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Articles

Immunogenicity Evaluation of N-Glycans Recognized by HIV Broadly Neutralizing Antibodies

Sachin S. Shivatare, Ting-Jen Rachel Cheng, Yang-Yu Cheng, Vidya S. Shivatare, Tsung-I Tsai, Hong-Yang Chuang, Chung-Yi Wu, and Chi-Huey Wong*

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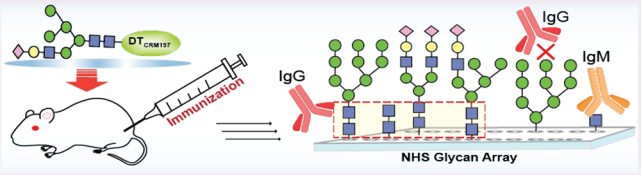
ABSTRACT: While the improved treatment of human immunodeficiency virus type 1 (HIV-1) infection is available, the development of an effective and safe prophylactic vaccine against HIV-1 is still an unrealized goal. Encouragingly, the discovery of broadly neutralizing antibodies (bNAbs) from HIV-1 positive patients that are capable of neutralizing a broad spectrum of HIV-1 isolates of various clades has accelerated the progress of vaccine development in the past few years. Some of these bNAbs recognize the N-glycans on the viral surface gp120 glycoprotein. We have been interested in using the glycan epitopes recognized by bNAbs for the development of vaccines to elicit bNAb-like antibodies with broadly neutralizing activities. Toward this goal, we have identified novel hybrid-type structures with subnanomolar avidity toward several bNAbs including PG16, PGT121, PGT128-3C, 2G12, VRC13, VRC-PG05, VRC26.25, VRC26.09, PGDM1400, 35O22, and 10-1074. Here, we report the immunogenicity evaluation of a novel hybrid glycan conjugated to carrier DT_{CRM197}, a nontoxic mutant of the diphtheria toxin, for immunization in mice. Our results indicated that the IgG response was mainly against the chitobiose motif with nonspecific binding to a panel of N-glycans with reducing end GlcNAc−GlcNAc (chitobiose) printed on the glass slides. However, the IgM response was mainly toward the reducing end GlcNAc moiety. We further used the glycoconjugates of $Man_3GlcNAc_2$, $Man_5GlcNAc_2$, and $Man_9GlcNAc_2$ glycans for immunization, and a similar specificity pattern was observed. These findings suggest that the immunogenicity of chitobiose may interfere with the outcome of N-glycan-based vaccines, and modification may be necessary to increase the immunogenicity of the entire N-glycan epitope.



INTRODUCTION

The human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein is composed of the gp120 and gp41 subunits, which exist as a trimer on the viral surface and is the prime target for the development of effective vaccines.[1−3] The extensive envelope glycosylation with half of its mass consisting of host-derived N-glycans shields much of the surface protein to evade the host immune response.[4] However, some of the most potent broadly neutralizing antibodies (bNAbs) recognize the glycan or glycopeptide components of HIV envelope glycoproteins.[5−8] Recent studies have shown that the glycosylation profile of the envelope isolated from the virions is significantly different from that of recombinant soluble forms (gp120 or gp41).[9,10] Ideally, the logic of glycan immunogen design should be based on the detailed understanding of glycosylation on gp120 generated in the primary infection site, i.e., human T-cells. However, the glycan structures on gp120 reported to date are not from human T-cells, raising a concern about the real glycan ligands of bNAbs. Since glycosylation is cell specific and human glycans are often nonimmunogenic due to self-tolerance, the precise characterization of HIV glycosylation is very important, especially that of gp-120 from human T-cells.

Approximately 10−30% of HIV infected individuals develop bNAbs after 2−3 years of infection.[11,12] These bNAbs are shown to protect macaques from infection in a SHIV challenge model upon passive administration at low serum concentration, suggesting that such antibodies could be elicited through vaccination.[13,14] The discovery of bNAbs has changed our views about how humans can deal with quickly mutating viruses such as HIV-1 and has provided an enormous impetus for HIV vaccine research. To date, a large number of bNAbs have been isolated via high-throughput screening of B-cell clones from HIV infected individuals and grouped into four categories depending on the location of their conserved epitopes on the viral spike (Figure 1):[15−17] (1) antibodies that recognize the site of CD4 interacting with gp120[18,19] (b12, VRCO1, and PGVO4); (2) antibodies that recognize variable region 1 and variable region 2 (V1/V2) glycopeptide epitopes including the N-linked glycan at residue Asn160 on gp120

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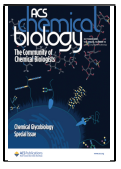

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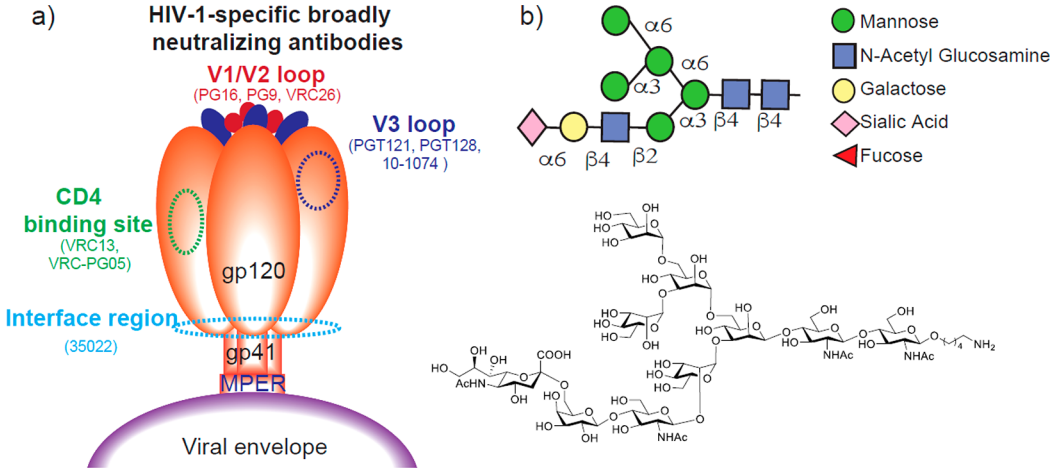

Figure 1. (a) Hybrid-type glycan reactive HIV-1 broadly neutralizing antibodies and the location of their epitopes on the HIV envelop. (b) Pictorial representation and chemical structure of hybrid-type glycan **I**.

