Peptide mimicry of carbohydrate epitopes on human immunodeficiency virus

Michael Agadjanyan, Ping Luo, M.A. Julie Westerink[1], Lisa A. Carey, Wendy Hutchins[1], Zenon Steplewski[2], David B. Weiner, and Thomas Kieber-Emmons*

*Department of Pathology and Laboratory Medicine, University of Pennsylvania, 36th and Hamilton Walk, Philadelphia, PA 19104-6082. [1]Department of Medicine, Medical College of Ohio, Toledo, OH. [2]Department of Medicine, Thomas Jefferson University, Philadelphia, PA. *Corresponding auther (e-mail: tom@xray.med.upenn.edu).*

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Cancer-related, mucin-type carbohydrate epitopes, principally mannose and sialo-syl residues, are expressed on the envelope protein gp160 of the human immunodeficiency virus (HIV). Anticarbohydrate antibodies directed toward these and other carbohydrate epitopes are known to neutralize HIV-1 infection by cell-free virus. Carbohydrates, however, being T cell–independent antigens, typically elicit diminished immune responses. To overcome this potential draw back, we have examined the ability of peptides that mimic such epitopes to elicit immune responses that cross-react with carbohydrate structures. We report that mouse polyclonal antisera generated against peptides that mimic mucin-related carbohydrate epitopes have anti–HIV-1 activity. Generation of antibodies was not Ir-gene restricted, as at least two different strains of mice, Balb/c (H-2^d) and C57Bl/6 (H-2^b), responded equally to the peptides. The antipeptide sera displayed neutralizing activity against HIV-I/MN and HIV-I/3B viral strains. This neutralization was as good as human anti-HIV sera. These results indicate that peptide mimics of carbohydrates provide a novel strategy for the further development of reagents that elicit immune responses to carbohydrate epitopes associated with many infectious organisms and tumor cells.

Keywords: applied immunology, peptide mimetic, carbohydrate, polysaccharide, HIV-1, Lewis Y

Protein-carbohydrate interactions mediate the initial steps in many bacterial and viral infections. The envelope (env) glycoprotein of human immunodeficiency viruses (HIV-1 and HIV-2) interacts with target cells through high mannose and/or N- and O-glycosylated regions of gp160 (ref. 1). Subsequently, certain lectins and anticarbohydrate antibodies display the capacity to neutralize HIV field and laboratory isolates in vitro[2–6]. O- and N-linked carbohydrates are one set of common saccharide subunits shared among bacteria, viruses, and tumor cells. Antibodies that target the Tn (GalNAc-Ser/Thr), sialo-syl-Tn (NeuAc-GalNAc-Ser/Thr), and the Histo-blood group antigen Lewis Y, (Fucα1→2 Galβ1→4(Fucα1→3) GlcNAcβ1→3Galβ1→4Glcβ1→R), occurring as surface antigens on most primary human breast carcinomas and their metastases, inhibit HIV infection and syncytium formation[5]. These observed cross-reactivities for HIV and tumor cell-associated carbohydrates suggest that the pathophysiology of infection and neoplasia are profoundly affected by the same or similar carbohydrate forms.

Viral-borne carbohydrates that are not structurally encoded by the viral genome represent a target for group-specific vaccine development as these antigens are unlikely to change dramatically with viral mutation. Unfortunately, carbohydrates are, per se, not immunogenic in humans and require extrinsic adjuvanticity, as they suffer from an inherent inability to generate antigen-specific T cell responses. Immune responses can be enhanced by coupling carbohydrates to immunologic-carrier proteins or administering them with adjuvants[7]; however, synthetic antigen-conjugates (representative of those on the HIV-1 env) do not always induce immune responses reactive with native antigens[8,9].

To overcome this and related deficiencies, surrogate peptide antigens might prove effective for eliciting immune responses reactive with natural carbohydrate forms[10]. In this context peptide mimetics have a significant conceptual advantage for vaccine design. As peptides, they have the ability to stimulate T cell help in an antigen specific manner. Ultimately, such a vaccine should be able to generate long-term immune responses, and would have advantages for manufacturing and vaccine production. The ability of a peptide or polypeptide to immunologically mimic a carbohydrate determinant[11–15] indicates that while mimicry is accomplished using amino acids in place of sugars, the specificity pattern can be precisely reproduced.

