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N-Acetyl-β-D-glucosaminyl-binding properties of the envelope glycoprotein of human immunodeficiency virus type1*

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ABSTRACT

The effect of carbohydrate structures on the adsorption of HIV-1 or of recombinant envelope glycoprotein gp 160 (rgp 160) to cells of the CEM line was investigated with an indirect immunofluorescence assay using gp 120-specific mouse monoclonal antibodies (mAbs) directed to envelope gp 120. The β-D-galactosyl, α-D-mannosyl, β-D-glucosyl, *N*-acetyl-β-D-glucosaminyl, sialosyl, and L-fucosyl derivatives tested had no effect on this binding. However, preincubation of HIV-1 (or rgp 160) with the neoglycoprotein, β-D-GlcNAc$_{47}$–BSA, specifically inhibited the labeling, by some of the mAb used, of HIV-1 (or rgp 160) bound at the cell membrane. This inhibition occurred only with mAbs that were specific for the immunodominant "neutralizing" third variable region (V3) of gp 120. Competition for the binding to rgp 160 between β-D-GlcNAc$_{47}$–BSA and mAb was further demonstrated by use of affinity matrices substituted with one of the relevant mAb (110-4), or with β-D-GlcNAc$_{47}$–BSA. Besides β-D-GlcNAc$_{47}$-BSA-Sepharose, rgp 160 also bound with low affinity, but high specificity, to two other *N*-acetyl-β-D-glucosaminyl affinity matrices, β-D-GlcNAc-divinylsulfone-agarose and asialoagalactothyroglobulin-agarose. Conversely, β-D-[^{125}I]GlcNAc$_{47}$–BSA bound specifically to gp 160-Sepharose. These results indicated that rgp 160 behaves as a *N*-acetyl-β-D-glucosaminyl-binding protein for GlcNAc residues presented at high density on a carrier, the carbohydrate-binding site of which is close to, or located on the V3 region of gp 120.

INTRODUCTION

The *env* gene of human immunodeficiency virus type 1 (HIV-1) encodes two glycoproteins, gp 120 and gp 41, which are cleavage products of a 160-kD precursor, gp 160[1]. External gp 120 binds with high affinity (K_d 3 × 10^{-9}M)[2,3] to CD4 molecule, the HIV-1 cellular receptor[4]. Fusion of virus envelope and cell membrane triggered by transmembrane gp 41[5,6] initiates viral penetration into the target cells[1,7]. Envelope glycoproteins are highly glycosylated molecules, and N-linked glycans, which represent

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approximately one-half of the molecular weight[8,9], are prominent structures on the HIV-1 surface. The precise role of these carbohydrate components, however, is poorly understood at present.

Lectin–carbohydrate interactions have been implicated in many aspects of the relationship between microorganisms and their host[10,11]. For example, they determine the efficiency and tropism of cellular infection for some viruses, as shown for influenza virus[12], whose hemagglutinin viral-attachment protein contains a sialic acid-binding pocket through which virions associate with sialoglycoprotein-binding sites on the surface of target cells. On the other hand, carbohydrate–lectin interactions may also serve for the recognition and ingestion of foreign particles by phagocytic cells, as first indicated by Sharon[10]. Therefore, lectins have been evaluated for their ability to bind *env* glycoproteins of HIV-1[13,14]. Concanavalin A, *Lens culinaris* lectin, and phytohemagglutinin, and more recently human serum D-mannose-binding protein[15], have been shown to attach to gp 120, which resulted in the neutralization of HIV-1 *in vitro*.

We hypothesized that lectins of the lymphocyte membrane, to which have been ascribed crucial biological properties as cell recognition molecules[16], might also be involved in the binding of gp 120 carbohydrates, and we examined whether this may play a role in the attachment of HIV-1 to $CD4^+$ cells. Therefore, we tested the inhibitory effect of different carbohydrates or carbohydrate derivatives on the binding of HIV-1 or soluble recombinant gp 160 (rgp 160) to $CD4^+$ lymphoid cells. None of these molecules showed any significant inhibitory effect under the experimental conditions used, indicating that lectin–carbohydrate interactions were not of major importance in the binding of HIV-1 to its target cells. Unexpectedly, however, we observed that gp 120 possesses *N*-acetylglucosaminyl-binding properties.

EXPERIMENTAL

Virus and rgp 160. — Purified viral pellet (1 mg/mL) of the prototype LAV_{BRU} strain of HIV-1[17] was purchased from Diagnostics Pasteur (Marnes-La Coquette, France). Because it has been demonstrated that rgp 160 and rgp 120 have the same properties for binding to CD4[2], only rgp 160, obtained from Transgene S.A. (Strasbourg, France), was utilized for logistical reasons. The recombinant protein was produced in BHK-21 cells infected with HIV-1-gp 160–vaccinia recombinant viruses as described by Kieny *et al.*[18].

Purification of anti-gp 120 murine monoclonal antibodies (mAbs). — mAb 110-3, 110-4, and 110-5 (Genetic Systems, Seattle, WA, U.S.A.), which recognize the third variable loop domaine of gp 120 (V3) of the LAV_{BRU} isolate of HIV-1 at position 303–338, a region that is not involved in the binding site of gp 120 to CD4[19,20], were used to label HIV-1 particles or rgp 160 previously complexed to the cells. Also used was mAb 110-1 (Genetic Systems), which recognizes the C-terminus of gp 120[19], and polyclonal human antisera to gp 120. IgGs from mAb-110-4 were purified in one step by filtration of ascitis fluid (1 mL) through a Sephadex G-200 column (Pharmacia Fine Chemicals, Uppsala, Sweden); the IgGs (10 mg) were coupled to CNBr-Sepharose CL 4B (4g; Pharmacia) according to the manufacturer's instructions (110-4-Sepharose).

Iodination of rgp 160. — As described by Fenouillet *et al.*[2], rgp 160 (5 μg) in phosphate-buffered saline solutine (50 μL, PBS), pH 7.4, and $Na^{125}I$ (9 MBq; 610.5 MBq/μg) (Amersham International PLC, Little Chalfour, Bucks, U.K.) were added to a tube coated with iodogen (5 nmol), and the mixture was incubated for 10 min at room temperature. The reaction was stopped by the addition of 0.9% L-tyrosine (10 μL). The iodinated glycoprotein was separated from $Na^{125}I$ by filtration through a Sephadex G-25 (PD 10) column (Pharmacia). The specific radioactivity of radiolabeled gp 160 ([^{125}I]gp 160) was ~1.3 MBq/μg.

CD4 lymphoid cells. — Cells of the CEM line (American Type Culture Collection, Rockville, MD, U.S.A.) were cultured at 37° in RPMI 1640 medium (Flow Laboratories Inc., Irvine, Scotland), supplemented with 10% of FCS, 1% of L-glutamine, and 1% of antibiotics (Gibco Laboratories, Paisley, Scotland) in humidified atmosphere containing 5% of CO_2.

