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Binding of High-Mannose-Type Oligosaccharides and Synthetic Oligomannose Clusters to Human Antibody 2G12: Implications for HIV-1 Vaccine Design

Lai-Xi Wang,* Jiahong Ni, Suddham Singh, and Hengguang Li
Institute of Human Virology
University of Maryland Biotechnology Institute
University of Maryland
Baltimore, Maryland 21201

Summary

Human antibody 2G12 broadly neutralizes human immunodeficiency virus type 1 (HIV-1) isolates and shows protective activity against viral challenge in animal models. Previous mutational analysis suggested that 2G12 recognized a novel cluster of high-mannose type oligosaccharides on HIV-1 gp120. To explore the carbohydrate antigen for HIV-1 vaccine design, we have studied the binding of 2G12 to an array of HIV-1 high-mannose type oligosaccharides by competitive ELISAs and found that Man_9GlNAc is 210- and 74-fold more effective than $Man_5GlcNAc$ and $Man_6GlcNAc$ in binding to 2G12. The results establish that the larger high-mannose oligosaccharide on HIV-1 is the favorable subunit for 2G12 recognition. To mimic the putative epitope of 2G12, we have created scaffold-based multivalent Man_9 clusters and found that the galactose-scaffolded bi-, tri-, and tetra-valent Man_9 clusters are 7-, 22-, and 73-fold more effective in binding to 2G12 than the monomeric $Man_9GlcNAc_2Asn$. The experimental data shed light on further structural optimization of epitope mimics for developing a carbohydrate-based HIV-1 vaccine.

Introduction

The worldwide epidemic of human immunodeficiency virus type 1 (HIV-1) urges the development of an effective HIV vaccine. Yet, it has been difficult to design effective immunogens that are able to elicit broadly neutralizing antibodies against HIV-1 primary isolates [1–3]. HIV-1 has evolved strategies such as frequent mutations of neutralizing epitopes, heavy glycosylations, conformational changes, and formation of envelope spikes to evade immune attacks. So far, only a few human monoclonal antibodies (MAbs) that neutralize a broad range of HIV-1 primary isolates in vitro have been identified. Neutralizing antibodies 2F5 and 4E10 were found to target epitopes on the inner-envelope glycoprotein gp41 [4–7], whereas neutralizing antibodies b12 and 2G12 recognize epitopes on the outer-envelope glycoprotein gp120 [8–12]. Passive immunization using these MAbs either alone or in combination has shown that the neutralizing antibodies protect against HIV-1 challenge in animal models when they are present at sufficient concentrations prior to or shortly after exposure [13, 14]. Therefore, further characterization of the neutralizing epitopes of these antibodies should provide valuable insights into HIV-1 vaccine design.

Among the broadly HIV-1-neutralizing antibodies so far identified, human monoclonal antibody 2G12 is the only one that directly targets the surface carbohydrate antigen of HIV-1. Several pieces of evidence suggest that the epitope of 2G12 is a unique cluster of high-mannose-type oligosaccharides (oligomannose) on HIV-1 gp120. Initial mutational studies indicated that the oligomannose sugar chains at the *N*-glycosylation sites N295, N332, N339, N386, N392, and N448 might be involved in 2G12 recognition [12]. Two recent studies further proposed that the epitope of 2G12 might consist of several Manα1-2Man-linked moieties contributed by the oligomannose sugar chains that form a unique cluster on gp120 at sites N295, N332, and N392 [15, 16]. Systematic mutational studies suggested that peptide portions of gp120 are not directly involved in the binding of 2G12 but serve primarily as a rigid scaffold to hold the oligomannose sugars in proximity to form a unique cluster [12, 15, 16]. Although high-mannose oligosaccharide moiety exists in some human glycoproteins, such a high-density, clustering oligomannose structure as that present on HIV-1 gp120 has not been found in any other human glycoproteins so far. Therefore, the unique carbohydrate antigenic structure on HIV-1 gp120 provides an ideal template for designing a vaccine that may generate HIV-neutralizing antibodies but will not raise cross-reactivity or autoimmune reactions in humans. As the first step toward a carbohydrate-based HIV-1 vaccine, we describe in this paper our studies on the binding of 2G12 to typical HIV-1 high-mannose-type oligosaccharides as well as synthetic oligomannose clusters that mimic the epitope of 2G12. We have found that $Man_9GlcNAc$ is the most efficient subunit for 2G12 binding among the high-mannose-type oligosaccharides tested. The synthetic tetravalent $Man_9GlcNAc$ cluster assembled on a galactose scaffold was found to inhibit the binding of 2G12 to gp120 more than 73-fold more effectively than the subunit $Man_9GlcNAc_2Asn$.