We show that immunization with peptides that mimic mannose and lactoseries carbohydrate subunits induce antibodies that cross-react with native HIV env proteins and can neutralize HIV-1 infection. These studies substantiate that induced antibodies to common carbohydrate subunits found on bacteria and tumor cells can also bind viral glycoprotein(s). Peptide antigens that are mimics of carbohydrate antigens thus provide an alternative vaccine strategy to elicit an appropriate immune response against natural polysaccharides.

Results

Choice of peptides. Peptides were chosen based upon the distribution of mucin-type and peripheral poly-N-acetylglucosamine carbohydrates on the major env protein of HIV-1 (refs. 4, 5, 16). Peptide motifs identified to mimic these carbohydrate forms are

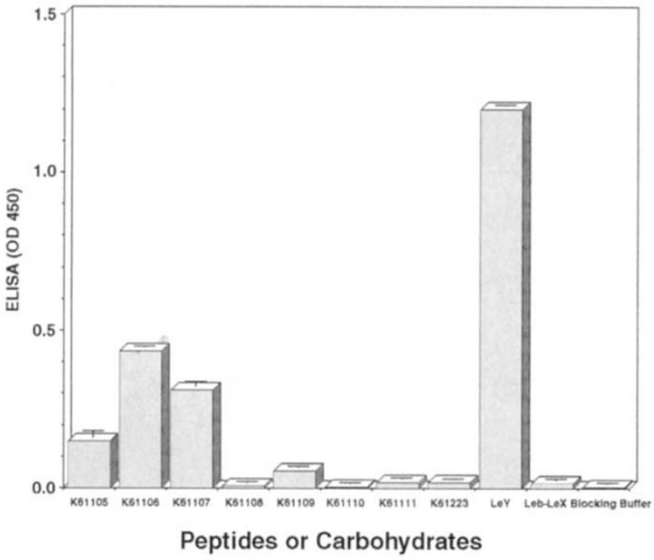

Figure 1. Binding of the LeY specific antibody BR55-2 to various peptides and LeY. The peptides K61105, K61106, and K61107 correspond to YYPYD, YYRYD, and YWRYD respectively. Other K series peptides were variants of the motifs or irrelevant peptides. Blocking buffer alone was also used as a control because anticarbohydrate antibodies have a tendency to adsorb to blocking agents, enhancing nonspecific binding.

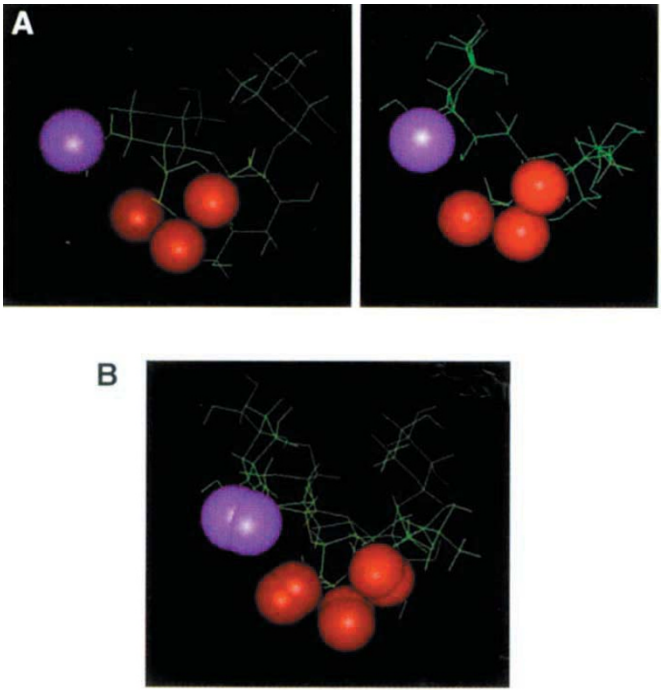

Figure. 2 Functional group similarities between LeY and MCP. (A) Low-energy conformers of LeY (on left side of panel A) and MCP (on right side of panel A) are compared highlighting the conserved spatial positions of the methyl group on GlcNAc (magenta colored sphere) and hydroxyl oxygens on the Fuc residue of Fuc1-3GlcNAc (red colored spheres) of the LeY structure and the methyl group of α2 sialic residue (magenta colored sphere) and hydroxyl oxygens (red colored spheres) of α9 MCP. (B) Superposition of LeY tetrasaccharide and MCP. In this orientation, the hydroxyl groups on the Fuc residue of Fuc1-3GlcNAc are spatially conserved with those of the α9 sialic residue of MCP, while the respective methyl groups on GlcNAc and on α2 sialic residue are spatially conserved.

