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A Bacterial Lipooligosaccharide that Naturally Mimics the Epitope of the HIV-Neutralizing Antibody 2G12 as a Template for Vaccine Design

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SUMMARY

The broadly neutralizing antibody 2G12 binds a fairly conserved cluster of oligomannose sugars on the HIV surface glycoprotein gp120, which has led to the hypothesis that these sugars pose potential vaccine targets. Here, we present the chemical analysis, antigenicity, and immunogenicity of a bacterial lipooligosaccharide (LOS) comprised of a manno-oligosaccharide sequence analogous to the 2G12 epitope. Antigenic similarity of the LOS to oligomannose was evidenced by 2G12 binding to the LOS and the inability of sera elicited against synthetic oligomannosides, but incapable of binding natural oligomannose, to bind the LOS. Immunization with heat-killed bacteria yielded epitope-specific serum antibodies with the capacity to bind soluble gp120. Although these sera did not exhibit specific anti-HIV activity, our data suggest that this LOS may find utility as a template for the design of glycoconjugates to target HIV.

INTRODUCTION

One of the greatest scientific hurdles still facing the development of an effective HIV vaccine is the design of an immunogen that elicits antibodies with the capacity to neutralize the infectivity of an antigenically diverse range of HIV strains (Burton et al., 2004; Johnston and Fauci, 2008). The target for HIV-neutralizing antibodies (nAbs) is the virus envelope spike, a trimeric complex formed by heterodimers of the glycoprotein subunits gp120 and gp41. Gp120, the larger of the two subunits, is the main target for nAbs. Roughly 50% of gp120's molecular mass is comprised of carbohydrate imparted by the host cell glycosylation machinery. Normally, such carbohydrates would not be expected to be sensed as foreign by the host immune system, except that their dense clustering on gp120 is atypical of host glycosylation (Wyatt et al., 1998). Indeed, clustered carbohydrates on gp120 are of interest for HIV vaccine design because of the description of the broadly neutralizing human monoclonal antibody (mAb) 2G12 (Trkola et al., 1996) and more recently of additional broadly neutralizing anticarbohydrate mAbs (Walker et al., 2011). 2G12 binds a specific cluster of oligomannose moieties (Sanders et al., 2002; Scanlan et al., 2002; Trkola et al., 1996) located within a ~1,000 $Å^2$ surface on gp120 covered densely with host-derived carbohydrates that protect the underlying protein surface from NAb recognition (Wyatt et al., 1998). Crystal structures of 2G12 in complex with various synthetic carbohydrates show that 2G12 binds preferentially to the distal α-D-Man-(1→2)- α-D-Man disaccharide found in the so-called D1 arm of oligomannose (Figure 1; Calarese et al., 2003, 2005; Lee et al., 2004).

Given that 2G12 binds an oligomannose cluster, the general strategy aimed at eliciting 2G12-like antibodies has been to immunize with antigenic clusters of oligomannose that are supposed to mimic the carbohydrate-covered gp120 surface (reviewed in Astronomo and Burton, 2010). Attempts to elicit 2G12-like antibodies have relied largely on glycochemistry based designs, ranging from simplified linear tetrasaccharide structures that encompass the 2G12 core mannosyl epitope to the presentation of branched oligomannosides that mimic those present on HIV gp120 (Astronomo et al., 2008, 2010; Doores et al., 2010c; Joyce et al., 2008; Li and Wang, 2004; Ni et al., 2006; Wang et al., 2004, 2007). Although several of these neoglyconjugates are recognized by 2G12, none so far have elicited carbohydrate-specific antibodies capable of binding recombinant gp120 or neutralizing HIV. A second strategy to elicit 2G12-like nAbs has been to utilize genetically engineered *Saccharomyces cerevisiae*, expressing mainly Man8 oligomannose (Agrawal-Gamse et al., 2011; Dunlop et al., 2010; Luallen et al., 2008, 2010; Scanlan et al., 2007). Whereas antibodies capable of binding monomeric gp120 have been reported upon immunization with engineered yeast cells (Agrawal-Gamse et al., 2011; Dunlop et al., 2010), only in one of these studies did the elicited antibodies display statistically significant, albeit very modest, neutralization of HIV-1 compared to control immune sera (Dunlop et al., 2010).

The limited success of current strategies aimed at eliciting 2G12-like antibodies prompted us to search for potential alternative avenues. Carbohydrate conjugate vaccines for protection

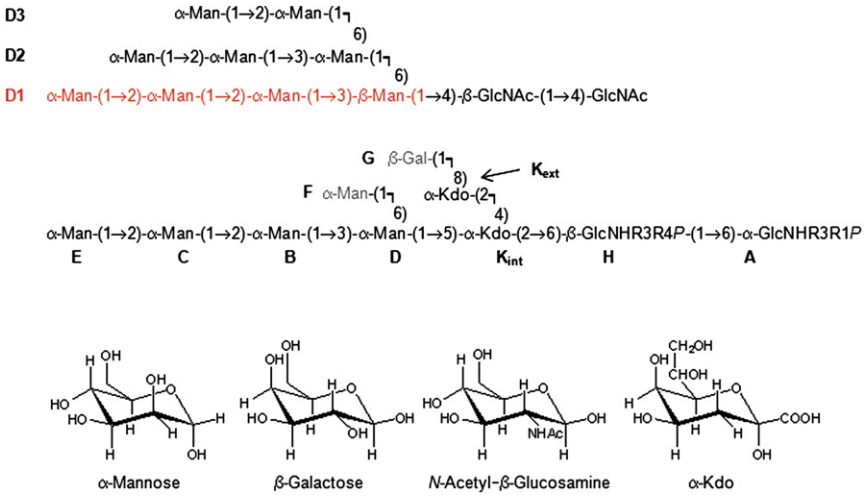

Figure 1. Chemical Structures of Oligomannose (Man9) and the Carbohydrate Backbone of *R. radiobacter* Rv3 LOS

(Top) Oligomannose Man9, with the three mannose oligosaccharide branches referred to as D1, D2, and D3 indicated on the left. All monosaccharides are D configured and in the pyranose form. 2G12 binds the D1 arm (orange-red highlight) preferentially (Calarese et al., 2003 and 2005).

(Middle) Rv3 LOS backbone structure. All monosaccharides are D configured and in the pyranose form. Residue labeling reflects the letters used during NMR attribution (Tables 1 and S1). This structure represents the major component of the LOS fraction, namely, OS1. The minor component of the LOS fraction (OS2) lacks the gray-colored residues. Note that the residue sequence (E)-(C)-(B)-(D) is analogous to the D1 arm of Man9 oligomannose. Furthermore, α-mannose residue (F) is (1→6)-linked to residue (D), which resembles the branching of the D2 arm of oligomannose. The generic substituents designated R on residues (A) and (H) represent the fatty acids of the lipid A moiety on Rv3 LOS.

(Bottom) Chemical structures of the monosaccharides found in Rv3 LOS.

against bacterial infections have proven very efficacious (reviewed in Astronomo and Burton, 2010; Finn, 2004; Lucas et al., 2010; Vliegenthart, 2006); for example, capsule-derived carbohydrates coupled to appropriate carrier proteins can provide durable protection against encapsulated bacteria (Blanchard-Rohner and Pollard, 2011; Heath, 2011). We reasoned that a bacterial polysaccharide with antigenic similarity to the 2G12 epitope might prove a better way to elicit the anticarbohydrate nAbs to HIV-1.

