Lack of Fucose on Human IgG1 N-Linked Oligosaccharide Improves Binding to Human FcγRIII and Antibody-dependent Cellular Toxicity*

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Lec13 cells, a variant Chinese hamster ovary cell line, were used to produce human IgG1 that were deficient in fucose attached to the Asn297-linked carbohydrate but were otherwise similar to that found in IgG1 produced in normal Chinese hamster ovary cell lines and from human serum. Lack of fucose on the IgG1 had no effect on binding to human FcγRII, Clq, or the neonatal Fc receptor. Although no change in affinity was found for the His131 polymorphic form of human FcγRIIA, a slight improvement in binding was evident for FcγRIIB and the Arg131 FcγRIIA polymorphic form. In contrast, binding of the fucose-deficient IgG1 to human FcγRIIa was improved up to 50-fold. Antibody-dependent cellular cytotoxicity assays using purified peripheral blood monocytes or natural killer cells from several donors showed enhanced cytotoxicity, especially evident at lower antibody concentrations. When combined with an IgG1 Fc protein variant that exhibited enhanced antibody-dependent cellular cytotoxicity, the lack of fucose was synergistic.

The nature and importance of the conserved, Asn297-linked carbohydrate in influencing immunoglobulin G effector functions has long been recognized (1–4). Variations in composition of the carbohydrate have shown to affect the affinity of IgG for the three classes of FcγR (3, 4) (FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16)) that link IgG antibody-mediated immune responses with cellular effector functions (5–7). Carbohydrate composition also influences the activity of IgG in the classical pathway of complement activation, which is initiated by IgG1 binding to Clq (3, 4, 8) and to mannose-binding protein (5–7). For IgG from either serum or produced ex vivo in hybridomas or engineered cells, the IgG are heterogeneous with respect to differences in oligosaccharide composition (1–3). Variation among individual IgG includes attachment of galactose and/or sialic acid at one or both of the terminal GlcNAc and/or attachment of a third GlcNAc arm (biisecting GlcNAc). The presence or absence of terminal galactose or sialic acid residues affects IgG function (9, 10), and attachment of galactose-and/or sialic acid at one or both of the terminal GlcNAc and/or attachment of a third GlcNAc arm (biisecting GlcNAc). The presence or absence of terminal galactose or sialic acid residues affects IgG function (11, 12–14), and attachment of a third arm to the carbohydrate via biisecting GlcNAc has been reported to improve antibody-dependent cellular cytotoxicity (ADCC) (15, 16). A connection between IgG glycosylation and some human diseases has been shown (e.g. the level of galactosylation appears correlated with rheumatoid arthritis) (17).

Several recombinant monoclonal antibodies (mAbs) are being used as human therapeutics (18). Some of these (e.g. mAbs that bind to a receptor or soluble ligand and thereby block ligand-receptor interaction) may function without utilizing antibody effector mechanisms. Abrogating immune system recruitment for these mAbs can be achieved by altering IgG residues in the lower hinge region (19, 20), using IgG2 or IgG4 subclasses, which are relatively less efficient in effector function, or using F(ab) or F(ab’2) fragments (although these may have undesirable clearance rates). Other mAbs may need to recruit the immune system to kill the target cell (31–34). In those circumstances where recruitment of immune effector cells is desirable for therapeutic mAb efficacy, engineering the IgG Fc portion to improve effector function (via improved binding to IgG receptors and/or complement) could be a valuable enhancement to antibody therapeutics. Currently, methods that improve the immune system recruitment include bispecific antibodies in which one arm of the antibody binds to an IgG receptor (21), IgG-cytokine fusion proteins (22), alteration of the IgG Fc sequence for improved binding to FcγR (23), and optimization of the Asn297-linked carbohydrate (24, 25, 26, 27). To evaluate the role of fucosylated oligosaccharide in IgG1 function, the Lec13 cell line (38) was utilized to express human IgG1. This Chinese hamster ovary (CHO) cell line is deficient in its ability to add fucose but provided IgG with oligosaccharide that was otherwise similar to that found in normal CHO cell lines and from human serum (18, 19, 39). The resultant IgGs were used to evaluate the effect of fucosylated carbohydrate on antibody effector functions, including binding to hu-
with 10 mg/liter recombinant human insulin and 1 mg/liter trace elements (43). Cells remained in serum-free production medium for 3–5 days. Supernatants were collected and clarified by centrifugation in 150-ml conical tubes to remove cells and debris. The protease inhibitors phenylmethylsulfonyl fluoride and aprotinin (Sigma) were added, and the supernatants were concentrated 5-fold on stirred cells using MWCO30 filters (Amicon, Beverly, MA) prior to immediate purification using protein A chromatography (Amersham Biosciences). All proteins were buffer-exchanged into phosphate-buffered saline using Centrifrap-30 concentrators (Amicon) and analyzed by SDS-polyacrylamide gel electrophoresis. Protein concentrations were determined using the Bio-Rad protein assay. The protein concentration of monomeric IgG was determined using a Bio-Rad assay and expressed as mg of mAb per 15-cm plate.

