibody molecule requires the synthesis of both heavy and light chains. Initial experiments[4] took advantage of the availability of the two independent, dominant selectible markers. The H chain was first introduced using pSV2-gpt and mycophenolic acid selection; using pSV2-neo L chain was subsequently introduced into the H chain producing transfectants and transfectants resistant to both mycophenolic acid and G418 and producing both heavy and light chains selected. This approach is workable, but it requires two steps.

More recently an approach has been developed in which double drug resistant lines can be selected in one step.[14] The original vectors used the pBR origin of replication. A second series of vector was constructed using the origin of replication from pACYC; pBR and pACYC are compatible plasmids and so both can non-competitively replicate within a bacterium. The pACYC vector contains the prokaryotic selectible marker Cm® while the pBR vector contains Amp®. Into one vector is placed a heavy chain and the *gpt* gene; into the other vector is placed the L chain gene and the selectible marker *neo.* When transfection is by protoplast fusion (see below), both vectors can be simultaneously transferred and selected in a recipient cell.

Methods of Gene Transfer

A common procedure for DNA transfer is to make calcium phosphate precipitates of the DNA.[15,16] However, this method does not work very well for lymphoid cells. It is possible to achieve transfection using this method, but only at low frequencies.[3,17]

Protoplast fusion has proven to be an effective way of transfecting lymphoid cells.[18] Using protoplast fusion, frequencies of $>10^{-3}$ can be achieved using the optimal vectors and recipient cell line, J558L.[19] A reduced transfection frequency is seen with other myeloma lines. Recent minor modifications of the basic protoplast fusion procedure have resulted in an increased transfection frequency. This increased frequency is especially important when dealing with cell lines with low transfection frequencies.

For the modified protoplast fusion procedure, protoplasts are prepared essentially as previously described except they are diluted into DME + 10% sucrose + 10 mM $MgCl_2$.[14] For fusion, 4×10^6 cells that had been washed once in DME are mixed with 5 ml of protoplasts (fewer cells are used for cell lines with a high transfection frequency). The cells and protoplasts are pelleted together, and medium removed by aspiration. The pellet is resuspended in 0.5 ml of 41.7% PEG, 12.5% DMSO, 100 mM Tris, pH 8.0 in DME at 37°C, and gently agitated for 1 min. The pellet is then diluted with 0.5 ml of 50% PEG, 100 mM Tris, pH 8.0 in DME at 37°C and gently agitated for 2 min. The pellet is then disrupted with 10 ml of warm DME (37°C), repelleted by centrifugation at room temperature, and resuspended in complete growth medium. Cells are plated into microtiter dishes at a concentration of 4×10^4 cells/well. Selective medium is added after 48 hr.

Representative data from protoplast fusion experiments are shown in TABLE 1. When bacteria with two compatible plasmids are used and selection is made with one drug, frequencies $>10^{-5}$ are achieved with the two non-producing myelomas, SP2/0 and $P_3X63Ag8.653$. Other cell lines (J558L, EL4) will give frequencies more than tenfold higher. However, when clones are simultaneously selected with both selective

TABLE 1. Transfection by Protoplast Fusion

	$P_3X63Ag8.653$		SP2/0	
Selective Medium	Wells with Surviving Clones	Approximate Transfection Frequency	Wells with Surviving Clones	Approximate Transfection Frequency
Mycophenolic acid	504/612	2×10^{-5}	282/622	1×10^{-5}
G418 + Mycophenolic acid	100/580	4×10^{-6}	56/586	2×10^{-6}

$P_3X63Ag8.653$ or SP2/0 cells were fused with bacteria containing compatible plasmids bearing either a *gpt* or *neo* gene and then plated into microtiter dishes (4×10^4 cells/well). After 48 hours, clones were either singly selected (mycophenolic acid) or doubly detected (mycophenolic acid + G418). The plasmids used were pSV2gpt with a chimeric heavy chain gene and pACYCneo with a chimeric light chain gene.

drugs, the frequency of stable transfectants drops about fivefold. It is not clear at this time if this reduced frequency results because only 20% of the cells integrate and express both markers, or if the drop is a consequence of the inherent toxicity of double selection.

