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Synthetic carbohydrate antigens for HIV vaccine design

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Abstract

The heavy glycosylation of HIV envelope constitutes a strong defense mechanism for the virus to evade host immune response, which accounts for a major barrier for HIV vaccine development. Nevertheless, the identification of a number of glycan-dependent broadly HIV-neutralizing antibodies from HIV-infected individuals, including 2G12, PG9, PG16, PGT121-123, PGT125-128, and PGT135, strongly suggests that the defensive viral “glycan shield” can be important targets of vaccines. The novel glycan recognition mode exhibited by these antibodies provides new templates for immunogen design. This review highlights recent work on the characterization of the glycan-dependent epitopes of these neutralizing antibodies and recent advances on the synthesis of the relevant carbohydrate antigens for HIV vaccine design.

Introduction

The infection by human immunodeficiency virus (HIV), the cause of AIDS disease, is still a serious global health problem. An effective prophylactic vaccine may provide the best hope to contain the global epidemic. Despite tremendous efforts in the past 25 years, however, a truly effective HIV vaccine capable of eliciting broadly neutralizing antibodies (bnAbs) is still elusive [1–4]. The failure can be partly attributed to a multitude of defense mechanisms that HIV develops to counter against immune surveillance. Among them, frequent sequence variation and heavy glycosylation of the viral envelope glycoproteins (gp120 and gp41) are two major barriers that an effective immunogen should overcome in order to mount broad, strong, and long-lasting immunity against HIV infection [5••].

Carbohydrates account for half of the molecular mass of the outer envelope glycoprotein gp120, which cover a large surface area of the envelope and play a major protective role in viral immune evasion. Nevertheless, there are strong grounds to consider the viral carbohydrate antigens as targets for vaccine. The initial identification of 2G12, a carbohydrate-specific broadly neutralizing antibody, suggests that the defensive carbohydrate shield of HIV is vulnerable for immune recognition. This notion was greatly reinforced by the recent discovery of more than a dozen of new glycan-dependent bnAbs,

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including PG9, PG16, PGT121-123, PGT125-128, and PGT135, which neutralize HIV-1 primary isolates with remarkable breadth and potency [6–8]. These findings have stimulated great interests in further characterization and reconstitution of the fine neutralizing epitopes, which are essential first steps in the design of an effective immunogen [5•,9]. Early work on the synthesis of oligosaccharide clusters as mimics of 2G12 epitope was covered in two previous reviews [10,11]. The present review highlights recent advances in the characterization and synthesis of the glycan-dependent epitopes of these bnAbs for vaccine design.

Structural features and functions of HIV glycosylation

HIV-1 has two envelope glycoproteins, gp120 and gp41, which form a trimeric complex of a heterodimer. A typical gp120 is glycosylated at more than 20 conserved N-glycosylation sites (the NXS/T motif) [12]. O-glycosylation was rarely found for HIV-1 envelope, although a recent report suggests the existence of O-glycans on some gp120 [13]. HIV-1 glycosylation is tremendously heterogeneous [12,14–18]. On top of the structural heterogeneity, one important feature of HIV-1 glycosylation is the unusually high numbers of high-mannose type glycans on gp120 [12]. This tendency was even greater for the virion-associated gp120 from primary HIV-1 isolates as well as the simian immunodeficiency virus (SIV) [16,18]. Another important feature is the clustering of glycans on gp120. Remodeling of the N-glycans on the de-glycosylated gp120 revealed two distinct glycan clusters, one consisting mainly of high-mannose type and the other of complex type N-glycans [14]. While individual viral N-glycans are similar to host glycans, the dense high-mannose clusters are rare for normal host glycoproteins, which form a basis for immune discrimination and thus vaccine design.

HIV-1 glycosylation exerts profound effects on the antigenicity and immunogenicity of the envelope glycoproteins. The dense and dynamic “glycan shield” constitutes a major defense mechanism for immune evasion, reducing the immunogenicity of the envelope and limiting the access of the protein antigens by neutralizing antibodies [19,20]. In addition, the dense high-mannose or fucosylated complex type N-glycans also play an active role in promoting HIV-1 infection and transmission, via their interactions with respective lectins such as DC-SIGN on dendritic cells or mannose-binding proteins on macrophages [21].

