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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 Fcγ receptors (FcγR) 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 FcγRIIIa 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.

Keywords: Antibodies; Glycosylation; Antibody-dependent cellular cytotoxicity; Sialic acid; Fcγ receptors

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 Fcγ receptors (FcγR), including the so-called high-affinity receptor, FcγRI (CD64) and the lower-affinity receptors, FcγRIIa (CD32a) and FcγRIIIa (CD16a) (Mimura et al., 2000; Krapp et al., 2003; Dijstelbloem 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; FcγR, Fcγ 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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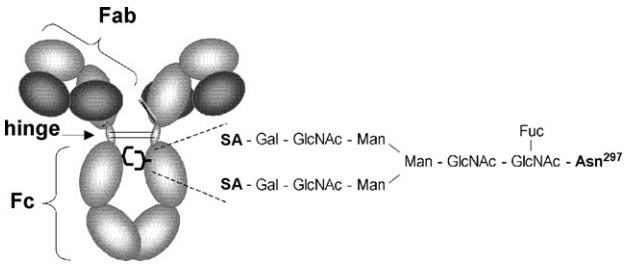

Fig. 1. Schematic depiction of an IgG Ab showing an Fc glycan structure. The position of the single Asn-linked glycosylation site at Asn^{297} of both heavy chains, and one of numerous possible glycan structures, are shown (SA, sialic acid; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Man, mannose; 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 $CaCl_2$ and 20 mM $MgCl_2$ 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