(PG9, PG16, and PGT145);[20−22] (3) antibodies that recognize glycan V3 epitopes including the N-linked glycan at residue Asn332 on gp120[23−26] (PGT121, -128, and -135); (4) antibodies that recognize the membrane-proximal external region (MPER) on gp41[27] (2F5, 4E10, and 10E8). Several of these glycan-specific bNAbs such as PG9 and PGT128 were able to neutralize >70% of the more than 2000 variants known to date.[17]

2G12 is the first antibody reported to bind the oligomannose ($Man_{8/9}GlcNAc_2$)-type glycans linked to Asn295, Asn332, Asn386, and Asn391[28,29] on gp120 with broad neutralization activity (EC_{50} < 50 μg/mL for ∼32% HIV isolates tested). Later, in 2009, two new antibodies in this class, PG9 and its close relative PG16, were isolated with higher breadth in specificity and neutralization potency than 2G12. Initial epitope mapping suggested that PG9 and PG16 engage two conserved N-glycans at Asn156 and Asn160 glycosylation sites and an adjacent β-strand.[22,30,31] The PGT series antibodies are relatively new in the class of glycan-specific bNAbs with higher neutralization potency than 2G12, PG9, and PG16. The crystal structures of PGT128 with a gp120 outer domain,[23] PGT135 with a gp120 core,[32] and PGT121 with soluble and cleaved gp140 trimer[24] have enabled the characterization of their glycan-dependent epitopes.[24] Interestingly, PGT128 recognizes two high mannose-type glycans at Asn301 and Asn332 and β-strand at the base of the gp120 V3 loop;[23] PGT135 interacts with a cluster of high mannose glycans at Asn332, Asn386, and Asn392 and an extensive β-sheet motif on the gp120 outer domain,[32] and PGT121 contacts the protein component at the V3 base, glycan at the Asn301 or Asn332 site, and a complex-type glycan from the V1/V2 domain.[24] A family of PGT151-series antibodies (PGTs151−158) specifically recognizes cleaved HIV Env trimer via a glycan-dependent epitope expressed on the prefusion form of gp41.[33] The common feature of all these bNAbs is their interactions with the epitope consisting of the peptide component of gp120 and the surrounding multiple glycans to achieve high affinity. Deciphering the exact glycan composition of the epitopes recognized by HIV-1 bNAbs is a critical step to a successful vaccine design. Accordingly, a glycoconjugate construct capable of eliciting a strong immune response to gp120 glycans could potentially emerge as a valuable candidate for vaccine design. However, it should be noted that none of the aforementioned structural studies used gp120 generated from human T-cell culture, so the glycans described in the study may not be precisely the same as that from human T-cells.

Since the discovery of HIV virus that caused AIDS, there have been only three vaccine approaches that have completed human efficacy trials:[34] a gp120 monomeric protein with alum adjuvant, which failed to prevent or control HIV infection,[35] a recombinant adenovirus type 5 vaccine containing HIV gag, pol, and nef genes, which also failed to provide any benefit,[36] and at last, in 2009, a prime-boost strategy vaccine (RV-144) utilizing a canarypox vector prime together with a monomeric gp120 boost, which provided the first signal for the prevention of HIV infection in humans with 30% efficacy.[37] Apart from protein-based vaccine candidates, several carbohydrate-based vaccines have been evaluated.[38−42] In all glycoconjugate immunization studies performed to date, the oligomannose containing glycoconjugates designed on the basis of the logic of 2G12 epitopes have failed to elicit broadly neutralizing antibodies against HIV or gp120, though the antibodies were able to recognize the oligomannose glycans. The reasonable explanations behind the failure are (i) the glycoproteins exist as numerous glycoforms in which different glycans can exist at a single site, thereby providing a heterogeneous array of antigens and diluting out the antiglycan immune response to a specific glycoform, (ii) the protein−glycan interactions are typically weaker than protein−protein interactions, potentially restricting the production of anticarbohydrate antibodies, (iii) the presentation of glycans on the carrier protein surface fails to achieve exact mimicry of the 2G12 epitope, and finally (iv), the glycan epitope selected is not precisely correct as the reason mentioned above or is not nonself to stimulate an immune response.

Recently, the binding studies of PG9 in complex with a gp120 glycopeptide showed that the antibody binds to a glycopeptide containing Man_5 and a sialylated biantennary N-glycan in close proximity.[43] Since these two glycans are basically self-glycans, we thought a vaccine design based on these two glycans may not elicit a strong immune response. However, a hybrid N-glycan containing a Man_5 motif and a sialylated complex-type motif identified in our recent study was found to closely resemble the complex in the X-ray structure with a similar distance between the two glycans yet with better

