

Higher levels of sialylated Fc glycans in immunoglobulin G molecules can adversely impact functionality

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Abstract

Although it is now clear that certain Fc glycan structures on immunoglobulin G (IgG) antibodies (Abs) can have a dramatic influence on binding to selected Fc γ receptors (Fc γ R) and on Fc-mediated immune functions, the effects of all known Fc glycan structures still have not been exhaustively studied. We report that *in vitro* analyses of pairs of monoclonal human IgG Abs that differ in the amount of sialic acid in their Fc glycans revealed that, for each of the three Ab pairs we examined, higher levels of sialylation were associated with reduced activity in Ab-dependent cellular cytotoxicity (ADCC) assays. This relationship between sialylation and ADCC activity was observed regardless of whether the differences in the extent of sialylation were derived by different Ab production processes, use of a lectin column to separate monoclonal Ab preparations into differentially sialylated fractions, or use of direct *in vitro* glycoengineering methods to convert a lesser sialylated Ab into a highly sialylated Ab. Subsequent investigations revealed that, depending on the individual Ab and how the differences in sialylation were derived, the lower ADCC potency of the more sialylated variants was apparently due to lower-affinity binding to Fc γ RIIIa on natural killer (NK) cells and/or, more interestingly, lower-affinity binding to cell-surface antigen. Our data provide the first example of an Fc glycan structure impacting antigen binding and suggest that avoiding Fc glycan sialylation can offer another means of optimizing ADCC activity of Abs.

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1. Introduction

The constant region of human IgG Abs contains a single site for asparagine (Asn)-linked glycosylation, i.e. the Asn residue at position 297 (EU numbering system) on the two identical heavy chains, which maps to the Fc domain (Fig. 1). These Fc glycans consist predominantly of the complex biantennary structures, which display a high degree of heterogeneity due to the presence or absence of various terminal sugars (reviewed by Jefferis, 2005; Raju, 2003). It has been known for two decades that the presence of this Asn-linked glycan is critical for Ab activity in immune effector function assays, such as complement activation and ADCC (Nose and Wigzell, 1983; Leatherbarrow et al.,

1985; Tao and Morrison, 1989). Multiple noncovalent contact sites between the glycan and the Fc backbone, along with interactions between the glycan on one heavy chain and the glycan on the other heavy chain, serve to define Fc conformation (Huber et al., 1976; Deisenhofer, 1981). Consequently, the absence of the Fc glycan, whether in Abs deglycosylated by enzymatic removal of the glycan or in aglycosylated Abs genetically engineered to lack the amino acid consensus sequence for Asn-linked glycosylation, results in a misconformation that perturbs the binding regions for C1q complement protein and the Fc γ receptors (Fc γ R), including the so-called high-affinity receptor, Fc γ RI (CD64) and the lower-affinity receptors, Fc γ RIIa (CD32a) and Fc γ RIIIa (CD16a) (Mimura et al., 2000; Krapp et al., 2003; Dijkstra et al., 2001). The affinity of such nonglycosylated Abs for their antigens, however, has been shown to remain essentially unchanged, which speaks to the apparent functional separation between the Fc structure of an Ab and its intrinsic affinity for antigen.

Differences in Fc functions of Abs that differ only in the fine structure of the Fc glycan are generally much less dramatic, with the exception being Abs that differ in their fucose con-

Abbreviations: Abs, antibodies; ADCC, antibody-dependent cellular cytotoxicity; Ag, antigen; Asn, asparagine; CPMs, counts per minute; Fc γ R, Fc γ receptor; GlcNAc, *N*-acetylglucosamine; IgG, immunoglobulin G; mAbs, monoclonal Abs; NK, natural killer; PBMC, peripheral blood mononuclear cells; WGA, wheat germ agglutinin

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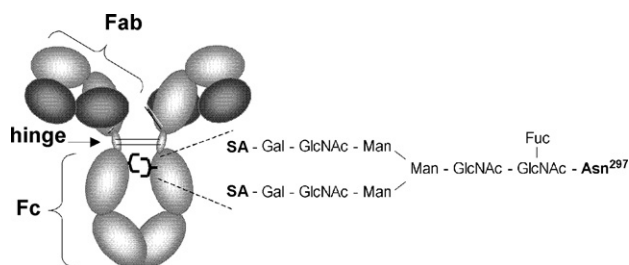


Fig. 1. Schematic depiction of an IgG Ab showing an Fc glycan structure. The position of the single Asn-linked glycosylation site at Asn²⁹⁷ of both heavy chains, and one of numerous possible glycan structures, are shown (SA, sialic acid; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Man, mannose; Fuc, fucose; Asn, asparagines).

tent. Compared to fully fucosylated IgG1 Abs, afucosylated versions of the same Ab show a 50-fold greater affinity for FcγRIIIa and a greater than 100-fold enhanced ADCC activity (Shields et al., 2003; Shinkawa et al., 2003; Yamane-Ohnuki et al., 2004). The same enhanced ADCC effects can be accomplished by co-expressing an IgG Ab with an enzyme that attaches a bisecting GlcNAc residue, which also serves to reduce fucose levels (Umaña et al., 1999). These observations took on increasing importance following the first report of clinical evidence that patients with an FcγRIIIa allotypic variant that binds IgG1 with greater affinity showed better clinical responses to the therapeutic IgG1 Ab, rituximab, compared to patients with a different FcγRIIIa allotypic variant that binds IgG1 with lower affinity (Cartron et al., 2002). Aside from the effects of fucose, functional effects of other variations in Fc glycan structure that have been reported appear to have much more subtle effects, such as the approximately two-fold reduced levels of *in vitro* complement lysis activity of Campath-1H and rituximab variants that have reduced levels of galactosylation (Boyd et al., 1995; Hodoniczky et al., 2005). Effects of Fc sialic acid on Ab functions have received relatively little attention, presumably in part due to purified Ab preparations typically containing only low amounts of sialic acid and also due to challenges associated with preparing variants of the same Ab that differ significantly in their sialic acid content but do not differ in other ways that might influence activity. Removal of sialic acid from IgG1 Ab Campath-1H was reported to have no effect on ADCC activity, but the Fc glycans on the original Ab preparation that was used as a reference appeared to be less than 5% sialylated, making it difficult to detect any effect (Boyd et al., 1995). The Fc activity of native (heterogeneous) IgG1 Fc and IgG1 Ab were compared to the same material after treatment with both sialidase and β-galactosidase, but not compared to material treated with sialidase only, which may have allowed an analysis of the effects of sialic acid (Mimura et al., 2000). A mutated human IgG3 Ab that was highly sialylated via an α2,3 linkage was observed to be two- to three-fold less active than its wild-type, lesser sialylated counterpart in FcγRI-dependent and FcγRII-dependent functional assays, but a variant of the same Ab that contained a mix of two sialic acid linkages (α2,3 and α2,6) showed activities comparable to the wild-type (Lund et al., 1996; Jassal et al., 2001). Although this demonstrated that the type of sialic acid