Here, we report on such a bacterial lipooligosaccharide (LOS) in which the epitope of 2G12 occurs naturally. Our investigation was prompted by knowledge of the chemical structure of the lipooligosaccharide (LOS) from the soil bacterium *Rhizobium radiobacter* Rv3 (De Castro et al., 2008), referred to here as simply Rv3. The detailed chemical analysis of the Rv3 LOS revealed that its carbohydrate backbone consists of a unique tetramannose segment that is analogous to the D1 arm of oligomannose (Figure 1). We found that 2G12 binds with reasonable affinity to purified Rv3 LOS and even stronger to Rv3 bacterial cells. These observations led us to examine whether immunization with heat-killed Rv3 bacteria might result in anticarbohydrate antibodies capable of neutralizing HIV. The elicited antibodies bound the core 2G12 epitope and monomeric gp120 but failed to exhibit significant anti-HIV neutralizing activity. Nevertheless, our data suggest that the unique presentation of an antigenic analog of the 2G12 epitope in Rv3 LOS may provide an alternate avenue for the design of glycoconjugates aimed at eliciting nAbs to the dense array of oligomannose on HIV-1.

RESULTS

Rv3 LOS Contains a Tetramannose Backbone Analogous to the D1 Arm of Mammalian Oligomannose

Bacterial LOS was sequentially extracted from dried bacterial cells in accordance with the petroleum ether chloroform phenol (PCP) and the hot phenol/water methods (Galanos et al., 1969; Westphal and Jann, 1965), and a pure LOS fraction was obtained from the PCP extract. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the LOS fraction gave a mobility profile characteristic of lipooligosaccharide species (Hitchcock and Brown, 1983). Combining the information from monosaccharide composition and methylation analysis, we determined that the LOS fraction contained mainly terminal, 2- and 3,6 substituted D-mannose, terminal D-galactose and minor amounts of 3-substituted D-mannose; 2-keto-3-deoxy-D-*manno*-octulosonic acid (Kdo) and phosphorylated glucosamine were not detected with the methylation protocol used. Fatty acids analysis showed the presence of C14:0 (3-OH), C16:0 (3-OH), C18:1 (3-OH), and C28:0 (27-OH)—lipids that are distinctive for this bacterial family (Silipo et al., 2004). The Rv3 LOS extracted from *R. radiobacter* Rv3 was completely delipidated, and the resulting oligosaccharide mixture purified by high-performance anion-exchange chromatography (HPAEC). Two oligosaccharide species, OS1 (~70%) and OS2 (~30%), were separated and their chemical structures determined by nuclear magnetic resonance (NMR).

NMR Spectroscopical Assigment of Rv3 LOS

The complete assignment of ^{1}H and ^{13}C resonances of oligosaccharides OS1 and OS2 was achieved by combining data obtained from 2D homo- and heteronuclear spectra (Figure 2A). For OS1 spectra attribution (Table 1; Figure 2B), the eight anomeric protons were labeled with a capital letter (A–H) in decreasing order of chemical shifts. The two couples of the diastereotopic methylene signals observed in the high-field region of the proton spectrum denoted the presence of two 2-keto-3-deoxy-D-*manno*-octulosonic acid (Kdo) residues, K_{int} and K_{ext}. Thus, a decasaccharide structure was identified for the OS1 species of the deacylated Rv3 LOS.

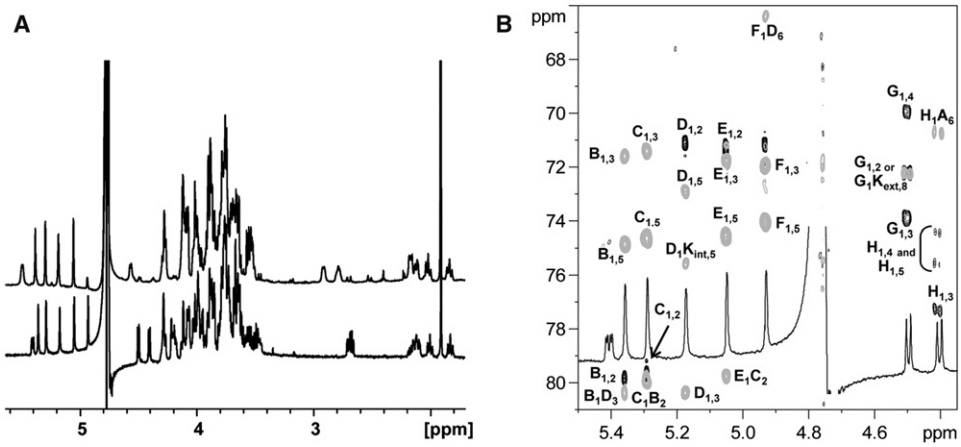

Figure 2. Spectroscopic Analysis of Rv3 Oligosaccharides Reveals a Carbohydrate Backbone that Is Analogous to the D1 Arm of Oligomannose

(A) Proton spectra of Rv3 OS1 (bottom; in 600 μl of NaOD 10 mM) and OS2 (top; in 600 μl of D_2O) measured at 600 MHz, 303 K.

(B) Attribution of anomeric region of the gHSQCTOCSY (black) and gHMBC (gray) spectra measured for OS1 (600 MHz, 303 K, 10 mM NaOD). Differently from TOCSY spectrum, magnetization propagation was not observed for GlcN1*P* residue A.

The anomeric protons of residues A and H showed six different TOCSY correlations, and on the basis of their ring, proton and carbon resonances were recognized as the two GlcN moieties of the lipid A moiety of the oligosaccharide. For residues B–F, the TOCSY and gHSQCTOCSY spectra of the anomeric region proved problematic for establishing connectivity after the H-2 proton because of the small $^3J_{H1,H2}$ and $^3J_{H2,H3}$ coupling constants values (Figure 2B). However, magnetization was propagated from the H-2 proton up to the H-6 s. Based on the value displayed from their *C*-5 residues, B–F were determined as

Table 1. ^{1}H- and ^{13}C NMR Spectral Assignments for the OS1 Oligosaccharide of *R. radiobacter* Rv3

	1	2	3	4	5	6
6)-α-GlcN1*P*	5.41	2.70	3.62	3.49	4.09	3.78; 4.28
A	*95.8*	*56.8*	*74.7*	*71.1*	*72.8*	*70.6*
2)-α-Man-(1→	5.36	4.08	3.99	3.69	3.80	3.88; 3.66
B	*102.1*	*79.8*	*71.6*	*68.3*	*74.8*	*62.3*
2)-α-Man-(1→	5.29	4.12	3.98	3.67	3.76	3.89 × 2
C	*101.9*	*79.7*	*71.3*	*68.3*	*74.6*	*62.3*
3,6)-α-Man-(1→	5.18	4.28	3.99	3.98	4.20	4.12; 3.66
D	*102.3*	*71.1*	*80.4*	*66.5*	*72.9*	*66.4*
t-α-Man-(1→	5.05	4.07	3.86	3.63	3.76	3.75 × 2
E	*103.4*	*71.3*	*71.6*	*68.1*	*74.5*	*62.4*
t-α-Man-(1→	4.93	4.03	3.88	3.66	3.67	3.77 × 2
F	*101.0*	*71.2*	*71.9*	*68.1*	*74.0*	*62.4*
t-β-Gal-(1→	4.50	3.54	3.72	3.95	3.76	3.80 × 2
G	*104.6*	*72.2*	*73.9*	*69.9*	*76.4*	*62.2*
6)-β-GlcN4*P*-(1→	4.41	2.67	3.57	3.64	3.64	3.78; 3.46
H	*104.2*	*57.5*	*77.3*	*74.5**	*75.6**	*70.5*
	3_{eq}-3_{ax}	4	5	6	7	8
8)-α-Kdo-(2→	2.13; 1.83	4.00	4.01	3.86	4.30	4.19; 3.93
K_{ext}	*36.0*	*67.4*	*68.5*	*73.0*	*70.8*	*72.1*
4,5)-α-Kdo-(2→	2.10; 2.00	4.05	4.22	3.73	3.74	3.86; 3.76
K_{int}	*35.8*	*72.6*	*75.4*	*73.0*	*70.9*	*64.4*

Spectra (shown in Figure 2) were recorded at 600 MHz (^{1}H; roman font) and 150 MHz (^{13}C; italics) at 303 K in 10 mM NaOD in D_2O (600 μl). The *C*-2 residues for K_{ext} and Kint were identified in the HMBC spectrum at 103.5 and 100.8 ppm, respectively. The *C*-1 for K_{ext} and K_{int} was not detected. Spectra assignments for the OS2 oligosaccharide are provided in Table S1.