Electroporation has also proven to be an efficient method of transfecting lymphoid cells. For electroporation, cells are pelleted and resuspended at a concentration of 10^6/ml in ice-cold PBS. Cells (0.8 ml) are placed along with 8 μg of linearized DNA from each plasmid into an electroporation cuvette (Biorad). A pulse of 200 V, 960 μF is delivered using a Biorad Gene Pulser. After the pulse, cells are removed from the cuvette, washed once in cold DME +10% horse serum, resuspended in complete medium, and plated into microtiter dishes at a concentration of approximately 1.6×10^4 cells/well. Selective medium is applied after 48 hr. Results from a representative experiment are shown in TABLE 2. In this experiment, cells were simultaneously transfected with two different plasmids bearing two different selectible markers. When selected for expression of one or the other, a frequency of $>10^{-5}$ was achieved, very similar to what is observed with protoplasts. With double selection, the frequency drops by a factor of 10–20. Therefore, for double selection under these conditions, electroporation is less efficient than using compatible vectors and protoplast fusion, but it is

TABLE 2. Transfection by Electroporation

Selective Medium	Wells with Surviving Clones	Approximate Transfection Frequency
Mycophenolic acid	14/48	1.8×10^{-5}
G418	24/48	3×10^{-5}
Mycophenolic acid + G418	1/48	1.3×10^{-6}

SP2/0 cells transfected by electroporation were plated into microtiter dishes at a concentration of 1.6×10^4 cells/well. Selective medium was added 48 hours after plating. The plasmids used were pSV2gpt with a chimeric heavy chain gene and pACYCneo with a chimeric light chain gene.

feasible. It is also possible that modifications of the conditions used for electroporation will improve the frequency.

Methods of Cloning Immunoglobulin Genes

The immunoglobulin (Ig) expression vectors that we have employed all use rearranged Ig genes driven by their own promoters and with their own regulatory sequences. The rationale for this approach is that Igs are expressed at high levels in plasma cells; therefore, if we can achieve comparable expression after transfection we will have high levels of protein available for functional studies.

In order for the approach of gene transfection to be broadly applicable, it must be feasible to clone the variable regions for the specificities of interest. The characteristics of Ig, the locus, facilitate this cloning. V regions must be rearranged to be expressed; expressed V regions are juxtaposed to J regions while unexpressed V regions are not. Therefore it is possible to use J region probes to identify restriction fragments bearing rearranged V's. Thus one does not need to know the exact characteristics of a rearranged V or its sequence to be able to clone it. A complication inherent in this approach is that hybridomas often contain aberrant rearrangements, some of which may be transcribed. Therefore, a frequent problem is the positive identification of the correct rearrangement.

Recently, we have also demonstrated the feasibility of reconstructing expressed V regions from cDNA clones. cDNA clones usually are easier to make than genomic clones, especially since the use of constant region primers enables one to selectively prime for Ig transcripts. The method we used to express the cDNAs took advantage of the fact that we had an expression vector with a variable region that shared restriction sites at the 5′ end of V and at J with the cDNA of interest. In this case, the expression vector (MPC-11)[20] and the anti-dextran cDNAs[21,22] we used belong to the very large J558 family. Members of this family are homologous and share many features. At amino acid four in V, there is a common *Pvu*II site; in J_3, present in both, is a *Pst*I site. Replacement of the *Pvu*II-*Pst*I fragment of MPC-11 with the same fragment from the cDNA, leads to the insertion of the anti-dextran variable region into the expression vector. The leader sequence and the first four amino acids of V are from MPC-11; however since these four amino acids are identical in MPC-11 and the anti-dextrans there is no alteration in specificity. The total V until J_3 is from the anti-dextran hybridoma. After J, the splice signals and regulatory sequences are derived from MPC-11 (FIG. 1). When this reconstructed variable region was joined to a constant region and transfected into a light chain–producing cell line (J558L), it was seen to direct the synthesis of an intact H chain, which assembled with the L chain and was secreted (FIG. 2).

The general expression vector systems we employ have several important properties. Firstly, H chains and L chains are on separate plasmids. Although it is feasible to include both H and L on the same plasmid and there is no inherent size limitation in these vectors, it becomes much more difficult to genetically manipulate large plasmids because of the paucity of novel restriction sites. Therefore, smaller plasmids are preferable if the objective is to be able to genetically engineer antibodies. A second feature is the presence of unique sites within the vectors that were introduced using linkers.[4] These sites facilitate transferring variable regions between plasmids; therefore once a variable region has been cloned into one expression vector, it becomes much simpler to then express it associated with different constant regions.