linkage can have at least some effect on Fc function of a mutant IgG3 Ab, the study did not compare Fc functions of an IgG with differing amounts of sialylation. Kumpel et al. (1994) did suggest that high levels of sialylation in Ab JAC10 may be a reason for its low activity in ADCC assays, but reasons for not being able to draw firm conclusions about sialic acid effects include the facts that (a) the same Ab with lower levels of sialylation was not included in the analyses to more clearly point to sialic acid effects, (b) the Ab lot with high sialylation was not the same Ab lot used in their ADCC assays, (c) data shown with another Ab with the same degree of sialylation was far more active, and (d) it was not known whether the Abs samples also differed in fucose levels, an important parameter to control for ADCC comparisons. Finally, it was reported that an IgG1 Ab expressed in a mutant CHO cell line that was defective in sialylation and the same Ab expressed in a wild-type CHO cell line showed no discernible difference in FcγRI binding, but the comparisons did not include binding to FcγRIIIa, the receptor primarily responsible for *in vitro* ADCC activity (Wright and Morrison, 1998). Given the lack of definitive comparisons in previous studies, and questions on the effect of the far-reaching, negatively charged sugar on Fc glycan and protein structure (see Fig. 1), we sought to more carefully test whether sialic acid content influences Ab function.

2. Materials and methods

2.1. Source, preparation, and analytical characterization of test Abs

Ab1, Ab2, and Ab3 are all monoclonal IgG Abs with human IgG1 and kappa constant regions that were stably expressed in transfected mouse myeloma cells as described (Knight et al., 1993). No post-purification modifications were performed with the sialic acid variants of Ab1 and Ab2 termed Ab1-20, Ab1-29, Ab2-0, and Ab2-26, which have 20%, 29%, 0%, and 26% sialylated Fc glycans, respectively (see Table 1).

Ab3 sialic acid variants were prepared after first enzymatically modifying the Fc glycan to be fully galactosylated prior to wheat germ agglutinin (WGA) lectin fractionation. The *in vitro* galactosylation reaction was performed using bovine β-1,4-galactosyltransferase as described by Raju et al. (2001). Fc glycans of the modified Ab, Ab3-GT, was confirmed to be fully galactosylated after release of glycans using PNGase F and characterization by MALDI-TOF-MS and HPLC. Highly sialylated Ab molecules were then separated from lesser sialylated Ab molecules by buffer-exchanging Ab3-GT (~10 mg) into 20 mM Tris-HCl buffer (pH 7.0) containing 20 mM CaCl₂ and 20 mM MgCl₂ and passing the Ab through a WGA lectin-agarose column. The column was washed with the same buffer and 1 ml fractions collected while monitoring OD at 280 nm. Bound antibody was eluted with 200 mM GlcNAc solution and buffer-exchanged into phosphate-buffered saline (PBS). Bound and unbound material contained 67% and 5% sialylated Fc glycans, respectively, and were termed Ab3-GT-WGA-67 and Ab3-GT-WGA-5. The same procedure was also used to fractionate Ab1-29 on a WGA lectin column, but without first treating with

Table 1
Summary of test Abs

Parent Ab	Name of sialic acid variant	% Fc glycans with sialic acid	Source of variation in Fc sialic acid content	% Fc glycans with fucose	Difference observed		
					ADCC	FcγRIIIa binding	Ag binding ^a
Ab1	Ab1-20	20	Production process	93.4	✓	✓	✓
	Ab1-29	29		94.8			
Ab2	Ab2-0	0	Production process	97.4	✓	✓	
	Ab2-26	26		96.5			
Ab3	Ab3-GT-WGA-5	5	GalT, WGA fractionation	94.8	✓		✓
	Ab3-GT-WGA-67	67		95.0			
Ab1	Ab1-WGA-29	29	WGA fractionation	94.7	✓		✓
	Ab1-WGA-41	41		94.5			
Ab1	Ab1-20	20	SialylT treatment	93.4	✓	Not tested	Not tested
	Ab1-GTST-90	90		94.9			

^a Refers to cell-surface antigen.

β-1,4-galactosyltransferase. The resulting fractions were termed Ab1-WGA-41 and Ab1-WGA-29. An *in vitro* glycoengineering method was used to prepare a highly sialylated sample of Ab1 by simultaneously treating purified Ab1-20 with bovine β-1,4-galactosyltransferase and rat liver α-2,3-sialyltransferase as described (Raju et al., 2001). The resulting Ab was found to contain 90% sialylated Fc glycans and was therefore termed Ab1-GTST-90. Enzymatically deglycosylated Abs, referred to as Gno variants, were prepared by treating Ab1, Ab2, and Ab3 with PNGase F and characterizations performed as described previously (Raju and Scallon, 2006). Each Gno Ab showed 100-fold reduced binding to FcγR on human U-937 cells in competition binding experiments (data not shown) performed as described below, with the trace binding believed to be due to incomplete deglycosylation. The negative control Ab (CNTO 3198) used in U-937 FcγR binding experiments is a recombinant, monoclonal mouse IgG1,k Ab with irrelevant antigen specificity that was prepared at Centocor.

To determine what proportion of glycans were sialylated in each Ab sample, PNGase F-released glycans were derivatized with anthranilic acid (2-aminobenzoic acid) and analyzed by normal phase HPLC as described (Papac et al., 1996, 1998; Raju et al., 2000; Anumula, 1995). The extent of Fc fucosylation was determined by digesting Abs (~50 μg) with PNGase F in 10 mM Tris–HCl buffer (50 μl) pH 7.0 for 4 h at 37 °C and analyzing the released glycans by MALDI-TOF-MS as described elsewhere (Papac et al., 1996, 1998; Raju et al., 2000). To quantitate aggregates, Ab samples were analyzed by SEC using Agilent 1100 HPLC system (Agilent, Palo Alto, CA). A TOSO HAAS TSK 3000SWXL (Tosoh Biosep LLC, Montgomeryville, PA) 7.8 mm × 30 cm column (5 μm particle size) was used at ambient temperature. The mobile phase was PBS, and the flow rate was 0.5 ml/min.