Glycan-dependent broadly neutralizing antibodies and their epitopes

Antibody 2G12

Human monoclonal antibody 2G12 was the first carbohydrate-reactive broadly neutralizing antibody identified from HIV infected patients. Its epitope was mapped to a high-mannose oligosaccharide cluster contributed from the N-glycans at the N295, N332, N386, and N392 sites, where a terminal Man α 1,2Man disaccharide moiety is essential for the binding [22–24]. Subsequent crystal structure study revealed an unusual Fab domain-swapped structure that created extended multivalent binding sites, providing a beautiful immunological solution to glycan cluster recognition [25,26]. Further characterization of the glycan specificity was provided by synthesis and binding study of well-defined oligosaccharide antigens [27–32]. These studies confirm the requirement of a terminal Man α 1,2Man subunit

for 2G12 binding and the necessity of a well-configured oligomannose cluster for high affinity interaction. These results laid the basis for designing 2G12 epitope-based immunogen.

Antibodies PG9 and PG16

Recently isolated from an HIV-infected donor, the PG9 and PG16 antibodies can neutralize 70–80% of circulating HIV-1 isolates and show 10-fold higher viral neutralization potency than 2G12 [33••]. Epitope mapping shows that PG9 and PG16 recognize a strand and two conserved N-glycans at the N156 and N160 (HXB2 numbering) glycosylation sites in the V1V2 region [34]. Recent crystal structures of PG9 in complex with a scaffolded V1V2 domain demonstrate that a $\text{Man}_5\text{GlcNAc}_2$ at N160 provides the major contacts, with additional contributions from the glycan at N156 (CAP45 strain) or N173 (ZM109 strain) and a strand of V1V2 peptide [35••]. This study suggests that a conserved glycopeptide at the V1V2 region might constitute the neutralizing epitope of PG9. Further characterization of the glycan specificity of PG9 and PG16 was accomplished by a synthetic glycopeptide approach [36••], which confirms the necessity of a $\text{Man}_5\text{GlcNAc}_2$ at N160 but also reveals a critical role of a sialylated N-glycan at N156 or N173 site for high-affinity binding. The importance of a sialylated N-glycan at the second site was confirmed by a recent crystal structure of PG16 in complex with a sialylated V1V2 domain [37]. Moreover, analysis of PG9 in complex with a HIV-1 gp120 trimer suggests that an additional N-glycan at N160 from an adjacent gp120 is also involved in PG9 recognition [38]. This asymmetric recognition mode partially explains why PG9 preferentially recognizes gp120 trimer over the monomer.

Antibodies PGT128, PGT121, and PGT135

The PGT series antibodies are a new class of glycan-dependent bNAbs identified from HIV-infected “elite neutralizers” [39••]. A majority of them neutralize HIV-1 isolates with remarkable breadth and with higher potency than PG9 and 2G12. The epitopes of these antibodies were mapped to the V3 domain and dependent on N332 glycosylation. A better picture of the epitope was provided by crystal structure of PG128 Fab in complex with $\text{Man}_9\text{GlcNAc}_2$ and a fully glycosylated gp120 outer domain [40••]. It shows that PGT128 can penetrate the glycan shield to recognize two high-mannose N-glycans at the N332 and N301 sites and a β -strand at the stem of the V3 loop. Thus the epitope of PGT128 may consist of a glycopeptide in the V3 region involving a high mannose glycan at N332 and another N-glycan at N301 which structure remains to be defined. The PGT127 has a very similar structure as that of PGT128.

The antigenic structure for PGT121 seems more complex. Initial epitope mapping suggests that PGT121 is dependent on glycosylation at N332. Recent structural and glycan microarray analysis indicates that PGT121 recognizes complex type N-glycan instead of high-mannose glycans [41•]. More recently, the structures of three PGT121 family bNAbs (PGT121-123) were solved [42••]. A reconstituted EM model suggests that the epitope may include the V3 base, the glycan at N332 or N301 site, and a complex type N-glycan that may come from the V1V2 domain. The crystal structure of another N332 glycan-dependent antibody, PGT135, was also solved [43••], which shows that PGT135 can interact with a

cluster of high-mannose glycans from N332, N392 and N386 sites and a strand on gp120. It is clear that all of these PGT antibodies recognize both glycans and peptide domains as integrated epitopes, but the fine structures of the epitope remain to be further defined.