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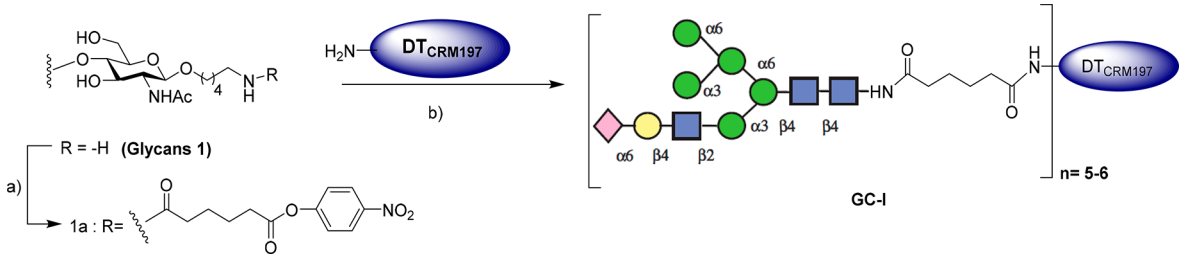
Scheme 1. Synthesis of Glycoconjugate-I[a]



[a]Reagents and conditions: (a) *p*-nitrophenyl ester (homobifunctional linker), DMF, RT, 5 h, 61%; (b) 10 mM PBS, pH 8, RT, overnight.

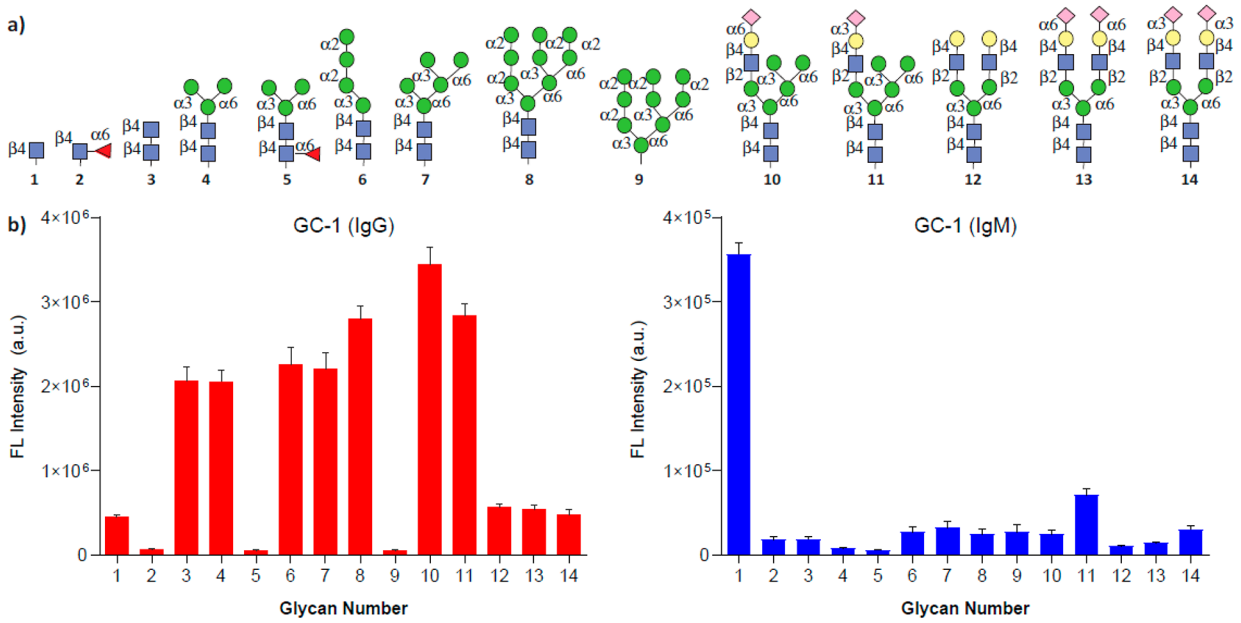


Figure 2. Glycan specificities of serum antibodies from mice immunized with GC-1. Female BALB/c mice were immunized with 2 μg of GC-**I** adjuvanted with 2 μg of glycolipid C34. Mouse serum was collected 2 weeks after the final vaccination, and the specificities of IgG (a) and IgM (b) antibodies against a panel of N-linked glycans were accessed after 100-fold dilution. Data of glycan binding represent the total intensity of five mice ± SEM.

avidity. This hybrid structure is considered to be unusual and more immunogenic, so we generated an array of hybrid-type glycans to map the binding specificity of several highly potent bNAbs, including PG9, PG16, PGT121, PGT128-3C, 2G12, VRC13, VRC-PG05, VRC26.25, VRC26.09, PGDM1400, 35O22, and 10-1074.[44] We found that hybrid-type glycan-1 (Figure 1) showed a significant binding affinity to most of the antibodies that we studied with K_d in the subnanomolar range. To study the immunogenicity of hybrid glycans, here, we report the design, synthesis, and immunological evaluation of hybrid glycan-1 conjugated to the DT_{CRM197} carrier as a vaccine. Our results indicated that a robust carbohydrate-specific IgG antibody response was elicited in comparison to IgM; however, a majority of the IgG response was nonspecific and directed toward the chitobiose motif. Therefore, the induced antibodies showed bindings to all N-glycans printed on the array surface. In contrast, the IgM response was mainly against the GlcNAc monosaccharide. We further reconfirm this outcome using various glycoconjugates containing Man_3, Man_5, and Man_9 glycans. In addition, a glycoconjugate containing only the chitobiose motif also induced the antibodies that recognized chitobiose and the glycans containing reducing end chitobiose. These results suggest that the strong immunogenicity of chitobiose may interfere with the outcome of glycan-based vaccines against HIV-1.