2.2. ADCC assays

Target cells for assays involving Ab1 and Ab3 were prepared at Centocor by transfecting Sp2/0 mouse myeloma cells

with cell-surface, transmembrane-anchored forms of human antigens Ag1 and Ag3, respectively. The Ag1-expressing and Ag3-expressing cell lines both expressed 50,000–70,000 Ag molecules per cell. The Ag1-expressing and Ag3-expressing cells were cultured in IMDM containing 5% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids. Target cells for assays involving Ab2 were a human cell line from ATCC that expresses 90,000–120,000 cell-surface, transmembrane-anchored human Ag2 molecules per cell. These cells were cultured in DMEM medium containing 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate and 0.1 mM nonessential amino acids.

ADCC assays were performed using a DELFIA EuTDA-based cytotoxicity assay (Blomberg et al., 1996) kit (Perkin-Elmer, Boston, MA) according to the manufacturer's instructions, with minor modifications. Briefly, target cells were loaded with BATDA (bis-(acetoxymethyl)-2,2':6',2''-terpyridine-6,6''-dicarboxylate) for 30 min at 37 °C, washed with PBS, and resuspended in the appropriate culture medium at 2×10^5 cells/ml. Peripheral blood mononuclear cells (PBMC) were isolated from blood of healthy donors by density gradient centrifugation on Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden) (Robinson et al., 2001), washed twice with PBS, and resuspended at 1×10^7 cells/ml in the same culture medium used for the Ag-expressing cells.

Ab dilutions in 100 μl culture medium were added in a round-bottom 96-well plate with 50 μl BATDA-loaded target cells and 50 μl PBMC at a ratio of 50:1. The plate was centrifuged for 5 min at $500 \times g$ and then incubated for 2 h at 37 °C. DELFIA europium solution was added to cells and then fluorescence was measured on an EnVision instrument (Perkin-Elmer). The percentage of specific cytotoxicity was calculated as (experimental release – spontaneous release)/(maximum release – spontaneous release) × 100. Spontaneous release was determined by incubating the targets with media instead of effector cells, and maximum release was determined by incubating target cells with 10 μl of DELFIA lysis solution. Data were fit-

ted to a sigmoidal dose–response curve using Graphpad Prism v4 (GraphPad Software, San Diego, CA).

2.3. Ab binding to cellular FcγRs

Human PBMCs were isolated as described above and NK cells isolated from the PBMCs by negative selection with magnetic beads (Miltenyi Biotec, Auburn, CA). NK cells were cultured overnight in 10% FBS at 37 °C with 5% CO₂. Anti-FcγRIIIa mouse IgG1 mAb 3G8 (Fleit et al., 1982) (BD Biosciences, San Diego, CA) was labeled with ¹²⁵I using Iodogen tubes (Pierce, Rockford, IL) to a specific activity of 11 μCi/μg. ¹²⁵I-3G8 was premixed with varying amounts of unlabeled competitor Ab in culture media and the Ab mixture added to the NK cells for a final concentration of 0.3 μg/ml ¹²⁵I-3G8. Cells were incubated at 4 °C for 16 h and unbound IgG removed by washing with PBS. Bound CPMs were quantitated using a gamma counter.

U-937 cells cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, and 10% FBS (U-937 medium) were seeded into 96-well plates with 3 × 10⁵ cells per well in 50 μl medium. Ab3 was labeled with ¹²⁵I to a specific activity of 17 μCi/μg. ¹²⁵I-Ab3 Ab was premixed with varying amounts of unlabeled competitor Abs in media. Fifty microliters of the Ab mix was added to 50 μl of U-937 cells for a final concentration of 0.2 μg/ml of ¹²⁵I-Ab3 in all wells. Cells were incubated at 4 °C for 16 h and the unbound Ab was removed by washing with medium. Bound CPMs were determined using a gamma counter.

2.4. Binding of Abs to cell-surface antigen

The same Ag-expressing cell lines used in the above-described ADCC assays were used in binding assays to test for differences among sialic acid variants in their antigen binding avidity. Assays were performed in a competition format, in which one of the radiolabeled Abs (either Ab1, Ab2, or Ab3), kept at a fixed concentration, was incubated with the Ag-expressing cells in the presence of varying amounts of unlabeled test Abs. ¹²⁵I-Abs, prepared by the Iodogen method, were at a specific activity of ~10 μCi/μg.

Ag1-expressing and Ag3-expressing cells were seeded in 96-well tissue culture plates at 50,000 cells per well, and Ag2-expressing cells at 180,000 cells per well, in IMDM medium with 5% FBS. The appropriate ¹²⁵I-Ab was premixed with titrating amounts of test Abs and the mixture added to the appropriate Ag-expressing cells and incubated for 2 h at RT. Cells were washed with medium to remove unbound Ab, and cell-bound CPMs determined using a gamma counter.

For the Ab2 variants, Ag2-expressing cells were seeded in 96-well tissue culture plates at 185,000 cells per well in 50 μl of DMEM, 10% FBS. ¹²⁵I-Ab2 was premixed with titrating amounts of test Ab and 50 μl of the mixture was added to the Ag-expressing cells. Plates were incubated for 16 h at 4 °C (to minimize internalization of Ag2 following Ab2 binding). Cells were then washed with medium, and cell-bound CPMs determined using a gamma counter.

2.5. Binding of plate-coated Abs to soluble targets

A recombinant soluble form of the extracellular domain of Ag1 was prepared at Centocor by expression in Sp2/0 mouse myeloma cells. A mouse IgG anti-idiotypic (Id) Ab specific for Ab3 variable region was made at Centocor and a Fab fragment of the anti-Id prepared by papain digestion. A 96-well break-apart plate (Nunc Maxisorp, Rochester, NY) was coated with test Ab by adding 50 μl of Ab at 5 μg/ml in PBS and incubating at 4 °C overnight. Soluble Ag1 and the Fab fragment of anti-Id3 were iodinated to specific activities of 14 and 10 μCi/μg, respectively. The Ab-coated plates were washed, blocked with 1% BSA in Hanks-buffered saline with calcium and magnesium (HBSS), and varying amounts of iodinated target (Ag1 or anti-Id3 Fab) in 50 μl 1% BSA in HBSS were added to the plates, and the plates incubated at RT for 2–3 h. Plates were washed with PBS and individual wells counted using a gamma counter. Nonspecific binding was determined using a 100-fold excess of unlabeled target.

2.6. Statistical analyses

A difference in potency between antibody variants was analyzed by comparison of the curves using simultaneous four-parameter logistic regressions with a common minimum, maximum, and slope following a preliminary test for slope and range given a common plateau for a zero concentration (i.e. always assumed without testing a common minimum for increasing curves and common top for decreasing curves). Significance testing was done with the extra sum of squares *F*-test in GraphPad Prism v4. A *p* value of <0.05 was considered to be significant. Analyses of CPMs were weighted inversely by square of CPMs because the standard deviation of CPMs increases proportionally to its mean.