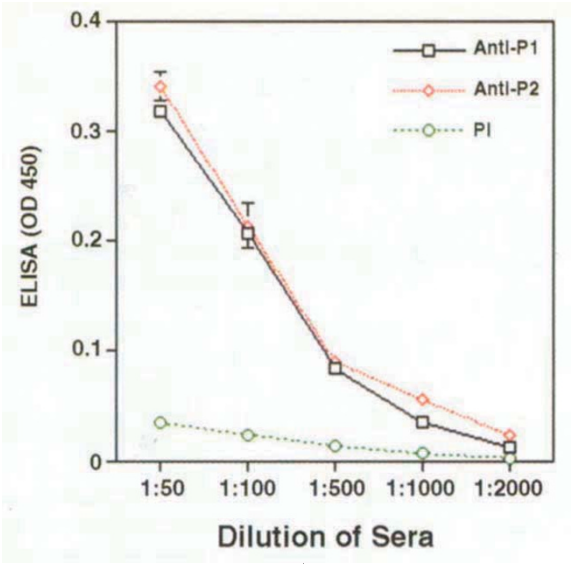

Figure 3 Binding of polyclonal antisera to LeY.

YPY, which has been found to mimic mannose[17,18]; WRY, which has been found to mimic α(1-4)glucose[19,20]; PWLY, found to mimic LeY[21]; and YRY, found to mimic the major C polysaccharide α(2-9) sialic acid (MCP) of *Neisseria meningitidis*[10]. It is noteworthy that these potential surrogate immunogens involve aromatic-aromatic interactions, suggesting that the motifs mimic carbohydrate subunits shared among a variety of carbohydrate forms.

The antigenic mimicry of LeY by the planar-X-planar motifs is shown in Figure 1. ELISA reactivities of YPY, YRY, and WRY motifs with the anti-LeY monoclonal antibody BR55-2 indicate that BR55-2 is specific for these motifs, displaying very little reactivity with other peptide sequences. BR55-2 displays high specificity for LeY, being made against an LeY-expressing tumor cell line[22]. We have recently determined the molecular recognition properties of LeY for BR55-2 (ref. 23), and that the LeY tetrasaccharide core structure is similar to the core structure of MCP (Fig. 2). The low energy-conformations of MCP and LeY structures overlap in their antigenic presentation, which may be mimicked by homologous peptides (Fig. 1).

Anti-LeY response to peptides. To determine the extent to which aromatic-aromatic motifs immunologically mimic the LeY antigen, we immunized Balb/c and C57Bl/6 mice with peptides containing YYPYD (P1) and YYRYD (P2) motifs. We also immunized with a peptide that changes the YYRYD sequence tract to YYRGD (P3). The RYD sequence has been shown to be a mimic for the adhesion motif RGD and its conformational

Table 1. Mean florescence of binding of antipeptide sera to different cells as measured by FACS.

Cell line	Anti-P1 (YYPY)	Anti-P2 (YYRYD)	Anti-P3 (YYRGD)
SKBR3	240.6	275.6	166.7
HS578 Bst (normal breast)	17.8	19.9	22.4
WM793	145.5	172.4	42.3
MT2	19.5	22.3	23.1

Background fluorescence (mean fluorescence) associated with nonspecific mouse sera is 24.2 for SKBR3, 24.4 for WM793, 17.3 for MT2, and 18.4 for HS578 (final sera concentration: 1:50).