RESULTS AND DISCUSSION

Glycoconjugate Antigens That Mimic PG9 Epitopes. The structure of the gp120-V1/V2 loop with the PG9 antigen-binding fragment (Fab) suggests the occurrence of $Man_5GlcNAc_2$ at N-linked glycosylation sites Asn160 and/or Asn156/173 and a short peptide strand.[22] Later, the binding studies of PG9 to a series of HIV-1 V1V2 glycopeptides by the Wang group[43] revealed the necessity of a $Man_5GlcNAc_2$ glycan at N160 for recognition by PG9 and a critical sialylated N-glycan at the secondary site (N156/N173). Danishefsky and colleagues reported the chemical synthesis of gp120 V1V2 glycopeptides bearing either $Man_3GlcNAc_2$ or $Man_5GlcNAc_2$ that binds to PG9 with surprisingly high affinities.[45] Recently, our group demonstrated the high affinity binding of PG9 to an α-2,6 sialylated hybrid-type glycan (Figure 1) having the high mannose-type D2/D3 arm and complex-type D1 arm, which is in fact a close mimic of the PG9 epitope. Furthermore, in accordance with structural studies, we characterized the heteroglycans binding of PG9 to mixtures of $Man_5GlcNAc_2$ and sialylated biantennary complex-type glycan on an

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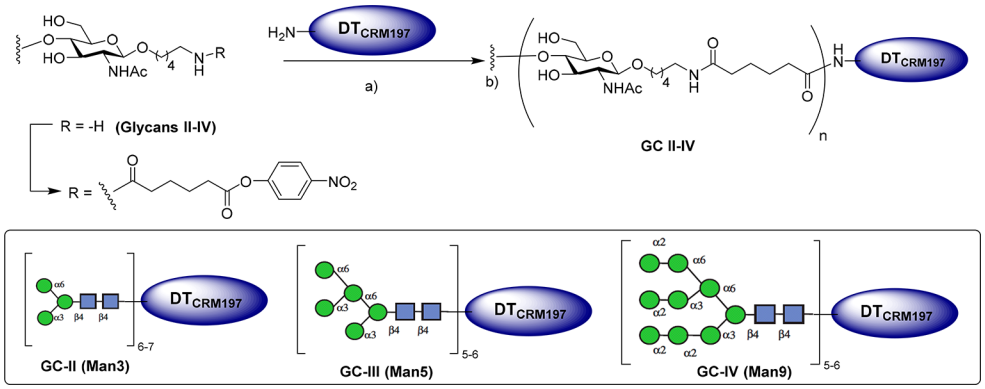
Scheme 2. Synthesis of Glycoconjugates II−IV[a]



[a]Reagents and conditions: (a) *p*-nitrophenyl ester (homobifunctional linker), DMF, RT, 5 h; (b) 10 mM PBS, pH 8, RT, overnight.

aluminum oxide coated-glass slide (ACG) array.[46] It was found that both the hybrid-type glycan and the mixed glycans have similar avidity toward PG9 with higher avidity to the hybrid type. All these efforts reached a conclusion that the glycan epitopes of PG9 consists of closely spaced $Man_5GlcNAc_2$ and complex-type glycan. On the basis of this result, we thought that a glycoconjugate containing either a hybrid-type glycan or a mixture of $Man_5GlcNAc_2$ and complex-type glycan may elicit PG9-like antibodies.

Generally, oligosaccharides are T-cell independent and have to be conjugated to a carrier protein to become immunogenic. After being taken up by antigen-presenting cells such as dendritic cells, the glycoconjugate is processed by proteases and glycosidases to glycopeptides and presented by MHC molecules to T-cells to stimulate long lasting immunity to the attached glycans.[47] Therefore, to increase the immunogenicity of glycans, we conjugated the hybrid-type glycan 1 to carrier protein DT_{CRM197} via amide bond formation between the activated oligosaccharide and the lysine residues on the protein surface. Because of its defined structure and well-proven clinical validation, DT_{CRM197} was selected as the carrier protein for glycoconjugate preparation. Depicted in Scheme 1, the hybrid-type glycan 1 with a five-carbon amine-containing linker at the reducing end was reacted with a 10-fold molar excess of a bifunctional linker diester to form a *p*-nitrophenyl half ester, so that the formation of dimer could be avoided (Scheme S1). After purification from the excess of bifunctional linker by P2-Gel (Biorad) column chromatography using water as eluent, the activated oligosaccharide was lyophilized and incubated with DT_{CRM197} at pH 8 to provide the desired glycoconjugate (GC-1), which was characterized by MALDI-TOF MS analysis to determine the average number of glycan epitopes on the carrier protein, and protein quantification was determined by the bicinchoninic acid (BCA) assay (Figure S1 and Table S1). Next, to determine the binding affinity of GC-1 to PG9, synthetic construct GC-1 was coated on an ELISA plate together with nonmodified carrier protein and HIV-1 gp120 (BG505) and titrated against PG9 serial dilutions. We found enhancement in the binding affinity of GC-1 to PG9 in comparison to nonmodified DT. However, the relative binding affinity of GC-**I** is much lower than PG9 binding to HIV-1 gp120 (Figure S7).

Immunological Evaluation of GC-I. A group of five mice were immunized with vaccines GC-**I** (2 μg of glycans) in the presence of α-galactosylceramide analog C34 as an adjuvant, which was also capable of inducing a class switch.[48] Three injections were performed at a two-week interval, and the sera were collected 2 weeks after the third injection (Figure S5). All mice remained healthy for the duration of the study, and no adverse reactions were observed. The serum binding titer was determined against a wider panel of a related N-linked oligosaccharide array on a glass slide (Figure 2a). Serum was assayed at a 1:100 dilution to facilitate the detection of low affinity interactions while minimizing nonspecific background binding.

