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ANTIBODY-DEPENDENT CYTOTOXICITY MEDIATED BY NATURAL KILLER CELLS IS ENHANCED BY CASTANOSPERMINE-INDUCED ALTERATIONS OF IgG GLYCOSYLATION*

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Abstract—Inhibitors of glycosylation and carbohydrate processing were used to probe the functional consequences of specific, differential alterations in glycosylation of monoclonal IgG secreted by hybridoma clones. Neither the absence of glycosylation nor the presence of atypical oligosaccharides significantly influenced binding of the monoclonal antibody to the cell surface antigen recognized. However, lymphocyte-mediated antibody-dependent cytotoxicity was enhanced significantly, as compared to native (unmodified) IgG-sensitized target cells, when target cells were sensitized with IgG bearing the atypical oligosaccharides induced metabolically by castanospermine, *N*-methyldeoxynojirimycin, deoxymannojirimycin or monesin, but not by swainsonine. The enhanced cytotoxicity was mediated by natural killer cells but not by monocytes or interferon-activated polymorphonuclear leukocytes. By contrast, antibody-dependent cytotoxicity mediated by activated polymorphonuclear leukocytes against target cells sensitized with the IgG glycosylation phenotypes induced by swainsonine and tunicamycin, but not by castanospermine, was decreased in comparison to cytotoxicity against target cells sensitized with native IgG.

The enhanced lymphocyte-mediated cytotoxicity was Fc receptor-dependent.

A panel of monoclonal antibodies directed against different human tumor target cells was used to demonstrate that the castanospermine-induced IgG phenotype generally enhanced antibody-dependent tumoricidal activity mediated by natural killer cells. However, differences in lymphocyte response to an alteration in IgG glycosylation were observed.

INTRODUCTION

A similar site of glycosylation is located within the Fc portion of IgG from different species (Howell *et al.*, 1967). Conservation of Fc glycosylation would suggest a functional importance for this moiety. The role of glycosylation has been studied by depleting IgG of its carbohydrates either enzymatically, by treatment of hyperimmune rabbit IgG with glycosidases (Winkelhake *et al.*, 1980; Koide *et al.*, 1977), or metabolically by treatment of IgG-secreting murine hybridomas and myelomas with tunicamycin (Leatherbarrow *et al.*, 1985; Nose and Wigzell, 1983).

Carbohydrates covalently bound to the IgG protein backbone are not necessary for either proper assemblage or secretion of IgG, as shown in studies using IgG from murine myeloma or hybridoma cells cultured in the presence of tunicamycin (Leatherbarrow *et al.*, 1985; Leatherbarrow and Dwek, 1983; Nose and Wigzell, 1983; Hickman and Kornfeld, 1978). Similar results have been obtained by Weitzman and Scharff (1976), using a mutant mouse myeloma that synthesized a polypeptide-deleted IgG heavy chain with a partial block in glycosylation.

Deglycosylation does not seem to alter significantly the antigen-binding properties of IgG antibodies, as aglycosylated IgG secreted by tunicamycin-treated hybridomas have been shown to maintain the same antigen-binding affinity as their glycosylated forms (Nose and Wigzell, 1983; Leatherbarrow and Dwek, 1983). Similarly, enzymatic deglycosylation of hyperimmune rabbit IgG with β-aspartyl-*N*-acetylglucosamidohydrolase has no effect upon antigen-binding (Winkelhake *et al.*, 1980).

However, depletion of carbohydrates, either from rabbit IgG or from murine IgG secreted by hybri-

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Abbreviations: FcR, receptor for the Fc fragment of IgG; ADCC, antibody-dependent cell-mediated cytotoxicity; Cs, castanospermine; DMM, deoxymannojirimycin; MdNM, *N*-methyldeoxynojirimycin; Mon, monensin; Sw, swainsonine; Tm, tunicamycin; NK, natural killer; PBL, peripheral blood lymphocyte; PMN, polymorphonuclear leukocyte; GFC, gel filtration chromatography; Endo H, endo-β-*N*-acetylglucosaminidase H; Con A, concanavalin A; LcH, *Lens culinaris* agglutinin; RIA, radioimmunoassay; mAb, monoclonal antibody; r-IFNγ, recombinant immune interferon; HI-FBS, heat-inactivated fetal bovine serum; PBS, 137 m*M* NaCl/3 m*M* KCl/8 m*M* Na_2HPO_4/1.5 m*M* KH_2PO_4 (pH 7.4); TBS, 150 m*M* NaCl/10 m*M* Tris/1 m*M* $CaCl_2$/1 m*M* $MgCl_2$/0.02% NaN_3 (pH 8).

domas, results in significantly lower binding to Fc receptors (FcR) expressed on human monocytes and murine macrophages (Leatherbarrow *et al.*, 1985; Nose and Wigzell, 1983; Koide *et al.*, 1977). Presumably, the decreased binding of carbohydrate-deficient IgG antibodies to FcR I accounts for the decreased antibody-dependent cytotoxicity (ADCC) mediated by murine macrophages and splenocytes against target cells sensitized with carbohydrate-deficient IgG (Nose and Wigzell, 1983; Koide *et al.*, 1977).

Although these studies suggest that glycosylation of the IgG antibodies used to sensitize the target cell plays a role in ADCC, these studies do not address the question of the functional significance of individual types of IgG oligosaccharides. In fact, an array of 31 structurally distinct, yet related, oligosaccharides are expressed on human IgG (Parekh *et al.*, 1985) and approximately half of these oligosaccharides are found on mouse IgG as well (Mizuochi *et al.*, 1987).

Recently, a series of carbohydrate processing inhibitors have become available. These inhibitors interfere with discrete steps involved in the maturation of protein-bound oligosaccharides (Elbein, 1987). Castanospermine (Cs) and *N*-methyldeoxynojirimycin (MdNM) are chemically dissimilar glucosidase inhibitors that interfere with an early step of carbohydrate processing. The next steps involve removal of different mannosyl residues by two distinct mannosidases. The first of these mannosidases is inhibited by deoxymannojirimycin (DMM), whereas the later-acting mannosidase is inhibited by swainsonine (Sw). These trimming reactions are necessary for the oligosaccharides to acquire structures able to interact subsequently with glycosyltransferases. These transferases catalyze the terminal elongations characteristic of the mature oligosaccharides found on secreted glycoproteins. Although the effect of monensin (Mon) is less certain, it appears to uncouple the trimming reactions from the terminal elongations of the oligosaccharides. Several of these inhibitors, tested on rat hybridomas, have no effect upon IgG secretion (Hashim and Cushley, 1987, 1988).