Binding of rgp 160 or of HIV-1 to CD4$^+$ cells. — The buffer solutions were those described by McDougal *et al.*[21]. Heat-inactivated HIV-1 (1 μg) or rgp 160 (50 ng) were incubated for 1 h at 37° with 1 × 10^6 CEM cells in buffer (25 μL) containing 0.5% of bovine serum albumin (BSA) (Sigma Chemical Co, St. Louis, U.S.A.). The cells were washed and resuspended in mAb (110-3, 110-4, 110-5, or 110-1) diluted (1:100) in buffer (25 μL); after 30 min at 4°, the cells were washed and incubated again for 30 min at 4° with 1:25 sheep antimouse IgGs coupled to biotin (Amersham, International). In some experiments, human anti HIV-1 IgG was used instead of mAb. After being washed, the cells were resuspended in streptavidin–phycoerythin (25 μL) (Becton Dickinson Microbiology Systems, Mountain View, CA, U.S.A.), diluted (1:25) in buffer, for 30 min at 4°. After two more washes, the cells were resuspended in buffer (500 μL) containing 1% of paraformaldehyde. The membrane-fluorescence intensity was measured with the FACS analyzer (Becton Dickinson).

To test the hypothesis that lectin-like interactions were involved in the binding of HIV-1 external glycoprotein to CD4$^+$ cells, various carbohydrates and carbohydrate derivatives were coincubated at different concentrations with CEM cells and HIV-1 (or rgp 160), before labeling (Table Ia). In parallel experiments, the carbohydrate compounds were preincubated either with CEM cells (Table Ib and Id) or with HIV-1 (or rgp 160) (Table Ic). D-Glucose, D-mannose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, lactose, 4-nitrophenyl β-D-galactopyranoside, and 4-nitrophenyl α-D-glucopyranoside (Sigma) were tested at a mM concentration, D-mannan and β-D-glucan (Sigma) at a 0.2–2 mg/mL concentration, and fetuin and asialofetuin (Sigma) at the μM concentration. Synthetic neoglycoproteins were obtained by conjugating BSA with various 4-nitrophenyl glycosides (Sigma), as described by Westphal and Schmidt[22]. The substitution ratio of sugars to BSA was 16 for α-D-Man-BSA, 23 for β-D-Gal-BSA, 12 for α-L-Fuc-BSA, 47 for β-D-GlcNAc-BSA, and 10 for β-D-lactosyl-BSA. These neoglycoproteins were tested at the μM concentration (0–33μM).

Binding of [^{125}I]gp 160 to various affinity matrices. — β-D-$GlcNAc_{47}$-BSA or β-D-Gal_{23}-BSA (8 mg) was coupled to CNBR-Sepharose Cl 4B (4 g) (Pharmacia) to give

β-D-$GlcNAc_{47}$-BSA- and β-D-Gal_{23}-BSA-Sepharose, respectively, according to the manufacturer's instructions. The other affinity matrices used were: β-D-GlcNAc-divinyl-sulfone-agarose, lactogel (E.Y. Laboratories Inc., San Mateo, CA, U.S.A.), and thyroglobulin-agarose (Sigma). In some experiments, thyroglobulin-agarose (5 mL) was pretreated with *Vibrio cholerae neuraminidase* (EC 3.2.1.18; 100 mU) (Behringwerke, Marburg, F.R.G.) dissolved in 50mM sodium acetate–0.150M NaCl–9mM $CaCl_2$ buffer, pH 5.5 (100 μL), and diluted with PBS (pH 7) to 400 μL; after incubation for 24 h at 37°, the matrix was centrifuged for 10 min at 1000*g*. The concentrations of free sialic acid in the supernatant solution, determined by the thiobarbituric reaction as described by Warren[23], was 59nM. α- and β-D-Galactosidases from coffee bean, *Aspergillus niger*, and *Escherischia coli* (Sigma) (50 mU of each) were added to the matrix in PBS, pH 5.5 (500 μL), and the mixture was incubated for 48 h at 37°. After centrifugation, the concentration of free D-galactose, determined in the supernatant solution by the enzymic method of Schachter[24], was 69nM. The matrix was washed and suspended in an equal volume of PBS, supplemented with 0.1% NaN_3, before being used with the appropriate buffer.

The binding capacity of [^{125}I]gp 160 to the affinity matrices was determined as follows. Various amounts of affinity matrices (5–100 μL), suspended in an equal volume of 0.02M Tris, 0.15M NaCl, 0.01M $CaCl_2$, and 0.05% BSA buffer, pH 7.4, (Tris–Ca^{2+}–BSA buffer), were incubated with various amounts (0.06–1.5 ng) of [^{125}I]gp 160 for 1 h at 37°. Unbound [^{125}I]gp 160 was eliminated by two washes with buffer (500 μL each). The solid-phase-bound radioactivity was counted in a gamma counter (Packard). In some experiments, in order to determine the calcium dependency of the interaction, the binding test between [^{125}I]gp 160 and the matrix was performed in buffer containing 10mM $CaCl_2$ with or without 10mM EDTA (Sigma). In order to determine the carbohydrate specificity of the interaction, [^{125}I]gp 160 was preincubated for 45 min at 37° with carbohydrates or carbohydrate derivatives diluted in the buffer prior to the binding test. The effect of various mAbs on the binding of *[^{125}I]gp* 160 to some of the matrices, the binding of [^{125}I]gp 160 to 110-4-Sepharose, and the effect of carbohydrate derivatives on the interaction between [^{125}I]gp 160 and 110-4-Sepharose were tested by the same procedure in parallel experiments.

Binding of β-D-[^{125}I]GlcNAc$_{47}$-BSA to rgp 160-Sepharose. — β-D-$GlcNAc_{47}$-BSA was iodinated by the same procedure as that used for [^{125}I]gp 160, the specific radioactivity being ~0.4 MBq/μg. Rgp 160 was coupled to CNBr-Sepharose CL4B (Pharmacia) according to the manufacturer's instructions (rpg 160–Sepharose). Investigation of the binding capacity of β-D-[^{125}I]$GlcNAc_{47}$-BSA to rgp 160-Sepharose and competition experiments between the binding of ^{125}I-labeled and unlabeled β-D-$GlcNAc_{47}$-BSA to rgp 160-Sepharose were performed under the same conditions as those described earlier for [^{125}I]rgp 160 and the various affinity matrices.

RESULTS AND DISCUSSION

Lectin–carbohydrate interactions are not involved in the binding of HIV-1 or rgp

160 to CD4⁺ cells. — Our aim was to investigate whether lectin–carbohydrates interactions play a role in the attachment of HIV-1 to CD4⁺ lymphoid cells. Various carbohydrates or carbohydrate derivatives were incubated with cells of the CEM line and HIV-1, or rgp 160, according to the experimental procedures described in Table I (a, b, and c). None of the lactosyl, D-galactosyl, *N*-acetyl-D-galactosaminyl, sialosyl, D-glucosyl, D-mannosyl, *N*-acetyl-D-glucosaminyl, and L-fucosyl structures tested had any effect on the binding of HIV-1 or rgp 160 to the CEM cell membrane (Table II). The observation that these structures do not interfere with the binding of HIV-1 envelope glycoprotein to its target is in line with the results of Fenouillet *et al.*[2,25] who have shown that neither total enzymic deglycosylation of soluble recombinant CD4 nor that of rgp

TABLE I

Outline of the assays for examining the binding of HIV-1 particles or rpg 160 to CD4⁺ CEM cells[a]

Conditions	*Components preincubated for 1 h at 37° with carbohydrate or carbohydrate derivative*	*Component added, followed by incubation for 30 min at 37°*
a	CEM cells + HIV-1	None
b	CEM cells	HIV-1
c	HIV-1 or rgp 160	CEM cells
d	CEM cells, followed by washing	HIV-1

[a] The incubation for 30 min at 37° was followed by a washing and addition of mAb.