*These signals can be interchanged.

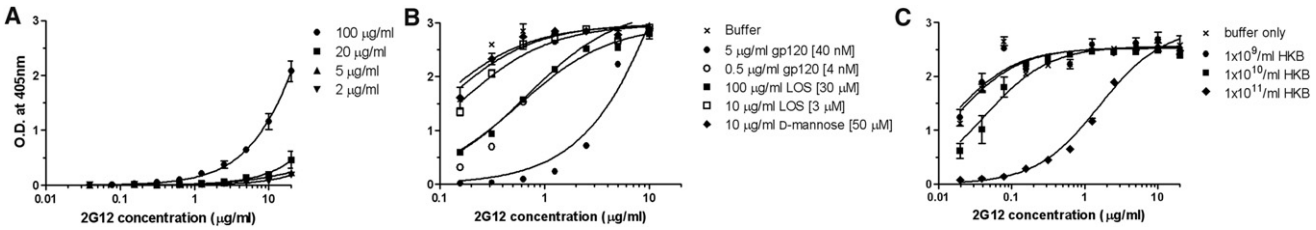

Figure 3. 2G12 Binds Rv3 LOS

(A) Binding of serially diluted antibody 2G12 to graded concentrations of Rv3 LOS coated directly onto microtiter plates. In contrast, rabbit sera raised against synthetic oligomannosides, comprising the D1 arm of mammalian oligomannose, do not recognize Rv3 LOS (Figure S1).

(B) Binding of serially diluted antibody 2G12 to recombinant gp120 in the absence (buffer control) or presence of fixed concentrations of inhibitors $gp120_{ADA}$, D-mannose and Rv3 LOS. Antibody was mixed for 1 hr at room temperature with buffer or inhibitor and then added to gp120-coated wells of a microtiter plate.

(C) 2G12 binding to gp120 is inhibited by heat-killed *R. radiobacter* Rv3 cells. Serially diluted antibody was mixed with buffer or graded amounts of heat-killed Rv3 bacteria for 1 hr at RT. The mixtures were then added to microtiter plate wells coated with recombinant monomeric gp120.

Error bars denote the signal ranges from replicate wells.

representing different types of α configured mannosyl residues, with residues B and C both glycosylated at *O*-2, residue D substituted at both *O*-3 and *O*-6, and both residues E and F terminal. Residue G was determined as being a β-configured Gal unit on the basis of its anomeric carbon chemical shift at 104.6 ppm and its coupling constant $^3J_{H1,H2}$ 7.8 Hz. The TOCSY spectrum related the anomeric proton up to H-4, whereas H-5 position was established analyzing the TROESY spectrum and assessing the spatial proximity of these two protons. Ring carbon values suggested the terminal location of this residue. For the two Kdo residues, the recognition of the internal unit K_{int} was made possible by a key NOE effect, relating the equatorial H-3 proton of this residue with H-6 of the external unit K_{ext} (Holst et al., 1995). Scalar connectivities between the other exocyclic Kdo protons were also clearly present and carbon chemical shift identified K_{ext} as an *O*-8 glycosylated Kdo, whereas K_{int} was substituted at both *O*-4 and *O*-5.

Residue sequence was established based on scalar gHMBC connectivities (Figure 2B). Starting from the terminal nonreducing end, residue E was determined as linked at *O*-2 of residue C, which was linked at *O*-2 of residue B, and this residue in turn linked at *O*-3 of D, which was the substituent at *O*-5 of K_{int}. The other terminal Man unit, residue F, was linked at *O*-6 of residue D, whereas residue G (Gal) was determined as located at *O*-8 of K_{ext}. The OS1 structure was completed by placing K_{int} at *O*-6 of residue H (GlcN).

For OS2, spectra interpretation followed the same approach as described above for OS1. The analysis was simplified because of the lower number of residues constituting the molecule. Complete spectra attribution (Table S1 available online) identified a structure differing from that of OS1 by the absence of the F and G units, corresponding to the terminal Man and Gal residues, respectively (Figure 1).

Rv3 LOS Is Antigenic for mAb 2G12

We next investigated whether 2G12 could bind Rv3 LOS by first examining the ability of 2G12 to bind Rv3 LOS directly in an enzyme-linked immunosorbent assay (ELISA). We found that 2G12 could indeed bind the LOS (Figure 3A), albeit that a fairly high concentration of LPS was required to observe strong binding (100 μg/ml, equivalent to 30 μM). To avoid the possible caveat of poor Rv3 LOS coating of microtiter plates, we also assessed the ability of Rv3 LOS to inhibit 2G12 binding to recombinant gp120 (Figure 3B). We observed that soluble Rv3 LOS at 30 μM, reduced the apparent affinity of 2G12 by nearly 10-fold relative to the buffer-only control. No significant inhibition of 2G12 binding to gp120 was observed with D-mannose at a similar concentration (50 μM), indicating that the inhibitory activity of Rv3 LOS is specific to its structural composition and not simply due to the presence of mannose residues. The level of inhibition observed for Rv3 LOS (85% reduction in apparent affinity relative to buffer-only control at 30 μM LOS) is better than that obtained using soluble synthetic oligomannosides or $Man_9GlcNAc_2$ (65%–80% inhibition at 2 mM; Lee et al., 2004) and in the same range as reported with oligomannose dendromers (10 μM; Walker et al., 2011); thus, this result strengthens the notion that the Rv3 LOS backbone is antigenically similar to the D1 arm in mammalian oligomannose and that presentation of the D1 analog in Rv3 LOS is antigenically favorable for 2G12 recognition.

We next examined whether 2G12 binding to Rv3 LOS would be enhanced if the LOS is displayed mutivalently, as observed with oligomannose (Astronomo et al., 2008, 2010). As a source of multivalently presented LOS, we prepared heat-killed bacteria and examined their ability to inhibit 2G12 binding to gp120. We found that 2G12 binding signals decreased with increasing concentration of bacteria, with the largest reduction in apparent antibody affinity observed with 10^{11} cells/ml (~2 log reduction relative to buffer-only control; Figure 3C). The concentration of 10^{11} cells/ml corresponds to an estimated LOS concentration of 15–150 μM, based on the yield of LOS from extracted bacteria. Given that the level of inhibition observed with the bacteria matches that of 2G12 inhibition by monomeric gp120, we concluded that the multivalent display of Rv3 LOS on bacterial cells resembles, at least to some extent, the oligomannose cluster on HIV-1 gp120 that constitutes the site of interaction with 2G12. This notion was strengthened by the inability of synthetic oligomannoside-specific rabbit sera, shown previously to be incapable of binding HIV gp120 (Astronomo et al., 2010), to recognize Rv3 LOS (Figure S1).

Immunization with Heat-Killed Rv3 Cells Elicits Tetramannose-Specific Antibodies that Recognize Monomeric HIV gp120

To further examine whether Rv3 LOS on the surface of bacteria approximates oligomannose clusters on HIV gp120, C57/BL6

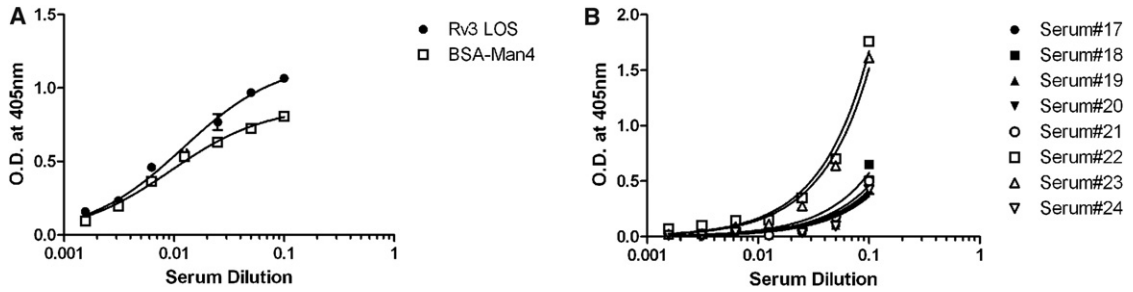

Figure 4. Immunization of Mice with Heat-Killed *R. radiobacter* Rv3 Elicits Antibodies Specific for the D1 Arm of Oligomannose with the Capacity to Bind Monomeric gp120

(A) Sera from immunized animals were collected two weeks following the second booster injection. The sera were pooled and a serial dilution thereof was examined for binding to Rv3 LOS (100 μg/ml) and BSA-Man4 (5 μg/ml) coated directly onto microtiter plates.