Results and Discussion

Binding of Homogeneous High-Mannose-Type Oligosaccharides to 2G12

A typical HIV-1 envelope glycoprotein gp120 bears 24 asparagine-linked glycans (N-glycans). Among them, 11 are high-mannose- and/or hybrid-type oligosaccharides, and 13 are complex-type oligosaccharides [17]. Structural analysis indicated that high-mannose-type oligosaccharides on HIV-1 gp120 are heterogeneous and range from Man_5 to Man_9 [18–20]. However, isolation of individual high-mannose oligosaccharides directly from HIV-1 gp120 is technically difficult. To evaluate the affinity of individual high-mannose-type glycoform for 2G12 interaction, we isolated three typical high-mannose-type oligosaccharides, namely $Man_5GlcNAc$, $Man_6GlcNAc$, and

*Correspondence: wangx@umbi.umd.edu

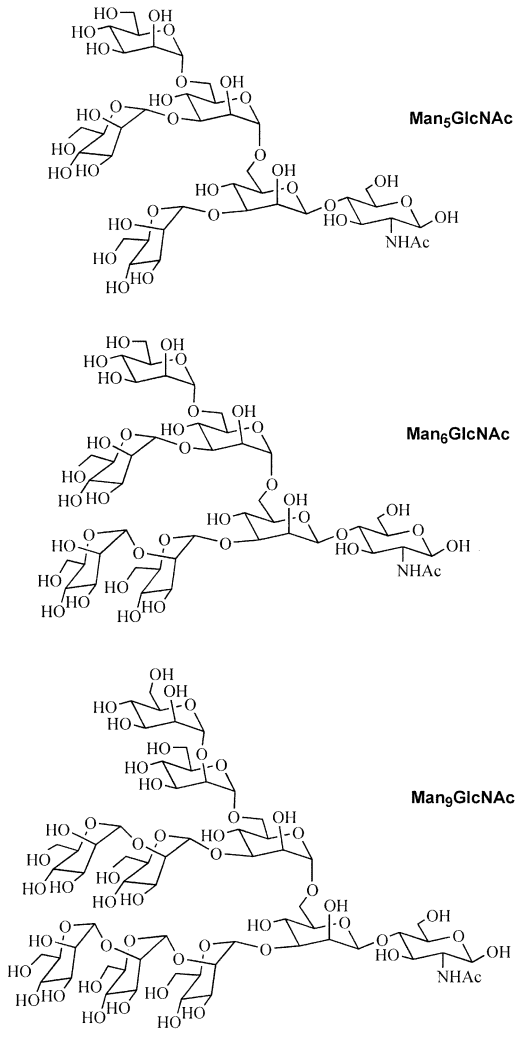

Figure 1. Structures of Typical HIV-1 High-Mannose-Type Oligosaccharides

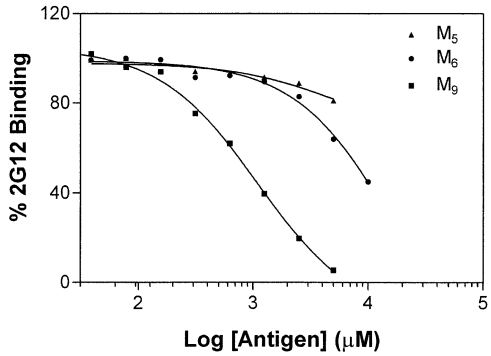

Figure 2. Inhibition of 2G12 Binding to gp120 by High-Mannose-Type Oligosaccharides

2G12 binding (percent) was plotted against the log of competing carbohydrate concentrations in micromolar units. Triangles indicate $Man_5GlcNAc$; solid circles indicate $Man_6GlcNAc$; and solid squares indicate $Man_9GlcNAc$.

$Man_9GlcNAc$ (Figure 1), with high purity from chicken ovalbumin and soybean agglutinin, respectively. The mixture of $Man_5GlcNAc$ and $Man_6GlcNAc$ obtained by sequential treatment of chicken ovalbumin with pronase and *Arthrobactor* endo-β-N-acetylglucosaminidase (Endo-A) was carefully separated on a Celite-Carbon chromatography to afford each oligosaccharide. Based on HPAEC-PED analysis, the $Man_5GlcNAc$ and $Man_6GlcNAc$ thus isolated are at least 98% pure without crosscontamination (data not shown). Similarly, ultrapure $Man_9GlcNAc$ was obtained through sequential digestion of soybean agglutinin with pronase and Endo-A and subsequent gel filtration on Sephadex G25 and reverse-phase HPLC purification.

The affinity of the purified high-mannose oligosaccharides was examined by competitive inhibition of 2G12 binding to immobilized gp120 (Figure 2). It was observed that the solubility of $Man_5GlCNAc$ and $Man_6GlcNAc$ in aqueous solution was unexpectedly low (less than 80 mM). As a result, the IC50 for $Man_5GlcNAc$ and $Man_6GlcNAc$ cannot be accurately determined. The IC_{50} (concentration for 50% inhibition) for $Man_9GlcNAc$ was found to be 0.96 mM, whereas the IC_{50} for $Man_6GlcNAc$ and $Man_5GlcNAc$ was estimated to be 70 and 200 mM, respectively. Therefore, the Man_9GlNAc is 74-fold and 210-fold more effective in inhibition of 2G12 binding than $Man_6GlcNAc$ and $Man_5GlcNAc$, respectively. On the other hand, it was also found that the two Man_9 structures, $Man_9GlcNAc$ and $Man_9GlcNAc_2Asn$, showed almost the same affinity for 2G12 binding (Table 1). This suggests that the GlcNAc-Asn moiety linking the oligosaccharide and the protein portion is not directly involved in the recognition with 2G12, which otherwise could not be revealed through mutagenesis studies. The much higher affinity of $Man_9GlcNAc$ to 2G12 than of $Man_5GlcNAc$ and $Man_6GlcNAc$ to 2G12 implies the importance of terminal Manα1,2Man linkages in antibody recognition. Our studies clearly indicate that antibody 2G12 preferably recognizes Man_9 moiety among the oligomannose glycoforms on HIV-1 gp120. In comparison, $Man_9GlcNAc$ contains three terminal Manα1,2Man linkages, and $Man_6GlcNAc$ contains one Manα1,2Man linkage, but $Man_5GlcNAc$ does not have any terminal Manα1,2Man linkage. It was previously reported that treatment of HIV-1 gp120 with mannosidases and high-mannose-specific endo-enzymes such as Endo H abolished the binding of gp120 to antibody 2G12, suggesting that the antibody binding is mannose dependent [15, 16]. Furthermore, treatment of gp120 with a special *Aspergillus saitoi* mannosidase, which selectively hydrolyzes Manα1,2Man linkage without removing Manα1,3 Man