The mice immunized with GC-**I** in the presence of C34 generated higher titers of IgG antibodies (4×10^6) than IgM (1×10^5). The antiglycan serum reactivity profiles indicated that the GC-1 elicited IgG antibodies bind strongly to glycan **10**, the antigen used in vaccine; however, it also recognizes a series of N-glycans of high mannose, hybrid, and complex types (Figure 2a, left panel). The IgG response toward GlcNAc (**1**), Fucα-1,6GlcNAc (**2**), Fucα-1,6$Man_3GlcNAc_2$ (**5**), and Man_9 (**9**) on the array suggested that the chitobiose motif is the main epitope for the high affinity recognition. In contrast, the majority of the IgM response was against GlcNAc (Figure 2b, right panel); however, all other glycans were also recognized by IgM antibodies with low affinity. These results led to our interest to study the immunogenicity of gp-120 related glycans.

Glycoconjugate Antigens That Mimic the Epitopes of PGT Series Antibodies. The crystal structure of PG128 Fab complexed with a fully glycosylated gp120 outer domain indicated that PGT128 can penetrate the glycan shield to recognize two high-mannose N-glycans at the Asn332 and Asn301 sites and a β-strand at the stem of the V3 loop.[23] PGT127 has a very similar structure as that of PGT128 with a strong affinity toward high mannose-type $Man_{8/9}$ glycans based on glycan array analysis.[23] The crystal structure of another Asn332 glycan-dependent antibody, PGT135, suggested that PGT135 can interact with a cluster of high-mannose glycans from Asn332, Asn392, and Asn386 sites and a strand on gp120.[32] Recently, our group characterized the glycan specificities of PGT141−144 antibodies and showed that the high mannose series $Man_{3/5/9}GlcNAc_2$ glycans could form the basis of their epitopes.[46] The glycan binding specificities of these PGT series antibodies suggest that vaccines containing high mannose series $Man_{3/5/9}$ glycans may induce PGT127−128 or 141−144 like antibodies. Therefore, $Man_{3/5/9}GlcNAc_2$ glycans were used for conjugation to the carrier protein to form glycoconjugates GC II−IV to compare their immune response with GC-1 (Schemes 2 and S2−S4).

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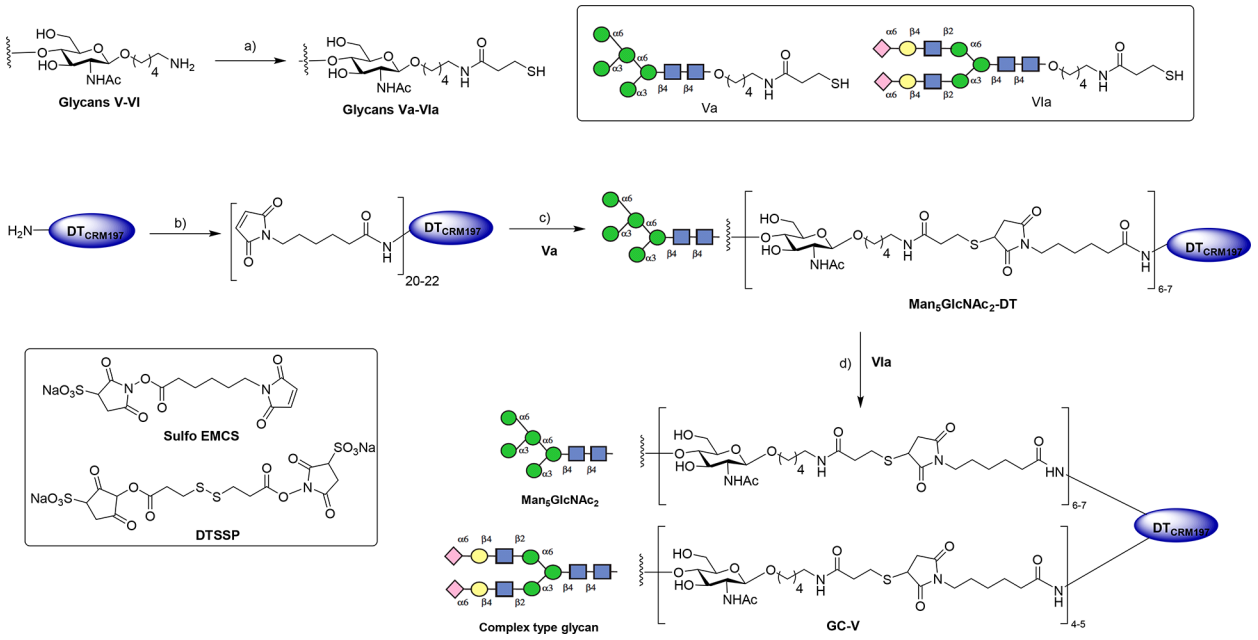
Scheme 3. Synthesis of GC-V[a]



[a]Reagents and conditions: (a) i. DTSSP, PBS buffer, pH 7.4, overnight; ii. DTT, 40 °C, 2 h; (b) Sulfo-EMCS, PBS buffer, pH 6.4, RT, 2 h; (c) **Va**, PBS buffer, pH 7.4, RT, overnight; (d) i. **VIa**, PBS buffer, pH 7.4, RT, overnight; ii. mercaptoethanol, PBS buffer, pH 7.4, overnight.