In this report, we describe the functional effects of alterations in IgG glycosylation induced by inhibitors of glycosylation and carbohydrate processing. The structural alterations have been characterized and correlated with their effect upon ADCC mediated by various populations of human peripheral blood leukocytes. Our data suggests a possible involvement of core fucosylation of IgG in NK cell-mediated ADCC.

MATERIALS AND METHODS

Materials

D-[U-^{14}C]-Glucosamine hydrochloride (284 Ci/mol), D-[6(*N*)-^{3}H]-glucosamine hydrochloride (23.4 Ci/mmol), $Na_2{}^{51}CrO_4$ and En^{3}Hance were purchased from New England Nuclear (Boston, MA). Sephadex G-50 (Superfine), Con A–Sepharose and Protein A–Sepharose were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). LcH (E-Y Laboratories, San Mateo, CA) was conjugated to Affigel 10 (Bio-Rad, Richmond, CA) (7 mg/ml resin) according to an established procedure (Knudsen *et al.*, 1981). Endoglycosidase H (endo-β-*N*-acetylglucosaminidase H) was purchased from Genzyme (Boston, MA), and Pronase (grade B) was from CalBiochem (San Diego, CA). r-IFNγ was purchased from Genentech, Inc. (San Francisco, CA).

Castanospermine was supplied initially by Dr R. J. Molyneux (Agricultural Research Service, USDA, Albany, CA), and later was purchased from Genzyme. Deoxymannojirimycin similarly was purchased from Genzyme; tunicamycin and monensin were from Sigma (St Louis, MO). *N*-Methyldeoxynojirimycin was a gift from Dr R. J. Schwarz (Institut für Virologie der Justus-Liebig, Giessen, F.R.G.). Swainsonine was obtained from Drs D. R. P. Tulsiani (Vanderbilt University, Nashville, TN) and P. R. Dorling (Murdoch University, Murdoch, Western Australia).

Murine hybridoma clones and monoclonal IgG

Description of hybridomas. 34-5-8S hybridomas were obtained from the American Type Culture Collection (Rockville, MD). These cells secrete an IgG2a mAb with specificity for the H-2D^{d} alloantigen (Ozato *et al.*, 1982).

A panel of hybridomas that secrete mAbs to human tumor antigens was previously described (Herlyn *et al.*, 1979, 1983, 1985; Mazauric *et al.*, 1982; Mitchell *et al.*, 1982). All mAbs were of IgG2a isotype. The mAbs ME-288, ME-5073 and ME-37-7 are directed against antigens expressed on WM-9, a human melanoma cell line. CO-17-1A is directed against an antigen expressed on SW-1116, a human colorectal carcinoma cell line, and LU-16B13 recognizes an antigen present on both tumor cell lines.

Culturing conditions. Hybridomas, seeded at 5×10^5/ml, were cultured in Dulbecco's Modified Eagle Medium with low glucose (Gibco, Grand Island, NY), supplemented with 10% HI-FBS, 10 m*M* sodium bicarbonate and 25 m*M* Hepes (pH 7.2). Parallel cultures were grown in the presence or absence of either Tm (0.25 μg/ml), Cs (10 μg/ml), MdNM (1 m*M*), DMM (1 m*M*), Sw (2 μg/ml), or Mon (0.25 μ*M*). For structural characterization studies, cultures were pre-incubated for 20 hr, and then metabolically labelled with either [^{3}H]-glucosamine (2 μCi/ml) or [^{14}C]-glucosamine (0.5 μCi/ml) for an additional 48 hr. Replicate cultures for each condition remained unlabelled for functional studies. In some instances, the cells were cultured in RPMI 1640 medium supplemented with 10% HI-FBS and 2 m*M* glutamine (complete RPMI).

Isolation of IgG. IgG in the spent culture medium were affinity-purified chromatographically on Protein

A–Sepharose by a modification of the procedure of Ey *et al.* (1978). Unlabelled, affinity-purified IgG used for functional studies were dialyzed against PBS. All manipulations were performed at 4°C. Protein concn in each IgG preparation was determined fluorimetrically by conjugation with fluorescamine (Bohlen *et al.*, 1973).

Pronase and Endo H digestions. For structural characterization studies, metabolically labelled, affinity-purified IgG were incubated with pre-digested pronase for 3 days according to an established procedure (Blithe *et al.*, 1980). The pronase digests were heat-inactivated by boiling for 5 min, and then lyophilized. In some instances, the residue was suspended in 200 m*M* sodium citrate/citric acid (pH 5.5), and incubated at 37°C for 21 hr with 10 mU of Endo H (50 mU/ml, final concn).

Structural characterization studies of IgG glycosylation

To insure consistency of elution profiles, a ^{14}C-labelled IgG pronase digest was added as an internal standard to all ^{3}H-labelled samples. The resulting doubly-labelled mixture of pronase digests was analyzed chromatographically using various techniques. The radioactive distribution in each chromatographic fraction, determined by liquid scintillation counting, was plotted by computer-aided graphics, which also provided peak area summations.

Alkaline borate gel filtration chromatography. Blue dextran and phenol red, added to the sample, served as exclusion and inclusion volume markers, respectively. Samples were analyzed on Sephadex G-50 columns (1 × 135 cm), equilibrated and developed with an alkaline borate buffer composed of 45.5 m*M* boric acid/4.5 m*M* sodium tetraborate/2 m*M* Na_2-EDTA/0.02% sodium azide (pH 8.2) (Rothman and Warren, 1988). The nominal flow rate was 3 ml/hr, and fractions of 0.7 ml were collected.

Lectin affinity chromatography. After boiling for 5 min, the pronase digests were analyzed on Con A–Sepharose or LcH–agarose columns (0.7 × 4 cm) according to the procedure of Cummings and Kornfeld (1982). Glycopeptides lacking lectin affinity were removed by washing the column with TBS buffer. Lectin-bound glycopeptides were eluted stepwise: first, glycopeptides with low lectin affinity were eluted with 10 m*M* α-methylglucoside in TBS. Afterwards, glycopeptides with high affinity were eluted with 100 m*M* α-methylmannoside in TBS, pre-warmed to 60°C.

Functional studies of the influence of IgG glycosylation

Target cell lines. The murine mastocytoma P815y of DBA/2 origin (H-2^{d}) was maintained in culture in complete RPMI medium.