Table 1. Carbohydrate Inhibition of 2G12 Binding to gp120

Carbohydrate Antigens	IC_{50} (mM)	Relative Affinity
$Man_5GlcNAc$	200 (estimated)	0.005
$Man_6GlcNAc$	70	0.014
$Man_9GlcNAc$	0.96	1.0
$Man_9GlcNAc_2Asn$	0.95	1.0
Man_9-dimer	0.40	2.4
Bi-Man_9	0.13	7.3
Tri-Man_9	0.044	22.0
Tetra-Man_9	0.013	73.0

or Manα1,6Man residues on gp120, also significantly reduced the binding of gp120 to 2G12, implying the importance of Manα1,2Man residue in the carbohydrate-antibody recognition [16]. Our direct binding studies with individual oligosaccharides clearly indicate that the larger high-mannose oligosaccharide $Man_9GlcNAc$, the affinity of which is 210- and 74-fold higher than that of $Man_5GlcNAc$ and $Man_6GlcNAc$, is the favorable subunit on gp120 for 2G12 recognition. Interestingly, another HIV-inactivating protein, cyanovirin-N, is also specific for larger high-mannose oligosaccharides such as Man_8 and Man_9 on gp120 [21, 22]. However, it should be pointed out that although 2G12 and cyanovirin-N are both specific for Man_9 structure on gp120 and show potent HIV-inactivating activity, cyanovirin-N binds to Man_9 with a dissociation constant at a nanomolar scale, whereas 2G12 binds to Man_9 only on a millimolar scale (Figure 2). It is clear that binding to a single subunit high-mannose oligosaccharide is not sufficient for 2G12 to neutralize HIV. It seems that 2G12 enhances its binding potency and specificity by recognizing a putative cluster of oligomannose sugar chains on gp120 [12, 15, 16]. This was further demonstrated by the fact that cyanovirin-N efficiently inhibits 2G12 binding to gp120, whereas 2G12 is not able to inhibit the binding of cyanovirin-N to gp120 [15, 16].

Design and Synthesis of Oligomannose Clusters as Mimics of 2G12 Epitope

Mutagenesis studies and carbohydrate analyses of 2G12 epitope suggested that 2G12 recognizes a unique oligomannose cluster contributed mainly from the high-mannose-type oligosaccharides at the glycosylation sites N295, N332, and N392, with other related oligosaccharides such as the glycans from N386, N339, or N448 on the flank of the clusters [12, 15, 16]. Our binding studies with an array of homogeneous high-mannose-type oligosaccharides have demonstrated that the Man_9 subunit is preferred over other HIV-1 high-mannose oligosaccharides for 2G12 recognition. Therefore, oligomannose Man_9 should be the "building block" of choice for creating mimics of the proposed epitope of 2G12. We reasoned that assembly of an oligomannose such as Man_9 on a suitable scaffold molecule should provide novel oligosaccharide clusters that may mimic or capture 2G12 epitope as present on HIV-1 gp120. To test the hypothesis, we synthesized bi-, tri- and tetra-valent Man_9 clusters based on a galactopyranoside scaffold (Figure 3). Compared to other types of molecules, monosaccharides have several advantages that help them serve as a scaffold. They have a rigid ring structure, possess multiple functionalities, and provide a defined three-dimensional spatial arrangement of substituents. When a galactopyranoside is used as the scaffold to present the oligosaccharides, the oligosaccharide chains being installed at the C-3, -4, and -6 positions will face up above the sugar ring to form a cluster, whereas the oligomannose sugar chain at position C-2 is likely to be located on the flank of the cluster. We expect that this arrangement will at least partially mimic the spatial orientation of the carbohydrate epitope of antibody 2G12. Based on the reported structure of gp120 core with remodeled N-glycans [23], the distances between the asparagine (Asn) side chains of the pairs N295-N332, N332-N392, and N295-N392 are estimated to be 5.8, 20.3, and 23.6 Å, respectively. We have remodeled a $Man_9GlcNAc_2Asn$ moiety on the previously synthesized galactose-based maleimide cluster [24] (MC-1, Figure 3) and found that the maleimide cluster can host four $Man_9GlcNAc_2Asn$ moieties, in which the distances among the Asn residues are in the range of 8–30 Å (data not shown). Therefore, the galactose-based maleimide cluster should be a reasonable starting point for constructing the desired oligomannose clusters. If the first set of synthetic oligosaccharide clusters were to show promising 2G12 binding properties, improved mimics could be readily achievable by changing the length of spacers on each arm and by reconfiguring the orientation of the sugar chains by using a different scaffold.