Production of Novel Proteins

In order to be able to use gene transfection to produce novel Ig molecules, the Ig produced must be a faithful representation of the Ig genes used. In the initial experiments using the S107A kappa light chain, it was shown that the gene expressed

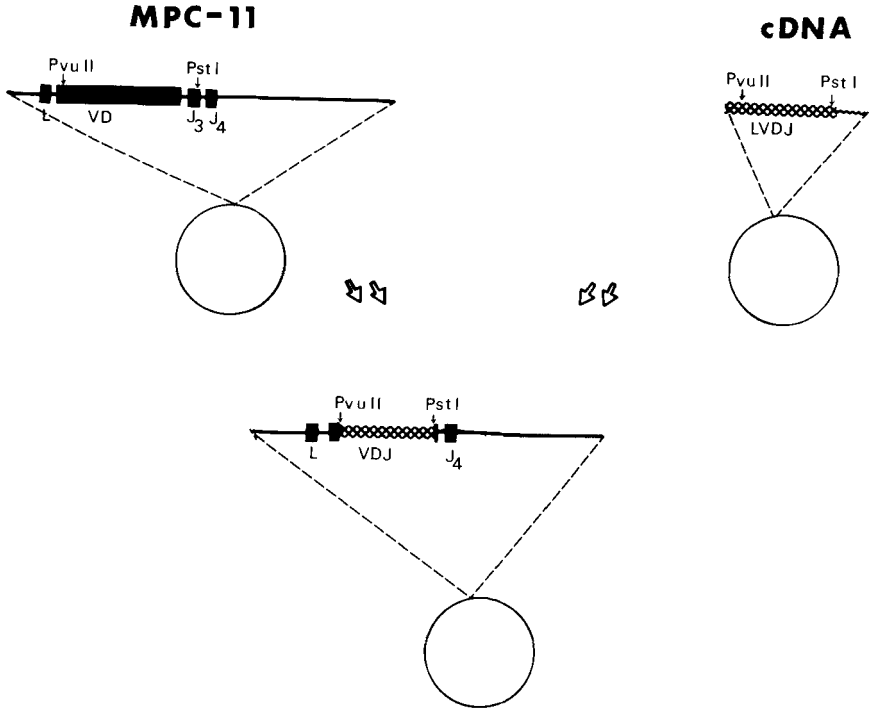

FIGURE 1. Construction of a vector capable of expressing a heavy chain cDNA. The coding sequences from the MPC-11 myeloma are shown as a solid black line. The coding sequences of the cDNA are indicated as a hatched area. The synthesis of the cDNA was initiated using an isotype-specific primer, which primed at the 5′ side of CH_1. To exchange the variable regions both the cDNA and MPC-11 plasmids were cleaved with *Pvu*II and *Pst*I. The *Pvu*II-*Pst*I fragment from the cDNA was then ligated into *Pvu*II-*Pst*I cleaved MPC-11. The reconstructed variable region was then inserted 5′ of a constant region in a complete H chain expression vector. The drawing is not to scale and all restriction sites are not shown.

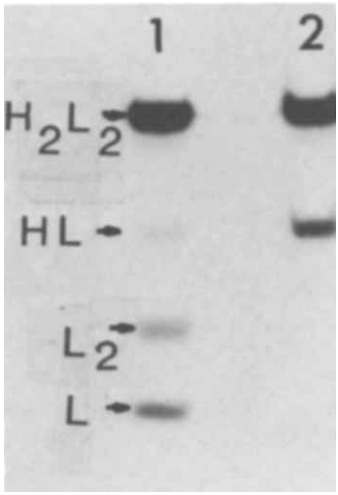

FIGURE 2. Synthesis, assembly, and secretion of an anti-dextran heavy chain constructed from a cDNA following transfection into the J558L (produces only λ light chains) myeloma. The anti-dextran variable region is joined to human γ_4 constant region. Cells were labeled with [^{35}S]methionine for 3 hours, secretions prepared, and immunoglobulin specifically immunoprecipitated and analyzed on SDS-PAGE. The reconstructed heavy chain gene directs the synthesis of a complete H chain that assembles with the λ light chain of the myeloma and is secreted as H_2L_2 molecules. Position of bands in marker proteins are indicated. Lane 1, myeloma (MPC-11); Lane 2, secreted Ig from the transfectant.

after transfection had the same apparent molecular weight and isoelectric point as the kappa light chain produced by the S107 myeloma.[3] In addition, the kappa light chain produced following transfection was like the kappa light chain produced by the parental myeloma in that it was secreted only when associated with a heavy chain.