3. Results

3.1. Test Abs

Our initial studies focused on two pairs of unmodified (collectively referred to here as ‘natural variants’) human IgG1 monoclonal Abs, Ab1 and Ab2, in which members of a pair differed in the extent of Fc sialylation, apparently due to the different production processes used to prepare them (but produced by the same host cell type). There were a number of differences in the production processes used, including the presence or absence of FBS, which correlated with production of lesser and higher sialylated Abs, respectively. The Ab1 variants, Ab1-20 and Ab1-29, contained 20% and 29% sialylated glycans, respectively, and Ab2 variants, Ab2-0 and Ab2-26, contained 0% and 26% sialylated glycans, respectively (see Table 1). Otherwise, members of each pair had the same amino acid sequences, the same levels of Fc fucosylation and bisecting GlcNAc content (MALDI-TOF mass spectrometry analyses), and the same low level of Ab aggregates (<1% by SEC-HPLC analyses). The antigens (Ag) recognized by Ab1 and Ab2 are referred to as Ag1 and Ag2, respectively.

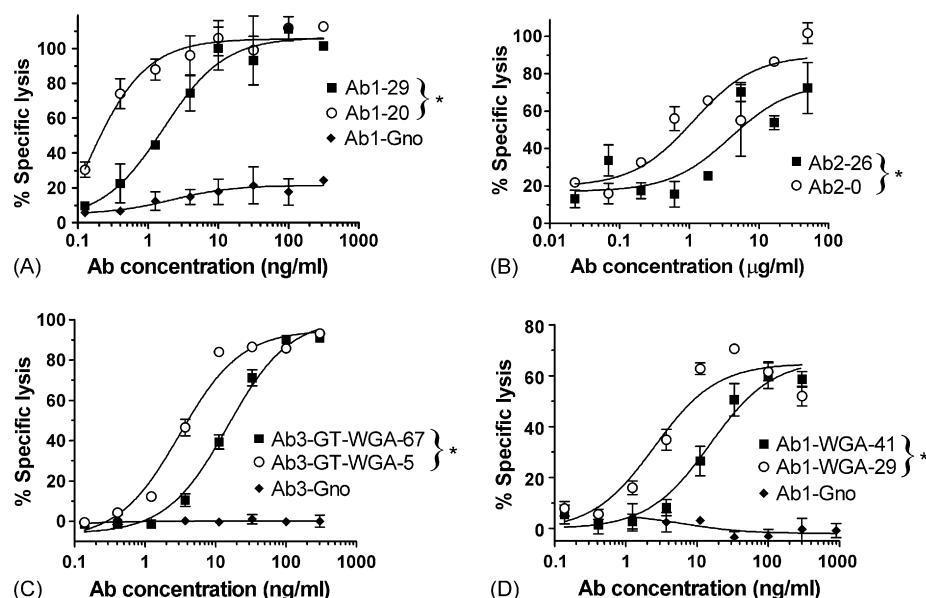


Fig. 2. ADCC activity of sialic acid variants. Titrating amounts of Ab variants were added to the appropriate Ag-expressing target cells, followed by the addition of freshly isolated human PBMC immune effector cells. The extent of target cell lysis was quantitated after 2 h. (A) Comparison of natural variants of Ab1 using Ag1-expressing target cells; (B) comparison of natural variants of Ab2 using Ag2-expressing target cells; (C) comparison of WGA lectin-derived variants of Ab3 using Ag3-expressing target cells; (D) comparison of WGA lectin-derived variants of Ab1 using Ag1-expressing target cells. Gno variants represent enzymatically deglycosylated Ab preparations that bind antigen but have greatly reduced affinity for Fc γ Rs. Samples were analyzed in duplicate or triplicate (error bars represent ranges or S.D., respectively) and the results shown are representative of three or four independent experiments for each Ab pair. For all four pairs of variants, the differences were statistically significant (asterisks) ($p < 0.0001$ for graphs A, C, and D; $p = 0.0016$ for graph B) as determined by extra sum of squares F -test.

Subsequently, two additional pairs of sialic acid variants were prepared by using lectin affinity chromatography to fractionate heterogeneous monoclonal Abs into higher and lower sialylated species. For one pair, unmodified Ab1-29 was passed directly over immobilized WGA lectin. The flow-through fraction and a weakly bound fraction were determined to contain 29% and 41% sialylated glycans, respectively, and were termed Ab1-WGA-29 and Ab1-WGA-41. A third Ab, Ab3, which contained ~5% sialylated Fc glycans, was first treated with β -1,4-galactosyltransferase to prepare fully galactosylated material prior to WGA column fractionation, resulting in a large amount of Ab3-GT-WGA-5 and a small amount of highly sialylated material called Ab3-GT-WGA-67. The antigen recognized by Ab3 is termed Ag3, and an anti-Id antibody specific for Ab3 is termed anti-Id3.

Another variant of Ab1 was later prepared using *in vitro* glycoengineering methods (Raju et al., 2001). The combination treatment of purified Ab1-20 with β -1,4-galactosyltransferase and α -2,3-sialyltransferase resulted in material found to be 90% sialylated, and termed Ab1-GTST-90.

3.2. Higher levels of Fc sialylation associated with reduced ADCC activity

Our initial evaluations of the effects of sialic acid focused on *in vitro* ADCC activity of the two pairs of natural variants. Ab1-29 and Ab1-20 at varying concentrations were incubated with Ag1-expressing target cells that had been labeled with an acetoxymethyl ester of fluorescence enhancing ligand, BADTA, which, when released after cytolysis, forms a highly fluores-

cent and stable europium chelate. As shown in Fig. 2A, there was a clear difference in cytotoxic activity, wherein Ab1-29, with higher levels of Fc sialylation, was required at approximately seven-fold higher concentrations than Ab1-20 in order to trigger cell lysis to the same extent. Experiments with Ag2-expressing target cells showed the same pattern for the pair of Ab2 natural variants. In order to achieve the same degree of cell lysis as the Ab2-0 variant with no detectable sialic acid, Ab2-26 was required at approximately six-fold higher concentrations (Fig. 2B).

To determine whether the association of higher sialic acid levels with lower ADCC activity would be observed with a third Ab, for which natural variants were not available, WGA lectin fractions prepared from Ab3 were evaluated in ADCC assays using Ag3-expressing target cells. Again, the higher sialylated material was less active, although there was only a four-fold difference in their EC₅₀ values despite their dramatic difference in sialic acid contents (5% versus 67%) (Fig. 2C). For comparison, WGA lectin fractions prepared from Ab1 showed that 41% sialylated material needed to be at approximately six-fold higher concentrations than 29% sialylated material to achieve the same degree of cell lysis (Fig. 2D).