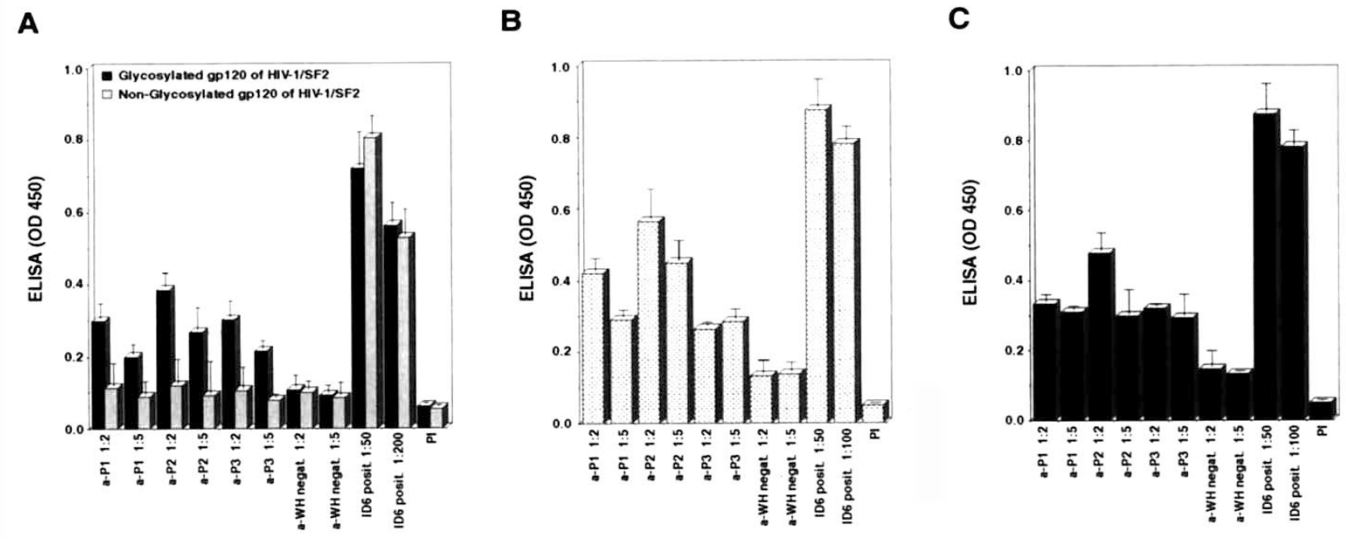

Figure 4. Binding of polyclonal antipeptide sera to HIV-1/SF2 and MN env protein. (A and B) Binding of sera derived from C57Bl/6 immunized mice; (C) Binding of sera derived from Balb/c mice. PI in (A and B) is preimmune C57Bl/6 sera. PI in (C) is preimmune Balb/c sera.