When chimeric mouse-human Igs were produced using gene transfection, these proteins were also shown to exhibit the expected specificity and properties. Anti-phosphocholine specific chimeric proteins were shown to assemble into H_2L_2 molecules, to bind antigen, and to be recognized by anti-idiotypic antibodies specific for the original mouse myeloma protein; the anti-idiotypic antibodies were either specific for the heavy chain alone or required the proper assembly of heavy and light chain to generate the antigenic determinant.[4] Similarly, anti-DNP specific, chimeric proteins exhibited the expected specificity.[9]

Subsequent experiments have reinforced the concept that chimeric Igs exhibit the expected binding specificity. Additional anti-hapten antibodies have been produced.[7,8] Chimeric antibodies binding tumor-associated antigen have been shown to exhibit reactivity patterns identical to the starting hybridoma.[23,24] Recently, chimeric Igs specific for the T cell antigen Leu3/T4 have also been shown to have the predicted reactivity.[25] All of these experiments demonstrate the feasibility of producing specific antibodies using gene transfection.

The first reports of chimeric Ig used human constant regions of the μ, ϵ, γ_1, or γ_2 isotypes.[4,9,8] Subsequently, the γ_3 and γ_4 isotypes have been produced.[26] More recently, it has been feasible to express human IgA. The dansyl-specific variable region was ligated 5′ of the human α_1 constant region gene (gift of Dr. Greg Hollis) in the

pSV2-gpt vector. The resulting construct was transfected into J558L cells and stable transfectants isolated. The chimeric α chain assembled with the endogenous λ light chain; H_2L_2 and higher polymers were present in the secretions (FIGURE 3). Thus, all isotypes of human Ig except δ have been expressed as chimeric proteins.

One potential application of transfectomas is to study structure-function relationships in antibody molecules. Human γ_3 has an extended hinge region consisting of four exons; human γ_4 exhibits quite different properties in spite of extensive sequence similarities (> 90% identical in CH_2 and CH_3) in their constant regions. It is now possible to define precisely the contribution of the hinge region to the properties of the molecules. IgG_3 genes have been constructed, expressed, and the proteins isolated that have one, two, three, or four (wild type) hinge exons (FIGURE 4); notice the change in molecular weight of the heavy chains with the subsequent hinge deletions. In addition, the γ_3 hinge has been placed in the γ_4 constant region and *vice versa*. These proteins will now be used to investigate the properties of the Ig, including its ability to fix complement and the segmental flexibility of the molecules. This example provides just one illustration of the potential uses of genetically engineered antibody molecules.

In order to maximize the usefulness of genetically engineered antibodies for such applications as drug delivery, it must be possible to make alterations in the antibody. The limitations are that the antibodies must be assembled and secreted and must retain their ability to specifically bind antigen.

To determine if we could define an upper limit on the size of an antibody molecule that could be produced, we constructed an IgG_3 heavy chain in which CH_1 and the hinge region were duplicated; in a second construct CH_1, hinge, and CH_2 were