These results showed a consistent pattern, for three out of three Abs tested, of reduced ADCC activity associated with higher levels of Fc sialic acid. Although there was not a neat correlation between the extent of the differences in sialic acid and the fold differences in ADCC activity across Abs, there was at least a consistent relationship within the panel of four Ab1 variants, where the EC₅₀ values were typically 0.3, 2, 2, and 10 ng/ml for Ab1-20, Ab1-29, Ab1-WGA-29, and Ab1-WGA-41, respec-

tively. The results with the lectin fractions also confirmed that sialylated Ab preparations can contain molecular species with varying levels of ADCC activity. It is noteworthy that, with the exception of Ab2-0 and Ab2-26, the variants analyzed here did not show differences in the maximum level of lysis achieved.

3.3. Increased sialylation associated with reduced FcγRIIIa binding

Because Ab activity in the ADCC assays involves simultaneous binding of Ab to FcγRIIIa on NK cells and to antigen on target cells, we analyzed both of these interactions to determine to what extent they were impacted by differences in Fc sialic acid content. To test whether Ab variants show differential affinity for FcγRIIIa, freshly isolated NK cells from healthy human donors were used in competition binding experiments involving 125 I-labeled mAb 3G8 (Fleit et al., 1982), an anti-FcγRIIIa Ab that competes with Fc domains for binding to FcγRIIIa, and unlabeled Abs as competitors. Free, uncomplexed Abs were used instead of immune complexes (which generally show much greater binding to FcγRIIIa) so that results would not be confounded by differences in stability of soluble immune complexes, which can be influenced by Fc sialic acid content (our unpublished data). Results showed that the higher sialylated natural variant of Ab1, Ab1-29, had a reduced affinity for FcγRIIIa on the NK cells, being required at four-fold higher concentration than Ab1-20 to achieve the same degree of binding (Fig. 3A). There was a similar difference with the natural variants of Ab2, where Ab2-26 needed to be at a five-fold higher concentration than Ab2-0 to compete against mAb 3G8 to the same extent (Fig. 3B). Similar results were obtained in each experiment when using NK cells from at least two other blood

donors (data not shown; FcγRIIIa allotype not determined). These results showed that higher levels of sialylation can reduce IgG affinity for FcγRIIIa and therefore almost certainly contributed to the observed reduction in ADCC activity.

When the same experiment was done with the pairs of variants derived by lectin fractionation, however, the higher sialylated variants were seen to bind FcγRIIIa just as well as the lesser sialylated variants (Fig. 3C and D). The reasons for the different results with the two pairs of natural variants and two pairs of lectin-derived variants are not known, but a good possibility is that there are differences in the locations of the sialic acid residues that are present, i.e. the relative amounts of sialic acid on the $\alpha(1,6)$ arm versus the $\alpha(1,3)$ arm of the biantennary structures may differ depending on how the variants were derived, and such differences may affect Ab behavior.

After observing that both Ab1-29 and Ab2-26 bound to NK cell FcγRIIIa with less affinity than their lesser sialylated counterparts, it was considered whether the effect could be due to increased electrostatic repulsion between the negatively charged sialic acid and the negatively charged cell-surface. However, we observed that binding to FcγRs on human U-937 cells did not follow the same pattern, arguing against an electrostatic effect (Fig. 3E). In fact, we usually observed slightly greater binding to U-937 cells by higher sialylated variants. Although U-937 cells express both FcγRI and FcγRII, only the high-affinity FcγRI receptor shows appreciable binding to single, monomeric IgG Abs. Therefore, our data from monomeric Abs suggests Fc sialylation either does not affect binding to FcγRI or may actually enhance binding to FcγRI. Nonetheless, neither the U-937 binding results nor the NK binding results with the lectin-derived variants lend support to the notion that the reduced binding to cellular proteins is due to electrostatic effects.

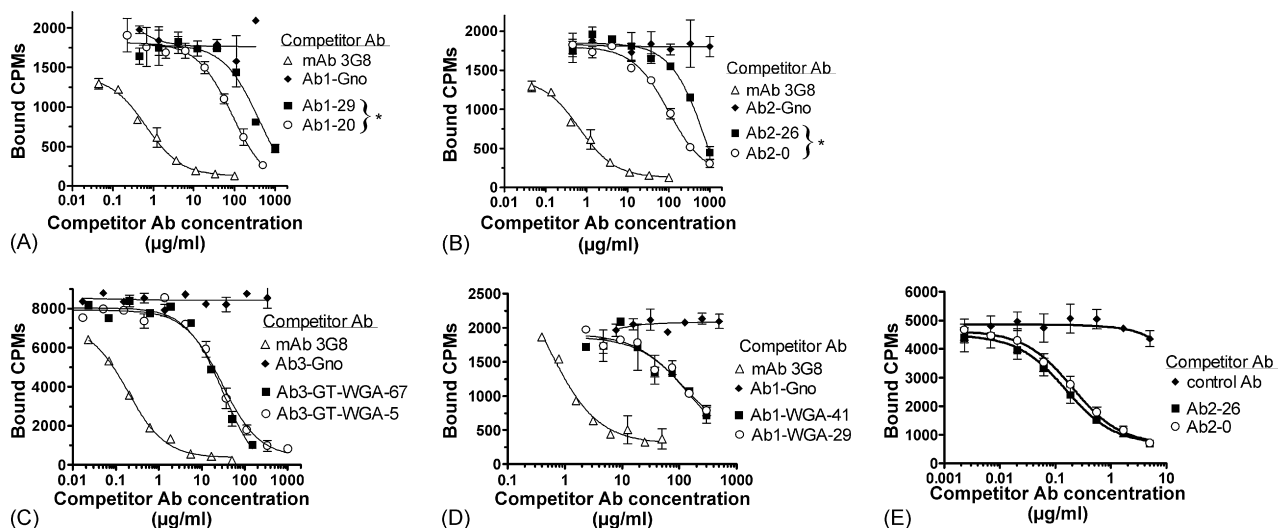


Fig. 3. Binding by Ab variants to cellular FcγRs. (A–D) Competitive binding of Abs to FcγRIIIa on freshly isolated human NK cells. 125 I-labeled anti-FcγRIIIa mAb 3G8 at a fixed concentration was incubated with NK cells in the presence of varying concentrations of unlabeled Ab sialic acid variants or control Abs as competitors. (A) Competitive binding by natural variants of Ab1; (B) competitive binding by natural variants of Ab2; (C) competitive binding by WGA lectin-derived variants of Ab3; (D) competitive binding by WGA lectin-derived variants of Ab1. (E) Competitive binding to FcγRI on human U-937 cells. Cells were incubated with an irrelevant 125 I-labeled human IgG1 mAb at a fixed concentration and varying concentrations of unlabeled natural variants of Ab2 or a mouse IgG1 control Ab as competitors. Samples for all five graphs were analyzed in duplicate or triplicate (error bars represent ranges or S.D., respectively) and the results shown are representative of two or three independent experiments for each Ab pair. The differences between the Ab variants in their binding were significant (asterisks) in graphs A and B ($p < 0.0001$) as determined by extra sum of squares F -test.

