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Identification of antibody glycosylation structures that predict monoclonal antibody Fc-effector function

Amy W. Chung^a, Max Crispin^b, Laura Pritchard^b, Hannah Robinson^a, Mirosław K. Gorny^c, Xiaojie Yu^b, Chris Bailey-Kellogg^d, Margaret E. Ackerman^e, Chris Scanlan^b, Susan Zolla-Pazner^{c,f}, and Galit Alter^a

^aRagon Institute of MGH, MIT, and Harvard, Boston, Massachusetts, USA

^bDepartment of Biochemistry, University of Oxford, Oxford, UK

^cNew York University School of Medicine, New York City, New York

^dDepartment of Computer Science, Dartmouth College, Hanover, New Hampshire

^eThayer School of Engineering, Dartmouth College, Hanover, New Hampshire

^fNew York Veterans Affairs Harbor Healthcare System, New York City, New York, USA

Abstract

Objective—To determine monoclonal antibody (mAb) features that predict fragment crystalizable (Fc)-mediated effector functions against HIV.

Design—Monoclonal antibodies, derived from Chinese hamster ovary cells or Epstein–Barr virus-immortalized mouse heteromyelomas, with specificity to key regions of the HIV envelope including gp120-V2, gp120-V3 loop, gp120-CD4⁺ binding site, and gp41-specific antibodies, were functionally profiled to determine the relative contribution of the variable and constant domain features of the antibodies in driving robust Fc-effector functions.

Methods—Each mAb was assayed for antibody-binding affinity to gp140^{SF162}, antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) and for the ability to bind to FcγRIIa, FcγRIIb and FcγRIIIa receptors. Antibody glycan profiles were determined by HPLC.

Results—Neither the specificity nor the affinity of the mAbs determined the potency of Fc-effector function. FcγRIIIa binding strongly predicted ADCC and decreased galactose content inversely correlated with ADCP, whereas N-glycolylneuraminic acid-containing structures exhibited enhanced ADCP. Additionally, the bi-antennary glycan arm onto which galactose was added predicted enhanced binding to FcγRIIIa and ADCC activity, independent of the specificity of the mAb.

Conclusions—Our studies point to the specific Fc-glycan structures that can selectively promote Fc-effector functions independently of the antibody specificity. Furthermore, we demonstrated antibody glycan structures associated with enhanced ADCP activity, an emerging Fc-effector function that may aid in the control and clearance of HIV infection.

Keywords

antibody-dependent cellular cytotoxicity; antibody-dependent cellular phagocytosis; glycan structure; monoclonal antibody

Introduction

Secondary immune correlate analysis of the moderately protective RV144 vaccine trial showed an association between the reduced risk of HIV acquisition and antibody-dependent cellular cytotoxicity (ADCC) in the absence of high serum IgA [1]. Similarly, mounting evidence suggests that ADCC activity correlates with enhanced HIV control [2], slower disease progression [3–7] and enhanced neutralizing antibody-mediated sterilizing protection from infection [3], strongly suggesting that antibody fragment crystalizable (Fc)-effector functions may play a critical role in protective immunity against HIV.

Previous studies in the monoclonal antibody (mAb) therapeutic field have demonstrated that the ADCC potential of an antibody is modulated by specific biochemical properties of the Fc-region of an antibody, including subclass selection and glycosylation [8]. N-linked glycans on antibodies can be classified according to their level of galactosylation: G0, G1, and G2, which, respectively, contain 0, 1 and 2 galactose residues. When a single galactose is added, it can be further identified as either being bound on the α -3 or α -6 mannose arm (α -3-arm G1 or α -6-arm G1, respectively) of the core conserved bi-antennary heptasaccharide structure. The glycan structure can be further altered by the presence or absence of fucose and sialic acid. In addition, mAbs produced from nonhuman cells, such as Chinese hamster ovary (CHO) cells and mouse myeloma cells, may generate distinct glycan structures due to the expression of enzymes that can add sugars and/or sugar linkages that are not present in humans, including a nonhuman sialic acid, N-glycolylneuraminic acid (Neu5Gc) and galactose in an α -1,3 linkage (α -1,3-Gal). Additionally, CHO cells lack GnT III and ST6GalII activity, resulting in a loss of glycan structures that otherwise would be produced in humans.