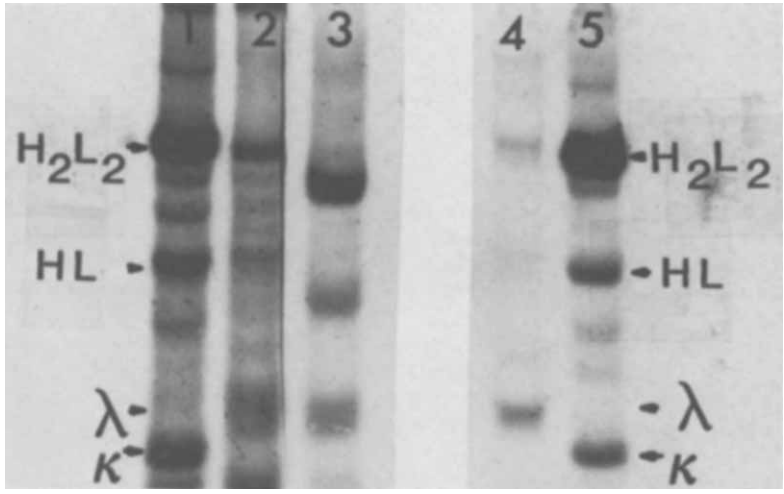

FIGURE 3. Synthesis, assembly, and secretion of a chimeric heavy chain with a human alpha 1 constant region. The anti-dansyl variable region was joined to human alpha 1 heavy chain and transfected into the J558L myeloma. Transfectants synthesizing the chimeric heavy chains were labeled with [^{35}S]methionine for 3 hours, cytoplasmic lysates and secretions prepared and immunoprecipitated. The immunoprecipitates were analyzed using SDS-PAGE. The chimeric alpha heavy chain assembles into H_2L_2 molecules; these are secreted both as monomers and polymers. Lanes 1 and 5, MPC-11 myeloma; Lane 2, cytoplasmic chimeric IgA; Lane 3, BALB/c mouse IgA; and Lane 4, secreted chimeric IgA.

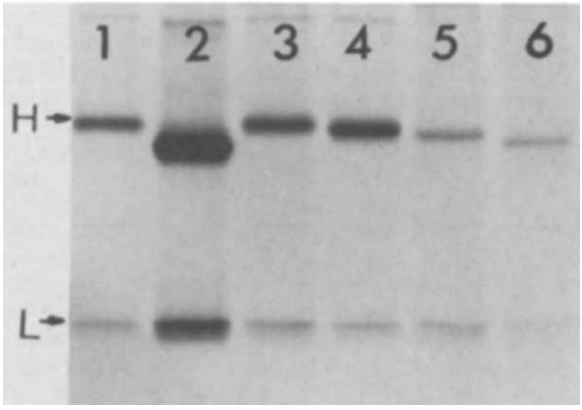

FIGURE 4. Synthesis of chimeric immunoglobulins with alterations in their hinge regions. Chimeric heavy chain genes were cotransfected with a chimeric light chain gene (mouse anti-dansyl with human kappa) into a non-producing myeloma. Transfectants producing immunoglobulin were labeled for 3 hours with [^{35}S]methionine, cytoplasmic lysates prepared and immunoprecipitated. The immunoprecipitates were analyzed on SDS-PAGE following treatment with β-mercaptoethanol. Lane 1, γ_4 with the complete hinge of γ_3; Lane 2, γ_4; Lane 3, γ_3 with an intact hinge; Lane 4, γ_3 lacking hinge exon 4; Lane 5, γ_3 lacking hinge exons 3 and 4; and Lane 6, γ_3 lacking hinge exons 2, 3, and 4.

duplicated. When the gene with CH_1 and the hinge duplicated was used for transfection a greatly reduced transfection frequency was seen; when the surviving transfectants were analyzed, none produced any detectable heavy chain protein. These results suggest, but do not prove conclusively, that the heavy chain encoded by this construct was toxic to the cells. Such heavy chain toxicity has been postulated by many groups;[27,28] indeed variants producing only heavy chains could be isolated from the MPC-11 myeloma only after a deletion was introduced into the heavy chain, which permitted its secretion in the absence of a light chain.[29]

In contrast, the gene encoding a heavy chain in which CH_1, hinge, and CH_2 are duplicated is expressed following transfection. It assembles with a mouse λ light chain and is secreted (FIGURE 5). These experiments suggest there will be limitations in the Ig molecules that can be produced, but that these limitations are not inherently related to size.

In another series of experiments, we set out to determine how deletions of various domains will affect the ability of Ig molecules to assemble, to be secreted, and to function. IgG_3 heavy chain genes encoding proteins with deletions of CH_2, hinge + CH_2, CH_1 + hinge and CH_1 + hinge + CH_2 were constructed and transfected into myeloma cells.

When the gene with the deletion of CH_2 was used, it was found to direct the synthesis of a protein of the expected molecular weight. This shortened heavy chain assembled with either mouse λ light chain or chimeric V-DNS-human κ light chain into an H_2L_2 molecule that was secreted (FIGURE 6).