3.4. Increased sialylation associated with reduced antigen binding

It remained possible that the different levels of activity between sialic acid variants in the ADCC assays were also due to effects of Fc sialylation on Ab binding to antigen on the target cells. Antigen binding experiments performed in a competition format with the same Ag-expressing target cells that were used in the ADCC assays indeed showed that Ab1-29 was required at three-fold higher concentration than Ab1-20 to compete for antigen binding to the same extent (Fig. 4A). Ab2-26, in contrast, was just as competitive as Ab2-0 (Fig. 4B). The same analyses performed with the two pairs of lectin-derived variants showed similar results to the Ab1 natural variants, i.e. the higher sialylated Ab3-GT-WGA-67 was required at four- to six-fold higher concentration than the lesser sialylated Ab3-GT-WGA-5 (Fig. 4C), and the higher sialylated Ab1-WGA-41 was required at four- to six-fold higher concentration than the lesser sialylated Ab1-WGA-29 to achieve the same degree of competitive binding (Fig. 4D). Interestingly, the unexpected differences in apparent affinity for cell-surface antigen by the Ab1 and Ab3 variants were also observed in experiments analyzing binding to soluble recombinant antigen or anti-Id Ab that were immobilized on 96-well EIA plates (data not shown). These results showed that differences in the extent of Fc sialylation may affect binding to antigen as well as binding to Fc γ RIIIa.

3.5. Fc sialylation did not affect intrinsic affinity for targets

From the above data, we hypothesized that increased Fc sialylation may serve to reduce Ab hinge region flexibility. In the case of Ab1 and Ab3 binding to their respective cell-surface antigens, this could lead to more monovalent binding and less bivalent (high avidity) binding to antigen. It is possible that the hinge region of the higher sialylated variant of Ab2 has reduced flexibility, but flexibility may not be needed for this Ab to achieve maximal binding to Ag2. This hypothesis would predict that the intrinsic affinity of each Fab arm of an Ab for antigen would not be affected by Fc sialylation. To test this prediction, EIA plates were coated with Ab and then incubated with varying amounts of a soluble target, i.e. either antigen or a Fab fragment of an anti-Id Ab, an experimental format designed to measure monovalent binding. The results indeed demonstrated that, for the three pairs of sialic acid variants which showed differences in binding to cell-surface antigen, there were no detectable differences observed between pairs of variants in their binding to soluble targets (Fig. 5). These results confirmed that differences in binding to immobilized targets were not due to differences in intrinsic affinity between each Fab arm and its respective target. Therefore, the combined observations of differences in soluble Ab binding to immobilized targets and the lack of detectable differences in immobilized Ab binding to soluble targets is consistent with the hypothesis that differences between the Ab1 and Ab3 sialic acid variants in their binding to immobilized

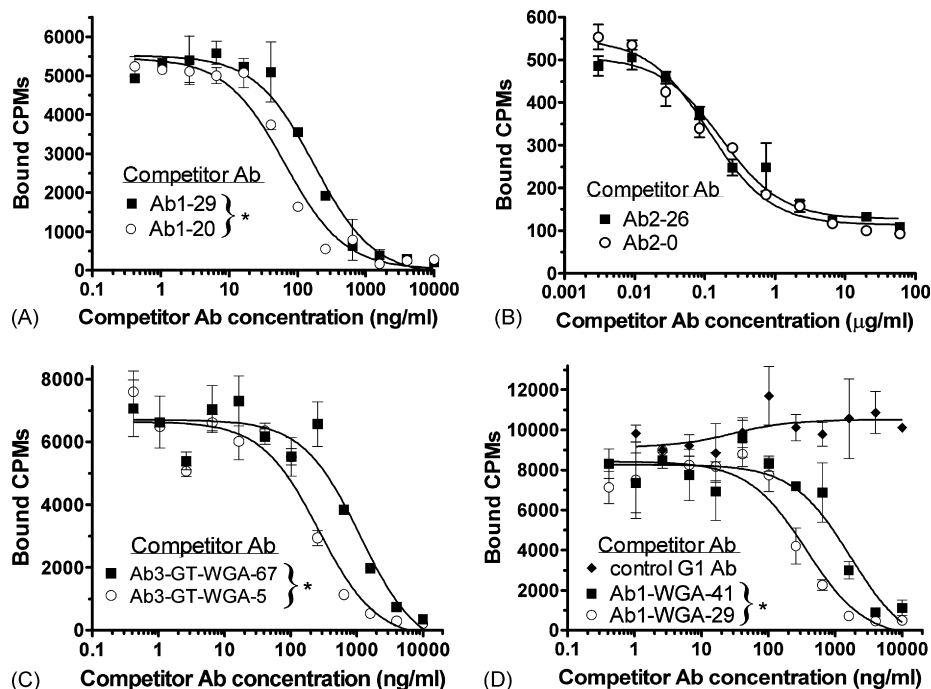


Fig. 4. Binding by Ab variants to cell-surface antigen. Competition binding using 125 I-labeled Abs at fixed concentrations incubated with the appropriate Ag-expressing cell lines in the presence of varying amounts of unlabeled Ab variants as competitors. (A) Binding of 125 I-labeled Ab1-29 to Ag1-expressing cells in the presence of unlabeled natural variants of Ab1; (B) binding of 125 I-labeled Ab2-26 to Ag2-expressing cells in the presence of unlabeled Ab2 natural variants; (C) binding of 125 I-labeled Ab3 to Ag3-expressing cells in the presence of unlabeled WGA lectin-derived variants of Ab3; (D) binding of 125 I-labeled Ab1-29 to Ag1-expressing cells in the presence of WGA lectin-derived variants of Ab1 or a control Ab. Samples were tested in duplicate or triplicate (error bars represent ranges or S.D., respectively) and the results shown are representative of two or three independent experiments for each Ab pair. The differences between Ab variants in binding were significant (asterisks) in graphs A, C, and D ($p < 0.0001$) as determined by extra sum of squares F -test.