TABLE II

Carbohydrates and carbohydrate derivatives that were devoid of significant effect on the binding of HIV-1 (or rgp 160) to CD4⁺ CEM cells

Compound	*Concentration (mM)*
Lactose	5
$Lactosyl_{10}$-BSA	0.003–0.033
β-D-Gal*p*$OC_6H_4NO_2$(4)	10
β-D-Gal_{23}-BSA	0.003–0.033
Asialofetuin	0.025
D-GalNAc	1–100
Fetuin	0.025
D-Glucose	100
β-D-Glucan	2[a]
α-D-Glc*p*$OC_6H_4NO_2$(4)	10
α-D-Glc*p*OMe	10
D-Mannose	100
α-D-Man*p*OMe	100
D-Mannan	2[a]
α-D-Man_{16}-BSA	0.003–0.033
D-GlcNAc	1–100
β-D-$GlcNAc_{47}$-BSA	0.003–0.033
α-L-Fuc_{12}-BSA	0.003–0.033

[a] mg/mL.

120 or of viral gp 120 does significantly modify the interaction between these two molecules. This indicated that carbohydrates are not involved in the respective binding of these molecules. However, the latter findings are at variance with those of Matthews *et al.*[26] who reported that enzymic deglycosylation of soluble, viral gp 120 markedly reduced its capacity to bind to membrane CD4 and to inhibit syncytium formation. This discrepancy may be due to differences in the gp 120 purification and deglycosylation methods: interestingly, full deglycosylation of gp 120 was performed in the absence of detergents and denaturing agents by Fenouillet *et al.*[2,25]. It has also been shown that the use of compounds that inhibit glycosylation processing markedly reduce HIV-1 infectivity and cytopathogenicity[27–29]. In this case, it is possible that aberrant glycosylation can affect processing and folding of gp 120, which may then result in alteration of its biological activity; this point is currently under investigation. From the present results, it may be concluded that lectin–carbohydrate interactions are not necessary for the first step of infection of $CD4^+$ cells by HIV-1, *i.e.*, the attachment of virions to the cell membrane. However, it still remains possible that such interactions may play a role in postbinding events that lead to virus penetration into the cells, which could perhaps explain, rather than mere steric hindrance, the *in vitro* anti-HIV-1-neutralizing activity of some lectins[13–15].

Competition between some mAb and β-D-GlcNAc$_{47}$-BSA for the binding to gp 120. — One of the derivatives, the neoglycoprotein β-D-$GlcNAc_{47}$-BSA, repeatedly inhibited (n 10), in a dose-dependent manner, the subsequent labeling by mAb 110-4 of cells mixed with HIV-1, in experiments conducted as described in Table I (a,b,c). The same

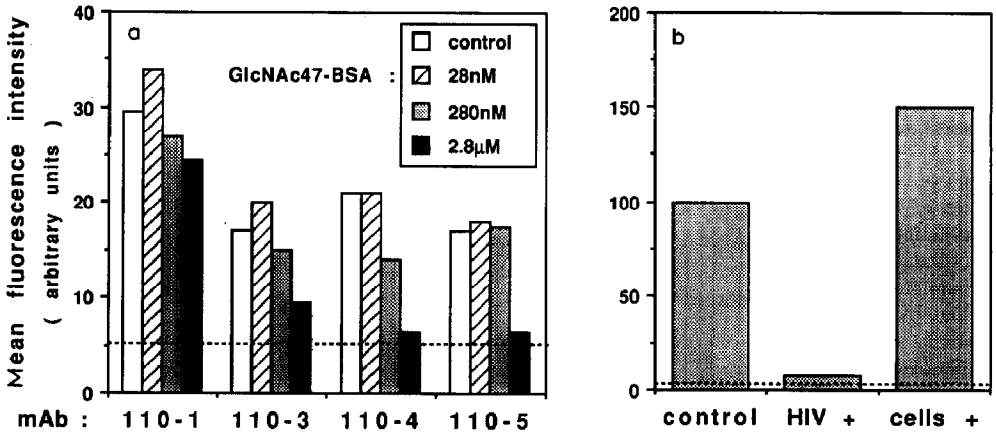

Fig. 1. Inhibition by β-D-$GlcNAc_{47}$-BSA of the binding of anti-gp 120 mAb to HIV-1 viral particles adsorbed on CEM cells: (a) Viral particles were preincubated with buffer (control) or with different concentrations of β-D-$GlcNAc_{47}$-BSA before the addition of CEM cells. The cells were then washed and mixed with the mAb as described in the Experimental section. (b) In an independent experiment, either virus was preincubated with (28µM) β-D-$GlcNAc_{47}$-BSA before addition of the cells (HIV $^+$) or CEM. The cells were preincubated with (28µM) β-D-$GlcNAc_{47}$-BSA, followed by a washing step before the addition of virus (cells +). The rest of the experiment was conducted as described in the Experimental section. The horizontal, dashed line at the bottom of the graphs indicates the control mean-fluorescence intensity obtained in the absence of mAb.

inhibition was observed for the two other mAbs (110-3 and 110-5), known to recognize the V3 region of gp 120, but not with mAb 110-1, which is specific for another region of gp 120 (Fig. 1a) or with polyclonal human anti HIV-1 IgG (data not shown). This suggested that β-D-$GlcNAc_{47}$-BSA interferes with mAb 110-3, 110-4, or 110-5 binding to viral gp 120 rather than with the attachment of viral gp 120 to the cells. It was clear that β-D-$GlcNAc_{47}$-BSA did not interact directly with the mAb, because under all conditions (Table Ia,b,c), a washing step removed the excess of the neoglycoprotein prior to the addition of the mAb for labeling. Moreover, the preincubation of CEM cells with β-D-$GlcNAc_{47}$-BSA, followed by washing prior to the addition of HIV-1 (Scheme Id), did not result in the inhibition of labeling with mAb 110-4 (Fig. 1b). This demonstrated that the neoglycoprotein interacts with viral gp 120 but not with the CEM cells, and that it competes with the fixation of the mAb to viral gp 120 but not with the binding of viral gp 120 to $CD4^+$ cells. The observation that other neoglycoproteins, such as β-D-Gal_{23}-BSA, $lactosyl_{10}$-BSA, and α-L-$fucosyl_{12}$-BSA, had no effect on the binding of mAb 110-4 to viral gp 120, whereas α-D-Man_{16}-BSA had a slight inhibitory effect (data not shown), testified nonetheless for the carbohydrate specificity of the competition observed with β-D-$GlcNAc_{47}$-BSA. However, free *N*-acetyl-D-glucosamine at high concentration (100mM) did not compete with mAb 110-3, 110-4, or 110-5 for the binding to HIV-1. Taken together, these results suggested that GlcNAc units, when present at high density on a protein carrier, may specifically bind to viral gp 120 whose *N*-acetyl-β-D-glucosaminyl-binding site may be the region recognized by these mAbs or close to it. Interestingly, these mAbs are known to bind to the immunodominant V3 loop of gp 120 that constitutes the principal target for type-specific neutralizing antibodies[30]. Actually, antibodies bound to this loop do not block binding of gp 120 to CD4[19,20,30], but rather they prevent infection by disrupting other events that occur after virus binding, such as