(B) Detergent-treated supernatant containing pseudotyped virus JRCSF was used as the antigen source for capture of solubilized gp120 onto microtiter plates. Serially-diluted mouse sera were then added and binding measured at 405 nm. Two of the 8 sera (serum #22 and #23) bound most strongly to the captured gp120. Error bars denote the signal ranges from replicate wells.

mice were immunized with heat-killed whole bacteria. Sera obtained two weeks after the final booster injection were used for further analyses. Despite the likelihood of having elicited antibodies to bacterial proteins, we observed reasonably strong binding of pooled sera to purified Rv3 LOS in ELISA (Figure 4A). The sera also bound to the bovine serum albumin (BSA)-Man4 glycoconjugate (Figure 4A), which is also recognized by 2G12 (Astronomo et al., 2008), suggesting that a large fraction of the antibodies were raised against the D1 analog portion of the molecule.

We next assessed whether the elicited antibodies could bind solubilized gp120 from pseudovirus lysates captured onto microtiter plate wells. This approach was used because of recent indications of differences between glycoforms found on recombinant gp120 and virus-associated gp120 (Doores et al., 2010a). Of the eight sera, two bound with moderate affinity to monomeric gp120, whereas the remaining sera bound substantially less strongly (Figure 4B). The ability of some but not all sera to bind with good affinity to gp120 suggests that not all elicited antibodies interacted with the D1 arm of oligomannose in a similar manner.

Antibodies Elicited with Heat-Killed Rv3 Cells Do Not Exhibit Neutralizing Activity

Despite the overall modest binding of sera to soluble gp120, we wished to examine whether the level of anticarbohydrate antibodies elicited with Rv3 cells might neutralize HIV. Two HIV-1 strains, JRCSF and TRO.11, were used that exhibit moderate, so-called tier-2, sensitivity to antibody-mediated neutralization (Binley et al., 2004; Seaman et al., 2010) and as such are representative of most circulating HIV-1 strains. Both viruses are sensitive to 2G12 neutralization, albeit that TRO.11 is slightly more sensitive to 2G12 neutralization than is JRCSF (Binley et al., 2004; Li et al., 2005). We also assessed neutralizing activity against SIVmac239, which, like HIV-1, is heavily glycosylated but is not recognized by 2G12 (Stansell and Desrosiers, 2010). At the lowest serum dilution tested (1:10), we observed what seemed like modest neutralization (~60%) against TRO.11 (Figure 5A); no activity was observed against JRCSF or SIVmac239. To assess whether the activity against TRO.11 was specific, we utilized the vesicular stomatitis virus (VSV), which expresses mainly complex carbohydrates on its surface (Hunt et al., 1978). We found that the sera also blocked VSV infectivity, with no statistical difference relative to the activity against TRO.11 (Figure 5B). From these results, we concluded that, despite the presence of antibodies in the sera that are specific for the 2G12 epitope, these antibodies are not of sufficient affinity and/or lack proper fine specificity to effectively neutralize HIV.

DISCUSSION

This study is, to the best of our knowledge, the first to report the elicitation of anticarbohydrate antibodies to HIV with a Gram-negative bacterium that naturally expresses an antigenic tetramannose structure that closely resembles the D1 arm of oligomannose. The most notable difference is the anomeric configuration of the mannosyl residue (residue D; Figure 1) at the reducing end of the D1 analog, being α rather than β as is found in oligomannose. Although bacteria with mannan-only polysaccharides have been described previously, none express structures that resemble the D1 arm of oligomannose as closely as it occurs in the Rv3 LOS. The nearest resemblance in chemical structure to D1 described to date is found in the O-polysaccharides of *Escherichia coli* O8 and O9, *Klebsiella pneumoniae* O3 and O5, and *Hafnia alvei* strain PCM 1223 (Curvall et al., 1973; Jansson et al., 1985; Katzenellenbogen et al., 2001; Lindberg et al., 1972; Prehm et al., 1976). However, unlike Rv3 LOS, none of these O-polysaccharides contain a terminal tetramannose structure similar to D1, and all are linear polymers.

The extent of antigenic similarity between Rv3 LOS and oligomannose, particularly as might be presented on HIV gp120, remains to be determined. Crystal structures of 2G12 in complex with Rv3 LOS should help to address this question and are planned. Notably, antimannose serum antibodies elicited by immunization with synthetic oligomannoside conjugates (Astronomo et al., 2010) failed to bind the Rv3 LOS. These serum antibodies, while specific for the synthetic oligomannosides, do not recognize (or neutralize) HIV. The lack of binding of these sera to the Rv3 LOS suggests that it does not present the neoepitopes found on synthetic oligomannosides.

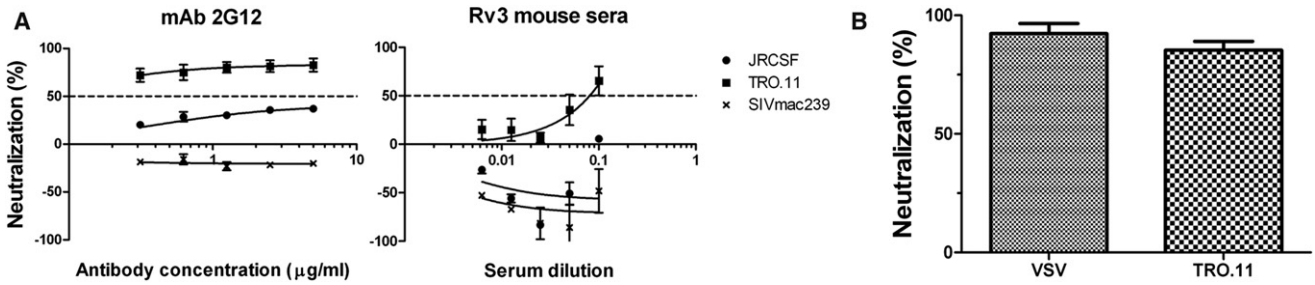

Figure 5. Rv3 Mouse Sera Do Not Specifically Inhibit HIV-1 Infectivity

(A) Pseudotyped HIV-1 strains TRO.11 and JRCSF were tested alongside virus SIVmac239 for their sensitivity to neutralization by antibody 2G12 (left) and serially diluted pooled mouse sera (right). Error bars denote the signal ranges from replicate wells.

(B) Pooled mouse sera was tested for nonspecific neutralizing activity utilizing vesicular stomatitis virus, which does not express high-mannose oligomannose on its surface. Pooled sera were tested at a single dilution of 1:10 alongside virus TRO.11. Error bars denote the standard error of measurement.