**RESULTS**

**Binding of mAbs to Human FcR**—Anti-HER2 Hu4D5 mAb (40) expressed in stably transfected Lec13 cells consistently had about 10% fusocylated carbohydrate (Table I). In contrast, Hu4D5 mAbs from stably transfected CHO-DP12 cells cultured in spinner flasks (CHO-S) or plates (CHO-P) showed about 98% fusocylated carbohydrate. The variation in galactosylation for the Lec13 mAbs was similar to that found for CHO-S mAbs (as opposed to CHO-P) (Table I); hence, CHO-S mAbs were used as reference in the binding assays.

Monomeric Hu4D5-Lec13 IgG1 bound to human FcγRI equivalent to binding of Hu4D5-CHO-S (Table II). The EC_{50} values (0.1 µg/ml) correspond to an affinity constant of 0.7 nM, at the lower end of the range of previously determined values (6, 7). In contrast to FcγRI, IgG binding to the low affinity FcγRII (FcγRII and FcγRIII) required the formation of dimers (Hu4D5 and HuE27) or trimers (HuE27) (12, 47) to detect binding. For FcγRIIA there are naturally occurring allopotypes at position 151 that result in different binding avidities for IgG2 (48, 49). Binding of Hu4D5-Lec13 dimers to the FcγRIIA(Arg^{151}) polymorphic form and to FcγRIIB exhibited only a slight improvement compared with Hu4D5-CHO-S (Fig. 2A). Lack of fusocyl did not affect binding to the FcγRIIA(His^{151}) polymorphic form (Fig. 2C). Note that in contrast to FcγRI and FcγRIIA, binding of the antibodies to FcγRIIB and FcγRIIC did not reach a plateau at the highest concentration used (10 µg/ml) (Fig. 2, A and B); hence, EC_{50} values could not be calculated. At concentrations above 10 µg/ml, aggregation of mAb was problematic.

**EXPERIMENTAL PROCEDURES**

**cDNA Constructs for Stable Cell Lines**—The heavy and light chains of Hu4D5 (40) and HuE27 (41) were subcloned into a previously described mammalian cell expression vector (42) with the modification of a puromycin site fused 5’ to the dihydrofolate reductase (DHFR) site to form a fusion gene. Puromycin is used as a selective marker in DHFR (+) cells such as the Lec13 cells and the DHFR site was retained for metotrexate amplification of the stable cell line.

**Transfection and Culturing of Lec13 and Wild-type CHO Cells**—The CHO cell line Pro-Lec13.6a (Lec13), was obtained from Professor Pamela Stanley of Albert Einstein College of Medicine of Yeshiva University. Parental lines were Pro/Pro-H11001 and Lec/Pro-H11002 (proline auxotroph) and GAT/Pro-H9262 (glycine, hypoxanthine, thymidine, low glucose Dulbecco’s F-12 without amino acids on the reference in the binding assays.

