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Fc-Glycosylation Influences Fcγ Receptor Binding and Cell-Mediated Anti-HIV Activity of Monoclonal Antibody 2G12

Donald N. Forthal,* Johannes S. Gach,† Gary Landucci,* Jakub Jez,‡ Richard Strasser,‡ Renate Kunert,§ and Herta Steinkellner‡

Interactions between the Fc segment of IgG and FcγRs on a variety of cells are likely to play an important role in the anti-HIV activity of Abs. Because the nature of the glycan structure on the Fc domain is a critical determinant of Fc–FcγR binding, proper Fc glycosylation may contribute to Ab-mediated protection. We have generated five different glycoforms of the broadly HIV-1-neutralizing mAb 2G12 in wild-type and glycoengineered plants and Chinese hamster ovary cells. Plant-derived 2G12 exhibited highly homogeneous glycosylation profiles with a single dominant N-glycan species. Using flow cytometry with FcγR-expressing cell lines, all 2G12 glycoforms demonstrated similar binding to FcγRI, FcγRIIa, and FcγRIIIa. In contrast, two glycoforms derived from glycoengineered plants that lack plant-specific xylose and core α1,3-fucose, and instead carry human-like glycosylation with great uniformity, showed significantly enhanced binding to FcγRIIIa compared with Chinese hamster ovary or wild-type plant-derived 2G12. Using surface plasmon resonance, we show that binding of 2G12 to FcγRIIIa is markedly affected by core fucose, irrespective of its plant-specific α1,3 or mammalian-type α1,6 linkage. Consistent with this finding, 2G12 glycoforms lacking core fucose (and xylose) mediated higher antiviral activity against HIV-1 or simian immunodeficiency virus as measured by Ab-dependent cell-mediated virus infection. This is, to our knowledge, the first demonstration that specific alterations of Fc glycosylation can improve antiviral activity. Such alterations may result in better immunotherapeutic reagents. Moreover, biasing vaccine-induced immune responses toward optimal Fc glycosylation patterns could result in improved vaccine efficacy. The Journal of Immunology, 2010, 185: 000–000.

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Address correspondence and reprint requests to Dr. Herta Steinkellner or Dr. Donald N. Forthal, Institute of Applied Genetics and Cell Biology, Muthgasse 18, 1190 Vienna, Austria (H.S.) or 3044 Hewitt Hall, University of California, Irvine, Irvine, CA 92697 (D.N.F.). E-mail addresses: heria.steinkellner@boku.ac.at (H.S.) or dnfortho@uci.edu (D.N.F.)

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Abbreviations used in this paper: ADCC, Ab-dependent cellular cytotoxicity; ADCVI, Ab-dependent cell-mediated virus inhibition; cells only, bare TZM-bl receptor cells; CHO, Chinese hamster ovary; FUT8, α1,6-fucosyltransferase; RT, room temperature; RU, resonance units; 2ndary, bare TZM-bl receptor cell lines treated with secondary Ab only; SHIV, simian immunodeficiency virus; SFR, surface plasmon resonance; WT, wild-type; AXTFT, glycosylation mutant.

Copyright © 2010 by The American Association of Immunologists, Inc. 0022-1767/10S16.00 toxicity (ADCC) activity of Abs elicited by an adenovirus-based vaccine (2). In humans, vaccination with recombinant gp120 resulted in an ADCVI response for which magnitude correlated with a reduced rate of sexually acquired HIV infection during the Vax004 trial (3). Thus, an effective HIV vaccine, as well as effective immunotherapy, may depend not only on interactions between Ab and Ag, but also on interactions between FcγRs and their Fc ligands. The binding of Fc to FcγRs, and the consequent Ab effector functions, are influenced by several factors, including the nature of the FcγR itself, the IgG subclass, and the pattern of Fc glycosylation (4, 5). Fc glycosylation occurs at a single conserved site, and the composition of these glycans has a substantial effect on the activity of Abs (5). It has been shown that the presence or absence of even a single sugar residue on a specific N-glycan structure has profound implications on Fc effector functions, mainly due to an alteration in the ability of Fc to bind to FcγRs. A prominent example in this respect is the finding that non-fucosylated anti-cancer mAbs, in comparison with their fucosylated counterparts, exhibit significantly enhanced ADCC activity through their enhanced binding to FcγRIIIa (6, 7). In addition, it has been reported that the synthesis of terminal sialic acid on Fc N-glycans reduces FcγR binding and converts IgG Abs to anti-inflammatory mediators through the acquisition of novel binding activities (8, 9). Despite the critical role of Fc glycosylation for anti-cancer Abs and anti-inflammatory activities, the influence of glycosylation on antiviral Abs is largely unknown. Recently, we generated different glycoforms of the two broadly HIV-1-neutralizing human mAbs 2G12 and 4E10 in the tobacco-related plant species Nicotiana benthamiana. Plant-derived mAbs carrying human-type glycosylation at great uniformity were generated, among them
We have shown that different glycosylation does not influence Ag binding of these Abs. However, highly galactosylated 2G12 and 4E10 exhibited enhanced virus neutralization activity as determined by a cell-based syncytium inhibition assay (11). In addition, recent findings that the in vivo and in vitro activity of mAbs against SHIV are dependent on Fc–FcR interactions point to the importance of proper Fc glycosylation (1). Nevertheless, no further immunological studies investigating the effect of Fc glycosylation on anti-viral Abs have been carried out so far.