Our most relevant findings—that heat-killed Rv3 bacteria were able to elicit antibodies that can bind HIV gp120—demonstrate the potential utility of Rv3 LOS as a design template for strategies aimed at eliciting 2G12-like antibodies. We do not know whether one or both Rv3 LOS species (OS1 or OS2; Figure 1) might be responsible for eliciting the response obtained. Given that the D1 analog is present in both oligosaccharides, there is some likelihood that both may be capable of inducing the D1-specific responses. One group reported very weak serum neutralizing activity upon immunization with a yeast strain engineered to express predominantly Man8 oligomannose, with IC_{50}'s at or around 1:5 on average (Dunlop et al., 2010), whereas another group only observed neutralizing activity against pseudotyped viruses produced in the presence of the mannosidase inhibitor kifunensine such that they unnaturally carry only oligomannose sugars on their surface (Agrawal-Gamse et al., 2011). Disappointingly, we did not observe specific neutralizing activity against any of the HIV strains tested here (Figure 5). Due to volume limitations as a result of doing our immunizations in mice, we were not able to assess whether our sera can neutralize viruses carrying only oligomannose sugars.

The specific reason for the lack of neutralizing activity is unclear but will require closer investigation in future studies. One of the most likely factors is the relatively low affinity of the elicited antibodies, as suggested by our ELISA binding data for monomeric gp120 (Figure 4). 2G12 achieves high-affinity binding as a result of its VH domain-exchanged structure, which allows it to interact multivalently with the dense array of oligomannose on gp120 (Calarese et al., 2003). Whereas an engineered Y-shaped 2G12 antibody failed to bind monomeric gp120 with high affinity (Doores et al., 2010b), domain-exchange is not the only solution for achieving high-affinity binding to oligomannose on gp120 as evidenced by the recent description of non-domain-exchanged antibodies with high affinity for oligomannose on HIV-1 (Walker et al., 2011). Immunization strategies that result in high-affinity anticarbohydrate antibodies will therefore need to be explored. Indeed, both the Dunlop et al. and Agrawal-Gamse et al. studies suggest that, whereas antimannose antibodies specific for oligomannose can be elicited in principle (Agrawal-Gamse et al., 2011; Dunlop et al., 2010), these antibodies bind most effectively when the oligomannose moieties are extensively arrayed—for example, as presented when monomeric gp120 is coated onto wells of a microtiter plate or on kifunensine-produced virus particles. Whereas experimentally elicited antimannose antibodies most likely bind bivalently to their target epitope under such conditions, these antibodies can likely only bind monovalently to oligomannose on native virus.

In summary, we show here that *R. radiobacter* Rv3 LOS, comprised of a tetramannose oligosaccharide backbone that is analogous to the epitope of the broadly neutralizing mAb 2G12, represents, to the best of our knowledge, a novel template for the elicitation of oligomannose-specific nAbs to HIV-1. Optimizing the presentation of Rv3 LOS will be required to elicit the desired antibody response, possibly along with tricks to boost the response, for example, using select adjuvants. Investigations into immune conditions conducive to the elicitation of antibodies that recognize oligomannose with high affinity will also be important.

SIGNIFICANCE

Antibodies with broad HIV-neutralizing activity serve to pinpoint conserved sites on the virus that are potential targets for vaccine design. The human mAb 2G12 neutralizes ~40% of HIV strains in vitro and provides sterilizing immunity at relatively modest serum titers in macaque models of HIV infection. Given that 2G12 recognizes the D1 arms of clustered oligomannose sugars on the surface of HIV gp120, strategies to elicit anticarbohydrate neutralizing-antibodies (nAbs) to HIV have focused mainly on trying to mimic oligomannose clusters on gp120 using presentations of oligomannosides; however, these approaches have had minimal success.

The glyco-immunogen presentations pursued so far may be too similar to oligomannose presentation on host cells and thus fail to elicit robust responses. We have reasoned that a bacterial polysaccharide with antigenic similarity to the 2G12 epitope might prove a better way to elicit the desired anticarbohydrate antibody response. In this paper we show that the LOS of *R. radiobacter* Rv3 contains a unique tetramannoside that is antigenically similar to the D1 arm of natural oligomannose, as judged by 2G12′s affinity for it. Furthermore, immunization with heat-killed Rv3 bacteria elicited carbohydrate-specific antibodies with

modest affinity for monomeric gp120 but no significant HIV-specific neutralizing activity. The results support investigating Rv3 LOS further as a possible means by which to elicit anticarbohydrate nAbs to HIV. The particular attractiveness of Rv3 LOS is its bacterial origin, which means that there is a reasonable likelihood of it being recognized as "foreign," yet the target structure is sufficiently similar to oligomannose so that many elicited antibodies are likely to be cross-reactive. Rv3 LOS is also attractive because of the proven efficacy of bacterial carbohydrate-based vaccines. Studies exploring the presentation of glycoconjugate immunogens that incorporate the Rv3 LOS should help to delineate the best presentation for eliciting robust levels of anticarbohydrate antibodies to HIV-1.

EXPERIMENTAL PROCEDURES

Bacterial Growth and LOS Extraction

R. radiobacter Rv3 strain DSM 30207 was grown in a shaker incubator (200 rpm) at 27°C in nutrient broth for 40 hr (early stationary phase). The bacterial suspension was pelleted (3500 *g* for 15 min) and the biomass then washed sequentially with 0.85% NaCl, ethanol, acetone, and diethyl ether. Dried cells (yield 275 mg/l) were subjected to extraction by the PCP method (Galanos et al., 1969), yielding an LOS fraction (2% g_{LOS}/g_{cells}). The PCP-extracted pellet was then subjected to a phenol-water extraction (Westphal and Jann, 1965), and LOS was found in the water phase only (7.5% g_{LOS}/g_{cells}). However, successive analyses demonstrated that this preparation was highly contaminated with cyclic β-glucans that are commonly present in the periplasmatic space of rhizobia (Breedveld and Miller, 1994). Therefore, only the PCP-extracted LOS was used in further analyses.

General and Analytical Methods

Rv3 LOS was stained with silver nitrate (Kittelberger and Hilbink, 1993) following SDS-PAGE using a 12% separating gel on a miniprotean gel system from BioRad (Hercules, CA, USA). Determination of fatty acid and sugar composition, including determination of the absolute configuration of monosaccharides, and glycosyl-linkage analysis was performed as described elsewhere (De Castro et al., 2010).

Isolation of Oligosaccharides OS1 and OS2

PCP-extracted LOS (60 mg) was dissolved in anhydrous hydrazine (25 mg/ml), stirred at 37°C for 30 min, cooled, poured into ice-cold acetone (15 ml), and allowed to precipitate. The solid was centrifuged (6000 *g* for 30 min), washed twice with ice-cold acetone, dried, and then dissolved in water and lyophilized (50 mg). The sample was de-*N*-acylated with 4 M KOH as described (Holst, 2000) and desalted by gel-permeation chromatography using a Sephadex G-10 (Pharmacia, New York, USA) column (50 × 1.5 cm, in water, flowrate 0.5 ml/min). The resulting oligosaccharide fraction (9 mg) was eluted in the void volume and further purified by HPAEC on a Carbopack PA-100 column (9 × 250 mm), followed by elution with a linear gradient of 30%–37% of 1 M sodium acetate in 0.1 M NaOH at 2 ml/min for 100 min. Under these conditions, two oligosaccharides, designated OS1 and OS2, with retention times of 41 and 62 min, respectively, were recovered. OS1 and OS2 were desalted as mentioned previously, yielding 1.5 mg and 0.7 mg, respectively.

NMR Spectroscopy

All NMR experiments were carried out on a Bruker DRX-600 spectrometer equipped with a cryo probe operating at 303 K. Chemical shift of spectra recorded in D_2O are expressed in δ relative to internal acetone (2.225 and 31.4 ppm). Two-dimensional spectra (DQ-COSY, TOCSY, TROESY, gHSQC, gHMBC, and gHSQCTOCSY) were measured using standard Bruker software. For all experiments, 512 free induction decays of 2048 complex data points were collected; 40 scans per FID were acquired for homonuclear spectra and mixing times of 120 ms and 250 ms for TOCSY and TROESY spectra acquisition, respectively. The spectral width was set to 10 ppm, and the frequency carrier was placed at the residual HOD peak. For the gHSQC spectrum, 50 scans per FID were acquired, and the GARP sequence was used for ^{13}C decoupling during acquisition. gHSQCTOCSY and gHMBC scans doubled those of gHSQC spectrum, and these sequences were not measured for OS2 because of the low amount of compound available. Data processing was performed using the Bruker Topspin 3 program, and the spectra were assigned using the computer program Pronto (Kjaer et al., 1994).