The human tumor cell lines WM-9 (melanoma) and SW-1116 (colorectal carcinoma) were cultured in Leibovitz's L-15 medium supplemented with 10% HI-FBS. Single-cell suspensions of these adherent cell lines were obtained after brief incubation at room temp with trypsin (1.25 mg/ml in 0.1% versene).

Peripheral blood leukocyte preparations. Peripheral blood, obtained from healthy donors by venipuncture and anticoagulated with heparin, was processed using an established protocol (Perussia *et al.*, 1987*a*). Blood was centrifuged on a Ficoll/Hypaque gradient (F/H, density = 1.077 g/ml). Mononuclear cells at the upper F/H interface were fractionated into PBL and monocytes by adherence to plastic surfaces after a 1-hr incubation at 37°C in complete RPMI. Adherent monocytes were recovered by scraping. Cells from the lower F/H interface were mixed with plasma, and diluted with 3% Dextran T500 in 0.15 *M* NaCl. After incubation at 37°C, the leukocyte-rich plasma was separated from agglutinated erythrocytes; PMN were isolated from this fraction after hypotonic lysis and a second centrifugation on F/H. All leukocytes were maintained in complete RPMI. In some cases, PMN (4×10^6/ml) were incubated overnight at 37°C with r-IFNγ (200 U/ml).

NK cells were purified from short-term bulk cultures of peripheral blood mononuclear cells co-cultured for 10 days with an irradiated B lymphoblastoid cell line (Daudi or RPMI-8866) (Perussia *et al.*, 1987*b*). NK cells were negatively selected from these lymphocyte cultures using a mixture of T cell- and monocyte-specific monoclonal antibody- and anti-globulin-rosetting as previously described in detail (Perussia *et al.*, 1983).

Binding studies. Triplicate samples of P815y cells (10^5 cells/50 μl/well) were incubated at room temp for 30 min in the presence or absence of serial log dilutions (initially, 10 μg/ml) of the various glycosylation phenotypes of 34-5-8S mAb, produced in the presence or absence of inhibitors of glycosylation and carbohydrate processing. After extensive washing, the cells were incubated for an additional hr at 4°C with ^{125}I-labelled goat Ig anti-mouse IgG. Cell-bound radioactivity was determined, after extensive washing, in a γ-counter. All incubations and washes were performed at 4°C with PBS/0.1% gelatin/0.1% sodium azide (pH 7.2).

The titer of anti-human tumor mAbs in the spent hybridoma culture fluid was determined similarly by indirect RIA binding studies using 1:3 dilutions of the culture fluid.

Antibody-dependent cell-mediated cytotoxicity. Cytotoxicity mediated by peripheral blood leukocytes was determined in a 3-hr ^{51}Cr release assay (Trinchieri *et al.*, 1984). After labelling overnight with ^{51}Cr (100 μCi/10^6 cells), P815y target cells (5×10^5/ml) were sensitized with 34-5-8S mAb (1 μg/ml) for 20 min at room temp, and then washed free of excess, unbound mAb. A constant number of these target cells (10^4/well) was added to serial dilutions of effector cells in a 96-well microtiter plate in complete RPMI. The plates were spun for 1 min at 800 rpm, and after a 3-hr incubation at 37°C, the ^{51}Cr released into the supernatant was measured in a

γ-counter. The percentage specific cytotoxicity was calculated as

$$\frac{\text{OBS} - \text{S.R.}}{\text{MAX} - \text{S.R.}} \times 100\%,$$

where OBS is the release observed in the presence of effectors; S.R. is the spontaneous release in the absence of effectors; and MAX, the maximal release in the presence of 1% Triton X-100. Typically, maximal release from target cells corresponded to 90% of the cell-incorporated isotope; and spontaneous release was less than 10% of maximal release. Values represent the mean of triplicate determinations.

ADCC of human tumor target cells was performed similarly, but with slight modifications. WM-9 and SW-1116 target cells were labelled overnight with ^{51}Cr (50 μCi/10^6 cells and 100 μCi/10^6 cells, respectively). The trypsinized suspensions of labelled target cells were sensitized with a 1:2 dilution of the spent hybridoma culture fluid. By indirect RIA binding studies, this concn had been determined previously to be saturating. Effector cells were NK cells that had been trypsin-treated in order to abrogate their ability to mediate spontaneous cytotoxicity (Perussia *et al.*, 1979). For this, NK cells (10^7/ml) in serum-free RPMI medium were incubated with trypsin (1 mg/ml) at 37 C for 20 min, and then washed with complete (serum-supplemented) medium. Release of ^{51}Cr from target cells was determined after a 6 hr incubation at 37 C.

RESULTS

Characterization of IgG glycopeptide alterations induced by inhibitors of carbohydrate processing

The effect of Cs, Mon or Sw upon glycosylation was determined by analyzing the glycopeptides derived from IgG secreted by 34-5-8S hybridomas cultured in the presence of these inhibitors. Although some similarities existed between glycopeptides derived from the different pharmacologically-induced glycosylation phenotypes, each altered phenotype was characterized by a unique set of IgG glycopeptides. For instance, when analyzed according to apparent size by alkaline borate GFC, only the glycopeptides derived from the Sw-induced phenotype were characterized by a relatively increased amount of class III glycopeptides (Fig. 1B). By contrast, culturing hybridomas in the presence of Cs or Mon induced small, but notably opposite, effects upon the apparent size of class IV glycopeptides (Fig. 1C and D). Relative to glycopeptides from the natively expressed phenotype (produced in the absence of inhibitors), Cs slightly increased (Fig. 1C), whereas Mon slightly decreased (Fig. 1D), the apparent size of class IV glycopeptides. Although small, these differences in apparent size induced by Mon and Cs probably are not artefactual, since in the absence of these inhibitors no differences in the elution profiles are apparent between class IV glycopeptides derived from IgG secreted by two parallel cultures differently labelled with ^{14}C and ^{3}H (Fig. 1A).

Unlike the IgG glycopeptides from the natively expressed phenotype, glycopeptides from each of the altered phenotypes were sensitive to Endo H digestion (Fig. 1E–H). However, comparison of the glycopeptide elution profiles after Endo H digestion would suggest that the Sw- and Mon-induced phenotypes are structurally related and distinct from the phenotype induced by Cs. Particularly apparent are differences in the late-eluting oligosaccharide fragment which is released from the glycopeptides upon Endo H digestion. When derived from glycopeptides of the Cs-induced phenotype, a lesser amount of the total radioactivity is released, and this fragment is eluted later than the analogous fragments released from glycopeptides of the other altered phenotypes.