Previous mAb therapeutic studies have linked the absence of core fucose [9] with elevated ADCC activity, whereas the role of galactosylation has been more controversial [10–13]. Notably, recent studies in HIV-infected individuals have demonstrated the enrichment of agalactosylated [14] and afucosylated glycans on both bulk and HIV-specific antibodies, and in particular in controllers who exhibit robust antibody-dependent cellular viral inhibition [15]. In contrast, the specific biophysical features of antibodies that drive more effective antibody-dependent cellular phagocytosis (ADCP), beyond differential binding to Fc-receptors (FcRs) [16,17], have yet to be defined.

With the growing interest in the delivery of HIV-specific mAbs for prophylactic treatment and ‘cure’ strategies [18–20], understanding the specific features of a mAb that promotes the

most effective innate immune responses may be of vital importance. However, although several studies have associated strong ADCC activity with antibodies against key epitope specificities [21–23], few studies have taken Fc-glycan modifications into account when comparing multiple mAbs in effector assays. Here we sought to determine whether antibody specificity to key regions of the HIV envelope could predict Fc-effector activity, and/or if the strength of Fc-activity was impacted by changes in glycosylation of the mAb Fc-region.

Methods

Monoclonal antibodies

A selection of mAbs with various specificities including CD4⁺ binding site: 559/64D, 1331–160E, b6, b12; gp41 cluster I: 50–69D, 240-D; gp41/MPER: 2F5, 4E10; V2: 697-30D, 2158; V3: 2191 and 2219. 559/64D, 1331-160E, 50-69D, 240-D, 697-30D, 2158, 2191 and 2219 – were produced by hybridoma cell lines obtained via fusion of Epstein–Barr virus-transformed peripheral blood mononuclear cells derived from HIV-seropositive individuals, with human × mouse heteromyeloma cells SHM-D33 [24]. Monoclonal antibodies b6 and b12, which were kindly provided by Dr Dennis Burton, and 2F5 and 4E10, provided by the NIH AIDS Reference Reagent Program, produced in CHO cells [25]. All mAbs were of an IgG1 isotype.

Enzyme-linked immunosorbent assay

Antigen avidity was determined by ELISA. Gp140^{SF162} was coated directly onto ELISA plates at a concentration of 0.5 mg/ml overnight, incubated with titrated mAbs at a dose range (1.0–0.0003 mg/ml) that could capture the three critical phases of mAb binding (saturation, a slope and no reactivity), followed by detection with alkaline phosphatase-conjugated goat antihuman IgG(Fc)-anti-body (Southern Biotech, Birmingham, Alabama, USA). ELISAs were repeated twice and in each experiment, mAbs were tested in duplicate. Relative affinity was determined by measuring the concentration of mAb that gave 50% maximal binding by regression analysis (using a curve fit) on Prism 5.

High-performance liquid chromatography

N-linked glycans from whole IgG were released from Coomassie blue-stained reducing SDS-PAGE gel bands by enzymatic release using PNGase F (New England Biolabs, Ipswich, Massachusetts, USA). Glycans were fluorescently labeled with 2-aminobenzoic acid (2-AA) [26,27] and the excess dye was removed using a Spe-ed Amide-2 column (Systematic Instruments, Stockport, UK). The 2-AA-labeled glycans were separated using LudgerSep N2amide HPLC column (Ludger, Oxford-shire, UK) and HPLC was carried out in a linear gradient of solvents at 30°C, as previously described [26,27].

Antibody-dependent cellular phagocytosis assay

A phagocytosis assay was performed as previously described [28]. Briefly, rgp140^{SF162} (Immune Technology, New York, New York, USA) bound to fluorescent beads were incubated with mAb and monocyte THP-1 cells. The cells were analyzed for bead uptake by flow cytometry. Each mAb was tested at 100 mg/ml, twice in replicate experiments.

Rapid fluorescent antibody-dependent cellular cytotoxicity assay

A modified rapid fluorescent ADCC (RFADCC) assay was used as previously described [29]. Briefly, CEM-natural killer resistant cell line (CEM-NKr) T-lympho- blast cells were pulsed with gp140^{SF162} (60 mg/ml), labeled with intracellular carboxyfluorescein succinimidyl ester (CFSE) and membrane Paul Karl Horan 26 dye (PKH26) dye. mAbs (100 mg/ml) were added to CEM-NKRs and healthy donor natural killer (NK) cells isolated using RosetteSep (Stem Cell Technologies, Vancouver, BC, Canada). Cells that maintained PKH26 but lost intracellular CFSE were analyzed by flow cytometry. mAbs were tested twice in replicate experiments.