Preparation of Heat-Killed bacteria

Single colonies of *R. radiobacter* Rv3 were cultured in Luria Bertani (LB) broth without antibiotics overnight at 25°C. Aliquots of the overnight cultures were then spread onto plain LB media plates and incubated overnight at 30°C. The bacteria were subsequently harvested and resuspended in 1 ml phosphate buffered saline (PBS) per plate. The cell concentration was determined spectrophotometrically using a standard conversion factor ($OD_{600} = 10^9$ cells/ml), and the total volume adjusted with PBS to 10^{11} cells/ml. Based on the yields for Rv3 LOS above, this concentration of bacteria corresponds to an LOS concentration of ~15–150 μM. To obtain heat-killed bacteria, cell suspensions were incubated for 1 hr at 100°C. Heat-killed bacteria were aliquoted and stored at −20°C until needed.

Mouse Immunizations

All animal immunizations were performed under contract at Spring Valley Laboratories, Inc., (Woodline, MD, USA) following approval of the submitted protocol. All experiments followed guidelines set out by the University Animal Care Committee. Immediately prior to each immunization, aliquots of heat-killed bacteria (1 ml) were mixed with an equal volume of PBS. Mice (C57/BL6; n = 8) were injected subcutaneously with 0.2 ml of the heat-killed bacteria/PBS mixture, corresponding to 10^{10} bacteria, on days 0, 28, and 84. The animals were exsanguinated 2 weeks after the last injection. Sera were obtained and stored at −20°C.

Monoclonal Antibodies, Rabbit Sera, and Purified Proteins

The antibody 2G12 was generously donated by Hermann and Dietmar Katinger (Polymun Scientific, Klosterneuburg, Austria). Two sets of rabbit antisera, raised against synthetic tetramannoside (Man4) and oligomannoside (Man9) (Astronomo et al., 2010), a BSA-Man4 glycoconjugate, recombinant gp120 derived from virus ADA, and mAb b12 were provided by the Burton laboratory at The Scripps Research Institute and IAVI Neutralizing Antibody Center (La Jolla, CA, USA). Recombinant gp120 derived from the virus JR-FL was made in-house from stably transfected CHO-K1 cells (Pantophlet et al., 2003). The construct was appended with a C-terminal HIS tag to facilitate purification of the overexpressed protein.

Pseudovirions and Solubilized gp120

Plasmids encoding the envelope glycoproteins of the viruses TRO.11 (TRO, clone 11 [SVPB12]; Li et al., 2005) and vesicular stomatitis virus (pHEF-VSVG; Chang et al., 1999) and the reporter plasmid pNL4-3.Luc.R-E- encoding HIV-1 structural proteins and a luciferase enzyme (Connor et al., 1995; He et al., 1995) were obtained from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program (NIH ARRRP). The plasmid pSVIII-JRCSF, expressesing the *env* of the primary isolate JRCSF, has been described previously (Zwick et al., 2001). A plasmid expressing codon-optimized, full-length *env* of SIVmac239 was from the Burton laboratory (The Scripps Research Institute). Pseudotyped viruses were generated in 293T cells as described (Zwick et al., 2001). Solubilized monomeric gp120 was obtained by adding detergent (Empigen, Sigma-Aldrich, St. Louis, MO, USA; 1% [v/v] final concentration) to the harvested culture supernatants to lyse virions. Detergent-treated supernatants were made fresh and used immediately. For neutralization assays, supernatant containing pseudotyped virus was harvested, aliquoted, and stored at −80°C until needed.

Neutralization Assays

Neutralization assays with single-round infectious pseudovirus were performed essentially as described elsewhere (Zwick ,et al., 2001), using U87.CD4+CCR5+ target cells (Björndal et al., 1997) obtained from the NIH ARRRP. The virus was mixed with an equal volume of antibody 2G12 or pooled heat-inactivated serum and incubated for 1 hr at 37°C. This mixture was then

added to the targets cells and incubated for a further three days. Luciferase activity in cell lysates was measured on a luminometer (Viktor X5; PerkinElmer, Ontario, Canada) using luciferase assay substrate (Promega, Madison, WI, USA). The percentage of virus neutralization at a given antibody concentration or serum dilution was determined by calculating the reduction in luciferase activity in the presence of antibody relative to virus-only wells and subtraction of luciferase background activity from cell-only wells.

ELISA

Enzyme-linked immunosorbent assays (ELISAs) were performed to measure the binding of 2G12, Qβ-Man4, and Qβ-Man9 rabbit sera, and anti-Rv3 mouse sera to antigen (LOS, solubilized gp120, BSA-Man4). Primary antibody binding was detected using relevant alkaline-phosphatase conjugated Fc-specific secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) in conjunction with a *p*-nitrophenyl phosphate substrate (Sigma, St. Louis, MO, USA). ELISA signals were measured at 405 nm. All ELISAs were performed in duplicate or triplicate.

Direct Antigen Binding ELISA

ELISAs to measure 2G12 and serum binding to LPS coated directly onto polyvinyl microtiter plates were performed essentially as described in a previous report (Pantophlet et al., 1998), except that the blocking step was performed with PBS supplemented with 3% BSA, and all antibody dilutions were made in PBS supplemented with 1% BSA (PBS-1% BSA). Serum antibody binding to BSA-Man4 glycoconjugate was performed as described elsewhere (Astronomo et al., 2008).

Antigen Capture ELISA

Antibody binding to solubilized gp120 was performed as described previously (Pantophlet et al., 2003) by capturing the gp120 onto microtiter plates coated with anti-gp120 antibody D7324 (Aalto Bioreagents, Dublin, Ireland). The remaining steps were performed as described above.

Inhibition ELISA

For ELISA inhibition experiments with antibody 2G12, serial twofold dilutions of the antibody in PBS-1% BSA were mixed with BSA-coated microtiter plates with an equal volume of antigen (fixed concentration) diluted in PBS-1% BSA. After incubation (1 hr at RT), 50 μl of the mixture was added to recombinant gp120-coated microtiter plates blocked with 3% BSA. Detection of antibody binding was performed as described previously.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and one table and can be found with this article online at doi:10.1016/j.chembiol.2011.12.019.

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REFERENCES

Agrawal-Gamse, C., Luallen, R.J., Liu, B., Fu, H., Lee, F.-H., Geng, Y., and Doms, R.W. (2011). Yeast-elicited cross-reactive antibodies to HIV Env glycans efficiently neutralize virions expressing exclusively high-mannose N-linked glycans. J. Virol. *85*, 470–480.

Astronomo, R.D., and Burton, D.R. (2010). Carbohydrate vaccines: developing sweet solutions to sticky situations? Nat. Rev. Drug Discov. *9*, 308–324.

Astronomo, R.D., Lee, H.-K., Scanlan, C.N., Pantophlet, R., Huang, C.-Y., Wilson, I.A., Blixt, O., Dwek, R.A., Wong, C.-H., and Burton, D.R. (2008). A glycoconjugate antigen based on the recognition motif of a broadly neutralizing human immunodeficiency virus antibody, 2G12, is immunogenic but elicits antibodies unable to bind to the self glycans of gp120. J. Virol. *82*, 6359–6368.

Astronomo, R.D., Kaltgrad, E., Udit, A.K., Wang, S.K., Doores, K.J., Huang, C.Y., Pantophlet, R., Paulson, J.C., Wong, C.H., Finn, M.G., and Burton, D.R. (2010). Defining criteria for oligomannose immunogens for HIV using icosahedral virus capsid scaffolds. Chem. Biol. *17*, 357–370.