Previously, we had shown that pronase-digested IgG from murine hybridomas can be resolved by apparent size into as many as five size classes of glycopeptides by GFC in the presence of an alkaline borate buffer (Rothman *et al.*, 1989; Rothman and Warren, 1988). Unlike class IV and V glycopeptides, a significant fraction of class III glycopeptides was sialylated. Moreover, class III glycopeptides were found to be relatively enriched in glycopeptides of low or no affinity for LcH-agarose. Additionally, class III glycopeptides of high affinity had qualitatively different LcH elution profiles than the analogous class IV glycopeptides.

Thus, structural alterations in glycosylation induced by Cs, Mon and Sw, as suggested by differences in GFC elution profiles, are apparent especially from the quantitative and qualitative differences in the lectin affinity chromatographic elution profiles of the IgG glycopeptides. In particular, the glycopeptides of low affinity for Con A derived from the altered phenotypes had a more retarded elution pattern than those of low affinity derived from the native phenotype (Fig. 2A–D). However, of all of the altered phenotypes, only the phenotype induced by Mon is characterized by an increase in the distribution of glycopeptides of high affinity for Con A (Fig. 2D).

Unlike the phenotype induced by Sw, both the Cs- and Mon-induced phenotypes are characterized by a five-fold increase in the distribution of glycopeptides which lack affinity for LcH (Fig. 2G and H). Of more importance, there is no comparable increase in the distribution of glycopeptides which lack affinity for Con A. Presumably, the majority of the glycopeptides derived from the Cs- and Mon-induced phenotypes which do not bind to LcH will bind to Con A.

Effect of alterations in glycosylation upon antigen-binding

The influence of the 34-5-8S IgG glycosylation phenotype upon binding to the target cell P815y was determined by indirect RIA (Fig. 3). Similar amounts

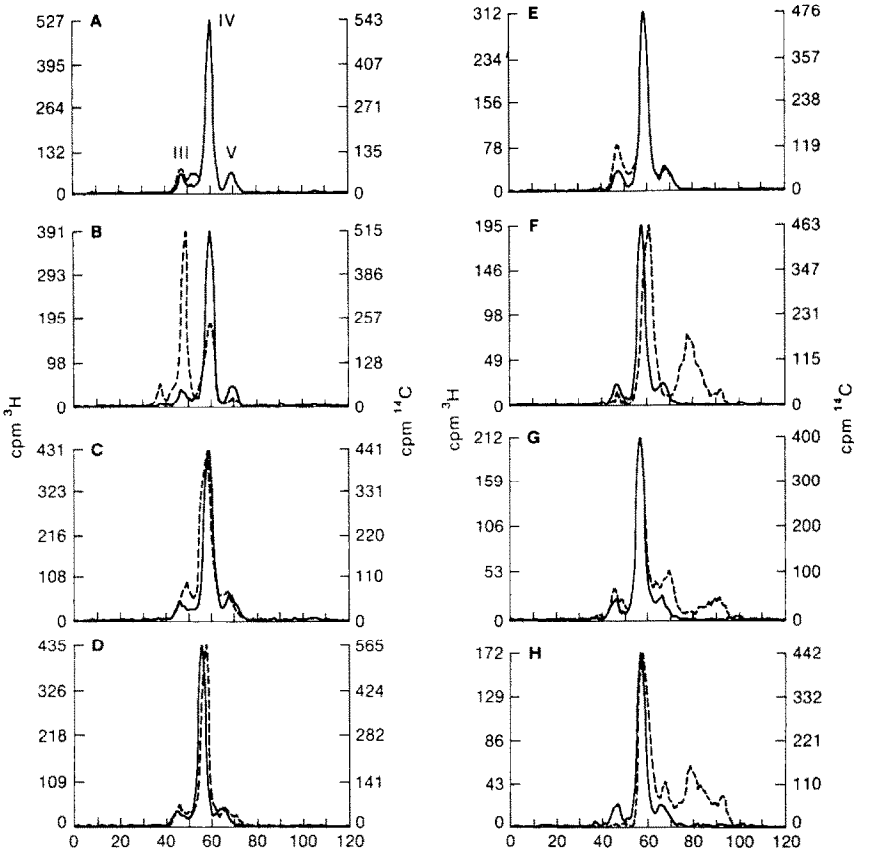

Fig. 1. Effect of carbohydrate processing inhibition upon alkaline borate GFC elution profiles of IgG glycopeptides. Parallel cultures of 34-5-8S hybridoma were metabolically labelled with [^{3}H]-glucosamine in the absence or presence of either Sw, Cs or Mon. An additional culture was labelled with [^{14}C]-glucosamine in the absence of inhibitors. The secreted IgG was affinity-purified and digested with pronase. The alterations in glycosylation were characterized on a column of Sephadex G-50 by alkaline borate GFC before (A–D) and after (E–H) Endo H digestion of the ^{3}H-labelled (---) pronase digest. The ^{14}C-labelled (——) digest, added before chromatography, served as a common internal standard. The elution profiles are shown for the glycopeptides derived from the native phenotype (A and E) as well as the Sw-induced (B and F), Cs-induced (C and G) and Mon-induced (D and H) phenotypes.

of mAb bound to P815y targets regardless of the nature, or even the absence, of the IgG oligosaccharide. Saturation of target cells occurred at a mAb concn of 1 μg/ml for all glycosylation phenotypes.

Effect of alterations in glycosylation upon antibody-dependent cell-mediated cytotoxicity

All effector cell populations tested mediated significant levels of ADCC against P815y target cells sensitized with a control polyclonal rabbit antiserum (data not shown). ADCC mediated by PBL and monocytes against 34-5-8S mouse monoclonal antibody-sensitized targets was lower than that mediated against polyclonal antibody-sensitized targets, whereas PMN mediated similar levels of ADCC against monoclonal or polyclonal antibody-sensitized target cells.

When their ability to promote ADCC were compared, the various altered phenotypes of 34-5-8S mAb were found to differ. Sensitizing P815y target cells with IgG produced in the presence of Mon, or of the chemically dissimilar glucosidase inhibitors Cs and MdNM, enhanced ADCC mediated by PBL (Fig. 4A). This enhancement in cytotoxicity was observed consistently with PBL obtained from various donors (lymphocytes from 24 donors assayed with the Cs-induced, and five donors with the Mon-induced phenotypes of 34-5-8S mAb) (data not shown).

In addition to being specific for certain altered phenotypes, the enhanced cytotoxicity was specific for the effector cell population. The Cs- and Mon-induced phenotypes did not enhance cytotoxicity mediated by monocytes or r-IFNγ-treated PMN obtained from the same donor of the PBL (Fig. 4B and C).