Fcg-receptor surface plasmon resonance analysis

Surface plasmon resonance (SPR) experiments were run on a Biacore 3000 to measure Fcg receptor (FcgR) binding activity. CM5 chips were coated with FcgRIIa, FcgRIIb, FcgRIIIa [Cat #1330-CD-050 (Histidine-131), #1875-CD-050, #4325-FC-050 (phenylalanine-158) R&D Systems, Minneapolis, Minnesota, USA] or buffer alone. mAb (100 mg/ml) binding to each FcgR was assessed, tested in replicate experiments and quantified as the relative response units of signal.

Statistics

Statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, California, USA). Spearman-rank correlations were used to examine bivariate associations between continuous outcomes. All P values were two-sided. GENE-E matrix visualization and analysis program was used to create heatmaps [30].

Results

Fragment antigen binding specificity does not influence monoclonal antibody Fc-effector responses

A panel of mAbs was selected based on their specificity to key immunogenic regions of gp120, including V2, V3, CD4⁺ binding site (CD4bs) and gp41. The mAb-binding avidity, and ADCP and ADCC activity were all assessed against the same gp140^{SF162} glycoprotein. Surprisingly, mAbs with similar specificities did not drive similar levels of antibody effector activity for either ADCC (Fig. 1a) or ADCP activity (Fig. 1b), for example, CD4bs-specific mAbs were among the highest and lowest mediators of ADCC and ADCP. Notably, however, gp41-specific mAbs did induce higher ADCP compared to other mAb specificities. Moreover, no association was observed between the capacities of the antibodies to drive both ADCC and ADCP (Fig. 1c). Gp140^{SF162} avidity was determined by ELISA and relative avidity was determined by measuring the concentration of mAb that gave 50% maximal binding by regression analysis (Fig. 1d). Similarly, gp140^{SF162} avidity did not predict Fc-effector function (Fig. 1e: ADCC $r = 0.2$, $P = 0.93$; Fig. 1f: ADCP $r = -0.35$, $P = 0.25$). These data suggest that for the specificities tested, other factors most likely dictated by the Fc-region may predominantly drive Fc-effector functions in in-vitro assays, in this set of mAbs ($n = 12$).

Monoclonal antibody Fc-effector function is determined by Fcγ-receptor binding

Numerous studies have shown that Fc-effector activity is driven by the antibody affinity to FcγRs [31]. In particular, NK cell-mediated ADCC is governed by FcγRIIIa activation [31], whereas phagocytes express a wider array of FcγRs, and therefore may be modulated by the synergistic triggering of multiple FcγRs simultaneously [28]. SPR technology was used to evaluate mAb binding to activating FcγRIIa (Fig. 1g), FcγRIIIa (Fig. 1i) and inhibitory FcγRIIb (Fig. 1h). ADCC correlated robustly with FcγRIIIa binding ($r = 0.71$, $P = 0.0032$, data not shown), confirming the previous studies [31]. In contrast, no single FcγR binding correlated with ADCC, suggesting that binding to a single FcγR alone may not predict this antibody Fc-activity.

Decreased galactosylation inversely correlates with antibody-dependent cellular phagocytosis, whereas N-glycolylneuraminic acid structures predict stronger antibody-dependent cellular phagocytosis

Monoclonal therapeutics have exploited antibody glycosylation as a mechanism to potentiate the Fc-effector activities of mAbs [32–34]. Therefore, mAb glycosylation was assessed to determine if glycan structures were more highly predictive of potent antibody effector function. Increased galactosylation correlated with stronger ADCC activity (Fig. 2a). Specifically, fully galactosylated glycans (total G2) positively correlated with increased ADCC ($r = 0.57$, $P = 0.02$), whereas agalactosylated structures (total G0) inversely correlated with ADCC ($r = -0.62$, $P = 0.01$). We further resolved the specific glycoforms that mediated these differential ADCC activities. Both G0 ($r = -0.65$, $P = 0.01$) and G0F ($r = -0.52$, $P = 0.04$) inversely correlated with ADCC activity, whereas divergent complex relationships were observed for G1 and G2-specific structures. Nearly all Neu5Gc-containing structures, regardless of galactosylation content [with the exception of a-G2F(3)Neu5Gc], significantly correlated ($r > 0.54$, $P < 0.05$) or trended towards elevated ADCC activity [a-G2F(6)Neu5Gc: $r = 0.52$, $P = 0.1$] (Fig. 2), indicating that agalactosylation reduces ADCC, whereas increased presence of Neu5Gc enhances ADCC activity.