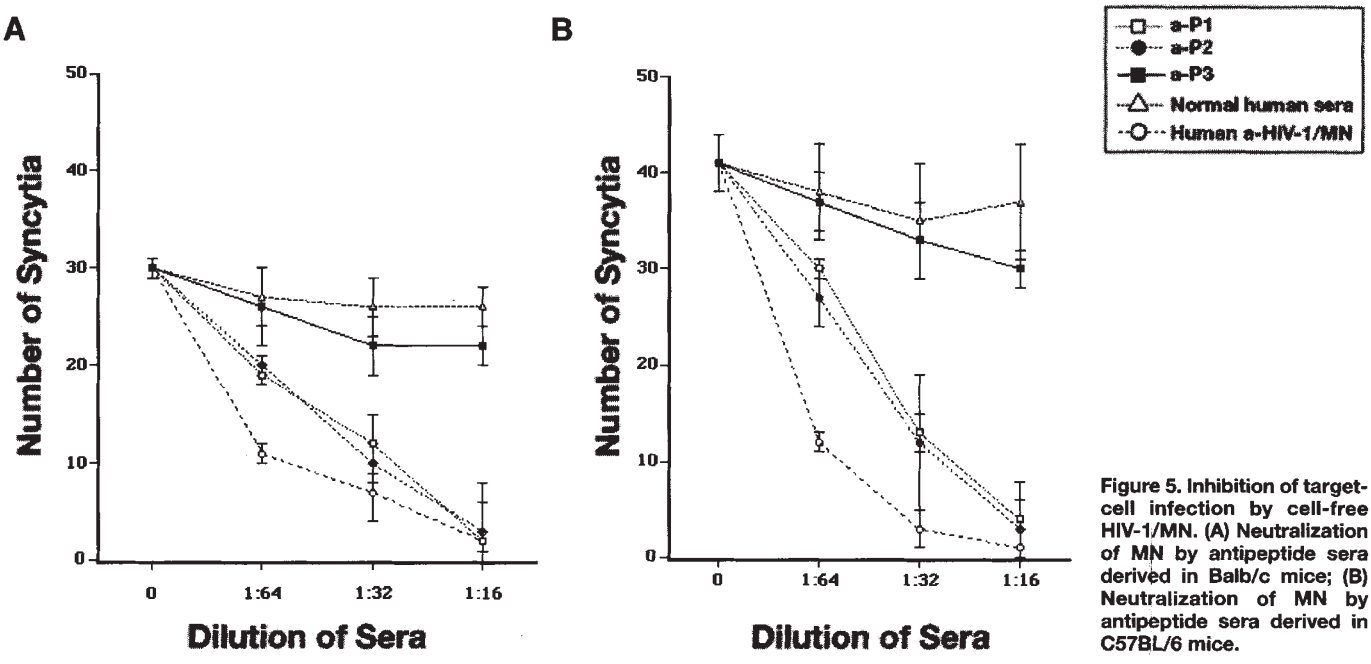

Figure 5. Inhibition of target-cell infection by cell-free HIV-1/MN. (A) Neutralization of MN by antipeptide sera derived in Balb/c mice; (B) Neutralization of MN by antipeptide sera derived in C57BL/6 mice.

properties correlate with bioactive RGD compounds[24]. Peptides had the form CARIYYXYDGFAY, which followed the structural properties of an antibody loop[10] or were synthesized as triple repeating units that emulate helical configurations, often observed for polysaccharides. It was observed that the reactivity of the IgG antisera to P1 and P2 titers out to 1:2000 against the LeY structure on solid phase ELISA (Fig. 3) with little reactivity observed from preimmune (PI) sera.

Envelope protein binding. We examined whether the polyclonal antipeptide sera derived from C57Bl/6 bound to glycosylated and nonglycosylated forms of HIV-1/SF2 (Fig. 4A). Negative-control groups included preimmune sera, a mouse polyclonal monospecific antisera directed to an irrelevant antigen[25] and a positive-control Balb/c monoclonal antibody (ID6) directed to HIV-1 glycosylated and nonglycosylated gp120 (ref. 26). Unexpectedly, we found that reactivity with the glycoslyated forms of HIV-1 env provided statistically significant O.D. readings only up to 1:5 dilution. Nevertheless, within this dilution range we observed that the sera bound approximately threefold better to the glycosylated form of SF2 over preimmune sera background, while binding to the nonglycosylated form was equivalent to that observed for the negative-control murine sera derived from immunization with an irrelevant antigen.

We also examined this sera for binding to glycosylated gp140 env protein of HIV-1/MN, lacking the transmembrane domain of gp41 (Fig. 4B). The antisera against all three peptide motifs bound

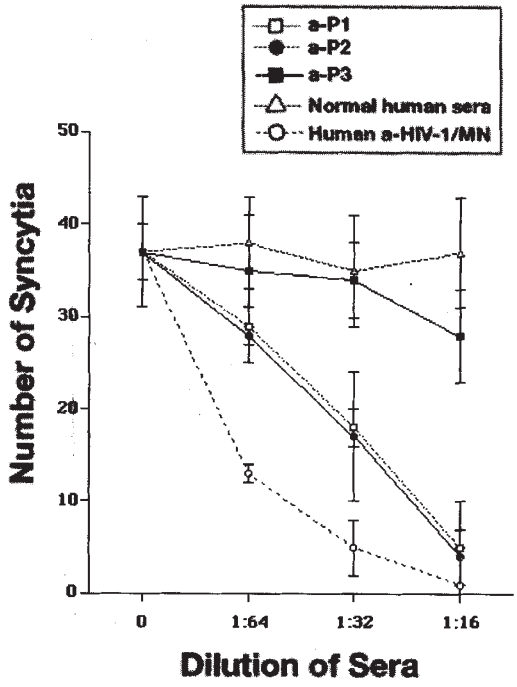

Figure 6. Inhibition of target cell infection by cell free HIV-1/3B isolate with sera derived from Balb/c mice.

to gp 140 HIV-I/MN when used at final dilution of 1:5. Binding was at least twofold above the preimmune sera and sera derived from immunization with irrelevant antigen as background. The same result was observed with sera derived from Balb/c animals (Fig. 4C). These results suggest that the antisera could recognize sugars on the HIV env protein, but perhaps the sugars were not presented in a proper orientation for maximum antisera reactivity to occur, or that the density of the carbohydrate expressed on the env protein surface was not high. In any event, our antisera bound to glycosylated but not to nonglycosylated gp120 derived from HIV-1 SF and MN.