In this study, we set out to explore the impact of Fc glycosylation on the broadly HIV-1–neutralizing mAb 2G12 (13). To this end, we generated five different 2G12 variants that differ in their Fc glycosylation pattern. The variants were expressed in N. benthamiana wild-type (WT) and glycosylation mutants and in Chinese hamster ovary (CHO) cells. Plant-derived 2G12 exhibited highly homogeneous glycosylation profiles with a single dominant N-glycan species. Purified glycovariants were subjected to FcγR binding studies using surface plasmon resonance (SPR) and flow cytometry with FcγR-expressing TZM-bl cells. We further evaluated the ability of the different 2G12 glycoforms to inhibit HIV-1 and SHIV strains in ADVCI assays.

Materials and Methods

Agroinfiltration and purification of 2G12

Agrobacterium strain GV3101::pMP90RK containing 2G12 binary vector (14) was grown overnight (OD_{600} 1.0–1.5) in the presence of appropriate antibiotics, diluted to an OD_{600} 0.3, and infiltrated into leaves as previously
described (12). Three days postinfiltration, 2G12 was purified by Protein A affinity chromatography as described recently (10). As a final step, purified 2G12 was dialyzed at 4˚C overnight against PBS.

N-glycosylation analyses

N-glycan analyses of purified 2G12 were carried out by liquid-chromatography electrospray ionization-mass spectrometry of tryptic glycopeptides as recently described (15). Briefly, the H chain band of purified, SDS-PAGE–separated IgGs was cut from the gel, S-alkylated, digested with trypsin, eluted from the gel fragment with 50% acetonitril, and separated on a BioBasic C18 column (150 × 0.32 mm, Thermo Electron, Wal-tham, MA) with a gradient of 1–80% acetonitrile containing 65 mM ammonium formate (pH 3). Positive ions were detected with a Q-TOF Ultima Global mass spectrometer (Waters, Milford, MA). Summed and deconvoluted spectra of the glycopeptides elution range were used for identification of glycoforms. This method generates two glycopeptides that differ by 482 Da (glycopeptide 1: EEQYNSTYR; glycopeptide 2: TKPREEQYNSTYR).

SPR experiments

The binding affinity of 2G12 glycoforms to CD16 was determined by SPR using the Biacore 3000 instrument (HBS-EP Puffer supplied by GE Healthcare Biosciences, Freiburg, Germany). The running buffer was 0.01 M HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P20 (HBS-EP Puffer supplied by GE Healthcare Biosciences). Protein A was immobilized on a research-grade CM5 sensor chip (Biacore, GE Healthcare Biosciences) by amine coupling (kit supplied by the manufacturer). Purified 2G12 was diluted with running buffer to a final concentration of 20 μg/ml and applied onto the CM5 chip at a flow rate of 20 μl/min. The CM5 sensor chip was washed with running buffer, and CD16 was applied in a concentration of 25 μg/ml and a flow rate at 20 μl/min. In addition, each sample was passed over a reference flow cell containing no ligand (Protein A). All binding experiments were performed at room temperature (RT).