The key step in the synthesis is the chemoselective maleimide cluster-thiol ligation reaction, which we have recently exploited for the synthesis of large multivalent peptides and glycoconjugates [24, 25]. To introduce a sulfhydryl (SH) tag into the oligomannose moiety, we first acylated the free amino group in $Man_9GlcNAc_2Asn$ with N-succinimidyl S-acetylthioacetate (SATA) [26] to give the N-(S-acetyl-thioacetyl) derivative. The S-acetyl group was then removed selectively by treatment with hydroxylamine to afford the SH-containing oligosaccharide $Man_9GlcNAc_2Asn$-Ac-SH. The synthesis of the tetravalent maleimide cluster MC-1 was previously reported. We synthesized the bi- and trivalent maleimide clusters MC-2 and MC-3 (Figure 3) in a similar way by starting with modified galactoside scaffold (details of the synthesis will be reported elsewhere). Chemoselective ligation between the $Man_9GlcNAc_2Asn$-Ac-SH and the maleimide cluster MC-1 was performed in a phosphate buffer (pH 6.6). HPLC monitoring indicated that the ligation was quantitative and was complete within 1 hr at room temperature to give the desired product, Tetra-Man_9 (Figure 3). Reverse-phase HPLC purification gave the tetravalent oligomannose cluster Tetra-Man_9 in 81% yield. The structure of Tetra-Man_9 was characterized by electron spray ionization mass spectroscopy (ESI-MS) (Figure 4). The ESI-MS spectrum revealed typical signals at 2435.35 $(M + 4H)^{4+}$, 2395.15 $(M - Man + 4H)^{4+}$, 1948.28 $(M + 5H)^{5+}$, 1915.85 $(M - Man + 5H)^{5+}$, and 1883.75 $(M - 2Man + 5H)^{5+}$, which are in agreement with the structure. Similarly, the bi- and trivalent Man_9 clusters Bi-Man_9 and Tri-Man_9 were synthesized through ligation of $Man_9GlcNAc_2Asn$-Ac-SH with the maleimide clusters MC-2 and MC-3, respectively. On the other hand, a dimer of $Man_9GlcNAc_2Asn$ was prepared through oxidation of $Man_9GlcNAc_2Asn$-Ac-SH to give the Man_9 dimer (Figure 3). It should be noted that many glycoclusters were synthesized in recent years for studying the multivalent interaction and clustering effects in carbohydrate-protein recognition, but only a few involve the synthesis of homogeneous, structurally defined glyco clusters of large oligosaccharides [27–31]. The method described here should be equally applicable for synthesizing various oligosaccharide clusters to probe the spatial requirement of subunit oligosaccharides in antibody recognition.

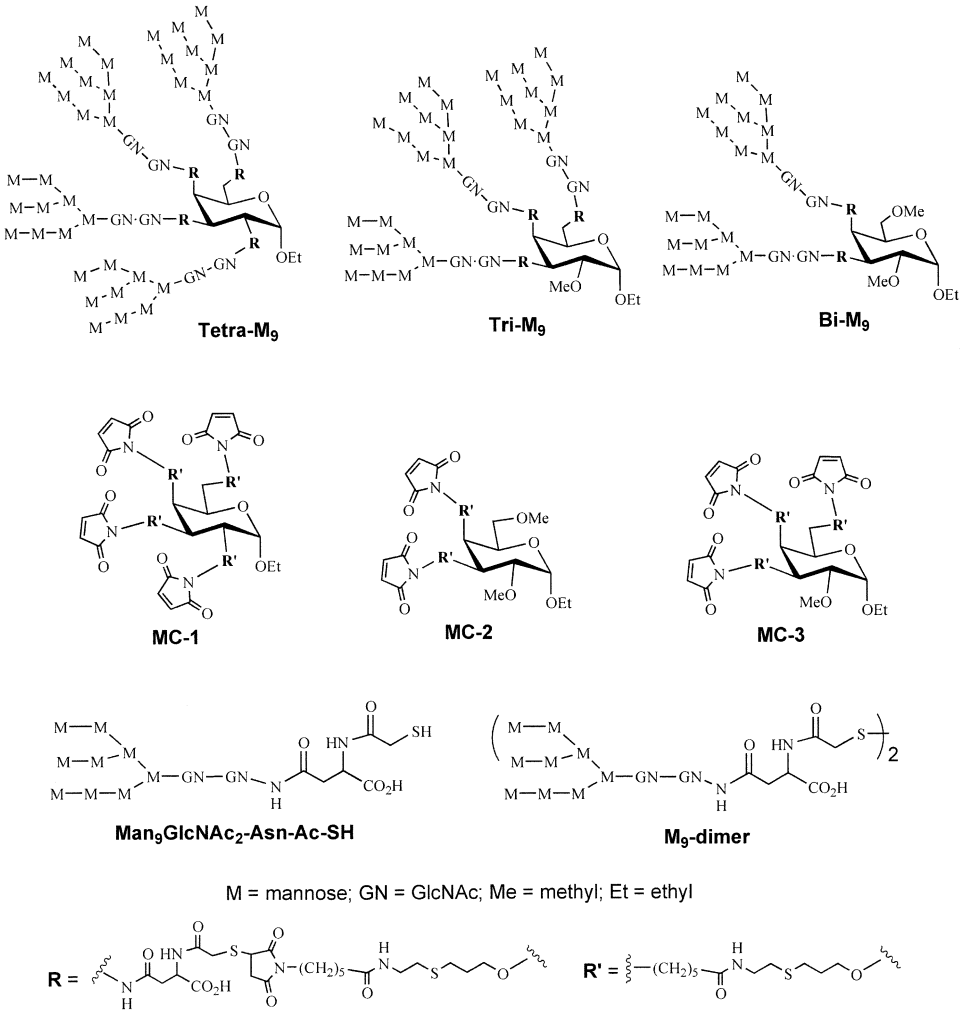

Figure 3. Structures of Galactose-Based Mmaleimide Clusters and Synthetic Oligomannose Clusters