**Human FcγR, human C1q, human FcRn, and ADCC using human effector cells.**

**FIG. 1. Schematic of IgG glycoforms.** The core structure (Galα1–3Gal[b] Fucose) normally is composed of Asn297–N2–GlcNAc(fucose)–GlcNAc–mannose–mannose–GlcNAc_{2}, where GlcNAc is N-acetylglucosamine. Variation among individual glycoforms includes attachment of one (Galα1–4) or two (Galα1–4Galα1–4) terminal galactose units, attachment of sialic acid to the terminal galactose, and/or attachment of acetylglucosamine. The core structure (Galα1–3Gal[b] Fucose) normally is composed of Asn297–N2–GlcNAc(fucose)–GlcNAc–mannose–mannose–GlcNAc_{2}, where GlcNAc is N-acetylglucosamine. Variation among individual glycoforms includes attachment of one (Galα1–4) or two (Galα1–4Galα1–4) terminal galactose units, attachment of sialic acid to the terminal galactose, and/or attachment of acetylglucosamine. The variation among individual glycoforms includes attachment of one (Galα1–4) or two (Galα1–4Galα1–4) terminal galactose units, attachment of sialic acid to the terminal galactose, and/or attachment of acetylglucosamine.
Human FcyRIIA has naturally occurring allotypes at position 48 (Leu, His, or Arg) and at position 158 (Val or Phe); the FcyRIIA(Val158) allotype interacts with human IgG better than the FcyRIIA(Phe158) allotype (12, 50, 51). Both the Phe158 and Val158 polymorphic forms of FcyRIIA exhibited significantly improved binding to IgG1 that lacked fucose. Binding of dimeric Hu4D5-Lec13 to FcyRIIA(Phe158) showed at least a 42-fold improvement (Fig. 3A; Table II), suggesting that the protein and carbohydrate alterations are synergetic. In addition to dimers, complexes consisting of three anti-IgE HuE27 and three IgE were used to test binding to FcyRIIA; these complexes are trimeric in anti-IgE HuE27 (12). Binding to FcyRIIA by the trimeric complexes was stronger than for the corresponding dimeric complexes (Fig. 4A, A and B; Table II), suggesting an appreciable avidity component. The avidity resulted in a smaller improvement in binding to FcyRIIA(Phe158) for trimeric HuE27-Lec13, only 13-fold, compared with 53-fold for dimeric HuE27. In a previous study, the improvement in binding of trimeric HuE27 S298A/E333A/K334A-IgG1 variant to FcyRIIA(Phe158) and FcyRIIA(Val158) was 2- and 1.1-fold, respectively; whereas the improvement in binding was small, the effect on ADCC was significant (12). In the current study, the trimeric complex of HuE27 S298A/E333A/K334A-IgG1 showed improved binding to both FcyRIIA polymorphic forms in line with the values from the previous study (Fig. 4, B and C; Table II).

The improved binding of fucose-deficient IgG1 to FcyRIIA was confirmed with FcyRIIA(Phe158) full-length α-chain co-expressed with γ-chain on CHO cells (Fig. 5; Table II). As for the α-chain fusion protein alone in ELISA format, fucose deficiency was synergistic with the S298A/E333A/K334A-IgG1 variant.

Binding of the native and fucose-deficient IgG1 to murine FcyRII and FcyRIII was also evaluated. As trimeric complexes, the fucose-deficient HuE27 showed slightly reduced binding to both receptors compared with fucosylated HuE27 (data not shown). The major histocompatibility complex class I-like neonatal Fc receptor (FcRn) is structurally unrelated to the FcyR (52) and has been proposed to be involved in a number of biological processes including clearance rate of IgG (53).
ing of nonfucosylated and fucosylated IgG1 to FcRn was equivalent (ratio of 0.98 at 5 μg/ml) (data not shown).

C1q Binding—Binding of C1q to antibodies is the first step in the classical pathway of complement activation (8). The nature of the carbohydrate on the IgG has been shown to influence its interaction with C1q (3, 4, 22, 24). However, the lack of fucose did not affect the ability of Hu4D5 to interact with human C1q (data not shown).

ADCC—The effect of fucosylated carbohydrate on ADCC was evaluated using Hu4D5-Lec13 IgG1 on the human breast cancer cell line SK-BR-3 (54). SK-BR-3 cells were opsonized with varying Hu4D5 concentrations, and PBMCs from three FcRIIIA(Phe158)/HuE27 dimers donors and three FcRIIIA(Phe158)/HuE27 trimers donors were used as effector cells at a 30:1 effector/target ratio; representative ADCC assays are shown in Fig. 6, A and B. For all donors studied, the fucose-deficient IgG1 exhibited significant improvement in ADCC compared with IgG1 with fucose, especially at the lower antibody concentrations (Fig. 6).