Flow cytometry

The different TZM-bl Fcγ receptor cell lines (16) were trypsinized, pelleted, and resuspended in FACS buffer (5% FCS, 1 mM EDTA in PBS) transfer into 96-well round-bottom plates (Corning Glass, Corning, NY). Postcentrifugation (1100 rpm at 4˚C), cells were resuspended and incubated for 1 h at RT with the serially diluted 2G12 variants starting at a concentration of 20 μg/ml. The cells were then washed twice with FACS buffer and finally resuspended with an 1:200 dilution of an Fab fragment goat anti-human IgG F(ab’)2 conjugated to PE (Jackson ImmunoResearch Laboratories, West Grove, PA). After 1 h at RT, the cells were subsequently washed twice in FACS buffer prior to FACS evaluation (BD LSR II, BD Biosciences, San Jose, CA). The binding curves were generated by plotting the mean fluorescence intensity of receptor binding as a function of Ab concentration. FlowJo software (Tree Star, Ashland, OR) and GraphPad Prism (GraphPad, San Diego, CA) were used for data interpretation and evaluation.

ADCVI Ab activity assay

ADCVI Ab activity was measured essentially as described previously (1, 17). Briefly, target cells were prepared by infecting CEM.NKr.CCR5 cells (National Institutes of Health AIDS Research and Reference Reagent...
Program, Germantown, MD) with the R5 HIV-1 clinical isolate 92US712 or with SHIV162p3 at a multiplicity of infection of ~0.1 for 48–72 h. The target cells were washed and incubated with Abs at indicated final concentrations and with fresh PBMCs or purified NK effector cells from normal healthy donors (E:T ratio of 10:1). The PBMCs were obtained from heparinized whole blood by Ficoll-Hypaque centrifugation. NK cells were then positively selected using CD56+ magnetic beads (StemCell Technologies, Vancouver, British Columbia, Canada). After 7 d, supernatant fluid from duplicate wells was assayed for p24 by ELISA (ZeptoMetrix, Buffalo, NY). Percent virus inhibition was calculated by referencing the fluid from duplicate wells was assayed for p24 by ELISA (ZeptoMetrix, Buffalo, NY).

**Results**

**Generation of different glycoforms of 2G12**

To generate different glycoforms of 2G12, an appropriate binary construct that contains the cDNA of the H and L chains (14) was infiltrated into *N. benthamiana* leaves. Leaves were harvested 3 d postinfiltration, and purified 2G12 was subjected to N-glycosylation analysis. The N-glycosylation profile of 2G12 derived from *N. benthamiana* WT (2G12WT) exhibited a largely homogeneous GnGnXF profile with plant-specific β1,2-xylene and core α1,3-fucose residues (Fig. 1A). Using an RNA interference knockdown approach, we were able to establish a glycosylation mutant (ΔXTFT) that lacks plant-specific glycan residues. 2G12 produced in this mutant exhibited humanlike GnGn structures (2G12ΔXT, Fig. 1B). Upon coexpression of a modified version of the human β1,4-galactosyltransferase targeted to a late Golgi compartment (11) with 2G12, a highly galactosylated mAb consisting of mainly mono- (MA) and digalactosylated (AA) structures was generated (2G12GalT, Fig. 1C). In addition, 2G12 was coinfiltrated with a binary construct that expresses mouse core α1,6-fucosyltransferase (FUT8), which resulted in the generation of GnGn structures (2G12FUT8, Fig. 1D). N-glycans carrying fucose in α1,6 linkage are the most abundant mAb glycoforms generated in CHO cells (2G12CHO, Fig. 1E) and are highly present in serum IgG. Notably, all four plant-produced Ab glycoforms exhibited a largely homogeneous N-glycosylation profile with a single dominant N-glycan species. This is in contrast to CHO-produced 2G12 (2G12CHO, Fig. 1E), in which five major and several minor glycoforms were present.

**FcγR binding affinity of 2G12 glycoforms**

The binding properties of the 2G12 glycoforms to different FcγRs were assessed by flow cytometry. For this purpose, four different TZM-bl cell lines expressing FcγRI, FcγRIIA, FcγRIIB, or FcγRIIIA were incubated with serially diluted 2G12 glycovariants, and binding was measured. Binding affinities to FcγRI, FcγRIIA, and FcγRIIB were similar for all glycovariants except 2G12WT, which bound with slightly lower affinity to all three receptors (Fig. 2A–C). In contrast, binding to FcγRIIIA was several-fold higher for the fucose-free 2G12galT and 2G12ΔXF variants compared with the fucose-containing Abs 2G12CHO, 2G12FUT8, and 2G12WT (Fig. 2D). In all experiments, negative controls (FcγR-bearing TZM-bl cells only, FcγR-bearing TZM-bl cells and secondary Abs, and non–FcγR-bearing TZM-bl cells) showed no specific signal.