Instead, the Cs-induced phenotype was as active as the native phenotype at promoting ADCC mediated by activated PMN. Additionally, PMN-mediated ADCC against target cells sensitized with the Tm- or Sw-induced phenotypes was significantly lower than that against native IgG-sensitized cells. However, unlike what is observed with PBL, all IgG phenotypes were effective in promoting ADCC by PMN.

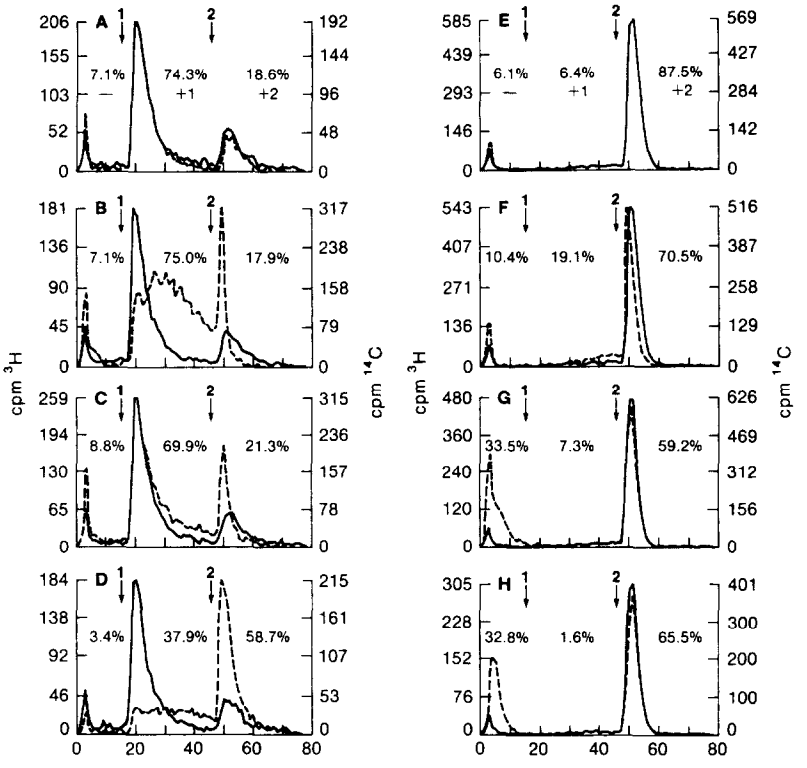

Fig. 2. Effect of carbohydrate processing inhibition upon lectin affinity elution profiles of IgG glycopeptides. The pronase digests of the various phenotypes of 34-5-8S IgG (described in Fig. 1) were analyzed by either Con A (A–D) or LcH (E–H) affinity chromatography. Arrows indicate the start of the stepwise application of 10 m*M* α-methylglucoside (1) and 100 m*M* α-methylmannoside (2). The percentage of ^3H recovered in each of the three lectin affinity fractions is also shown. The elution profiles are shown for the glycopeptides derived from the native phenotype (A and E), as well as the Sw-induced (B and F), Cs-induced (C and G) and Mon-induced (D and H) phenotypes.

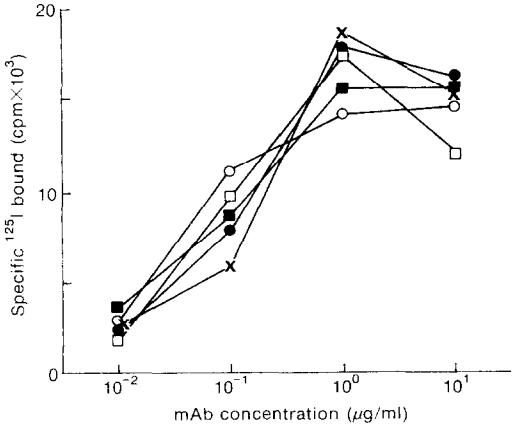

Fig. 3. Effect of IgG glycosylation phenotype upon antigen-binding. Serial log dilutions of equal amounts of 34-5-8S mAb of the various glycosylation phenotypes were incubated with a constant number of P815y cells. The amount of mAb bound at each dilution was determined by indirect RIA with ^{125}I-labelled goat Ig anti-mouse IgG. This was corrected for the amount of label bound non-specifically in the absence of 34-5-8S mAb, approximately 5–7% of maximal bound. The IgG glycosylation phenotypes assayed were the natively expressed (□), and the altered phenotypes induced by Sw (○), Tm (×), Cs (■) or Mon (●).

Virtually all ADCC observed with PBL is mediated by FcR-bearing NK cells (Perussia *et al.*, 1983). For further studies, NK cells were purified to homogeneity from bulk cultures of peripheral blood mononuclear cells. As previously described (Perussia *et al.*, 1987*b*), these purified NK cells had the phenotype and functions of freshly obtained NK cells, and mediated high levels of ADCC against polyclonal antibody-sensitized P815y target cells (data not shown). As observed with fresh PBL, these NK cell preparations mediated minimal levels of ADCC against P815y targets sensitized with native 34-5-8S murine IgG2a monoclonal antibodies, but were able to mediate high levels of ADCC against P815y target cells which had been sensitized with either the Cs- or Mon-induced phenotype of 34-5-8S mAb (Fig. 5A). A similar enhancement in NK-mediated ADCC also was observed using target cells sensitized with the DMM-induced phenotype of 34-5-8S mAb (Fig. 5B), and was observed consistently with NK cells from a total of five donors (data not shown). As described for the other phenotypes, indirect RIA binding studies revealed that the enhanced cytotoxicity promoted by the DMM-induced phenotype could not be ascribed to a significantly greater binding of this IgG phenotype to the target cell (data not shown).