Galactosylation arm linkages predict antibody-dependent cellular cytotoxicity activity

No difference was observed between total galactosylation levels (total G0 or G2) and ADCC activity (Fig. 2a). Interestingly, however, a difference in galactose arm linkages was associated with differential antibody functionality. Specifically when looking at bulk galactosylation, the total a-3-arm G1 levels inversely correlated with ADCC activity ($r = -0.65$, $P = 0.011$) and FcγRIIIa binding ($r = -0.75$, $P = 0.002$) (Fig. 2b and c), whereas the total a-6-arm G1 structures enhanced ADCC ($r = 0.45$, $P = 0.1$) and FcγRIIIa binding ($r = 0.58$, $P = 0.029$) (Fig. 2d and e). Upon further analysis, a-3-arm G1F inversely correlated with ADCC ($r = -0.54$, $P = 0.04$), whereas a-6-arm G1F positively correlated with ADCC ($r = 0.51$, $P = 0.05$). These data suggest that beyond gross galactosylation levels, the arm upon which galactose is added to the core glycan structure may contribute to FcγRIIIa binding and thus Fc-effector functions, and provide a potential explanation of conflicting findings regarding the influence of galactose content on ADCC [10–13].

a-G2F(6)Neu5Gc structures broadly induce Fc-effector activity

As mentioned above, the presence of Neu5Gc correlated with enhanced phagocytosis (Fig. 2a). Of interest, a-G2F(6)Neu5Gc was the only glycan structure that correlated with both ADCC ($r = 0.8571$, $P = 0.01$) and ADCP activity ($r = 0.52$, $P = 0.1$) (Fig. 2a). Not surprisingly, presence of a-G2F(6)Neu5Gc also correlated highly with FcγRIIIa ($r = 0.90$, $P = 0.004$), FcγRIIb ($r = 0.76$, $P = 0.03$) and trended with FcγRIIIa binding ($r = 0.69$, $P = 0.07$). However, the presence of a-1,3-Gal structures has been associated with anaphylaxis in previous mAb therapeutic studies, suggesting that while potentially functional, they may be undesirable due to risks of adverse events in vivo [35]. Importantly, a-1,3-Gal structures were only observed on mAbs derived from mouse heteromyeloma cells, but not CHO cells, pointing toward variability in mAb functionality derived from different cell lines.

Despite multiple studies pointing to a critical role of afucosylation in enhancing ADCC and FcγRIIIa binding, we did not observe this relationship in our relatively small study of mAbs ($n = 12$). In contrast, as mentioned above, we observed a strong correlation between the incorporation of a-G2F(6)Neu5Gc and ADCC and FcγR binding (Fig. 2a). We therefore speculated that the functional effect of afucosylation on ADCC may have been masked by the presence of a-G2F(6)Neu5Gc. To evaluate this hypothesis, glycan structures containing fucose were further divided into those with and without a-G2F(6)Neu5Gc structures to examine the effect of total fucose without the heavy influence of a-G2F(6)Neu5Gc. Upon separation of the structures, the expected trend was observed for fucose, with increased fucose correlating with decreased ADCC activity ($r = -0.48$, $P = 0.08$; Fig. 2f) and FcγRIIIa binding ($r = -0.43$, $P = 0.10$; Fig. 2g). These data confirm the importance of afucosylation in enhancing ADCC activity, highlight the importance of other functional glycan modifications, and point toward the complex and context-dependent impacts of these individual hexose structures on the overall activity of a mixed population of glycovariant mAbs.

Discussion

Whereas multiple studies have associated strong Fc-innate immune recruiting activity to specific mAbs targeting key immunogenic specificities in the setting of HIV infection [21–23], limited attention has been paid to differences in Fc-glycosylation, and its impact on Fc-effector activity. The study presented here suggests that the impact of variant glycan structures may dominate over the variable domain characteristics in driving Fc-effector activity. Hence, future studies examining mAb Fc-mediated control should consider not just antibody epitope-binding properties, but also FcR-binding capacity and glycan structures to fully predict antibody-mediated antiviral control.