Binding of 2G12 glycoforms to FcγRIIIA (CD16) was further measured by SPR. First, the binding of 2G12 glycoforms to Protein A was determined. All glycoforms bound in a similar range (Supplemental Material). Using CD16, 2G12WT, 2G12CHO, and 2G12FUT8 exhibited similar binding, whereas fucose-free glycoforms (2G12ΔXF and 2G12galT) showed significantly higher binding (Fig. 3). Moreover, the difference in binding of 2G12FUT8, compared with 2G12ΔXF, which vary only by the presence of fucose in 2G12FUT8, clearly demonstrates that Fc fucose is a major determinant of enhanced binding to FcγRIIIA. This alteration of receptor binding does not depend on the linkage of fucose, as demonstrated by similar binding of 2G12WT and 2G12FUT8, which carry fucose in α1,3 and α1,6 linkages, respectively (Fig. 3). The elongation of GnGn structures with β1,4-galactose does not seem to alter binding to any FcγR, as indicated by similar binding of 2G12ΔXF and 2G12galT in cytometry and SPR analyses.

**Determination of antiviral activities of 2G12 glycoforms**

We determined the anti–HIV-1 and anti–SHIV activity of the glycovariants in ADCVI assays. ADCVI is a measure of the ability of Abs, in the presence of FcγR-bearing effector cells, to inhibit

**FIGURE 3.** Characterization of binding affinities of 2G12 glycoforms to FcγRIIIA (CD16) using SPR. Abs were captured by Protein A immobilized on a CM5 sensor chip. After washing, CD16 was applied in a concentration of 25 µg/ml and a flow rate of 20 µl/min followed by an injection of plain buffer. Association and dissociation phases are shown in the sensograms. Signal of an uncoated reference cell was subtracted from all measurements. RU, resonance units.
virus yield from infected target cells. The assay relies on a number of FcγR-mediated mechanisms to inhibit virus, including killing of infected target cells (ADCCs), release of antiviral β-chemokines, and, if phagocytic effector cells are present, phagocytosis of infected cells or of Ab-opsonized cell-free virus. For these assays, HIV-1_{US712}, an R5 clinical isolate, and SHIV_{162p3}, an R5 isolate often used in monkey challenge experiments, were used. With fresh PBMCs as effector cells, we found that the two nonfucosylated forms of 2G12 (2G12\_ΔXF and 2G12\_GalT) resulted in stronger antiviral activity than either the CHO-expressed or the WT plant-expressed variants (Fig. 4\_A, 4\_B); when combining results for both viruses tested, there was significantly greater antiviral activity with the nonfucosylated than with the fucosylated forms of 2G12 at concentrations of 50 μg/ml (p = 0.012; Mann–Whitney U test), 25 μg/ml (p = 0.006), and 12.5 μg/ml (p = 0.043). The 2G12\_ΔUT8 variant was not evaluated for ADCVI activity. For all of the glycovariants assayed, antiviral activity was generally slightly better against the SHIV than against the HIV-1 strain (compare Fig. 4\_A, 4\_C, and 4\_E with 4\_B, 4\_D, and 4\_F). The PBMC effector cells used to generate the results described above contain both NK cells, which express FcγRIIIa (FV genotype), and monocytes, which express FcγRI, FcγRIIa, FcγRIIb, and, to a much lesser extent, FcγRIIa. Because the glycovariants were best distinguishable by their binding affinities to FcγRIIIa by SPR and cytometry, we next measured ADCVI activity using purified NK effector cells at an E:T ratio of 1:1. For this assay, effector cells from two different donors were used, one with the FcγRIIIa FF genotype and the other with the VV genotype. Several studies have shown that the FF receptor binds more poorly to IgG and mediates less potent ADCC (4, 18, 19). Anti-cancer Abs lacking fucose have been previously shown to have increased binding affinity and increased ADCC activity with both genotypes (20). Consistent with these previous studies, the nonfucosylated variants provided better virus inhibition in the ADCVI assay with NK cells of both genotypes (Fig. 4\_C–F), which was more pronounced using 2G12\_ΔXF.