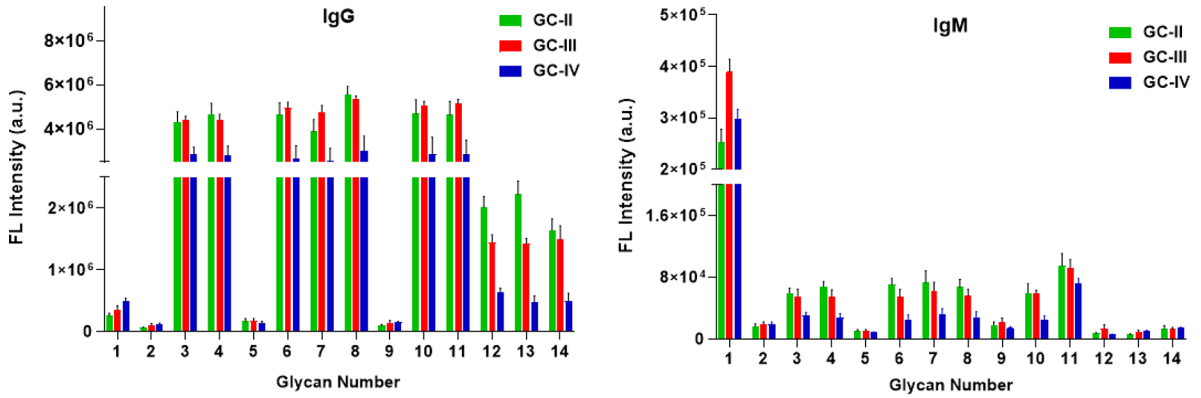


Figure 3. Glycan specificities of serum antibodies from mice immunized with glycoconjugates **II**−**IV**. Data represent the total intensity of five mice ± SEM.

Alternatively, we constructed the PG9 epitopes into a glycoconjugate by heterogeneous chemical glycosylation of a carrier protein with $Man_5GlcNAc_2$ **V** and sialylated biantennary complex-type glycan **VI**. The strategy of using a homobifunctional linker for the preparation of such glycoconjugates resulted in lower glycan loadings, which prompted us to explore other conjugation chemistries. Conjugate addition of thiol to Michael acceptors is a reliable way for the selective modification of proteins.[49,50] Because of its excellent reaction efficiency, we utilized maleimide as a Michael acceptor for the selective modification of the lysine residues on the carrier protein. The reaction of DT_{CRM197} with sulfo-EMCS at pH 6.4 resulted in the attachment of 20−25 copies of maleimide linkers on the protein surface (Scheme 3). Next, the glycans **V** and **VI** were reacted with 2 equiv of 3,3′-dithiobis(sulfosuccimidylpropionate) (DTSSP) in phosphate buffer, pH 7.2, followed by DTT mediated disulfide bond cleavage to afford thiolated glycans **Va** and **VIa**, respectively. Maleimide modified DT_{CRM197} was first incubated with thiolated $Man_5GlcNAc_2$ **Va** followed by dialysis to remove an excess of glycan. MALDI TOF analysis showed 6−7 Man_5 glycans incorporation onto DT_{CRM197} (Schemes 3 and S5−S7). Man_5 conjugated DT_{CRM197} was next reacted with thiolated complex-type glycan **VIa** overnight. Intact maleimide functional groups on the protein surface were capped by mercaptoethanol. Finally, GC-**V** was purified; carbohydrate loading was confirmed by MALDI-TOF, and protein quantification was done by the bicinchoninic acid (BCA) assay (Figures S3 and S4 and Table S1).

Up to now, several oligomannose-based glycoconjugates have been used for *in vivo* HIV vaccine studies, including the bivalent $Man_9GlcNAc_2$ mounted on a rigid cyclic peptide scaffold, which is further conjugated to the outer membrane protein complex (OMPC),[51−54] Man_9-V3 glycoptide that closely mimics an HIV-1 V3-glycan bnAb epitope,[55] three-component trivalent HIV-1 V3 glycopeptide immunogen,[56−58] and the galactose-based tetravalent $Man_9GlcNAc_2$ cluster conjugated to tetanus toxoid or T-helper peptide.[59,60]

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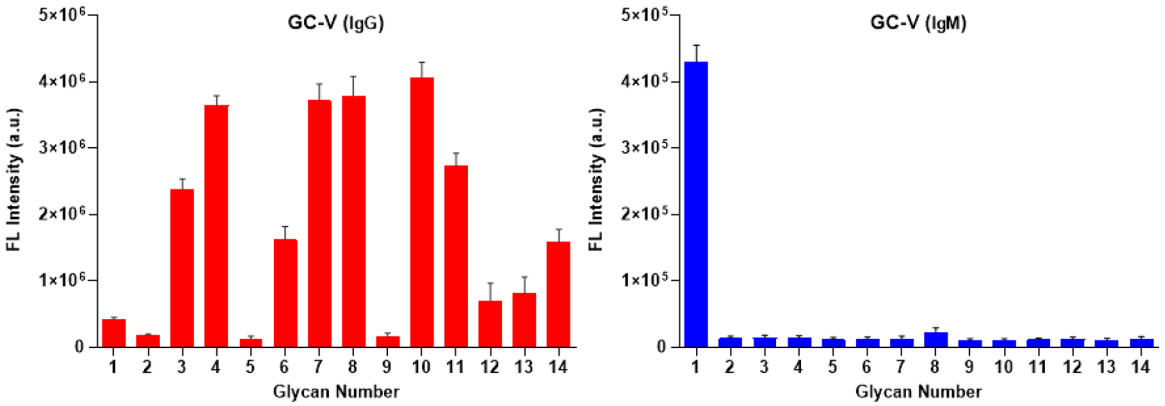

Figure 4. Glycan specificities of serum antibodies from mice immunized with glycoconjugate **V**. Data represent total intensity of five mice ± SEM.