Binley, J.M., Wrin, T., Korber, B., Zwick, M.B., Wang, M., Chappey, C., Stiegler, G., Kunert, R., Zolla-Pazner, S., Katinger, H., et al. (2004). Comprehensive cross-clade neutralization analysis of a panel of anti-human immunodeficiency virus type 1 monoclonal antibodies. J. Virol. *78*, 13232–13252.

Björndal, A., Deng, H., Jansson, M., Fiore, J.R., Colognesi, C., Karlsson, A., Albert, J., Scarlatti, G., Littman, D.R., and Fenyö, E.M. (1997). Coreceptor usage of primary human immunodeficiency virus type 1 isolates varies according to biological phenotype. J. Virol. *71*, 7478–7487.

Blanchard-Rohner, G., and Pollard, A.J. (2011). Long-term protection after immunization with protein-polysaccharide conjugate vaccines in infancy. Expert Rev. Vaccines *10*, 673–684.

Breedveld, M.W., and Miller, K.J. (1994). Cyclic beta-glucans of members of the family *Rhizobiaceae*. Microbiol. Rev. *58*, 145–161.

Burton, D.R., Desrosiers, R.C., Doms, R.W., Koff, W.C., Kwong, P.D., Moore, J.P., Nabel, G.J., Sodroski, J., Wilson, I.A., and Wyatt, R.T. (2004). HIV vaccine design and the neutralizing antibody problem. Nat. Immunol. *5*, 233–236.

Calarese, D.A., Scanlan, C.N., Zwick, M.B., Deechongkit, S., Mimura, Y., Kunert, R., Zhu, P., Wormald, M.R., Stanfield, R.L., Roux, K.H., et al. (2003). Antibody domain exchange is an immunological solution to carbohydrate cluster recognition. Science *300*, 2065–2071.

Calarese, D.A., Lee, H.K., Huang, C.Y., Best, M.D., Astronomo, R.D., Stanfield, R.L., Katinger, H., Burton, D.R., Wong, C.H., and Wilson, I.A. (2005). Dissection of the carbohydrate specificity of the broadly neutralizing anti-HIV-1 antibody 2G12. Proc. Natl. Acad. Sci. USA *102*, 13372–13377.

Chang, L.J., Urlacher, V., Iwakuma, T., Cui, Y., and Zucali, J. (1999). Efficacy and safety analyses of a recombinant human immunodeficiency virus type 1 derived vector system. Gene Ther. *6*, 715–728.

Connor, R.I., Chen, B.K., Choe, S., and Landau, N.R. (1995). Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes. Virology *206*, 935–944.

Curvall, M., Lindberg, B., Lönngren, J., and Nimmich, W. (1973). Structural studies on the *Klebsiella* O group 3 lipopolysaccharide. Acta Chem. Scand. *27*, 2645–2649.

De Castro, C., Molinaro, A., Lanzetta, R., Silipo, A., and Parrilli, M. (2008). Lipopolysaccharide structures from *Agrobacterium* and *Rhizobiaceae* species. Carbohydr. Res. *343*, 1924–1933.

De Castro, C., Parrilli, M., Holst, O., and Molinaro, A. (2010). Microbe-associated molecular patterns in innate immunity: extraction and chemical analysis of gram-negative bacterial lipopolysaccharides. Methods Enzymol. *480*, 89–115.

Doores, K.J., Bonomelli, C., Harvey, D.J., Vasiljevic, S., Dwek, R.A., Burton, D.R., Crispin, M., and Scanlan, C.N. (2010a). Envelope glycans of immunodeficiency virions are almost entirely oligomannose antigens. Proc. Natl. Acad. Sci. USA *107*, 13800–13805.

Doores, K.J., Fulton, Z., Huber, M., Wilson, I.A., and Burton, D.R. (2010b). Antibody 2G12 recognizes di-mannose equivalently in domain- and nondomain-exchanged forms but only binds the HIV-1 glycan shield if domain exchanged. J. Virol. *84*, 10690–10699.

Doores, K.J., Fulton, Z., Hong, V., Patel, M.K., Scanlan, C.N., Wormald, M.R., Finn, M.G., Burton, D.R., Wilson, I.A., and Davis, B.G. (2010c). A nonself sugar mimic of the HIV glycan shield shows enhanced antigenicity. Proc. Natl. Acad. Sci. USA *107*, 17107–17112.

Dunlop, D.C., Bonomelli, C., Mansab, F., Vasiljevic, S., Doores, K.J., Wormald, M.R., Palma, A.S., Feizi, T., Harvey, D.J., Dwek, R.A., et al. (2010). Polysaccharide mimicry of the epitope of the broadly neutralizing anti-HIV antibody, 2G12, induces enhanced antibody responses to self oligomannose glycans. Glycobiology *20*, 812–823.

Finn, A. (2004). Bacterial polysaccharide-protein conjugate vaccines. Br. Med. Bull. *70*, 1–14.

Galanos, C., Lüderitz, O., and Westphal, O. (1969). A new method for the extraction of R lipopolysaccharides. Eur. J. Biochem. *9*, 245–249.

He, J., Choe, S., Walker, R., Di Marzio, P., Morgan, D.O., and Landau, N.R. (1995). Human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G2 phase of the cell cycle by inhibiting p34cdc2 activity. J. Virol. *69*, 6705–6711.

Heath, P.T. (2011). An update on vaccination against group B streptococcus. Expert Rev. Vaccines *10*, 685–694.

Hitchcock, P.J., and Brown, T.M. (1983). Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. *154*, 269–277.

Holst, O. (2000). Deacylation of lipopolysaccharides and isolation of oligosaccharide phosphates. Methods Mol. Biol. *145*, 345–353.

Holst, O., Bock, K., Brade, L., and Brade, H. (1995). The structures of oligosaccharide bisphosphates isolated from the lipopolysaccharide of a recombinant *Escherichia coli* strain expressing the gene *gseA* [3-deoxy-D-manno-octulopyranosonic acid (Kdo) transferase] of *Chlamydia psittaci* 6BC. Eur. J. Biochem. *229*, 194–200.

Hunt, L.A., Etchison, J.R., and Summers, D.F. (1978). Oligosaccharide chains are trimmed during synthesis of the envelope glycoprotein of vesicular stomatitis virus. Proc. Natl. Acad. Sci. USA *75*, 754–758.

Jansson, P.E., Lönngren, J., Widmalm, G., Leontein, K., Slettengren, K., Svenson, S.B., Wrangsell, G., Dell, A., and Tiller, P.R. (1985). Structural studies of the O-antigen polysaccharides of *Klebsiella* O5 and *Escherichia coli* O8. Carbohydr. Res. *145*, 59–66.

Johnston, M.I., and Fauci, A.S. (2008). An HIV vaccine—challenges and prospects. N. Engl. J. Med. *359*, 888–890.

Joyce, J.G., Krauss, I.J., Song, H.C., Opalka, D.W., Grimm, K.M., Nahas, D.D., Esser, M.T., Hrin, R., Feng, M., Dudkin, V.Y., et al. (2008). An oligosaccharide-based HIV-1 2G12 mimotope vaccine induces carbohydrate-specific antibodies that fail to neutralize HIV-1 virions. Proc. Natl. Acad. Sci. USA *105*, 15684–15689.

Katzenellenbogen, E., Kocharova, N.A., Zatonsky, G.V., Kübler-Kiełb, J., Gamian, A., Shashkov, A.S., Knirel, Y.A., and Romanowska, E. (2001). Structural and serological studies on *Hafnia alvei* O-specific polysaccharide of alpha-D-mannan type isolated from the lipopolysaccharide of strain PCM 1223. FEMS Immunol. Med. Microbiol. *30*, 223–227.

Kittelberger, R., and Hilbink, F. (1993). Sensitive silver-staining detection of bacterial lipopolysaccharides in polyacrylamide gels. J. Biochem. Biophys. Methods *26*, 81–86.