To verify that the combination of fucose deficiency and pro-
tein sequence variant exhibited a synergistic effect, as seen in the ELISA and cell binding assays (Figs. 3–5), immunofluorescence staining and ADCC assays were performed comparing native Hu4D5 and S298A/E333A/K334A-IgG1 variant with and without fucose against NK cells from two FcγRIIIA(Phe158/Phe158) donors. The intensity of staining showed the pattern: Hu4D5 S298A/E333A/K334A-IgG1 Lec13/H11022 Hu4D5 Lec13/H11022 Hu4D5 CHO-S (Fig. 7A). For the ADCC assays, SK-BR-3 cells were opsonized with 1 ng/ml mAb, and NK cells were used as effector cells. Both donors showed similar results; the fucose-deficient S298A/E333A/K334A-IgG1 was more potent than either fucose-deficient Hu4D5 or fucosylated S298A/E333A/K334A-IgG1 (Fig. 7B) and correlated with the immunofluorescence staining.

**DISCUSSION**

Although the presence of carbohydrate is necessary for binding to FcγRII (12, 55), the equivalent binding of IgG1 regardless of differences in fucose content (Lec13 versus CHO-S) or galactose content (CHO-P versus CHO-S) suggests that IgG1 binding to FcγRI is insensitive to the presence of these moieties on the carbohydrate. The effect of galactosylation on binding of IgG to human FcγRII has been previously studied (11, 22–24), and review of the data suggests that if galactosylation affects binding to FcγRI, it is subtle and may be isotype-dependent (11).

The small improvement in binding of fucose-deficient IgG1 to both FcγRIIA(Arg131) and FcγRIIB, each having arginine at position 131, versus no effect on FcγRIIA(His131) suggests that the fucose may either directly interact with the FcγR residue at position 131 or alter the IgG1 conformation so as to effect a subtle, negative influence on binding when arginine is present at FcγR position 131. As with FcγRI, the IgG1 galactose content did not seem to affect binding to FcγRII.

In contrast to the FcγRII class of receptors, lack of fucose effected a significant improvement in binding of IgG1 to FcγRIIIA. The absence of fucose not only increased binding of native IgG1 to FcγRIIIA but also augmented binding of the S298A/E333A/K334A-IgG1 variant. For both protein and carbohydrate variants of HuE27 (i.e. S298A/E333A/K334A-IgG1 and Lec13-derived), the improvement in binding of the trimeric complex was much smaller than that observed for the dimeric
complex. For example, binding to FcγRIIA α-chain and γ-chain (representative plot for one assay). Open circles, Hu4D5 CHO-S; open squares, Hu4D5 Lec13-D; open diamonds, Hu4D5 Lec13-E; open triangles, Hu4D5 Lec13-F; filled circles, Hu4D5 HEK293-AAA; filled squares, Hu4D5 Lec13-AAA-B; filled diamonds, Hu4D5 Lec13-AAA-C.

Fig. 6. ADCC assays using anti-HER2 Hu4D5 mAbs, SK-BR-3 cells as target, and PBMCs as effector cells. The effector/target ratio was held constant at 30:1, and [mAb] varied. A, representative plot for one of three FcγRIIA(Val158/Phe158) donors. B, representative plot for one of three FcγRIIA(Phe158/Phe158) donors. Open squares, Hu4D5 Lec13-A; filled circles, Hu4D5 CHO-S; open circles, spontaneous lysis. Each assay was performed in duplicate with error bars shown.

Fig. 7. A, immunofluorescence staining of purified NK cells from an FcγRIIIA(Phe158/Phe158) donor by Hu4D5 variants. NK cells were purified by negative selection. Cells were adjusted to 2 × 10^6/ml and incubated with 2 μg/ml Hu4D5 variants for 30 min in ice-cold phosphate-buffered saline containing 0.1% bovine serum albumin, 0.01% sodium azide. Cells were then washed, and antibody binding was detected by incubating with fluorescein isothiocyanate-conjugated F(ab')₂ goat anti-human IgG and phycoerythrin-conjugated anti-CD56 for 30 min. The plot shows the fluorescence intensity on gated CD56⁺ cells for the secondary detecting antibody (shaded histogram) (1), Hu4D5-CHO-S (2), Hu4D5 Lec13-A (3), and Hu4D5 Lec13-AAA (4) (relative mean fluorescence intensities of 4.82, 15.8, 190, and 372, respectively). B, ADCC assay using anti-HER2 Hu4D5 mAbs, SK-BR-3 cells as target, and NK cells from an FcγRIIIA(Phe158/Phe158) donor as effector cells. SK-BR-3 cells were opsonized with 1 ng/ml mAb, and the effector/target ratio was varied. Closed circles, Hu4D5 CHO-S; filled squares, Hu4D5 Lec13-A; filled diamonds, Hu4D5 HEK293-AAA; filled triangles, Hu4D5 Lec13-AAA-C; open circles, spontaneous lysis. The assay was performed in duplicate with error bars shown.