When the hinge + CH_2 were deleted, the heavy chain apparently assembled into HL half molecules, which were secreted (FIGURE 7). However, it is impossible to say conclusively that these are not H_2. However, when CH_1 + the hinge were deleted, no assembly of the heavy chain with either other heavy chains or with L chain occurred. This is not surprising since the free cysteine that forms the interchain disulfide bonds is

present in CH_1. However, even in the absence of interchain disulfide bonds, the shortened heavy chain is secreted.

It is also possible to produce a heavy chain in which the variable region is directly joined to CH_3. This heavy chain is secreted when cotransfected with a chimeric light chain into a non-producing myeloma. It does not form covalent bonds with either light chain or another heavy chain.

DISCUSSION AND PROSPECTS

The feasibility of producing genetically engineered antibody molecules has now been demonstrated in many laboratories. The experiments reported here show that it is possible to produce immunoglobulin with greatly altered structures. Somewhat surprisingly, secretion of these structurally altered Igs generally seems to occur, so it is possible to obtain them in adequate quantities for study. The usefulness of these molecules will be determined by their biologic properties.

The potential application of antibody molecules is determined not only by their specificity, but also by their effector functions. That is, their serum half-life, tissue distribution, and then ability to fix complement and participate in antibody-dependent cellular cytotoxicity, will, among other things, determine their *in vivo* function. Antibodies have evolved to perform many different functions. With genetic engineer-

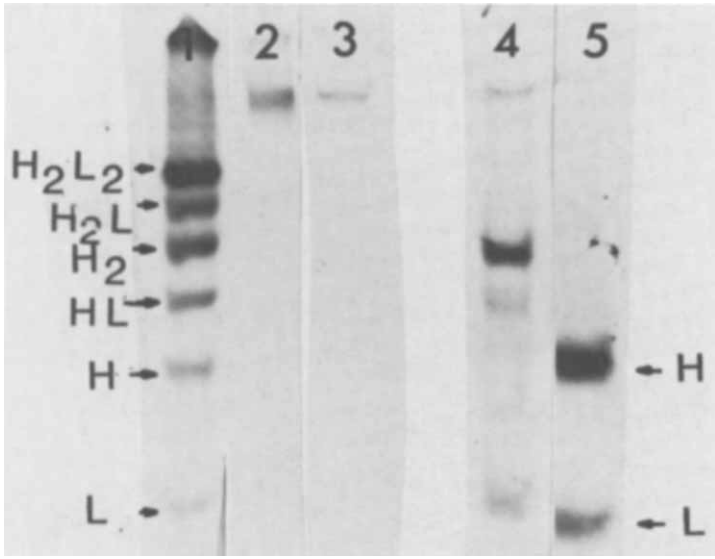

FIGURE 5. Synthesis, assembly, and secretion of a chimeric heavy chain gene in which the CH_1 hinge, CH_2 region has been duplicated. J558L cells were transfected with the chimeric gene and transfectomas synthesizing the heavy chain isolated. Cells were labeled for 3 hours using [^{35}S]methionine, cytoplasm and secretions prepared, the immunoglobulin immunoprecipitated with an anti-heavy chain antiserum, and analyzed by SDS-PAGE. Lane 1, MPC-11; Lane 2, cytoplasm from the transfectant; Lane 3, secretion from the transfectant; Lane 4, cytoplasmic Ig from transfectant treated with β-mercaptoethanol; and Lane 5, MPC-11 treated with β-mercaptoethanol. Arrows mark the positions of the marker Ig in MPC-11.