**Discussion**

Most successful vaccines elicit neutralizing Abs, and this property is a high priority when developing an HIV vaccine. mAbs against
HIV exhibit improved efficacy in preventing lentivirus infections when Fe–FcγR interactions occur (1), and Fe glycosylation is a critical determinant of Fe–FcγR binding. In this study, we have focused on elucidating the impact of glycosylation on the antiviral activity of the broadly neutralizing HIV-1 Ab 2G12. We show the efficient generation of glycoengineered 2G12 that exhibits largely homogeneous N-glycosylation profiles; such homogeneity cannot be achieved by mammalian cell-based expression platforms. Plant-derived 2G12 glycoforms differ in one or two glycan residues, thus allowing precise investigations of the impact of single glycan residues on Ab activity. We show a similar binding profile for all five 2G12 glycoforms to TZM-bl cells expressing FcγRIIa, FcγRIIbb, or FcγRIIIC. In contrast, two 2G12 glycoforms derived from glycoengineered plants that lack plant-specific xylose and core α1,3-fucose, and instead carry human-type GnGn (2G12 Δ1,3fuct) and terminally galactosylated (AA, 2G12GalT) structures, show significantly enhanced binding to TZM-bl cells expressing FcγRIIa compared with CHO or WT plant-derived 2G12 (2G12CHO, 2G12WT). These results were confirmed by SPR analyses in which fucose-free 2G12 (2G12 Δfucose, 2G12Δfuct) exhibited enhanced binding to FcγRIIa compared with glycoforms that carry core fucose (2G12 CHO, 2G12α1,6, and 2G12α1,6,α2,3). Interestingly, core fucose-containing 2G12 variants exhibited similarly weak binding, whether plant-typical α1,3 (2G12 WT) or mammalian-core α1,6 (2G12α1,6,α2,3) linkages were present. Notably, it seems that elongation of GnGn with terminal galactose does not further alter FcγR binding, as indicated by similar binding properties of 2G12 GalT and 2G12Δfuct. This is in contrast to observed enhanced HIV-1 neutralization of galactosylated 2G12 and 4E10 using a cell-based syncytium inhibition assay (11).

We have demonstrated that removing core fucose from 2G12 improves its ability to inhibit lentivirus infection by ADCVI. ADCVI, which is a measure of the ability of Ab to reduce virus yield from infected cells in the presence of FcγR-bearing effector cells, has been shown to correlate inversely with viral load during acute HIV infection (17). Moreover, higher levels of vaccine-induced ADCVI Ab activity were associated with lower rates of sexually acquired HIV infection during the Phase III Vax004 trial of recombinant gp120 vaccine (3). Finally, the level of protection of macaques from vaginal SHIV challenge by passive infusion of IgG1b12 or its Fc mutants could be attributed to ADCVI or other FcγR-mediated Ab activity (1). Our results demonstrate that modifying the glycosylation pattern of an HIV-1 mAb results in improvements in potentially critical antiviral activities such as ADCVI. Although we did not specifically measure other Fc–FcγR-mediated activities, such as ADCC or phagocytosis, it is likely that these functions would also be improved by proper Fc glycosylation.

The mammalian (CHO cell)-expressed 2G12 does not have particularly strong ADCVI activity against the lentiviral strains used in our assays. Nonetheless, the nonfucosylated variants provided consistent, though at times rather modest, improvements in antiviral function. When NK effector cells from an FcγRIIa Fκ donor were used, ADCVI activity was weaker than that observed with NK cells from an FcγRIIa Vκ donor. However, increased ADCVI activity was evident for both FcγRIIa genotypes with the nonfucosylated variants compared with the other variants. These results are consistent with a previous study examining the influence of glycosylation and FcγRIIa genotype on the ADCVI activity of anti-cancer mAbs (20).

In summary, we describe the binding and antiviral properties of different glycoforms of a broadly neutralizing anti–HIV-1 Ab. Our results have immediate implications for passive immunotherapy or passive immunoprophylaxis of lentiviral infections and suggest the need for in vivo studies comparing different glycovariants. Our results also suggest that vaccine-elicted Abs might afford a greater degree of protection if fucosylation is limited. Further research exploring the mechanisms by which Fe glycosylation can be controlled during the course of immunization will be required to achieve an Ab response with an optimal Fc glycosylation pattern.

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Disclosures

The authors have no financial conflicts of interest.

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