A previous study of protein sequence variants of human IgG1 found that alanine (and other) substitutions at some Fc posi-
tions could reduce or improve binding to FcγR as well as enhance ADCC (12). Interestingly, some of these substitutions were not near the interaction interface found in crystal structures of human IgG Fc-human FcγRIIA complex (12, 56, 57). For example, of the three alanine substitutions S298A/E333A/K334A used in this study, only Ser298 is at the Fc/FcγRIIA interface in the crystal structures. Likewise, in the co-crystal structures, neither of the fucose residues on the two heavy chains interact with FcγRIIA. Inspection of crystal structures of human and rodent Fc or IgG shows that the fucose can adopt varying conformations and exhibits high B-factors (56–60), suggesting a high degree of mobility. Although the presence or absence of fucose definitely affects the interaction of an IgG with FcγRIIA (and, to a lesser extent, FcγRIIA/Arg131) and FcγRIIB, the current study cannot discern whether this is due to direct interaction of the fucose with receptor or to a conformational influence on the IgG itself.

Improved binding to FcγRIIA translated into improved ADCC in vitro. Notably, for all donors the enhancement in cytotoxicity was more apparent as the concentration of antibody was reduced. This may reflect the larger improvement in FcγRIIA binding seen for dimers compared with that for trimers (i.e. fucose-deficient IgG1 may require fewer mAbs on the surface of the target cell in order to effect binding/activation of an effector cell). If this is the case, it suggests that for therapeutic antibodies that utilize ADCC, unfucosylated antibody could conceivably be given at lower doses to effect an equivalent cell kill as higher doses of fucosylated antibody.

In addition to the human FcγR, the influence of fucose on binding to C1q and FeRn were also investigated. Previous studies have shown that differences in IgG glycosylation can affect C1q binding, although the results of this study show that the presence/absence of fucose had no discernible effect. Likewise, the presence or absence of fucose did not affect IgG binding to FeRn. This is not surprising, since aglycosylated IgG1 binds this receptor similarly to glycosylated IgG1 (12–14).

Rothman et al. (61) tested the ADCC function of monoclonal murine IgG purified from hybridomas treated with glycosidase inhibitors that act at different stages in the carbohydrate processing pathway. Treatment with castanospermine, which inhibits removal of glucose residues from the nascent oligosaccharide (62), generated IgG that showed enhanced ADCC mediated by NK cells, which express only FcγRIIA, but not by other types of effector cells such as monocytes. Lectin-binding analysis suggested that the castanospermine-treated IgG lacked fucose (61); however, the IgG resulting from such treatment probably had other carbohydrate structure, such as hypermannosylation as well as terminal glucose residues (4, 63, 64), not routinely found on IgG secreted from nontreated cells or from human serum. In the current study, Lec13 cells were utilized to produce fucose-deficient IgG1. Other than the lack of fucose, the oligosaccharide on the IgG1 was similar to that found in normal CHO cell lines and from human serum (18, 19, 39) and did not suffer from the hypermannosylation or hyperglycosylation resulting from the use of castanospermine. The improvement in FcγRIII binding and ADCC by the fucose-deficient IgG1 suggests that it is possible to generate monoclonal antibody with otherwise normal oligosaccharide that would have enhanced effector function.

Normal CHO and HEK293 cells add fucose to IgG oligosaccharide to a high degree (80–98% in this study). IgGs from sera are also highly fucosylated (18, 19, 27, 39). Unfortunately, the Lec13 cell line is not robust enough to consider as a production cell line, because the expression levels of the anti-HER2 Hu4D5 and anti-IgE HuE27 antibodies in this cell line were low compared with the standard CHO cells used. Treatment of fucosylated IgG with fucosidases (65) may be useful for generating enough afucosyl IgG to use for assays but might be impractical for production. Hence, a production cell line deficient in fucosylation of IgG remains a challenge.
Binding of Fucose-deficient IgG1 to Human FcγR

References:

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