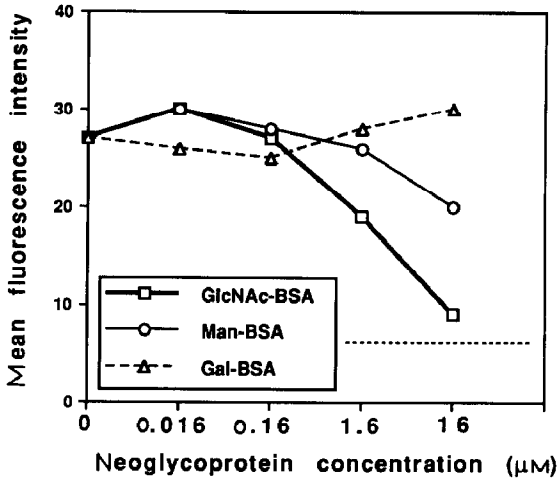

Fig. 2. Inhibition by β-D-$GlcNAc_{47}$-BSA of the binding of mAb 110-4 to rgp 160. Rgp 160 was preincubated with 0–16μM neoglycoprotein before the addition of CEM cells, and mAb 110-4 was added after washing. The horizontal dashed line at the bottom of the graph indicates the control mean-fluorescence intensity obtained in the absence of mAb.

the interaction between gp 120 and gp 41, or membrane fusion. Therefore, it is possible that the attachment of a neoglycoprotein to the same region, if the affinity of interaction were strong enough, might result in the same neutralization as antibody binding. However, our preliminary results indicated that this does not appear to be the case (data not shown). At any rate, the existence of carbohydrate-binding properties in this region might open new approaches for drug targeting.

Under the same experimental conditions (Fig. 2), β-D-$GlcNAc_{47}$-BSA but not β-D-Gal_{23}-BSA also inhibited the binding of mAb 110-4 to rgp 160 in a dose-dependent manner, whereas α-D-Man_{16}-BSA displayed a moderate effect similar to that we observed when using HIV-1, thus suggesting that rgp 160 may present α-D-mannosyl-binding properties (Fig. 2). These results confirmed that *N*-acetyl-D-glucosamine units specifically compete with the binding of mAb'110-4 to rgp 160, as well as to viral gp 120.

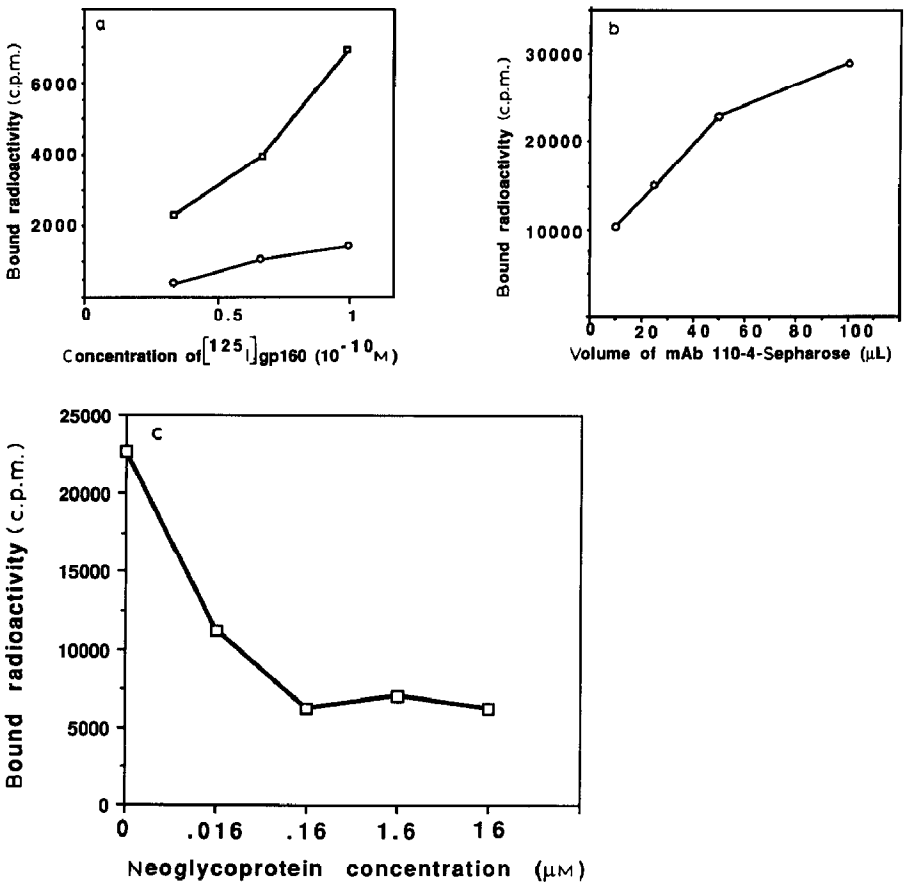

Fig. 3. Binding of rgp 160 to 110-4-Sepharose: (a) [^{125}I]gp 160 (0.25–0.75 ng; 0.032–0.1 nM; 10 000–30 000 c.p.m.) was preincubated in buffer in the presence (O) or in the absence (□) of 30 µM β-D-$GlcNAc_{47}$-BSA before being added to the affinity matrix (50 µL). (b) [^{125}I]gp 160 (1.5 ng; 0.1 nM; 46 000 c.p.m.) was added to various quantities of affinity matrix. (c) [^{125}I]gp 160 (1.5 ng; 0.1 nM; 46 000 c.p.m.) was preincubated in buffer in the presence or in the absence of β-D-$GlcNAc_{47}$-BSA before being added to the affinity matrix (50 µL).

Therefore, rgp 160 constitutes an appropriate model for examining at the molecular level the *N*-acetyl-β-D-glucosaminyl-binding properties detected on viral gp 120.

To confirm these observations, the competition between β-D-$GlcNAc_{47}$-BSA and mAb 110-4 for the binding to rgp 160 was further examined at the molecular level with 110-4-Sepharose as an affinity matrix. In these experiments, the binding of [^{125}I]gp 160 to 110-4-Sepharose depended on the amounts of [^{125}I]gp 160 and matrix (Figs. 3a and b). It was inhibited in a dose-dependent manner by competition with β-D-$GlcNAc_{47}$-BSA (Fig. 3c), 30μM β-D-$GlcNAc_{47}$-BSA giving 84 ± 2% of inhibition (*n* 6); no inhibition occurred with β-D-Gal_{23}-BSA. Thus β-D-$GlcNAc_{47}$-BSA and mAb 110-4 compete at the molecular level for binding to rgp 160.