Neutralization of HIV. Sera from Balb/c (Fig. 5A) and C57Bl/6 (Fig. 5B) mice immunized with P1 or P2, neutralized HIV-1/MN at final dilution's up to 1:64. Normal human sera and the anti-P3 sera were ineffective at blocking syncytia formation, whereas human α-HIV-1 sera from four different infected patients neutralize cell-free virus at dilutions up to 1:64. The isolate specificity was further determined by cell-free neutralization of the HIV-1/3B isolate (Fig. 6). As with MN, anti-P1 and anti-P2 Balb/c sera were effective in neutralizing virus infection in vitro. Collectively, these results suggest that the production of HIV-neutralizing antibodies by the peptide-proteosome complexes induces humoral immune responses in divergent haplotypes that can be as effective as sera from HIV-1–infected individuals in neutralizing HIV-1 cell-free infection.

Binding of immune sera to cells. To further confirm that the peptide-induced antisera were reacting with virus but not target cells used in the neutralization assay, we investigated binding of the sera to different cell lines including the MT-2 human T cell line by FACS. All three antisera did not bind to the MT-2 line at all (Table 1). Importantly, the antisera also did not bind to normal breast cells, HS578, which suggests that there is little to no tissue adsorption of the antipeptide sera in normal tissue. All three sera reacted very strongly with the LeY-expressing SKBR3 human breast-cancer line. Two out of three antisera also bound the human melanoma cell line WM793, which expresses sialyated GD2/GD3 gangliosides. While the antisera recognized overly expressed carbohydrate antigens on two control cell lines, no binding to the target MT-2 cells was observed, suggesting that the mechanism of inhibition of viral infection is connected with binding of the antipeptide sera to viral particles.

Discussion

Comparison of oligosaccharide profiles on the HIV env protein reveals that different virus isolates, propagated in the same host cells, yield very similar glycan patterns, whereas cultivation of an isolate in different host cells results in markedly divergent oligosaccharide maps[27]. Variations concern the proportion of high-mannose-type, hybrid-type, and complex-type substituents, as well as the state of charge and structural parameters of the complex-type species. As a characteristic feature, complex-type glycans of monocyte-derived–macrophage-derived viral glycoprotein are almost exclusively substituted by lactosamine repeats.

We and others have found that certain peptides mimic carbohydrate subunits, inducing cross-reactive in vivo anticarbohydrate-like antibody responses. We have shown that aromatic containing peptide motifs can mimic salient features of at least one lactosamine form, LeY. Unlike synthetic carbohydrate forms[8,9], sera induced with these peptide motifs bound to an LeY-expressing cell line and can neutralize HIV-1 cell-free infection in vitro. The specificity in the neutralization profile is illustrated by considering that a change in one amino acid in an immunizing peptide (YYRYD to YYRGD) can affect the neutralizing ability of the antipeptide sera. We have found that the production of HIV-neutralizing antibodies was not severely major histocompability complex-restricted, and cross-reacts and cross-neutralizes with at least two divergent isolates within clade B; the major North American and Western European clade. We do not know at this time if the antipeptide sera inhibit primary isolates of HIV-1, which are more resistant to neutralization[27–30].

It was not the intent of these studies to advocate a new vaccine that displays broad HIV-1 neutralization ability, but rather to draw attention to the possibility of developing peptides that mimic HIV-1–associated carbohydrate forms. We found that aromatic-aromatic interactions are a major driving force in mimicking carbohydrate subunits. Polymerization of aromatic residue-containing peptides can structurally mimic the helical shape of many carbohydrate forms. While carbohydrate-conjugate vaccines are certainly effective and are viewed as superior to peptide mimics, peptide mimics might be used as priming or boosting agents, being formulated to develop longer-lasting responses after booster immunization. As peptides have the ability to stimulate T cell help in an antigen-specific manner, peptide mimetics would be of importance as novel agents for adjuvant therapy. Peptides that mimic carbohydrates may be further designed and manipulated to develop specific immune responses against a variety of polysaccharides on bacteria, viruses, and tumors that might be associated with their pathobiology.