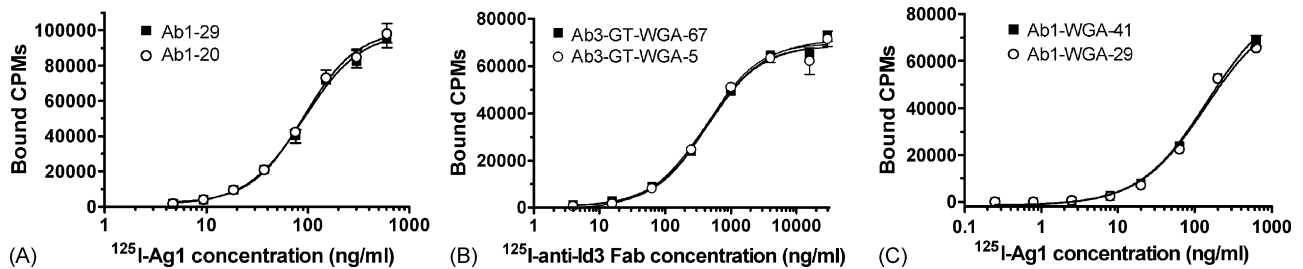


Fig. 5. Binding of plate-coated Ab variants to soluble targets. A 96-well plate was coated with sialic acid variants of Ab1 or Ab3 and then incubated with varying amounts of ^{125}I -labeled soluble target (antigen or anti-Id Ab specific for Ab3). (A) Binding of ^{125}I -labeled soluble Ag1 to plate-coated Ab1 natural variants; (B) binding of ^{125}I -labeled Fab fragment of anti-Id3 Ab to plate-coated Ab3 WGA lectin-derived variants; (C) binding of ^{125}I -labeled soluble Ag1 to plate-coated Ab1 lectin-derived variants. Samples were tested in duplicate or triplicate (error bars represent ranges or S.D., respectively) and the results shown are representative of two or three independent experiments for each Ab pair. Ab2 variants were not analyzed due to unavailability of soluble Ag2.

targets are due to differences in the extent of bivalent binding to the cells. Further experimentation, such as binding comparisons to $F(\text{ab}')_2$ and Fab fragments of the Abs, would test this hypothesis.

3.6. Increasing sialylation by direct glycoengineering also reduced ADCC potency

Although data from the WGA lectin fractions described above showed that our monoclonal Ab preparations contained subpopulations of differentially sialylated molecules that differed in ADCC potency and Ag binding affinity, such data implicating sialic acid as being responsible is primarily correlative in nature. To obtain more direct evidence for a role of sialic acid in ADCC potency, we pursued two additional approaches. In one approach, a sample of purified Ab1-20 was treated with β -1,4-galactosyltransferase and α -2,3-sialyltransferase to prepare a highly sialylated variant, Ab1-GTST-90, and then the original Ab was compared to the glycoengineered derivative in the same ADCC assay that was used for other Ab1 variants. As shown in Fig. 6A, the highly sialylated Ab1-GTST-90 was 10–20-fold less potent than the Ab1-20 from which it derived. Mass spectrometry and SDS-PAGE analyses showed no indications that the reduced ADCC potency might be due to Ab degradation resulting from the glycoengineering treatment (data not shown). As an additional demonstration that the glycoengineering did not result in unknown, inadvertent modifications, a competition binding analysis was performed with Fc γ R on U-937 cells. That comparison showed that the highly sialylated glycoengineered variant bound to Fc γ R on U-937 cells even more competitively than the starting material (Fig. 6B), making it unlikely that the reduced ADCC activity was due to unintended effects on Ab structure. The significantly enhanced binding to Fc γ R on U-937 cells by the highly sialylated variant is consistent with the suggestion of enhanced binding by Ab2-26 compared to Ab2-0 shown in Fig. 3E and, as stated above, calls for more experimentation to test whether Fc sialylation enhances binding to the high-affinity Fc γ RI receptor. These results with the enzymatically sialylated Ab are consistent with the ADCC data described previously and provide the first direct evidence that sialic acid in Fc glycans can adversely impact ADCC potency of an Ab. Limited availability of Ab1-GTST-90 and the sialyltransferase used

to prepare it precluded further characterizations in Fc γ RIIIa and antigen binding assays.

For a second direct approach, we sought to use sialidase to enzymatically remove sialic acid from a highly sialylated Ab, and then compare the activity of the desialylated Ab to the orig-

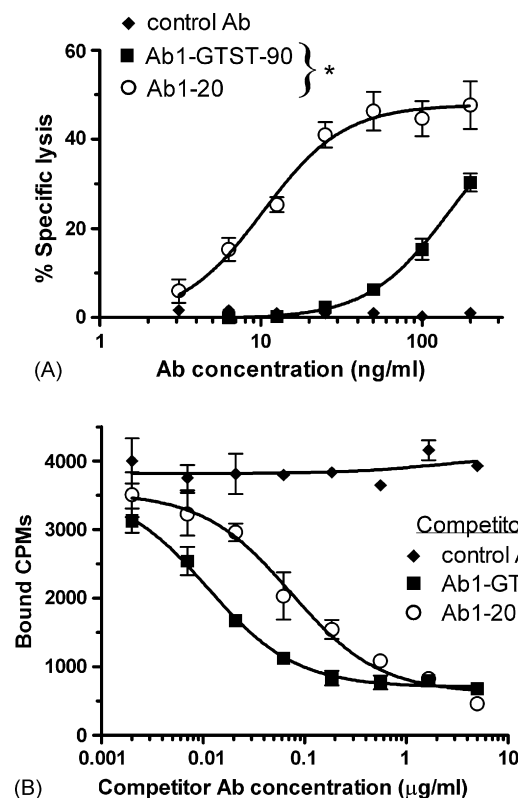


Fig. 6. Analyses of Ab1 engineered to be highly sialylated using sialyltransferase. (A) ADCC comparison of Ab1-GTST-90 and Ab1-20. Ab1-GTST-90 was prepared from Ab1-20 by enzymatic addition of sialic acid residues *in vitro*. Samples were analyzed in triplicate and the results shown are representative of three independent experiments. (B) Competitive binding of Ab1-GTST-90 and Ab1-20 to Fc γ R on human U-937 cells. Cells were incubated with an irrelevant ^{125}I -labeled human IgG1 mAb at a fixed concentration and varying concentrations of unlabeled test Abs as competitors. Samples were analyzed in duplicate (error bars represent ranges) and the results shown are representative of two independent experiments. The differences between the Ab variants were significant (asterisks) in both graphs A and B ($p < 0.0001$) as determined by extra sum of squares *F*-test.

inal sialylated Ab. However, we found this approach to be less reliable due to resulting changes in the proportion of other glycan species following sialidase treatment, particularly the proportion of fucosylated to nonfucosylated glycans. Because small changes in fucose levels dramatically impact ADCC activity and Fc γ RIIIa binding, we did not pursue this approach any further.