Synthetic oligosaccharide clusters as mimics of 2G12 epitope

The characterization of 2G12 epitope as a unique oligomannose cluster on gp120 has stimulated tremendous interests in design and synthesis of epitope mimics. As the first attempt, Wang and co-workers selected a galactoside moiety as the scaffold to construct glycan clusters displaying 2–4 display Man₉GlcNAc₂ subunits [28]. An efficient chemoselective ligation between thiol and maleimide moieties was used for making the glycoconjugates. The affinity of the tetra-valent Man₉GlcNAc₂ cluster (Figure 1a) for 2G12 was 70-fold higher than Man₉GlcNAc₂Asn. In an design, cholic acid was selected as a more rigid scaffold to attach three Man₉GlcNAc₂ at the 3, 7 and 12 positions, respectively [44]. The resulting trivalent glycan cluster (Figure 1b) demonstrated a 46-fold higher affinity than that of the Man₉GlcNAc₂Asn. These studies demonstrate a clear glycan clustering effect in 2G12 binding. However, the affinity of the synthetic glycan clusters (at μ M level) was still much lower than the affinity of HIV-1 gp120 for 2G12 (IC₅₀ at nM range). Further work is required to optimize the design of the glycan clusters. Functionalized cyclic peptides were also used as scaffold to make glycan clusters. Danishefsky and co-workers applied a cyclic peptide scaffold to display synthetic Man₉GlcNAc₂ (Figure 1c) [45]. Wang and co-workers synthesized a cyclic peptide template to construct clusters of the D1 Man₄ and its selectively fluorinated derivative [46]. Copper(I)-catalyzed 1,3-dipolar azide–alkyne cycloaddition (CuAAC) was used to attach four Man₄ subunits to one face of the cyclic peptide and two T-helper peptides to the other face (Figure 1d). Again, these glycan clusters show enhanced affinity for 2G12 over the oligosaccharide subunit as evaluated by SPR analysis.

Polyvalent glycan clusters were designed and synthesized based on dendron scaffolds to further enhance the affinity. Wong and co-workers devised an AB₃ type dendrimeric skeleton as the scaffold and used the CuAAC click reaction to attach 3, 9, and 27 copies of synthetic Man₄ or Man₉ oligosaccharide to obtain the oligomannose dendrons (Figure 1e) [47]. The dendrimers could efficiently bind to 2G12, and the 9-valent dendromer shows optimized affinity for 2G12 and lectin DC-SIGN (IC₅₀ at nM). This high affinity is well comparable to the affinity of HIV-1 gp120 for 2G12 and DC-SIGN binding, suggesting that the synthetic glyco-dendrimers may be further developed as candidate vaccines or anti-HIV microbicides. The use of polyamidoamine (PAMAM) as scaffold led to the generation of 4- and 8-valent oligomannose clusters carrying Man₄, Man₆ and Man₉ (Figure 1f) [48]. The 8-valent dendrimer of Man₉ showed the highest affinity for 2G12. Gold nanoparticles were also used as scaffold to display Man₄ at different density [49]. In addition, directed evolution was explored for evolving DNA-based multivalent glycoclusters containing Man₄ subunits, leading to generation of μ M binders for 2G12 [50].

Synthesis and immunization studies of 2G12 epitope-based immunogens

Since carbohydrate antigens are poorly immunogenic and are usually difficult to trigger T cell-dependent immune response to produce IgG antibodies, a conventional approach for

vaccine design is to conjugate the carbohydrate antigens to a T cell-helper epitope such as a carrier protein to afford functional immunogens [51]. Accordingly, Wang and co-workers conjugated the galactoside-based, tetravalent Man₉GlcNAc₂ cluster to keyhole limpet hemocyanin (KLH, a well-known carrier protein that can provoke strong immune response) to provide the immunogen (Figure 2a) [52]. Immunization of the glycoconjugates in rabbits raised moderate anti-carbohydrate antibodies that demonstrate weak cross-reactivity to HIV-1 gp120, but the antisera did not show HIV-neutralizing activity. Further analysis indicates that majority of the antibodies were raised against the maleimide-based linkers. Conjugation of the cyclic peptide-scaffolded Man₉GlcNAc₂-containing glycopeptide to the outer membrane protein complex (OMPC) of *Neisseria meningitidis* gave a glycoconjugate (Figure 2b) and its immunogenicity was tested in guinea pigs and rhesus macaques [53]. The glycoconjugate induced high titers of carbohydrate-specific antibodies in both animal models, but the antibodies showed only weak affinity for recombinant HIV-1 gp160 and failed to neutralize a panel of viral isolates. Conjugation of the PAMAM dendron-based Man₄ and Man₉ clusters to CRM197, a non-toxic mutant of diphtheria toxin gave the CRM197-glycoconjugates (Figure 2c), which were tested in rabbits and mice [48]. Similarly, a Man₄-BSA conjugate (Figure 2d) was synthesized and tested in rabbits [54]. These synthetic glycoconjugates raised mannose-specific antibodies. However, none of the antibodies were able to recognize the high-mannose type N-glycans in gp120.