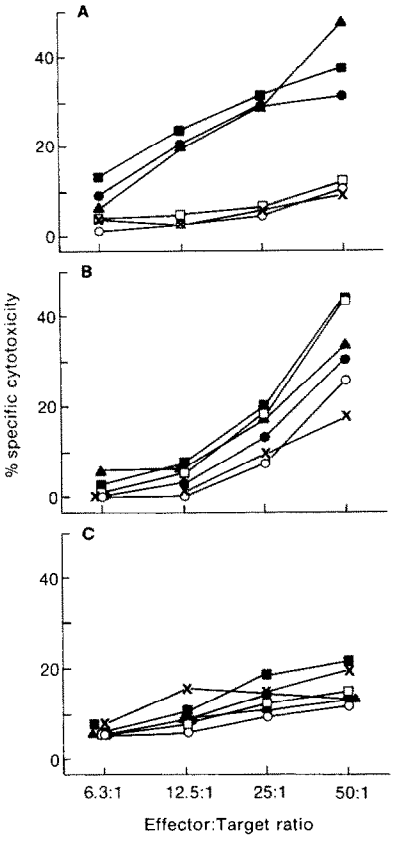

Fig. 4. Effect of IgG glycosylation phenotype upon ADCC mediated by different leukocyte populations. A constant number of ^{51}Cr-labelled P815y cells, sensitized with saturating amounts of 34-5-8S mAb of the various glycosylation phenotypes, was added to serial dilutions of various populations of human peripheral blood leukocytes. Specific ADCC mediated by either PBL (A), PMN (activated with r-IFNγ) (B) or monocytes (C) was then determined in a 3-hr ^{51}Cr release assay. The IgG glycosylation phenotypes assayed were the natively expressed (□), and the altered phenotypes induced by Sw (○), Tm (×), Cs (■), MdNM (▲) or Mon (●).

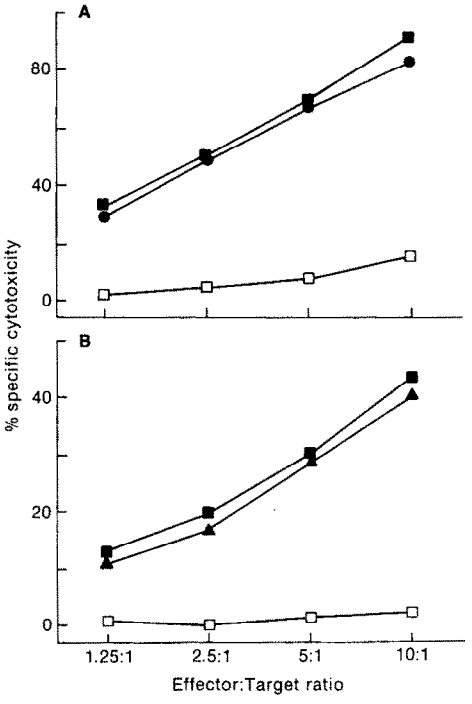

Fig. 5. Effect of IgG glycosylation phenotype upon NK-mediated ADCC. P815y target cells labelled with ^{51}Cr were sensitized with saturating amounts of 34-5-8S mAb of either native (□) or the Cs- (■), Mon- (●) or DMM-induced (▲) phenotype. The effect upon ADCC was determined in a 3-hr ^{51}Cr release assay using serial dilutions of NK effector cells purified from two donors, (A) and (B).

The enhanced lymphocyte-mediated cytotoxicity was found to be FcR-dependent (Fig. 6). ADCC was specifically abrogated by pre-incubation of PBL with 3G8, an anti-FcR mAb (Perussia and Trinchieri, 1984; Fleit *et al.*, 1982), but not by other irrelevant monoclonal antibodies (data not shown). Pre-treatment of NK cells with trypsin did not abrogate ADCC (Fig. 7), although FcR-independent spontaneous cytotoxicity of K562 or human tumor target cells was abrogated (data not shown). Trypsin resistance is a charactersitic feature of the FcR expressed on NK cells (Perussia *et al.*, 1979).

Generality of the influence of the Cs-induced phenotype upon ADCC mediated against tumor target cells sensitized with a panel of mAbs

The generality of the phenomenon of enhanced NK cell-mediated ADCC was explored using various human tumor target cell lines sensitized with a panel of murine mAbs. As observed with P815y target cells, the native IgG phenotype of relevant antigenic specificity induced only minimal ADCC by NK cells. ADCC activity generally was enhanced when WM-9 melanoma cells were sensitized with the

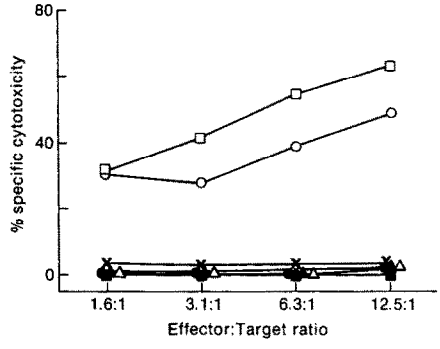

Fig. 6. Fc receptor-dependence of enhanced PBL-mediated ADCC. Serial dilutions of PBL (initially, 1.25×10^5/well) were pre-incubated at room temp for 1 hr in the presence (closed symbols) or absence (open symbols) of 3G8 ascites fluid (1:100 dilution). The effect upon ADCC was determined after the addition of ^{51}Cr-labelled P815y target cells (10^4/well) which had been sensitized with saturating amounts of 34-5-8S mAb of either native (△, ▲), Cs- (□, ■) or Mon-induced (○, ●) phenotype. Spontaneous cytotoxicity against labelled P815y targets which had not been sensitized with 34-5-8S mAb was determined in the absence of 3G8 ascites fluid (×).

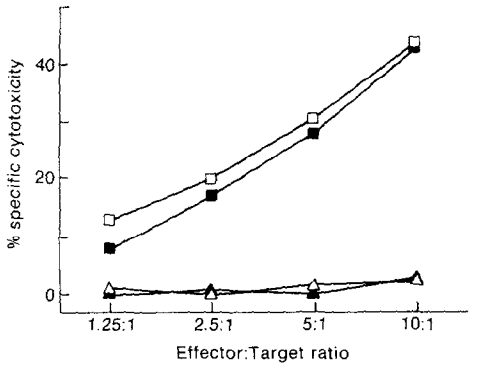

Fig. 7. Effect of trypsin treatment of NK cells upon enhanced NK-mediated ADCC. Purified NK cells were incubated at 37°C for 30 min with trypsin (1 mg/ml/10^7 cells). The effect of the treatment upon ADCC was determined in a 3-hr ^{51}Cr release assay using P815y target cells that had been sensitized with saturating amounts of either the native (△, ▲) or Cs-induced (□, ■) phenotype of 34-5-8S mAb. Trypsin-treated NK cells are indicated by closed symbols; untreated NK cells by open symbols.

Cs-induced phenotype of mAbs of relevant antigenic specificity (Fig. 8). From a total of five donors assayed, NK cells from four donors mediated significantly greater ADCC when WM-9 cells were sensitized with the Cs-induced phenotype of either LU-16B13 or ME-288 mAb (data not shown). Although the level of ADCC was too low to be conclusive, ADCC of WM-9 cells also appeared to be enhanced by sensitizing target cells with the Cs-induced phenotype of either ME-5073 or ME-37-7 mAb (enhancement observed with NK cells from three donors; total of four donors assayed; data not shown).