4. Discussion

The data presented here demonstrate that higher levels of Fc sialylation in IgG Abs is associated with reduced activity in ADCC assays. This was routinely observed with all three of the human IgG1 Abs we examined, including three independently derived pairs of variants for Ab1, including a variant derived by *in vitro* glycoengineering methods. However, our analyses of these three Abs reveal that the reasons for the reduced ADCC activity may vary depending on the Ab, and apparently depending on how the sialic acid variants are derived, i.e. whether due to different Ab production processes (the natural variants) or lectin-based fractionation. In the case of the higher sialylated natural variant of Ab1, binding data suggested that both reduced Fc γ RIIIa binding and reduced cell-surface antigen binding probably contributed to reduced ADCC activity. For Ab2, the reduced ADCC activity may be solely due to reduced Fc γ RIIIa binding. For the higher sialylated lectin-derived variants of Ab1 and Ab3, reduced cell-surface antigen binding appears to be the sole reason for reduced ADCC activity.

Our working hypothesis regarding the impact of Fc sialylation on ADCC functions is that higher levels of sialic acids can reduce flexibility of the Ab molecule, presumably around the otherwise highly flexible hinge region, and that reduced hinge region flexibility may result in either reduced binding to Fc γ RIIIa, reduced bivalent binding to antigen, or both. Although crystal structures of sialylated Abs or Fc domains have not been reported to date, it seems clear based on structures of glycosylated Fc fragments that certain of the negatively charged, far-reaching, and somewhat bulky sialic acid sugars may localize in proximity to the hinge region. Because each glycan moiety can have 0, 1, or 2 sialic acids, and we have no information on which of the two positions the sialic acid residues in our test Abs occupy, i.e. on the α (1,6) arm of the biantennary glycan structure or on the α (1,3) arm, one of the prevailing questions is which sialic acid residues have the most impact on ADCC functions. Such differences in sialic acid distribution may be the reason for the appreciable differences between Abs in binding affinity despite having relatively subtle differences in the amounts of sialylation. For example, it is possible that Ab1-29 has four-fold less affinity than Ab1-20 for Fc γ RIIIa on NK cells because a greater proportion of the sialic acid residues in Ab1-29 are in close proximity to the hinge region. Given existing models that show IgG molecules needing to assume a sharp 90° bend at the hinge region in order to bind Fc γ RIIIa (Sondermann et al., 2001), it would not be surprising for a structural modification that reduces hinge flexibility to reduce that affinity. Interestingly, we observed significantly higher binding to Fc γ RI on U-937 cells by the higher sialylated glycoengineered variant of Ab1 than its lesser sialylated variant, i.e. the opposite effect

observed for Fc γ RIIIa binding by the other pairs of variants. Slightly higher binding (although not statistically significant) to Fc γ RI by the higher sialylated natural variant of Ab2 compared to its lesser sialylated counterpart may support that observation. The apparent Fc γ R-specific sensitivities to Fc sialylation may reflect the effects of unique glycosylation on Fc γ RIIIa (Ferrara et al., 2006).

Reduced hinge flexibility may also explain reduced binding to cell-surface antigen, since it may lead to less bivalent binding and more monovalent binding as a result of Ab molecules not being able to orient themselves to enable both Fab arms to simultaneously bind antigen. Such a difference in binding valency would be expected to result in reduced apparent affinity, since avidity effects associated with bivalent binding would be reduced. Because the capacity of an Ab to bind bivalently to two antigen epitopes will depend on epitope accessibility, orientation, density, and mobility, in addition to Ab concentration, it seems likely that individual Abs will show varying degrees of sensitivity to Fc sialylation. However, the relative flexibility of higher versus lesser sialylated variants is still not known—additional studies would help clarify at a molecular level why Fc sialylation impacts ADCC functions. It should be noted that the antigen binding effect of sialylation may also be relevant to Abs that recognize viral or bacterial surface antigens, and even soluble antigens that are homopolymers, since Ab flexibility can determine to what extent individual Ab molecules bind bivalently within a soluble immune complex, where not only might some of the Abs bind more than one antigen but some of the antigens may be bound by more than one Ab.

There are numerous questions that remain to be answered. With the test Abs described here having been expressed in mouse myeloma cells, the sialic acid present in these Abs was the *N*-glycoylneuraminic acid isomer of sialic acid and not the *N*-acetylneuraminic acid isomer present in CHO cell and human cell-derived Abs. Although it seems likely that the effects of Fc sialylation will be the same regardless of sialic acid isomer, this remains to be confirmed. We are currently testing whether sialylation impacts complement activation, and whether the extent of sialylation of the Fc glycans affects circulating half-life of IgG Abs. It is possible that the relatively low abundance of sialylated IgG in human serum (~15%) reflects a faster clearance of more highly sialylated Abs. Because sialic acid is known to be acid labile (Varki and Diaz, 1984; Raju and Davidson, 1994), it is possible that purification and subsequent processing steps that expose Abs to low pH could alter the sialic acid content, and therefore the ADCC activity, of an Ab. Finally, there is evidence that commonly used culture medium components contain one or more sialidase enzymes that enzymatically remove sialic acid from the Fc glycan of Abs that have been secreted from production cells. These issues may not be concerns for Abs that do not depend on bivalent binding to antigen or on Fc γ RIIIa binding, but Abs that do may be adversely impacted by production processes which result in higher levels of Fc sialic acid. Numerous approaches for preparing Abs with little or no sialic acid could be considered, including culturing the production host cells in the presence of a sialidase enzyme, blocking sialyltransferase enzyme activity in the host cells, blocking the addition of the

Gal residues to which these sialic acid residues are attached, or expressing in plants or other transgenic systems that do not produce sialylated glycans.

In summary, our data show that therapeutic IgG Abs which depend on Fc γ RIIIa binding and/or binding to immobilized antigen as part of the mechanisms of action may show higher activity if their Fc glycans are not sialylated. This is the first report demonstrating that the presence or absence of Fc sialylation can affect ADCC activity and, to our knowledge, the first time that any Fc glycan structure of an Ab has been reported to affect binding to antigen. These findings show that there are still more ways to optimize Fc glycan structure for this very important class of therapeutic molecules.

Note: following submission of this manuscript, Kaneko et al. (2006) reported that Fc sialylation of mouse IgG Abs is associated with anti-inflammatory properties, at least in part due to reduced binding to Fc γ Rs by the more highly sialylated IgG. Results shown here with human IgG and human receptors appear to be generally consistent with their findings.

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