Finn, Burton and co-workers explored virus-like particles (VLPs) such as bacteriophage Q β as a scaffold to present multiple copies of oligomannose in a highly organized fashion to mimic the clustering presentation of high-mannose N-glycans on gp120 (Figure 2e) [55•]. In a relevant design, Davis and co-workers ligated a C-6 methylated Man₄ to Q β , providing a novel multivalent nonself sugar mimic of the HIV glycan shield (Figure 2f) [56•]. For the synthesis, surface-exposed amine groups on Q β was functionalized with alkyne groups and then reacted with oligomannose-azides via an efficient CuAAC click reaction to afford the glycoconjugates carrying Man₄, Man₈, and/or Man₉ (Figure 3). Three Q β variants (Q β , Q β K16M, and Q β -HPG) were used as scaffolds to investigate the effects of varied number and geometry of oligomannose attachments. The conjugates of Q β and Q β K16M carrying Man₄ and Man₉ showed high affinity for 2G12. Surprisingly, Q β -Man₈ was poorly recognized by 2G12. However, the mixed Q β HPG-Man₈/Man₉ glycoconjugate showed the highest affinity for 2G12. Immunization of the Q β K16M-Man₄ and Q β K16M-Man₉ conjugates in rabbits elicited high-titers of IgGs against Man₄ and Man₉. But none of the anti-sera could cross-react with gp120, neither did the anti-sera show HIV-neutralizing activity. Glycan microarray analysis indicates that the mannose-specific antibodies do not cross-react with natural high mannose N-glycans, despite their binding to synthetic oligomannose structures. The C6-methylated mannose containing Q β -Man₄ constructs showed a higher antigenicity for 2G12 than the unmodified Q β -Man₄ and could induce enhanced anti-oligomannose responses in comparison with the non-modified Q-Man₄, supporting the hypothesis that appropriate nonself modification could improve the immunogenicity. However, similar to the other synthetic oligomannose-containing glycoconjugates, the anti-sera did not recognize gp120 and failed to neutralize HIV [56•].

Given the fact that the glycoconjugates containing natural high-mannose N-glycan such as Man₉GlcNAc₂ could raise antibodies moderately cross-reactive to HIV-1 gp120 or gp160

[52,53], the failure of the anti-mannose antibodies raised by the synthetic oligomannose-conjugates to recognize natural N-glycans of gp120 may be due to the lack of the chitobiose core in the synthetic oligomannoses, which may play a role in modulating the orientation, antigenicity, and immunogenicity of the glycans. This notion was reinforced by a recent report, showing that immunization with yeast-derived glycoproteins carrying high-dense natural Man₈GlcNAc₂ glycans was able to elicit carbohydrate-specific IgG antibodies that are cross-reactive with HIV-1 gp120, and can neutralize virions expressing exclusively high-mannose N-glycans [57].