A second tumor cell line SW-1116 also was used as a target (Fig. 9). The Cs-induced phenotype of LU-16B13 was found to augment cytotoxicity against both tumor lines by NK cells derived from the same donor. Cytotoxicity by the same NK cells, however, was enhanced, though only modestly, when SW-1116 target cells were sensitized with the Cs-induced

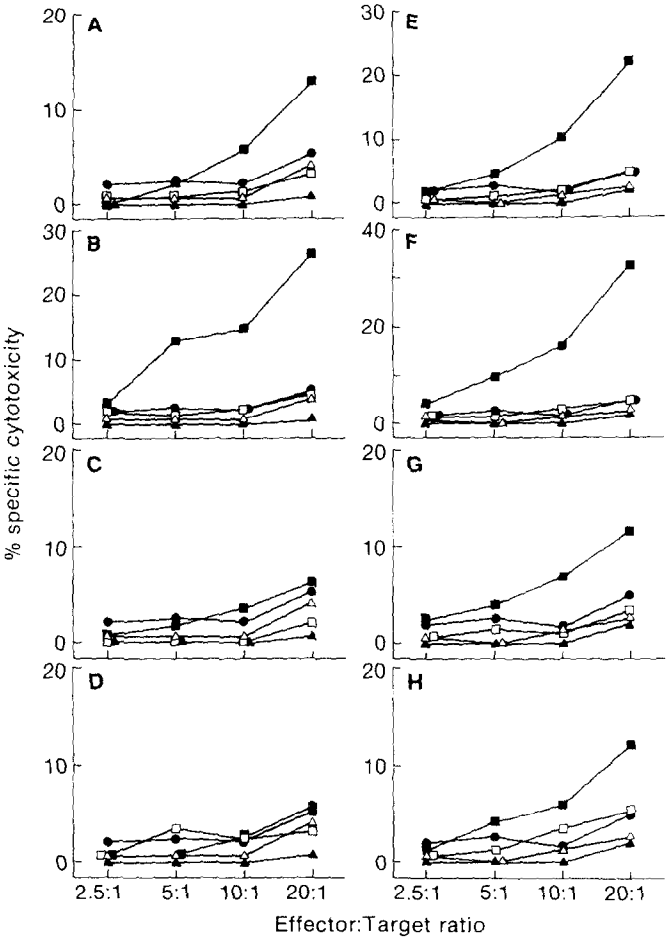

Fig. 8. Effect of the Cs-induced phenotype of a panel of anti-WM-9 mAbs upon NK-mediated ADCC. WM-9 target cells were sensitized with saturating amounts of either the native (□) or Cs-induced (■) phenotype of LU-16B13 (A and E), ME-288 (B and F), ME-5073 (C and G), or ME-37-7 (D and H) mAbs (1:2 dilution of spent hybridoma culture fluid). The effect of the altered IgG phenotype upon ADCC activity was measured in a 6-hr ^{51}Cr release assay using serial dilutions of purified NK effector cells from either donor A (A–D) or donor B (E–H). To assess spontaneous cytotoxicity, WM-9 cells either were not sensitized with mAb (●); or were sensitized with CO-17-1A, an isotype-matched mAb of irrelevant antigenic specificity, of either the native (△) or Cs-induced (▲) phenotype.

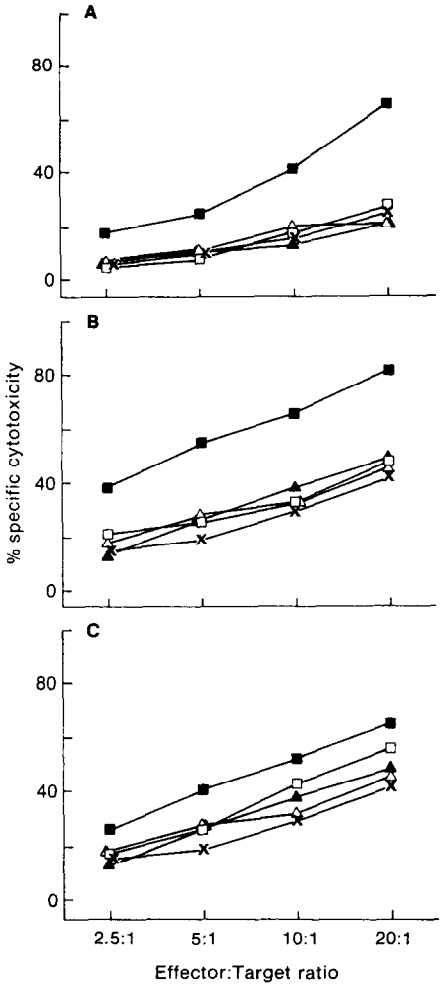

Fig. 9. Effect of the Cs-induced IgG phenotype upon NK-mediated ADCC of several human tumors. SW-1116 target cells were sensitized with saturating amounts of either the native (□) or Cs-induced (■) phenotype of LU-16B13 (B) or CO-17-1A (C) mAb (1:2 dilution of spent hybridoma culture fluid). WM-9 target cells were sensitized similarly with LU-16B13 mAb (A). The effect of the altered IgG phenotype upon ADCC activity was measured in a 6-hr ^{51}Cr release assay with serial dilutions of purified NK cells from the same donor. To assess spontaneous cytotoxicity, target cells either were not sensitized with mAb (×); or were sensitized with an isotype-matched mAb of irrelevant antigenic specificity, of either the native (△) or Cs-induced (▲) phenotype. ME-288 and CO-17-1A mAbs were used as irrelevant mAbs for SW-1116 and WM-9 target cells, respectively.

phenotype of CO-17-1A mAb instead. Sensitizing SW-1116 cells with the Cs-induced phenotype of either LU-16B13 or CO-17-1A mAb enhanced ADCC mediated by NK cells from three donors (total of five donors assayed; data not shown).

In these latter ADCC studies, human tumor cells were sensitized with saturating amounts of the spent culture medium of the hybridoma, rather than with purified mAb. However, the use of the unpurified mAb preparations did not seem to interfere with the cytotoxicity assays. Dialysis of the spent culture medium to deplete it of the carbohydrate processing inhibitors did not influence ADCC (data not shown). Also, it should be noted that the cytotoxicity assays were done in the absence of the inhibitors, as target cells, after brief sensitization with mAb, routinely were washed free of the sensitizing hybridoma culture medium prior to incubation with NK cells. Furthermore, sensitization of tumor target cells with the spent culture medium from Cs-treated hybridomas that secrete an isotype-matched mAb of irrelevant antigenic specificity did not enhance spontaneous cytotoxicity (Figs 8 and 9). Rather, it is specifically the relevant mAbs of the Cs-induced phenotype which account for the enhanced ADCC activity mediated by NK cells.