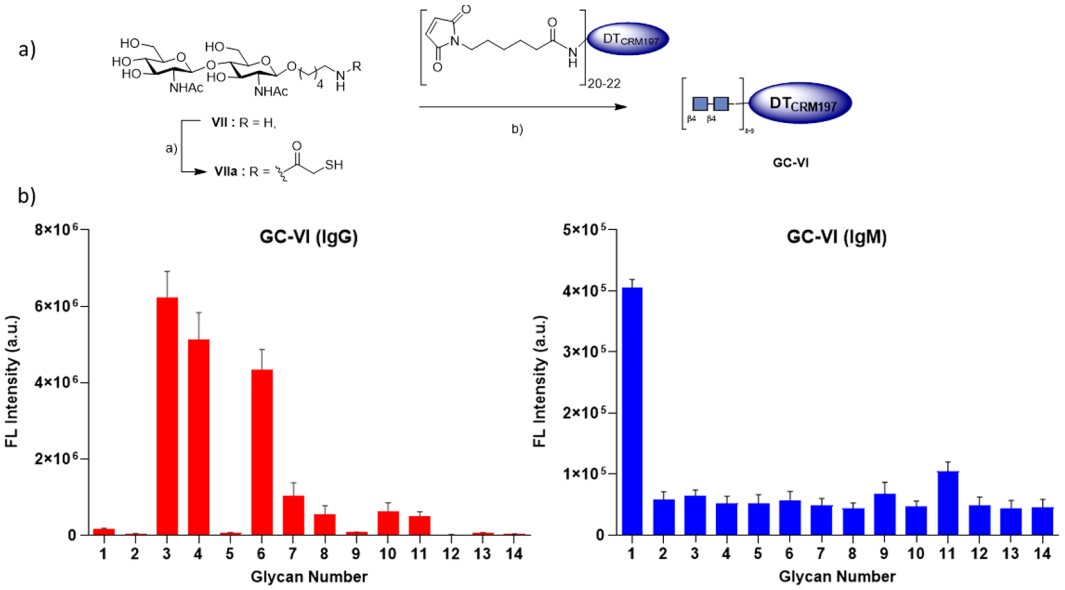

Figure 5. Synthesis of glycoconjugate **VI** (a) and its immunological evaluation (b). (a) (1) DTSSP, PBS, pH 7.4, RT, overnight; (2) DTT, PBS, pH 7.4, 40 °C, 2 h, 70% over two steps; (b) (1) PBS, pH 7.2, RT, 2 h; (2) thioethanol, PBS, pH 7.2, RT, overnight. The glycan specificities of serum antibodies from mice immunized with glycoconjugate **VI**. Data represent the total intensity of five mice ± SEM.

However, most of the synthetic oligomannose vaccines studied so far are not successful, though some are able to induce oligomannose-specific antibodies that can cross react with HIV-1 gp120 but failed to neutralize HIV-1 virions. Recently, bacterially derived synthetic oligomannose mimetic glycoconjugates reported by Pantophlet et al. showed an induction of bNAbs with specificity and neutralization activity equivalent to existing bNAbs.[61] In another study, macaques were immunized with Env expressing V3-high mannose glycans to elicit antibodies that neutralized HIV-1 expressing only high-mannose glycans.[62] In our study, the mice immunized with glycoconjugates **II**–**V** in the presence of C34 generated a robust IgG (4×10^6) response compared to IgM (8×10^4) (Figures 3 and 4). Interestingly, the specificity of IgG and IgM antibodies showed a similar pattern that was seen for GC-**I**. The IgG response was reduced for the glycans lacking the chitobiose motif (e.g., glycans **1** and **9**) and also for the glycans having core fucose (e.g., glycans **2** and **5**). These results suggest that, despite the glycan structure, the glycan density on the carrier, and coupling chemistry, the antibody response was still mainly against the chitobiose motif. In contrast, the IgM antibodies were specific for the reducing end GlcNAc moiety. The possible reasons behind this induction of nonspecific antibodies could be (1) that the multivalency of the conjugated glycans on the carrier protein failed to achieve the exact mimicry of the native carbohydrate epitope; (2) due to the strong immunogenicity of the reducing end di-GlcNAc motif, which was not addressed before.

Next, to address the immunogenicity of the chitibiose motif, we conjugated the GlcNAc-β-1,4-GlcNAc disaccharide to DT_{CRM197} via thiol-maleimide coupling chemistry to achieve a glycoconjugate with 8–9 glycans conjugated to the protein (Figure 5a, Scheme S8, Figure S6, and Table S2). A group of five mice were vaccinated with GC-**VI** (2 μg of glycans) in the presence of C34. The antiglycan serum reactivity profiles suggest that GC-**VI** elicited an IgG response mainly to chitobiose (**3**) with a significant cross-reaction with the chitobiose linked to a trimannose (**4**) or a tetramannose (**6**) through the 1,3-arm (Figure 5b). The antibodies also reacted with other glycans with a significant reduction in binding.

In summary, we have generated a series of glycoconjugate antigens based on the recognition motifs of HIV-1 bNAbs. The immunological evaluation of these glycoconjugates suggests that the induced IgG response was robust but nonspecific to

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the glycan antigens that were specifically incorporated in the vaccines. The serum antibodies showed binding to a series of N-glycans, indicating that the immune response was directed toward the chitobiose motif of N-glycans. We notice the possibility of the glycosidase-mediated cleavage of glycans in plasma or in antigen-presenting cells leaving behind the chitobiose as the main epitope exposed to the immune response, as shown in a related study of the oligomannose-conjugate vaccine.[63] However, the antibodies elicited from the conjugate of the hybrid type, or a mixture of high mannose and complex type, or various high-mannose types containing the chitobiose moiety were shown to bind various glycans strongly as long as they contain the chitobiose, and the binding affinity is decreased when the chitobiose is fucosylated, while the antibodies elicited from the chitobiose-conjugate only recognized chitobiose and two other mannose-containing glycans on the array. Considering this differential recognition pattern, we believe that the glycan-conjugate containing chitobiose is not likely cleaved by glycosidases to chitobiose for presentation; instead, the glycan-conjugate may elicit the immune response biased toward the chitobiose moiety. However, a partial cleavage of glycans cannot be ruled out. This finding is in agreement with the recently published study by Nguyen et al., where the specificity of rabbit antibodies elicited by oligomannose-based immunogens were primarily toward the glycan core rather than the outside D1/D2 mannose arm or the whole glycan.[64] This robust immunogenicity of the chitobiose motif could drastically change the direction of future carbohydrate-based vaccines against HIV-1 or other viruses. One possible approach to improve the immunogenicity of the N-glycan epitope is to modify the rest of the N-glycan moiety to increase its immunogenicity, as demonstrated in our previous study in which the replacement of certain hydroxyl groups in Globo-H saccharide with the azide group would increase the immunogenicity and thus enhance the immune response to the unmodified glycan.[65]