Kjaer, M., Andersen, K.V., and Poulsen, F.M. (1994). Automated and semiautomated analysis of homo- and heteronuclear multidimensional nuclear magnetic resonance spectra of proteins: the program Pronto. Methods Enzymol. *239*, 288–307.

Lee, H.K., Scanlan, C.N., Huang, C.Y., Chang, A.Y., Calarese, D.A., Dwek, R.A., Rudd, P.M., Burton, D.R., Wilson, I.A., and Wong, C.H. (2004). Reactivity-based one-pot synthesis of oligomannoses: defining antigens recognized by 2G12, a broadly neutralizing anti-HIV-1 antibody. Angew. Chem. Int. Ed. Engl. *43*, 1000–1003.

Li, H., and Wang, L.X. (2004). Design and synthesis of a template-assembled oligomannose cluster as an epitope mimic for human HIV-neutralizing antibody 2G12. Org. Biomol. Chem. *2*, 483–488.

Li, M., Gao, F., Mascola, J.R., Stamatatos, L., Polonis, V.R., Koutsoukos, M., Voss, G., Goepfert, P., Gilbert, P., Greene, K.M., et al. (2005). Human immunodeficiency virus type 1 *env* clones from acute and early subtype B infections for standardized assessments of vaccine-elicited neutralizing antibodies. J. Virol. *79*, 10108–10125.

Lindberg, B., Lönngren, J., and Nimmich, W. (1972). Structural studies on *Klebsiella* O group 5 lipopolysaccharides. Acta Chem. Scand. *26*, 2231–2236.

Luallen, R.J., Lin, J., Fu, H., Cai, K.K., Agrawal, C., Mboudjeka, I., Lee, F.-H., Montefiori, D., Smith, D.F., Doms, R.W., and Geng, Y. (2008). An engineered *Saccharomyces cerevisiae* strain binds the broadly neutralizing human immunodeficiency virus type 1 antibody 2G12 and elicits mannose-specific gp120-binding antibodies. J. Virol. *82*, 6447–6457.

Luallen, R.J., Agrawal-Gamse, C., Fu, H., Smith, D.F., Doms, R.W., and Geng, Y. (2010). Antibodies against Manalpha1,2-Manalpha1,2-Man oligosaccharide structures recognize envelope glycoproteins from HIV-1 and SIV strains. Glycobiology *20*, 280–286.

Lucas, A.H., Rittenhouse-Olson, K., Kronenberg, M., Apicella, M.A., Wang, D., Schreiber, J.R., and Taylor, C.E. (2010). Carbohydrate moieties as vaccine candidates: meeting summary. Vaccine *28*, 1121–1131.

Ni, J., Song, H., Wang, Y., Stamatos, N.M., and Wang, L.-X. (2006). Toward a carbohydrate-based HIV-1 vaccine: synthesis and immunological studies of oligomannose-containing glycoconjugates. Bioconjug. Chem. *17*, 493–500.

Pantophlet, R., Brade, L., Dijkshoorn, L., and Brade, H. (1998). Specificity of rabbit antisera against lipopolysaccharide of *Acinetobacter*. J. Clin. Microbiol. *36*, 1245–1250.

Pantophlet, R., Ollmann Saphire, E., Poignard, P., Parren, P.W., Wilson, I.A., and Burton, D.R. (2003). Fine mapping of the interaction of neutralizing and nonneutralizing monoclonal antibodies with the CD4 binding site of human immunodeficiency virus type 1 gp120. J. Virol. *77*, 642–658.

Prehm, P., Jann, B., and Jann, K. (1976). The O9 antigen of *Escherichia coli* Structure of the polysaccharide chain. Eur. J. Biochem. *67*, 53–56.

Sanders, R.W., Venturi, M., Schiffner, L., Kalyanaraman, R., Katinger, H., Lloyd, K.O., Kwong, P.D., and Moore, J.P. (2002). The mannose-dependent epitope for neutralizing antibody 2G12 on human immunodeficiency virus type 1 glycoprotein gp120. J. Virol. *76*, 7293–7305.

Scanlan, C.N., Pantophlet, R., Wormald, M.R., Ollmann Saphire, E., Stanfield, R., Wilson, I.A., Katinger, H., Dwek, R.A., Rudd, P.M., and Burton, D.R. (2002). The broadly neutralizing anti-human immunodeficiency virus type 1 antibody 2G12 recognizes a cluster of $\alpha 1 \rightarrow 2$ mannose residues on the outer face of gp120. J. Virol. *76*, 7306–7321.

Scanlan, C.N., Ritchie, G.E., Baruah, K., Crispin, M., Harvey, D.J., Singer, B.B., Lucka, L., Wormald, M.R., Wentworth, P., Jr., Zitzmann, N., et al. (2007). Inhibition of mammalian glycan biosynthesis produces non-self antigens for a broadly neutralising, HIV-1 specific antibody. J. Mol. Biol. *372*, 16–22.

Seaman, M.S., Janes, H., Hawkins, N., Grandpre, L.E., Devoy, C., Giri, A., Coffey, R.T., Harris, L., Wood, B., Daniels, M.G., et al. (2010). Tiered categorization of a diverse panel of HIV-1 Env pseudoviruses for assessment of neutralizing antibodies. J. Virol. *84*, 1439–1452.

Silipo, A., De Castro, C., Lanzetta, R., Molinaro, A., and Parrilli, M. (2004). Full structural characterization of the lipid A components from the *Agrobacterium tumefaciens* strain C58 lipopolysaccharide fraction. Glycobiology *14*, 805–815.

Stansell, E., and Desrosiers, R.C. (2010). Fundamental difference in the content of high-mannose carbohydrate in the HIV-1 and HIV-2 lineages. J. Virol. *84*, 8998–9009.

Trkola, A., Purtscher, M., Muster, T., Ballaun, C., Buchacher, A., Sullivan, N., Srinivasan, K., Sodroski, J., Moore, J.P., and Katinger, H. (1996). Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. J. Virol. *70*, 1100–1108.

Vliegenthart, J.F.G. (2006). Carbohydrate based vaccines. FEBS Lett. *580*, 2945–2950.

Walker, L.M., Huber, M., Doores, K.J., Falkowska, E., Pejchal, R., Julien, J.-P., Wang, S.-K., Ramos, A., Chan-Hui, P.-Y., Moyle, M., et al; Protocol G Principal

Investigators. (2011). Broad neutralization coverage of HIV by multiple highly potent antibodies. Nature *477*, 466–470.

Wang, L.X., Ni, J., Singh, S., and Li, H. (2004). Binding of high-mannose-type oligosaccharides and synthetic oligomannose clusters to human antibody 2G12: implications for HIV-1 vaccine design. Chem. Biol. *11*, 127–134.

Wang, J., Li, H., Zou, G., and Wang, L.X. (2007). Novel template-assembled oligosaccharide clusters as epitope mimics for HIV-neutralizing antibody 2G12. Design, synthesis, and antibody binding study. Org. Biomol. Chem. *5*, 1529–1540.

Westphal, O., and Jann, K. (1965). Bacterial lipopolysaccharides: extraction with phenol-water and further applications of the procedure. In Methods in Carnohydrate Chemistry, R.L. Whistler, ed. (New York: Academic Press), pp. 83–91.

Wyatt, R., Kwong, P.D., Desjardins, E., Sweet, R.W., Robinson, J., Hendrickson, W.A., and Sodroski, J.G. (1998). The antigenic structure of the HIV gp120 envelope glycoprotein. Nature *393*, 705–711.

Zwick, M.B., Wang, M., Poignard, P., Stiegler, G., Katinger, H., Burton, D.R., and Parren, P.W. (2001). Neutralization synergy of human immunodeficiency virus type 1 primary isolates by cocktails of broadly neutralizing antibodies. J. Virol. *75*, 12198–12208.