Binding of the Synthetic Man_9 Clusters to 2G12

The synthetic Man_9 clusters were examined for competitive inhibition of 2G12 binding to immobilized gp120 (Figure 5). A significant clustering effect was observed for the Man_9 clusters in the binding studies. If IC_{50} is taken as an indication for relative affinity (Table 1), the Tetra-Man_9 was found to inhibit 2G12 binding to gp120 73-fold more effectively than the monomeric $Man_9GlcNAc_2Asn$ does on a molar basis. On the other hand, the tri- and bivalent oligomannose clusters are 22- and 7-fold more effective than the monomeric $Man_9GlcNAc_2Asn$ in inhibition of 2G12 binding to gp120. The enhanced affinity for the clusters with higher valency suggests that antibody 2G12 may have multiple binding sites for the carbohydrate antigen. During the preparation of this manuscript, Calarese et al. [32] reported the crystal structures of Fab 2G12 and its complex with oligosaccharide $Man_9GlcNAc_2$. The X-ray structural studies revealed that two Fab fragments in 2G12 assemble into an extraordinary V_H domain-swapped dimer, in which the V_H domain is exchanged with V'_H domain to interact with V'_L and vice versa. This unusual configuration of antibody domains provides an extended surface (e.g., the new V_H and V'_H interface) and creates new binding sites for high-affinity multivalent interaction with a conserved cluster of oligomannose sugars on gp120. The crystal structure suggested that a total of four high-mannose oligosaccharide chains could be bound to the domain-exchanged Fab dimer [32]. The new crystal structure, together with molecular modeling studies, further suggested that an oligomannose cluster composed of sugar chains on N-glycosylation sites N332, N339, and N392 was best fit for the binding to 2G12 [32]. Our observed enhancement in 2G12 binding for the higher-valent oligomannose clusters also suggests the existence of additional binding sites on 2G12 for carbohydrate antigen. Another interesting finding in our binding studies came from the two bivalent oligomannose compounds, Bi-Man_9 and Man_9 dimer. The Bi-Man_9 is 3-fold more effective than Man_9 dimer in inhibition of 2G12 binding to gp120. The results suggest that the control of geometry and distance between the subunits is important for a tight, multivalent interaction between the carbohydrate antigen and the antibody. However, it should be noted that the relative affinity of the current synthetic clusters is still much lower than the affinity of

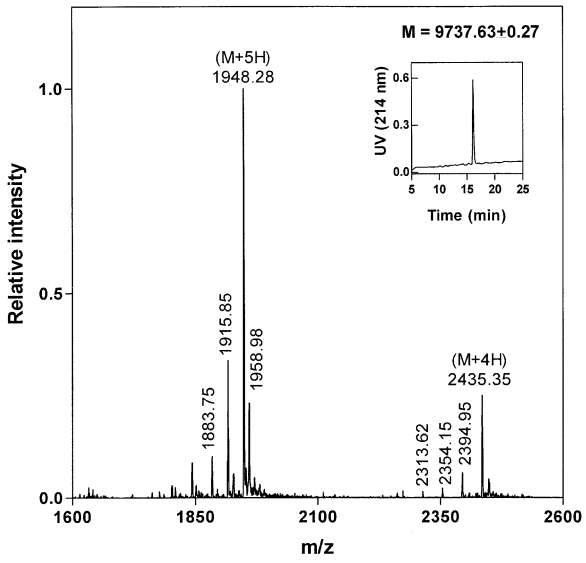

Figure 4. The HPLC and ESI-MS Profiles of the Synthetic Tetravalent Oligomannose Cluster Tetra-Man_9

gp120 for 2G12. The IC_{50} for the best mimic, Tetra-Man_9, is at *micromolar* level, whereas the IC_{50} for gp120 is in the middle of the *nanomolar* range in our assay (data not shown). Therefore, the present study provides the first-generation synthetic oligosaccharide clusters in an attempt to mimic the putative epitope of 2G12 for HIV vaccine design. Apparently, the logical next step should optimize the spatial orientation of oligomannose sugar chains by changing the length of spacers, as well as the rigidity and configuration of the scaffold. The availability of the crystal structure of 2G12 now will facilitate more rational design of template-assembled oligomannose clusters. Finally, because the synthetic oligomannose clusters themselves are likely to be poorly immunogenic, they will be conjugated to a carrier protein or to a universal T-helper epitope to provide a functional immunogen. Work along this line is in progress and will be reported in due course.