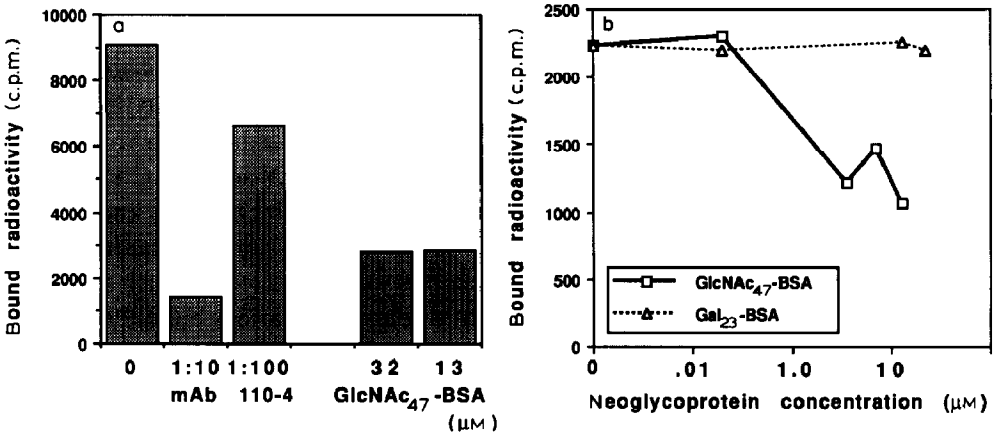

Fig. 4. Inhibition of the binding of rgp 160 to β-D-$GlcNAc_{47}$-BSA-Sepharose: (a) [^{125}I]gp 160, (1.2 ng; 0.075nM; 60000 c.p.m.) was preincubated with buffer (control), or with mAb 110-4, or soluble β-D-$GlcNAc_{47}$-BSA diluted in buffer, and then added to the affinity matrix (25 μL). (b) [^{125}I]gp 160 (0.6 ng; 0.05nM; 20000 c.p.m.) was preincubated with the neoglycoprotein diluted in buffer, and then added to the affinity matrix (25 μL).

TABLE III

Inhibition of the binding of rgp 160 to β-D-$GlcNAc_{47}$-BSA-Sepharose[a]

[^{125}I]gp 160 preincubation with		*Inhibition (%)*	*Number of experiments*
Compound	*Concentration (mM)*		
β-D-$GlcNAc_{47}$-BSA	0.013	57 ± 6	3
β-D-$GlcNAc_{47}$-BSA	0.032	70 ± 2	7
β-D-Gal_{23}-BSA	0.032	15 ± 6	7
β-D-Gal*p*$OC_6H_4NO_2$(4)	30	0	3
α-D-Glc*p*$OC_6H_4NO_2$(4)	30	0	3
D-GlcNAc	50–250	0	3
D-GalNAc	50–250	0	3

[a] The results are reported as means ± S.E.M.

Binding of [^{125}I]gp 160 to β-D-GlcNAc$_{47}$-BSA-Sepharose. — The interaction between rgp 160, β-D-GlcNAc$_{47}$-BSA, and mAb 110-4 was then examined by use of β-D-GlcNAc$_{47}$-BSA-Sepharose. Preincubation with soluble mAb 110-4 significantly inhibited the binding of [^{125}I]gp 160 to β-D-GlcNAc$_{47}$-BSA-Sepharose (Fig. 4a) (% of inhibition of the mAb diluted 1:10 was 87 ± 0.5; n 3), whereas other antibodies unrelated to HIV-1, such as antiscorpion-toxin mAb, had no effect (data not shown). In addition, preincubation of [^{125}I]gp 160 with soluble β-D-GlcNAc$_{47}$-BSA was also able to repeatedly inhibit binding to β-D-GlcNAc$_{47}$-BSA-Sepharose (Figs. 4a, 4b, and 5a; and Table III), as compared to β-D-Gal$_{23}$-BSA (Fig. 4b), and 4-nitrophenyl β-D-galactopyranoside and α-D-glucopyranoside (Table III). Binding of rgp 160 to β-D-GlcNAc$_{47}$-BSA-Sepharose was shown to be saturable, depending on the quantities of [^{125}I]gp 160 and β-D-GlcNAc$_{47}$-BSA-Sepharose added, on the temperature, and on Ca^{2+} concentration (Figs. 5a and 5b).

These data demonstrated that rgp 160 specifically interacts with the *N*-acetyl-β-D-glucosaminyl-matrix. However, even high concentrations of free *N*-acetyl-D-glucosamine had no effect on this interaction (Table III), as for the binding of mAb 110-4 to viral gp 120. Therefore, viral gp 120, as well as rgp 160, binds *N*-acetyl-D-glucosamine units, but only where they are presented on a protein carrier. Indeed, it is known that lectin-binding affinity for free sugar is much lower than for complex carbohydrate derivatives[31]. In this respect, HIV-1 gp 120 resembles some other lectins, such as that of *Vicia graminea*, that do not bind to free mono- or oligo-saccharides but only to more complex D-galactosyl–*N*-acetyl-D-galactosaminyl structures presented on a carrier[32].

Binding of [^{125}I]β-D-GlcNAc$_{47}$-BSA to rgp 160-Sepharose. — Conversely, soluble, ^{125}I-labeled β-D-GlcNAc$_{47}$-BSA was shown to bind to rpg 160-Sepharose in a dose-dependent manner with respect to the quantities of β-D-[^{125}I]GlcNAc$_{47}$-BSA (Fig. 6a) and of the matrix, and to calcium concentration (binding being 1.75 times higher in

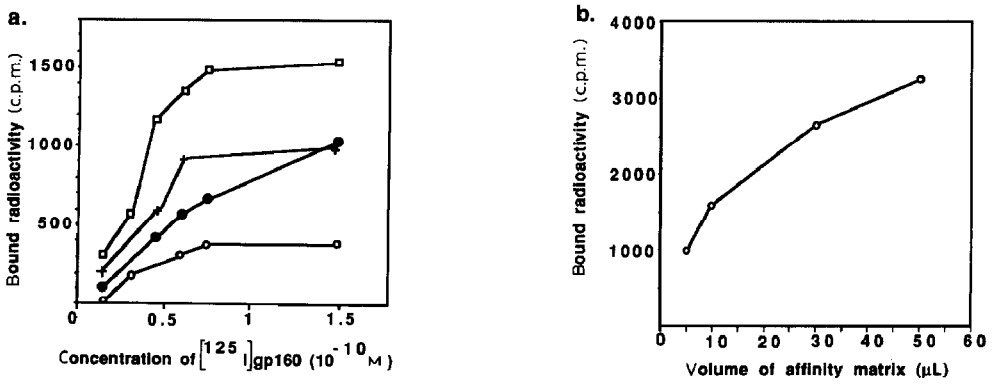

Fig. 5. Binding of rgp 160 to β-D-GlcNAc$_{47}$-BSA-Sepharose: (a) Before incubation for 1 h at 37° with the affinity matrix (5 µL), [^{125}I]gp 160 (0.06–0.6 ng; 0.015–0.15 nM; 4000–40 000 c.p.m.) was preincubated at 47° in Tris–Ca^{2+}–BSA buffer (□), in Tris–Ca^{2+}–BSA–EDTA buffer (●), and in 30µM β-D-GlcNAc$_{47}$-BSA diluted with Tris–Ca^{2+}–BSA buffer (○); [^{125}I]gp 160 was incubated in Tris–Ca^{2+}–BSA buffer for 1 h at 4° with the affinity matrix (5 µL) (+). (b) [^{125}I]gp 160 (0.6 ng; 0.15nM, 40 000 c.p.m.) was incubated in Tris–Ca^{2+}–BSA buffer for 1 h at 37° with various amounts of the affinity matrix.