METHODS

General Procedures. *Preparation of Oligosaccharide Half Esters I–IV.* To a solution of oligosaccharide (2–5 μmol) in 0.5 mL of anhydrous dimethylformamide (DMF) solution was added *p*-nitrophenyl ester linker (10–25 μmol), and the mixture was stirred for 5 h at RT. The reaction was monitored by thin layer chromatography (TLC) (5:3:1:1, MeOH/EtOAc/H_2O/AcOH). The disappearance of free amine indicated the completion of the reaction. The reaction mixture was evaporated under reduced pressure without heating to remove DMF. The reaction mixture was dissolved in 500 μL of H_2O; an insoluble excess of linker was separated by filtration, and the water-soluble fraction was purified by Bio-Gel P2 (Bio-Rad) column chromatography and gradually eluted with H_2O. The solution was then lyophilized to afford the desired respective half esters (**I–IV**) as a light yellow colored solid.

Synthesis of Glycoconjugates I–IV. The salt of commercial DT_{CRM197} (1.0 mg) was removed by being dissolved in water and dialyzing (10 kDa, Ultra-0.5; Amicon) for 3–4 times. Protein was dissolved in phosphate buffer, pH 8.0 (~1 mg mL^{-1}), and 40–50 equiv of half esters **I–IV** were added to the solution. The mixture was stirred gently for 24 h at RT. The mixture was then diluted with deionized water and dialyzed against five changes of deionized water. The obtained glycan–protein conjugates **I–IV** were characterized by MALDI-TOF analysis to determine the carbohydrate loading on the protein.

Preparation of Maleimide Conjugated DT. The salt of commercial DT-CRM_{197} (1.0 mg) was removed by being dissolved in water and dialyzing (10 kDa, Ultra-0.5; Amicon) for 3–4 times. The residue was dissolved in PBS buffer (pH 6.5, 1.0 mL) and transferred into a sample vial. Sulfo-EMCS (1.0 mg, 1:1 weight ratio) was added to the solution, and the reaction was stirred at RT for 2 h. The mixture was purified by Amicon Ultra-0.5 (10 kDa). MALDI-TOF MS analysis was performed to determine the loading, and the bicinchoninic acid (BCA) assay was done to calculate the amount of protein. The difference between the molecular weights of DT_{CRM197} before and after the modification was used to determine the number of linkers conjugated. The DT_{CRM197}-maleimide was stored in PBS buffer (pH 7.2, 1.0 mg mL^{-1}) for further use at 4 °C.

Preparation of Oligosaccharides with Thiol Linker V–VIIa. To a solution of glycans **V–VII** (~5 μmol) in PBS buffer (pH 7.4, 1.0 mL) was added 3,3′-dithiobis[sulfosuccinimidylpropionate] (DTSSP) (10 μmol) at RT. The reaction mixture was adjusted to pH 7.4 by the slow addition of 1N NaOH. After the reaction was stirred overnight, DTT (40 μmol) was added to the reaction, and it was kept stirring at 40 °C for 2 h. The solvent was then evaporated under reduced pressure, and the reside was purified by Bio-Gel P2 chromatography using water as eluent to afford the desired compounds **V–VIIa** as white colored powders.

MALDI-TOF MS Analysis for Glycoconjugates. The primary carrier protein DT_{CRM197}, maleimide modified DT_{CRM197}, and glycoconjugates were reconstituted with ddH_2O (~1 μg/μL). The matrix, sinapinic acid, was freshly prepared with acetonitrile and deionized water (1:1), making a final matrix concentration of 10 mg mL^{-1} including 0.1% TFA. Matrix solution and glycoconjugates were gently loaded, mixed, and air-dried in the plate. Calibration was imperative using BSA before measurement. Each glycoconjugate and primary protein sample were detected under linear positive mode. The average molecular weight allows the calculation of the average numbers of carbohydrates incorporated on the carrier protein.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.1c00375.

Detailed experimental procedures, synthetic methods, immunological evaluation, glycan array analysis, and spectroscopic and analytical data for new compounds (PDF)

AUTHOR INFORMATION

Corresponding Author

Chi-Huey Wong − *Genomics Research Center, Academia Sinica, Taipei 115, Taiwan; The Scripps Research Institute, La Jolla, California 92037, United States;* orcid.org/0000-0002-9961-7865; Email: wong@scripps.edu

Authors

Sachin S. Shivatare − *The Scripps Research Institute, La Jolla, California 92037, United States;* orcid.org/0000-0002-7512-752X

Ting-Jen Rachel Cheng − *Genomics Research Center, Academia Sinica, Taipei 115, Taiwan*

Yang-Yu Cheng − *Genomics Research Center, Academia Sinica, Taipei 115, Taiwan*

Vidya S. Shivatare − *The Scripps Research Institute, La Jolla, California 92037, United States*

Tsung-I Tsai − *The Scripps Research Institute, La Jolla, California 92037, United States;* orcid.org/0000-0002-8355-6017

Hong-Yang Chuang − *Genomics Research Center, Academia Sinica, Taipei 115, Taiwan*

Chung-Yi Wu − *Genomics Research Center, Academia Sinica, Taipei 115, Taiwan;* orcid.org/0000-0002-5233-4454

Complete contact information is available at:

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Notes

The authors declare no competing financial interest.

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