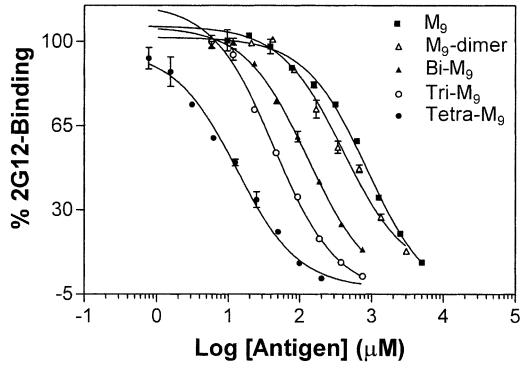

Figure 5. Inhibition of 2G12 Binding to gp120 by Synthetic Oligomannose Clusters

2G12 binding (percent) was plotted against the log of competing carbohydrate concentrations in micromolar units. Solid diamonds indicate $Man_9GlcNAc_2Asn$; open triangles indicate Man_9-dimer; solid triangles indicate Bi-Man_9; open circles indicate Tri-Man_9; and solid circles indicate Tetra-Man_9.

Significance

It has been proposed that the broadly neutralizing antibody recognizes a unique cluster of high-mannose oligosaccharides on HIV-1 gp120 [12, 15, 16]. The unique oligomannose cluster formed on the so-called immunologically "silent face" of gp120, which was not found on normal human glycoproteins, may be regarded as "non-self" and, therefore, provides an attractive target for HIV-1 vaccine design. However, HIV-1 gp120 expresses heterogeneous high-mannose oligosaccharides ranging from Man_5 to Man_9 on these glycosylation sites [18–20]. The diverse oligomannose glycoforms of HIV-1 gp120 may dilute any potential immune response to the epitope. This may partially explain why gp120 itself can hardly raise 2G12-like antibodies. As an initial step toward the design of a vaccine that mimics 2G12's epitope, we first studied the binding of an array of homogeneous high-mannose oligosaccharides with 2G12 and found that the Man_9 structure is the best subunit for 2G12 recognition, implying the structural importance of terminal Manα1,2Man linkages in 2G12's epitope. To mimic the clustering epitope of 2G12, we then designed and synthesized several structurally defined oligomannose clusters based on a galactose scaffold. Our binding studies with the synthetic oligomannose clusters revealed an apparent clustering effect of the oligomannose clusters, with the higher-valent clusters binding to 2G12 better than the lower-valent oligomannose clusters. The enhanced affinity for the clusters with higher valency suggests that antibody 2G12 may have multiple binding sites for the carbohydrate antigen. Coincidently, the most recently solved X-ray structure of 2G12 reveals that 2G12 creates additional binding sites for multivalent interaction with the carbohydrate antigen by taking an extraordinary, V_H domain-swapped antibody structure [32]. The first generation of synthetic oligomannose clusters described in this paper serves as a basis for further optimization of synthetic mimics of 2G12 epitope. Conjugation of the synthetic epitope mimics to a carrier protein should provide a functional immunogen that may raise neutralizing antibodies specific for the high-mannose oligosaccharides on gp120, which may eventually lead to a carbohydrate-based synthetic vaccine for HIV-1.

Experimental Procedures

Materials

Monosaccharides, pronase, Sephadex, trifluoroacetic acid, and reagents for ELISAs and buffers were purchased from Sigma-Aldrich and used as received. N-succinimidyl S-acetylthioacetate was from Pierce Chemical Company. HPLC grade acetonitrile was purchased from Fisher Scientific. The immobilized endo-β-N-acetyl-glucosaminidase from *Arthrobactor* (Endo-A) was overproduced and purified according to the literature [33].

High-Performance Liquid Chromatography

Unless otherwise specified, analytical high-performance liquid chromatography (HPLC) was carried out on a Waters 626 HPLC instrument under the following conditions: column, Waters Nova-Pak C18

(3.9 × 150 mm); temperature, 40°C; and flow rate, 1 ml/min. The column was eluted with a linear gradient of acetonitrile (0%–50%) containing 0.1% TFA in 25 min with UV detection at 214 nm. Preparative HPLC was performed on a Waters 600 HPLC instrument with a preparative C18 column (Waters Symmetry 300, 19 × 300 mm). The column was eluted with a suitable gradient of water-acetonitrile containing 0.1% TFA.

High-Performance Anion Exchange Chromatography Coupled with Pulsed Electrochemical Detection

The analytical anion-exchange chromatography was performed on a Dionex DX600 chromatography system (Dionex Corporation, Sunnyvale, CA) equipped with an electrochemical detector (ED50, Dionex Corporation, Sunnyvale, CA). The following conditions were used: column, CarboPac-PA1 (4 × 250 mm); eluent A, 0.1 M NaOH; eluent B, 1 M sodium acetate (NaOAc) in 0.1 M NaOH; gradient: 0–5 min, 0% B; 5-25 min, 0%–15% B; flow rate, 1 ml/min.