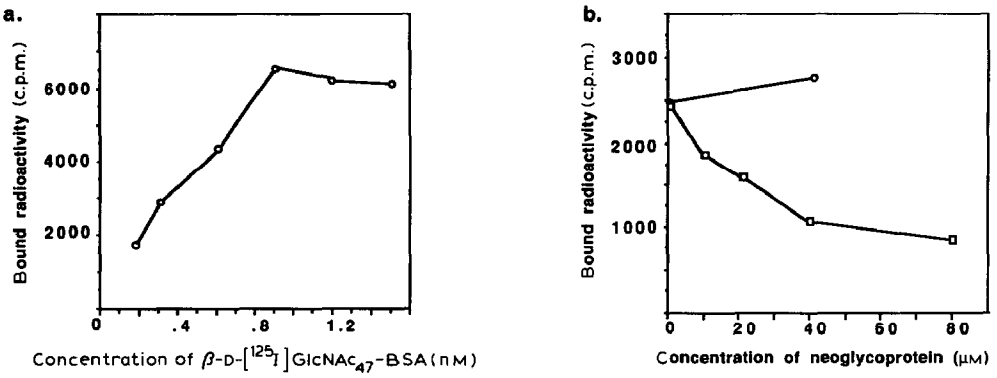

Fig. 6. Binding of soluble β-D-[^{125}I]GlcNAc$_{47}$-BSA to rgp 160-Sepharose: (a) β-D-[^{125}I]GlcNAc$_{47}$-BSA (0.2–1.5 nM; 8600–65 000 c.p.m.), diluted in Tris–Ca^{2+}–BSA buffer, was added to the affinity matrix (10 μL). (b) The affinity matrix (20 μL) was preincubated for 45 min at 37° with 0–80 μM unlabeled β-D-GlcNAc$_{47}$-BSA (□) or with α-D-Man$_{16}$-BSA (○), diluted in buffer, before the addition of β-D-[^{125}I]GlcNAc$_{47}$-BSA (0.36nM; 60 000 c.p.m.).

Tris–Ca^{2+}–BSA buffer than in Tris–Ca^{2+}–EDTA–BSA buffer), but it was not influenced by Mg^{2+} or Mn^{2+}. Binding was saturable (Fig. 6a) and inhibited by unlabeled β-D-GlcNAc$_{47}$-BSA or, to a lesser extent, by very high concentrations of *N*-acetyl-D-glucosamine but nog by α-D-Man$_{16}$-BSA, β-D-Gal$_{23}$-BSA, or *N*-acetyl-D-galactosamine (Fig. 6b, Table IV), which indicated its specificity.

Altogether, the results indicated that the external envelope glycoprotein of HIV-1 behaves as an *N*-acetyl-β-D-glucosaminyl-specific lectin and that the V3 region of gp 120 may be involved in the carbohydrate-binding site.

Binding of [^{125}I]gp 160 to β-D-GlcNAc-divinyl sulfone-agarose and to asialo-

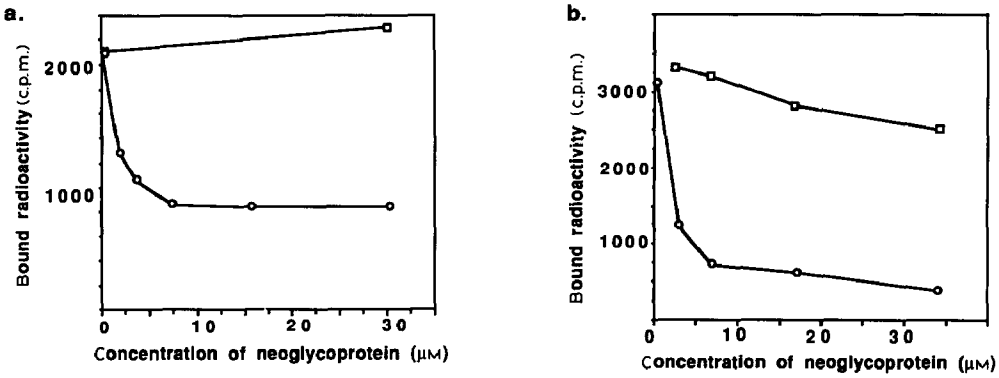

Fig. 7. Binding of rgp 160 to *N*-acetyl-β-D-glucosaminyl-affinity matrices. (a) Inhibition of binding of rgp 160 to β-D-GlcNAc-divinyl sulfone-agarose by β-D-GlcNAc$_{47}$-BSA; [^{125}I]gp 160 (0.3 ng, 0.075nM; 20 000 c.p.m.) was preincubated with β-D-GlcNAc$_{47}$-BSA (○) or with β-D-Gal$_{23}$-BSA (□) in Tris–Ca^{2+}–BSA buffer, before addition to the affinity matrix (5 μL). (b) Inhibition of binding of rgp 160 to asialoagalactothyroglobulin-agarose by β-D-GlcNAc$_{47}$-BSA; [^{125}I]gp 160 (0.6 ng; 0.1nM; 30 000 c.p.m.) was preincubated with β-D-GlcNAc$_{47}$-BSA (○) or with β-D-Gal$_{23}$-BSA (□) in Tris–Ca^{2+}–BSA buffer, before addition to the affinity matrix (30 μL).

agalactothyroglobulin-agarose. — In order to confirm these findings, it was necessary to investigate whether [^{125}I]gp 160 could actually interact with other *N*-acetyl-β-D-glucosaminyl-negative- and -positive-affinity matrices different from β-D-GlcNAc$_{47}$-BSA-Sepharose. As expected, [^{125}I]gp 160 did not specifically bind to lactogel or to β-D-galactosyl$_{23}$-BSA-Sepharose. It bound to β-D-GlcNAc-divinyl sulfone-agarose, and this binding was inhibited by β-D-GlcNAc$_{47}$-BSA (inhibition by 30μM β-D-GlcNAc$_{47}$-BSA was 62 ± 17%; n 4), but not by β-D-Gal$_{23}$-BSA (inhibition by 30μM β-D-Gal$_{23}$-BSA was 10 ± 5%; n 4) (Fig. 7a) nor by 16mM 4-nitrophenyl β-D-galactopyranoside. In addition, [^{125}I]gp 160 did also bind to asialoagalactothyroglobulin-Agarose, and this binding was specifically blocked by β-D-GlcNAc$_{47}$-BSA but not by β-D-Gal$_{23}$-BSA (Fig. 7b), and 16mM 4-nitrophenyl β-D-galactopyranoside and α-D-glucopyranoside (data not shown).