DISCUSSION

IgG secreted by the same hybridoma clone, but cultured in the presence of various inhibitors of glycosylation or carbohydrate processing, were used to probe the functional consequences of alterations in glycosylation. Neither the absence of glycosylation (resulting from inhibition by Tm) nor the presence of atypical oligosaccharides (as a consequence of inhibition by Cs, MdNM, Mon or Sw) significantly influenced antigen-binding of mAb to their target cells. Despite this similarity in the degree of sensitization of target cells with mAb, a correlation was observed between the efficiency of promoting ADCC and the glycosylation phenotype of the mAb.

The effect of the phenotype upon ADCC varied with the type of cytotoxic effector cell. Sensitization of target cells with mAb of either the Cs-, MdNM-, DMM- or Mon-induced phenotypes significantly enhanced ADCC mediated by PBL (and NK cells purified from this fraction), but not by fresh monocytes or r-IFNγ-treated PMN. However, sensitization of target cells with the native phenotype was as efficient as the Cs-induced phenotype at promoting ADCC mediated by PMN which had been activated by r-IFNγ. Regardless of the glycosylation phenotype, the various mAbs all promoted ADCC with activated PMN. Because NK cells bear only one FcR type (CD16), it is likely that alterations of glycosylation play a major role in binding of IgG Fc to this receptor. PMN share with NK cells FcR (CD16); however, like monocytes but unlike NK cells, they also constitutively express a distinct FcR (gp40) (Shen *et al.*, 1987) which has been shown to mediate ADCC. An enhancing effect of the altered IgG phenotypes on CD16-mediated ADCC could be masked on PMN if efficacy of this second FcR type in mediating ADCC is not altered by these IgG glycosylation phenotypes.

The enhanced lymphocyte-mediated ADCC observed with the Cs- and Mon-induced phenotypes cannot be attributed simply to a functional inactivity of the other altered phenotypes because all

phenotypes bound equally well to their target cells when assayed by RIA, and all phenotypes promoted PMN-mediated ADCC. Furthermore, the enhanced cytotoxicity depends on specific alterations of IgG glycosylation, as the Sw-induced phenotype, although also characterized by a radical alteration in glycosylation, was ineffective at enhancing lymphocyte-mediated ADCC.

Enhancement of NK cell-mediated ADCC correlates with the expression of phenotypes characterized by IgG glycopeptides which bind to Con A but not to LcH. Glycopeptides from human myeloma IgG with similar lectin-binding properties have been identified as complex-type oligosaccharides in which core fucosylation is absent (Kornfeld *et al.*, 1981). In addition to these complex-type structures, high mannose-type structures also would be expected to lack fucosylation, as these oligosaccharides are not substrates for the core fucosyl transferase (Hubbard and Ivatt, 1981).

The mere exposure of peripheral mannosyl residues, however, seems to be insufficient to enhance ADCC, as the Sw-induced phenotype did not alter lymphocyte-mediated ADCC even though peripheral mannosyl residues are expressed in this phenotype. Although functionally distinct, both the Sw- and Mon-induced phenotypes appear related structurally, as evidenced by their similar susceptibility to Endo H digestion as a probe for exposure of mannosyl residues. However, of more importance is the fact that they also differ structurally, as the hybrid structures induced by Sw are core fucosylated, unlike a subset of those oligosaccharides expressed in the Mon-induced phenotype.

Glucosylation, characteristic of the phenotypes induced by the chemically dissimilar glucosidase inhibitors Cs and MdNM, likewise would appear to be unrelated to ADCC activity. Despite the putative absence of glucosylation, the DMM-induced phenotype was as effective as the glucosylated Cs-induced phenotype at enhancing NK cell-mediated ADCC. Moreover, incubation of the Cs-induced IgG phenotype with α-glucosidase did not affect ADCC of targets sensitized with the glucosidase-modified mAb (data not shown). Thus, absence of core fucosylation itself would appear to be a likely candidate as a structural feature necessary for enhancement of NK cell-mediated ADCC.

Because NK cells, unlike monocytes and PMN, bear only one FcR type (CD16), our data suggest that such alterations of glycosylation mainly affect the functional consequences of IgG binding to FcR (CD16) and not to other FcR types. Also in support of this hypothesis is the observation that murine monoclonal antibodies are as efficient as rabbit polyclonal antisera in inducing ADCC by PMN, whereas rabbit polyclonal antisera are more efficient than murine mAbs in promoting antibody-dependent cytotoxicity mediated by human NK cells that express only CD16 (Ortaldo *et al.*, 1987; Christiaansen and Sears, 1984). Several obvious reasons may account for this observation, such as recognition by polyclonal IgG of numerous antigens and epitopes, each with a different orientation, organization and binding affinity (Christiaansen *et al.*, 1987; Christiaansen and Sears, 1984), but perhaps glycosylation also may be involved.

Although the absence of core fucosylation may not be expressed natively in the murine hybridoma repertoire for glycosylation of IgG (Rothman *et al.*, 1989), species-specific differences exist in its expression. Approximately 15% of human IgG, and 68% of rabbit IgG, lack core fucosylation (Mizuochi *et al.*, 1987). Thus, it is tempting to speculate that polyclonal variability in the expression of core fucosylation may confer a functional advantage to host defense by diversifying the effector activity of IgG.

The influence of the glycosylation phenotype may have implications for immunotherapy of neoplasias with murine mAbs. Its possible general applicability is demonstrated by our findings with a panel of anti-tumor mAbs. Typically, expression of the Cs-induced phenotype enhanced the ADCC activity of human NK cells against two human tumor cell lines.

Differences in reactivity are apparent between the Cs-induced phenotypes of the various anti-tumor mAbs of the panel. Glycosylation of the mAb might have to act in concert with other features, such as the orientation and organization of the immune complex on the tumor cell, for the efficient presentation of a determinant that will elicit an immune response. Thus, it may be either the recognition of this determinant or else its presentation which is perceived differently by NK cells.

Perhaps other glycosylation phenotypes will be detected which may result in their preferential interaction with different populations of cytotoxic leukocytes and this may provide a means by which the immune response can be fine-tuned.

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