Competitive Enzyme-Linked Immunosorbent Assays

We performed competitive ELISAs to determine the relative inhibition potency of various carbohydrate antigens against the binding of 2G12 to gp120. Microtiter plates were coated with human cell line 293-expressed HIV-1_{IIIB} gp120 (100 ng/ml) overnight at 4°C. After plates were washed, nonspecific binding was blocked with 5% BSA in PBS for 1 hr at room temperature. The plates were then washed three times with 0.1% Tween-20/PBS. Serial dilutions (1:2) of various carbohydrate antigens were mixed with an equal volume of mAb 2G12 (fixed final concentration of 5 ng/ml) and added to the plates. The plates were incubated for 1 hr at 37°C and washed with washing buffer. To the plates was added a 100 μl solution of 1:3000 diluted horseradish peroxidase-conjugated goat anti-human IgG in 0.5% BSA/PBS. After incubation for 1 hr at 37°C, the plates were washed again, and a 100 μl solution of 3,3′,5,5′-tetramethyl benzidine (TMB) was added. Color was allowed to develop for 5 min, and the reaction was quenched by the addition of a 100 μl solution of 0.5 M H_2SO_4 to each well. The optical density was then measured at 450 nm.

Preparation of Homogeneous High-Mannose-Type Oligosaccharides

$Man_9GlcNAc_2Asn$ and $Man_9GlcNAc$ were prepared by enzymatic digestion of soybean agglutinin and subsequent chromatographic purification. Crude soybean agglutinin (3.2 g) was obtained from 500 g of soybean flour (Sigma) through fractional precipitation with ammonium sulfate and digested thoroughly with pronase (2 × 15 mg, Sigma) according to the literature [34]. The digestion was filtered, and the filtrate was lyophilized. The residue was loaded onto a column (1.5 × 70 cm) of Sephadex G50 (Sigma), which was preequilibrated and eluted with 0.1 M AcOH. The fractions containing $Man_9GlcNAc_2Asn$ were pooled and lyophilized. The material was finally purified by reverse-phase HPLC to afford homogeneous $Man_9GlcNAc_2Asn$ (55 mg) as a white powder after lyophilization. Treatment of $Man_9GlcNAc_2Asn$ (20 mg) with immobilized *Arthrobactor* endo-β-N-acetylglucosaminidase (Endo-A) in an acetate buffer (pH 6.0), and subsequent gel filtration on a column (1.5 × 50 cm) of Sephadex G25 gave pure $Man_9GlcNAc$ (12 mg), which was characterized by compositional analysis, HPLC, and electron spray ionization mass spectrometry (ESI-MS).

Homogeneous $Man_5GlcNAc$ and $Man_6GlcNAc$ were obtained from pronase digestion of chicken ovalbumin and subsequent chromatographic purification. Chicken ovalbumin (Sigma) was digested with pronase to provide a crude mixture of Man_5- and Man_6-containing glycopeptides, according to the literature [35]. A crude glycopeptide (350 mg) was treated with immobilized Endo-A to release $Man_5GlcNAc$ and $Man_6GlcNAc$ as a mixture. The two oligosaccharides were then separated by chromatography on a column (1 × 125 cm) of Celite-Charcoal (1:1, w/w), which was eluted by a gradient of 0%–20% aqueous ethanol to give pure $Man_5GlcNAc$ (25 mg) and pure $Man_6GlcNAc$ (30 mg). The purity of the above isolated oligosaccharides was confirmed by HPAEC-PED, and their identity was characterized by ESI-MS.

$Man_9GlcNAc_2Asn$

HPAEC-PED, t_R 17.1 min; ESI-MS: calculated for $C_{74}H_{124}N_4O_{58}$: 1997.77. Found: 1998.73 $(M + H)^+$, 999.69 $(M + 2H)^{2+}$, 918.65 $(M - Man + 2H)^{2+}$, 837.68 $(M - 2Man + 2H)^{2+}$, 756.70 $(M - 3Man + 2H)^{2+}$, 675.52 $(M - 4Man + 2H)^{2+}$, 594.61 $(M - 5Man + 2H)^{2+}$.

$Man_9GlcNAc$

HPAEC-PED, t_R 16.9 min; ESI-MS, calcd. for $C_{62}H_{105}NO_{51}$: 1679.57. Found: 1680.80 $(M + H)^+$, 1518.64 $(M - Man + H)^+$, 1356.72 $(M - 2Man + H)^+$, 1194.54 $(M - 3Man + H)^+$, 1032.60 $(M - 4Man + H)^+$, 841.36 $(M + 2H)^{2+}$.

$Man_6GlcNAc$

HPAEC-PED, t_R 15.9 min; ESI-MS, calculated for $C_{44}H_{75}NO_{36}$: 1193.41. Found: 1216.84 $(M + Na)^+$, 1194.81 $(M + H)^+$, 608.99 $(M + 2Na)^{2+}$.

$Man_5GlcNAc$

HPAEC-PED, t_R 15.3 min; ESI-MS, calculated for $C_{38}H_{65}NO_{31}$: 1031.35. Found: 1054.70 $(M + Na)^+$, 1032.79 $(M + H)^+$, 528.07 $(M + 2Na)^{2+}$.