Thus, rgp 160 can specifically bind not only to β-D-GlcNAc$_{47}$-BSA-Sepharose but also to two other *N*-acetyl-β-D-glucosaminyl affinity matrices, such as β-D-GlcNAc-divinylsulfone-Agarose, which presents 2-acetamido-2-deoxy-β-D-glucopyranosyl groups *via* a divinylsulfone bridge, and asialoagalactothyroglobulin-Agarose in which enzymic removal of sialic acid and D-galactose units resulted in the exposure of nonreducing, terminal 2-acetamido-2-deoxy-D-glucopyranosyl groups linked to a D-mannosyl residue, similarly to many glycoproteins of animal origin[3].

Affinity between soluble β-D-GlcNAc$_{47}$-BSA and rgp 160. — It was possible to calculate the intrinsic dissociation constant of the soluble rgp 160–β-D-GlcNAC$_{47}$-BSA complexes (K_d) by use of the precedure of Hořejší *et al.*[34], when applying to the results of the binding assays between [^{125}I]gp 160 and two different *N*-acetyl-β-D-glucosaminyl-affinity matrices, β-D-GlcNAc$_{47}$-BSA-Sepharose and β-D-GlcNAc-divinyl sulfone-agarose. The results obtained with the two matrices gave approximately the same estimate of the K_d value for the soluble [^{125}I]gp 160–β-D-GlcNAc$_{47}$-BSA complexes, *i.e.*, 14–20μM. In addition, the affinity of soluble β-D-GlcNAc$_{47}$-BSA for immobilized rgp 160 was

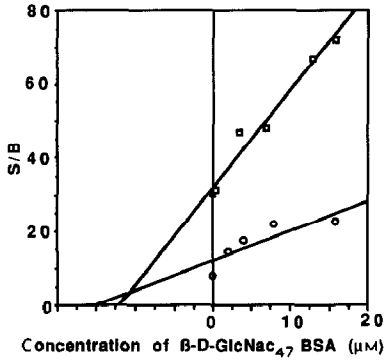

Fig. 8. Determination of the K_d value for the interaction between [^{125}I]gp 160 and soluble β-D-GlcNAc$_{47}$-BSA: (□) [^{125}I]gp 160 (0.6 ng; 0.05nM; 20000 c.p.m.), preincubated with Tris–Ca^{2+}–BSA buffer or with β-D-GlcNAc$_{47}$-BSA, diluted in buffer, was applied to β-D-GlcNAc$_{47}$-BSA-Sepharose (25 μL). (○) [^{125}I]gp 160 (0.3 ng; 0.04nM; 20000 c.p.m.), preincubated with Tris–Ca^{2+}–BSA buffer or with β-D-GlcNAc$_{47}$-BSA diluted in buffer, was applied to β-D-GlcNAc-divinyl sulfone-Agarose (5 μL). S/B, Ratio of soluble to bound gp.

further estimated by use of the competition experiments of unlabeled *vs.* ^{125}I-labeled β-D-GlcNAc$_{47}$-BSA for the binding to rgp 160–Sepharose (Fig. 6b); the results enabled us to calculate the K_d value by the method of Muller[35]. From the data of Fig. 6b, the $K_{0.5}$ value, *i.e.*, the concentration of unlabeled β-D-GlcNAc$_{47}$-BSA required to decrease by 50% the β-D-[^{125}I]GlcNAc$_{47}$-BSA binding was 33μM, from which a K_d value of 13μM was derived. Therefore, both estimates of the K_d values of β-D-GlcNAc$_{47}$-BSA–rgp 160 complexes are within the same order of magnitude. These results indicated that the affinity of *N*-acetyl-D-glucosaminyl groups presented on a protein carrier for gp 120 is much weaker, by a factor of 10^4, than that of gp 120 for CD4[2,3].

In conclusion, the results reported herein showed that no lectin-like interactions could be demonstrated at the initial step of infection of lymphoid CD4$^+$ cells by HIV-1, *i.e.*, the virus binding to the cell membrane. However, one neoglycoprotein, β-D-GlcNAc$_{47}$-BSA, repeatedly inhibited the labeling of HIV-1, or of rgp 160, already bound to CD4$^+$ cell membranes by murine mAb (that are specific for the V3 region of gp 120[19]) but not by other mAb or polyclonal antibodies. Such inhibition was carbohydrate-specific because the other neoglycoproteins tested, except α-D-Man$_{26}$-BSA, had no effect. However, no competition between free *N*-acetyl-D-glucosamine and mAb for their binding to HIV-1 or to rgp 160 could be observed. These results suggested that (a) HIV-1 gp 120 presented specific binding properties for *N*-acetyl-D-glucosamine units where present at high density on a carrier, and (b) the V3 region or a region close to it could be involved in the *N*-acetyl-β-D-glucosaminyl-binding site of gp 120.

Competition for the binding, to rgp 160, of β-D-GlcNAc$_{47}$-BSA and of one of the mAb involved, mAb 110-4, was further determined at the molecular level by use of affinity matrices substituted either by mAb 110-4 or by β-D-GlcNAc$_{47}$-BSA. The *N*-acetyl-β-D-glucosaminyl-binding properties of rgp 160 were further demonstrated by showing that [^{125}I]gp 160 bound in a specific manner to three different *N*-acetyl-β-D-glucosaminyl-affinity matrices (β-D-GlcNAc$_{47}$-BSA-Sepharose, β-D-GlcNAc-divinyl sulfone-agarose, and asialoagalactothyroglobulin-agarose) that present their *N*-acetyl-β-D-glucosaminyl groups on a BSA carrier, on a divinylsulfone bridge, or linked to a D-mannosyl residue, respectively. Conversely, β-D-[^{125}I]GlcNAc$_{47}$-BSA specifically bound to a rgp 160-Sepharose matrix.

These results allowed the estimation that the K_d value of the complex formed between rgp 160 and β-D-GlcNAc$_{47}$-BSA was in the 10^{-5}M range, showing that the affinity of rgp 160 for *N*-acetyl-D-glucosaminyl groups is 10^4 weaker than that of gp 120 for CD4. This finding might well explain the lack of detection of the lectin-like interactions between HIV-1 gp 120 and CD4$^+$ cells, even though they might be present. In addition, this low K_d value for the interaction between rgp 160 and a highly substituted neoglycoprotein could account for the observation that free *N*-acetyl-D-glucosamine does not compete with the binding of [^{125}I]gp 160 to the various *N*-acetyl-β-D-glucosaminyl-affinity matrices, as well as for the limited inhibition by free *N*-acetyl-D-glucosamine of the binding of β-D-[^{125}I]GlcNAc$_{47}$-BSA to rgp 160-Sepharose (Table IV).

The carbohydrate-binding property herein described, which does not appear to

TABLE IV

Inhibition of the binding of β-D-[^{125}I]GlcNAc$_{47}$-BSA to rpg 160-Sepharose[a]

Rgp 160-Sepharose preincubated with		*Inhibition (%)*
Compound	*Concentration (mM)*	
D-GlcNAc	170	41 ± 5
D-GalNAc	170	0
β-D-GlcNAc$_{47}$-BSA	0.044	62 ± 2
β-D-Gal$_{23}$-BSA	0.044	15 ± 9
α-D-Man$_{16}$-BSA	0.044	0

[a] Results are reported as means ± S.E.M. of three experiments.

play any role in HIV-1 attachment to CD4$^+$ cells, might nevertheless be involved in postbinding events or in some interactions between immature gp 160 synthetized in the host cells and some of the intracellular components of the infected cells that are necessary for the intracellular routing of the molecule.