Experimental protocol

Generation of polyclonal sera. Peptides were synthesized with the addition of a tripeptide YGG spacer, and a cysteine at the amino terminus conjugated to a lauroyl group (Bio-Synthesis, Lewisville, TX) and then complexed with proteosomes[10]. For generation of polyclonal sera, Balb/c mice (H-2^d) (four per group) and C57Bl/6 (H-2^b) (four per group) 4 to 6 weeks of age, were immunized intraperitoneally on a weekly basis for 3 weeks with 50 μg of peptide-proteosome complex. Sera were collected within 7 to 14 days after the last immunization and analyzed for binding against LeY by ELISA .

Binding of immune sera and BR55-2 to LeY and peptides. Solid-phase ELISA was performed to asses the binding activity of the generated sera to LeY incorporated into polyacrylamide (PAA) matrix, creating 30 kDa multivalent polymer (GlycoTech Inc., Rockville, MD). Immunlon 2 plates were

coated with the multivalent LeY-PAA probe overnight at 4°C. The plates were blocked with 0.5% FCS/0.2% tween , 200 µl/well, 37°C, for 1 h. Serial dilutions of the respective antisera were added and incubated at 37°C for 2 h and resolved with 100 µl/well of 1:10,000x goat antimouse IgG isotype matched HRP (Sigma, St. Louis, MO) diluted in blocking buffer, incubated at 37°C for 1 h, and read at OD_{450}. This same protocol was used in assessing BR55-2 binding to various peptides and carbohydrate probes. Peptides were presented as MAP peptides (Genetics Research, Huntsville, AL) coated at 2 µg/well. Multivalent LeY and Leb-LeX probes were coated at 0.1 µg/well and the monoclonal antibody BR55-2 (IgG3) concentration used was 0.1 µg.

Binding of immune sera to cells. Cells, with FACS buffer (1% BSA, 0.01% Na azide, 25 mM EDTA) were washed, scraped and transferred to 15-ml centrifuge tubes. Viability of cells was checked by trypan blue and 100 µl of 1 to 2×10^6/ml cells in FACs buffer were used for analysis. Ten microliters of experimental or control sera were added to sample tubes and incubated on ice for 30 min, washed twice and 10 µl of FITC Ab (goat antimouse IgG conjugate FITC-labeled (Sigma) diluted 1:20 with PBS) was added to the sample. Cells were fixed with 1% paraformaldelhyde, followed by FACS measurement.

Binding of immune sera to HIV envelope protein. Oligomeric soluble gp140 , a truncated env of the HIV-1/MN, was produced by BS-C-1 cells infected with VPE12B, recombinant vaccinia virus, and was purified[31]. Bacteria produced nonglycosylated SF2 gp120 and Chinese hamster ovary cells produced glycosylated SF2 gp120 were obtained from the AIDS Research and Reference Reagent Program (Rockville, MD). Binding of sera were determined by ELISA as previously described[26]. Briefly, 2 µg/ml of recombinant proteins were adsorbed onto microtiter wells. Serial dilutions of experimental or control antisera were added to these antigen-coated plates. Wells were washed, incubated with goat antimouse IgG conjugated with HRPO (Sigma), and developed with 3,3′,5,5′-tetramethylbenzidine dihydrochloride (TMB).

Viral neutralization assay. Cell-free HIV-1/MN and HIV-I/3B, obtained from the AIDS Research and Reference Reagent Program, were propagated in H9 cells. Cell-free virus neutralization was performed as previously described[32,33] with minor modifications. One hundred $TCID_{50}$ of HIV-1/MN or HIV-1/3B cell-free virus (50 µl) were preincubated with serial dilutions of experimental antisera or controls (preimmune mouse sera, normal human sera, or mixture of four sera of HIV-1 positive patients) for 1 h at 37°C. Following incubation, the pretreated virus was then plated on 4×10^4 HTLV-I/MT-2 target cells (50 µl), for 1 h at 37°C . The target cells were then washed three times and incubated at 37°C with 5% CO_2. Neutralization was detected as inhibition of syncytia 3 days later, assessed by counting the number of multinuclear cells[33].

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