Preparation of the SH-Tagged Man_9 Oligosaccharide

A solution of N-succinimidyl S-acetylthioacetate [26] (22 mg) in acetonitrile (0.5 ml) was added to a solution of $Man_9GlcNAc_2Asn$ (32 mg) in a phosphate buffer (3 ml, pH 7.4) containing 20% acetonitrile. The mixture was stirred at room temperature for 1 hr and lyophilized. The product was purified by reverse-phase HPLC to give the N-(S-acetyl-thioacetyl) $Man_9GlcNAc_2Asn$ derivative (26 mg): analytical HPLC (gradient: 0%–30% acetonitrile containing 0.1% TFA in 25 min; flow rate, 1 ml/min), t_R 6.3 min; ESI-MS, 2114.55 $(M + H)^+$, 1057.66 $(M + 2H)^{2+}$, 976.55 $(M - Man + 2H)^{2+}$, 895.45 $(M - 2Man + 2H)^{2+}$, 815.54 $(M - 3Man + 2H)^{2+}$, 733.43 $(M - 4Man + 2H)^{2+}$, 652.39 $(M - 5Man + 2H)^{2+}$, 571.34 $(M - 6Man + 2H)^{2+}$]. A solution of the N-(S-acetyl-thioacetyl) derivative (20 mg) in a phosphate buffer (2 ml, 50 mM, pH 7.4) containing hydroxylamine (50 mM) was stirred at room temperature for 2 hr, and the De-S-acetylated product was directly purified by reverse-phase HPLC to give the SH-tagged oligosaccharide $Man_9GlcNAc_2Asn$-Ac-SH (15 mg), which was characterized by HPLC and ESI-MS. Analytical HPLC (gradient: 0%–30% acetonitrile containing 0.1% TFA in 25 min, flow rate, 1 ml/min), t_R 2.7 min; ESI-MS, 2072.56 $(M + H)^+$, 1036.71 $(M + 2H)^{2+}$, 955.68 $(M - Man + 2H)^{2+}$, 874.71 $(M - 2Man + 2H)^{2+}$, 793.66 $(M - 3Man + 2H)^{2+}$, 712.56 $(M - 4Man + 2H)^{2+}$, 631.51 $(M - 5Man + 2H)^{2+}$, 550.66 $(M - 6Man + 2H)^{2+}$.

Synthesis of Tetravalent Oligomannose Cluster

To a solution of $Man_9GlcNAc_2Asn$-Ac-SH (7.60 mg, 3.67 μmol) in a phosphate buffer (pH 6.6, 50 mM, 1.2 ml) was added a solution of the galactose-based maleimide cluster MC-1 (0.67 mg, 0.46 μmol) in acetonitrile (0.8 ml). The mixture was gently shaken at room temperature under nitrogen atmosphere for 1 hr. The mixture was then lyophilized. The ligation product was purified by reverse-phase HPLC to afford Tetra-Man_9 (3.60 mg, 81%). Analytical HPLC, t_R, 16.1 min; ESI-MS, 2435.35 $(M + 4H)^{4+}$, 2395.15 $(M - Man + 4H)^{4+}$, 1948.28 $(M + 5H)^{5+}$, 1915.85 $(M - Man + 5H)^{5+}$, and 1883.75 $(M - 2Man + 5H)^{5+}$, which are in agreement with its structure.

Synthesis of Trivalent Oligomannose Cluster

The trivalent maleimide cluster MC-3 (1.0 mg) and $Man_9GlcNAc_2Asn$-Ac-SH (8.0 mg) were reacted in the same way as for the preparation of Tetra-Man_9. The ligation product was purified by reverse-phase HPLC to give the Tri-Man_9 (7.3 mg, 82%). Analytical HPLC, t_R, 15.5 min; ESI-MS: 2457.90 $(M+3H)^{3+}$, 1843.64 $(M + 4H)^{4+}$, 1802.92 $(M - Man+4H)^{4+}$, 1762.68 $(M - 2Man + 4H)^{4+}$, 1722.10 $(M - 3Man + 4H)^{4+}$, 1681.79 $(M - 4Man + 4H)^{4+}$.

Synthesis of Bivalent Oligomannose Cluster

The bivalent maleimide cluster MC-2 (1.3 mg) and $Man_9GlcNAc_2Asn$-Ac-SH (9.4 mg) were reacted in the same way as for the preparation of Tetra-Man_9. The ligation product was purified by reverse-phase HPLC to give Bi-Man_9 (6.1 mg, 80%). Analytical HPLC, t_R, 15.4 min; ES-MS, 2502.12 $(M+2H)^{2+}$, 1668.22 $(M + 3H)^{3+}$, 1614.19 $(M - Man + 3H)^{3+}$, 1560.24 $(M - 2Man + 3H)^{3+}$, 1506.01 $(M - 3Man + 3H)^{3+}$, 1452.54 $(M - 4Man + 3H)^{3+}$.

Preparation of Man_9 Dimer

$Man_9GlcNAc_2Asn$-Ac-SH (8 mg) was dissolved in a phosphate buffer (2 ml, 50 mM, pH 7.5), and air was bubbled into the solution for 10 min. The solution was kept at room temperature overnight. The oxidized product thus formed was purified by reverse-phase HPLC to give the Man_9 dimer (5.6 mg). Analytical HPLC (gradient: 0%–30% acetonitrile containing 0.1% TFA in 25 min; flow rate, 1 ml/min), t_R 5.3 min; ESI-MS, 2072.0 $(M + 2H)^{2+}$, 1381.6 $(M + 3H)^{3+}$, 1327.5 $(M - Man + 3H)^{3+}$, 1273.4 $(M - 2Man + 3H)^{3+}$, 1219.45 $(M - 3Man + 3H)^{3+}$, 1165.41 $(M - 4Man + 3H)^{3+}$, 1111.2 $(M - 5Man + 3H)^{3+}$.

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