Interestingly, these data highlight previous observations that antibody glycosylation can vary between different producer cell lines [9,34,36]. Within this study, immunogenic a-1,3-Gal structures were only present on mAbs derived from mouse heteromyelomas, whereas nonhuman sialic acid Neu5Gc structures were produced by both heteromyelomas and CHO cell lines. Although the presence of Neu5Gc structures were associated with increased ADCP activity, mounting evidence suggests that humans may contain high levels of anti-Neu5Gc-specific IgG [37], bringing into question the potential therapeutic utility of these

structures, given both their potential immunogenicity and the impact that pre-existing immunity could have in dampening the efficacy of mAbs containing these structures. Thus, the selection of the most functional glycan structures must be balanced with the potential immunogenic nature of some of the unusual, but most highly functional glycan structures.

Unexpectedly, we observed that decreased galactosylation (G0 and G0F) reduced ADCC activity, whereas, in contrast, fully agalactosylated structures (G0) correlated with increased FcγRIIIa binding. Additionally, specific galactosylation arm linkages were associated with ADCC activity (a-6-arm G1 was positively associated with enhanced ADCC, whereas a-3-arm G1 did not), independent of the presence of fucose, potentially explaining the conflicting observations from numerous groups on the importance of galactosylation on ADCC activity [12,13].

Overall, mAb therapeutics used in HIV therapeutic treatment [18,19], prevention, and potential cure strategies [20] should take into consideration Fc-glycan modification in order to boost the antiviral ability of the most potent fragment antigen binding domains in their battle against HIV. Over 30 distinct glycan structures have been identified in healthy human serum [38]; however, only a limited number are being actively exploited by mAb therapeutics. Thus, future efforts should aim to develop understanding of how other novel glycan structures may naturally tune antibody activity in order to define a new set of Fc modifications that may be exploited in the setting of mAb therapeutic functional optimization efforts.

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Fig. 1.

Correlation between specificity and Fc-effector function. (a) The ADCC (% gp140 specific lysis) and (b) ADCP (phagocytic score) activity induced by mAb ordered by increasing strength of responses. (c) Scatter-plot of antibody functional activity by specificity. (d) Gp140^{SF162} avidity was determined by ELISA. mAb 1418(B19) and 860-55D (CMV) were used as negative controls. 50% maximal binding was calculated for all mAb. Relationship of effector activity and binding avidity was presented as scatter- plots of ADCC (e) and ADCP (f) activity versus gp140^{SF162} ec50 binding avidity. SPR analysis is shown for (g) FcγRIIIa,

(h) FcγRIIb, and (i) FcγRIIIa. Relative response to the respective FcγRs was calculated by determining the maximal relative response compared to baseline (time = 0) response during the passing of each mAb sample over each FcγR-coated flow cell between time 25 and 90 s. ADCC, antibody-dependent cellular cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; FcγRs, Fcγ receptors; mAb, monoclonal antibody.

Fig. 2.

Correlation between Fc-glycan structures and Fc-function/Fc-receptor binding. (a) Heatmap displaying correlations between Fc-glycan structures and Fc-effector function (ADCP and ADCC)/FcR binding. Nomenclature of glycans: G0, G1, G2, which, respectively, contain 0, 1, 2 galactose residues; single galactose residues are identified as being bound on a-3 or a-6 mannose arm of the core conserved heptasaccharide structure (a-3-arm G1 or a-6-arm G1). Additional residues may include fucose (F), sialic acid (S), bisecting N-acetylglucosamine (B) and N-glycolylneuraminic acid (Neu5Gc). Additionally, galactose can be incorporated in an alpha-1,3 linkage (a-G). Positive correlations are red in color and negative correlations are blue. Significant Spearman rank co-efficient values are identified as *P < 0.05, **P < 0.01.

0.01. Correlation plots showing: ADCC activity (% gp140-specific lysis) versus (b) total % of G1 glycans bound on the a-3-arm of mannose (a-3-arm-G1), (d) total % of G1 glycans bound on the a-6-arm of mannose (a-6-arm-G1) and (f) total % of fucose minus a-G2F(6)Neu5Gc glycan structures, FcγRIIIa binding versus (c) % total a-3-arm-G1 glycan, (e) % total a-6-arm-G1 glycan and (g) total % of fucose minus a-G2F(6)Neu5Gc glycan structures. ADCC, antibody-dependent cellular cytotoxicity; FcRs, Fc-receptors.