Synthesis of HIV-1 V1V2 glycopeptides as mimics of the epitopes of PG9 and PG16

Structural studies suggest that PG9 recognizes a glycopeptide epitope located in the V1V2 region, but the precise structure of the glycans remains to be defined. To further characterize the glycan specificity and to reconstitute the neutralizing epitopes, Wang and co-workers designed cyclic V1V2 glycopeptides derived from two HIV-1 strains (CAP45 and ZM109) based on the reported crystal structure, and synthesized over 25 homogeneous glycopeptides to probe the nature of the epitopes [36••]. A chemoenzymatic approach was used, as demonstrated for the synthesis of three typical CAP45 V1V2 glycopeptides (Figure 4a). Glycosynthase-catalyzed transglycosylation using excess Man₅GlcNAc oxazoline substrate gave the lycopeptide carrying two Man₅GlcNAc₂ glycans. To install different glycans at the N156 and N169 sites, glycosylation was controlled to give two monoglycosylated intermediates, which were separated by HPLC. Then the two intermediates were glycosylated to attach a distinct N-glycan at the remaining site. The overall yield was excellent. SPR and ELISA binding analysis indicate that both the precise glycan structure and the glycosylation site in the right context of the polypeptide are important for antibody recognition, as free glycans, nonglycosylated, or mis-glycosylated V1V2 peptides do not bind. These studies confirmed the importance of a Man₅GlcNAc₂ glycan at N160 for recognition by PG9 and PG16, and further revealed a critical role of a sialylated N-glycan at the N156 or N173 site for high affinity binding, which was not revealed by the original PG9 structural study [35••]. The best glycopeptides show a μ M affinity for the Fab of PG9 and PG16 in SPR analysis, and are able to detect PG9 and PG16 antibodies at 50 pM to 1.5 nM concentrations in an ELISA format. Independently, Danishefsky and co-workers synthesized several V1V2 glycopeptides with a sequence from another HIV-1 strain (A244) [58•]. A convergent chemical assembly approach was employed for the construction of the V1V2 glycopeptides carrying two Man₅GlcNAc₂ glycans at the N156 and N160 sites (Figure 4b). The successful native chemical ligation between the two fragments carrying bulky N-glycans around the ligation site, which gave a notable 55% yield, was impressive. The Man₅GlcNAc₂-containing glycopeptide shows high affinity ($K_D = 300$ nM) for PG9. Surprisingly, the glycopeptide carrying a truncated Man₃GlcNAc₂ at the N156 and N160 sites also demonstrates comparable affinity for PG9 in SPR analysis, implicating some flexibility in glycan recognition. It is expected that synthesis of defined carbohydrate antigens will continue to play an important role in further characterization and reconstruction of the fine neutralizing epitopes for HIV vaccine development.

Conclusion

HIV glycosylation constitutes a strong defense mechanism for viral immune evasion. Nevertheless, the discovery of 2G12 and the new glycan-dependent broadly neutralizing antibodies (e.g., PG9, PG16, and PGT128) strongly suggests that the viral carbohydrate antigens can also be attractive targets for vaccines. Significant progresses have been made in the design and synthesis of scaffold-based oligosaccharide clusters to mimic the proposed epitope of 2G12. The binding and animal immunization studies have yielded valuable information that helps define the structural requirement for 2G12 recognition and the immunogenicity of the antigens. But it should be noted that the 2G12 epitope-based synthetic immunogens have so far failed to elicit HIV-neutralizing antibodies, reflecting the general challenges in HIV vaccine design. Future work should be focused on further characterization of the neutralizing epitopes, on innovative approaches for immunogen design, and on exploration of novel immunization protocols to test candidate vaccines.

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Highlights

HIV glycosylation has novel structural and functional features

A new class of HIV-neutralizing antibodies recognizes glycan-dependent epitopes

Synthetic oligomannose clusters can partially mimic the epitope of antibody 2G12

X-ray structures reveal novel mode of recognition by glycan-dependent antibodies

Synthetic carbohydrate antigens enable the characterization of glycan specificity

Figure 1. Structures of synthetic oligosaccharide clusters based on small molecule scaffolds

a) a Man₉GlcNAc₂ cluster on a galactopyranoside scaffold; **b)** a Man₉GlcNAc₂ cluster on a cholic acid scaffold; **c)** a Man₉GlcNAc₂ cluster on a cyclic peptide scaffold; **d)** a Man₄ and fluorinated Man₄ cluster on a cyclic peptide scaffold; **e)** a synthetic Man₉ dendrimer based on an AB3 dendron; **f)** a synthetic Man₉ dendrimer based on a PAMAM dendron.

Figure 2. Structures of synthetic 2G12 epitope-associated immunogens for immunization studies
 a) the $\text{Man}_9\text{GlcNAc}_2$ -cluster/KLH conjugate; b) the glycoconjugate between the synthetic cyclic peptide-based bivalent $\text{Man}_9\text{GlcNAc}_2$ and the OMPC; c) the glycoconjugate of PAMAM dendron-based oligomannose and protein CRM197; d) the Man_4 -BSA glycoconjugate; e) bacteriophage Q β -based immunogens carrying $\text{Man}_8/\text{Man}_9$; f) Q β -based immunogens carrying nonself C6-methylated mannose moiety.

Figure 3.
Synthesis of the Qβ-based oligomannose clusters

Figure 4. Synthesis of HIV-1 V1V2 glycopeptides corresponding to the epitopes of PG9 and PG16

a) a chemoenzymatic synthesis involving enzymatic transglycosylation; b) chemical synthesis involving native chemical ligation.