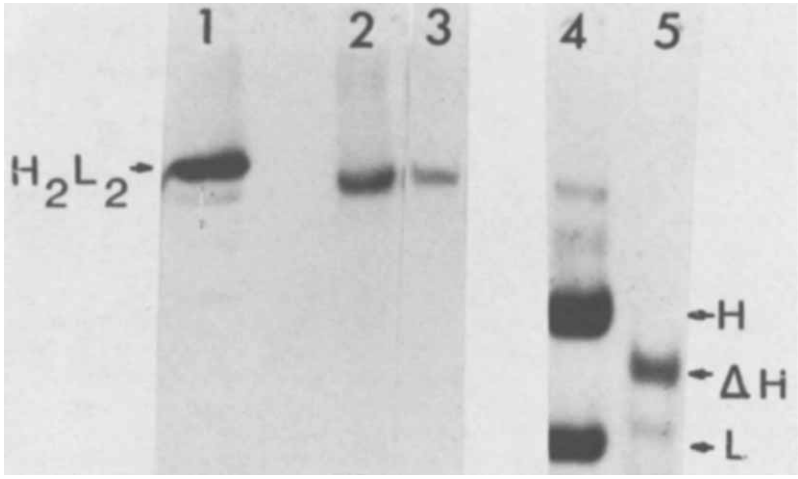

FIGURE 6. Synthesis of a chimeric heavy chain in which CH_2 has been deleted. J558L was transfected with the chimeric heavy chain gene and transfectants synthesizing protein were isolated. Transfectants were labeled for 3 hours using [^{35}S]methionine and cytoplasmic and secreted Ig prepared by immunoprecipitating with anti-heavy chain. The immunoprecipitates were analyzed by SDS-PAGE. Lane 1, MPC-11; Lane 2, cytoplasmic Ig of transfectant; Lane 3, secreted Ig of transfectant; Lane 4, MPC-11 treated with β-mercaptoethanol; Lane 5, cytoplasmic Ig of transfectant treated with β-mercaptoethanol.

ing we can now produce antibody molecules in which the desired effector function has been maximized and unwanted function has been eliminated. It is also possible to make an antibody molecule in which a drug is part of the molecule (e.g., a chimeric antibody ricin gene). Antibodies can also be modified so as to facilitate their *in vitro* manipulation. Antibodies could be altered so that it is easier to add more molecules of a radioactive isotope without changing their binding specificity.

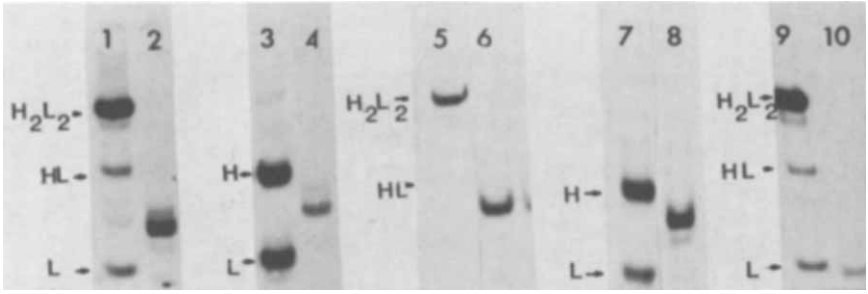

FIGURE 7. Secretions from cells transfected with heavy chains bearing domain deletions. Cells were labeled with [^{35}S]methionine for 3 hours, secretions prepared, and immunoprecipitated. The immunoprecipitated proteins were analyzed by SDS-PAGE. Lane 1, MPC-11; Lane 2, γ_3 with CH_1 and hinge deleted; Lane 3, MPC-11 treated with β-mercaptoethanol; Lane 4, CH_1 and hinge deleted protein treated with β-mercaptoethanol; Lane 5, MPC-11; Lane 6, γ_3 with hinge and CH_2 deletion; Lane 7, MPC-11 treated wtih β-mercaptoethanol; Lane 8, hinge and CH_2 deleted protein treated with β-mercaptoethanol; Lane 9, MPC-11; and Lane 10, γ_3 with CH_1, hinge and CH_2 deleted.

The specificity of antibody molecules can also be manipulated using genetic engineering. In certain cases, antibodies with either higher or lower affinities are desirable; *in vitro* mutagenesis of the variable regions can be used to change the binding specificity or affinity of the antibody.

Modern techniques permit us to make antibody molecules with virtually any structure. The challenge now is to determine what structures correlate with optimal function.

SUMMARY

Immunoglobulin genes can be efficiently expressed following transfection into myeloma cells. Using protoplast fusion, transfection frequencies greater than 10^{-3} can be achieved. Compatible plasmids containing two different selectible markers are used to simultaneously deliver heavy and light chain genes to the same cell. To produce molecules with differing specificities the rearranged and expressed variable regions can be cloned from the appropriate hybridoma. In some cases, variable regions from cDNAs can be inserted into the expression vectors. It is possible to manipulate the immunoglobulin genes and produce novel antibody molecules. Antibodies have been produced in which the variable regions from mouse antibodies have been joined to human constant regions. In addition, antibodies with altered constant regions have been produced. These genetically engineered antibodies provide a unique set of reagents to study structure-function relationships within the molecule. They also can potentially be used in the diagnosis and therapy of human disease.

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