Anyway, the observation that the *N*-acetyl-β-D-glucosaminyl-binding site of gp 120 is close to, or located at, its immunodominant V3-neutralizing loop might be of possible therapeutic value. Finally, the problem whether lectin–carbohydrate interactions may be used by HIV-1 to attach to cell membranes independently of CD4 and, thus, may account for the infection by HIV-1 of CD4-negative cells under some circumstances[36–38], is currently under investigation.

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REFERENCES

1 W. A. Haseltine, *J. AIDS*, 1 (1988) 217–225.
2 E. Fenouillet, B. Clerget-Raslain, J. C. Gluckman, D. Guetard, L. Montagnier, and E. Bahraoui, *J. Exp. Med.*, 169 (1989) 807–822.
3 L. A. Lasky, M. S. Singer, T. A. Yednock, D. Dowbenko, C. Fennie, H. Rodriguez, T. Nguyen, S. Stachel, and S. D. Rosen, *Cell*, 56 (1989) 1045–1055.
4 D. Klatzmann, E. Champagne, S. Chamaret, J. Gruest, D. Guetard, T. Hercend, J. C. Gluckman, and L. Montagnier, *Nature (London)*, 312 (1984) 767–769.
5 F. Dimarco Veroneze, A. L. Devico, T. D. Copeland, S. Oroszelan, R. C. Gallo, and M. G. Sarngadharan, *Science*, 229 (1985) 1402–1405.
6 W. R. Gallaher, *Cell*, 50 (1987) 327–328.

7 D. Klatzmann and J. C. Gluckman, *Immunol. Today*, 7 (1986) 291–296.
8 H. Geyer, C. Holschbach, G. Hunsmann, and J, Schneider, *J. Biol. Chem.*, 263 (1988) 11760–11767.
9 T. Muzuochi, M. W. Spellman, M. Larkin, J. Solomon, L. J. Basa, and T. Feizi, *Biochem. J.*, 254 (1988) 599–603.
10 N. Sharon, *Immunol. Today*, 5 (1984) 143–147.
11 D. C. Wiley and J. J. Skehel, *Annu. Rev. Biochem.*, 56 (1987) 365–394.
12 W. Weis, J. H. Brown, S. Cusack, J. C. Paulson, J. J. Skehel, and D. C. Wiley, *Nature (London)*, 332 (1988) 426–431.
13 W. E. Robinson, J. R. D. C. Montefiori, and W. M. Mitchell, *AIDS Res. Human. Retrov.*, 3 (1987) 265–282.
14 J. Lifson, S. Coutre, E. Huang, and E. Engleman, *J. Exp. Med.*, 164 (1986) 2101–2106.
15 R. A. B. Ezekowitz, M. Kuhlman, J. E. Groopman, and R. A. Byrn, *J. Exp. Med.*, 169 (1989) 185–196.
16 N. Sharon and H. Lis, *Science*, 246 (1989) 227–234.
17 F. Barre-Sinoussi, J. C. Chermann, F. Rey, M. T. Nugeyre, S. Chamaret, J. Gruest, C. Dauguet, C. Axler-Blin, F. Vezinet-Brun, C. Rouzioux, W. Rozenbaum, and L. Montagnier, *Science*, 220 (1983) 868–871.
18 M. P. Kieny, R. Lathe, Y. Rivier, K. Dott, M. Girard, L. Montagnier, and J. P. Lecoq, *Prot. Eng.*, 2 (1988) 219–225.
19 E. M. Bahraoui, B. Clerget-Raslain, F. Chapuis, R. Olivier, C. Parravicini, M. Yagello, L. Montagnier, and J.C. Gluckman, *AIDS*, 2 (1988) 165–170.
20 P. S. Linsley, J. A. Ledbretter, E. Kinney-Thomas, and S. L. Hu, *J. Virol*, 62 (1988) 3695–3701.
21 J. S. McDougal, A. Mawle, S. P. Cort, J. K. A. Nicholson, G. D. Cross, J. A. Scheppler-Campbell, C. D. Hicks, and J. Sligh, *J. Immunol.*, 135 (1985) 3151–3162.
22 O. Westphal and H. Schmidt, *Justus Liebigs Ann. Chem.*, 574 (1951) 84–90.
23 L. Warren, *J. Biol. Chem.*, 234 (1959) 1971–1975.
24 M. Schachter, *Anal. Methods*, 31 (1969) 296–305.
25 E. Fenouillet, J. C. Gluckman, and E. Bahraoui, *J. Virol.*, 164 (1990) 2841–2848.
26 T. J. Matthews, K. J, Weinhold, H. K. Lyerly, A. J. Langlois, H. Wigzell, and D. P. Bolognesi, *Proc. Natl. Acad. Sci. U.S.A.*, 84 (1987) 5424–5428.
27 R. A. Gruters, J. J. Neefjes, M. Tersmette, R. E. Y. de Goede, A. Tulp, M. G. Huisman, F. Meidema, and H. L. Ploegh, *Nature (London)*, 330 (1987) 74–77.
28 D. C. Montefiori, W. E. Robinson, and W. Mitchell, *Proc. Natl. Acad. Sci. U.S.A.*, 85 (1988) 9248–9252.
29 B. D. Walker, M. Kowalski, W. C. Goh, K. Kosarsky, M. Krieger, C. Rosen, L. Rohrschneider, W. A. Haseltine, and J. Sodroski, *Proc. Natl. Acad. Sci. U.S.A.*, 84 (1987) 8120–8124.
30 K. Javaherian, A. J. Langlois, C. McDanal, K. L. Ross, L. I. Ecker, C. L. Jellis, A. T. Profy, J. R. Rusche, D. P. Bolognesi, S. D. Putney, and T. J. Matthews, *Proc. Natl. Acad. Sci. U.S.A.*, 86 (1989) 6768–6772.
31 H. Leffler and S. H. Barondes, *J. Biol. Chem.*, 261 (1986) 10119–11124.
32 M. J. Prigent, V. Verez-Bencomo, P. Sinaÿ, and J. P. Cartron, *Glycoconj. J.*, 1 (1984) 73–80.
33 J. Montreuil, *Biol. Cell.*, 51 (1984) 115–132.
34 V. Hořejši, V. Matousek, M. Ticha, and T. Trnka, in T. C. Bøg-Hansen (Ed.), *Lectins*, Walter de Gruyter, Berlin, 1985, pp. 298–306.
35 R. Muller, *J. Immunol. Methods.* 34 (1980) 345–352.
36 P. R. Clapham, J. N. Weber, D. Whitby, K. McIntosh, A. G. Dalgleish, P. J. Maddon, K. C. Deen, R. W. Sweet, and R. A. Weiss, *Nature (London)*, 337 (1989) 368–370.
37 J. M. Harouse, C. Kunsh, H. T. Hartle, M. A. Laughlin, J. A. Hoxie, B. Wigdahl, and F. Gonzalez-Scarano, *J. Virol.*, 63 (1989) 2527–2533.
38 M. Tateno, F. Gonzalez-Scarano, and J. A. Levy, *Proc. Natl. Acad. Sci. U.S.A.*, 86 (1989) 4287–4290.