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Marked Depletion of Glycosylation Sites in HIV-1 gp120 under Selection Pressure by the Mannose-Specific Plant Lectins of *Hippeastrum* Hybrid and *Galanthus nivalis*

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ABSTRACT

The plant lectins from *Hippeastrum* hybrid (HHA) and *Galanthus nivalis* (GNA) are 50,000-D tetramers showing specificity for α-(1,3) and/or α-(1,6)-mannose oligomers. They inhibit HIV-1 infection at a 50% effective concentration of 0.2 to 0.3 μg/ml. Escalating HHA or GNA concentrations (up to 500 μg/ml) led to the isolation of three HIV-1(III_B) strains in CEM T cell cultures that were highly resistant to HHA and GNA, several other related mannose-specific plant lectins, and the monoclonal antibody 2G12, modestly resistant to the mannose-specific cyanovirin, which is derived from a blue-green alga, but fully susceptible to other HIV entry inhibitors as well as HIV reverse transcriptase inhibitors. These mutant virus strains were devoid of up to seven or eight of 22 glycosylation sites in the viral envelope glycoprotein gp120 because of mutations at the Asn or Thr/Ser sites of the *N*-glycosylation motifs. In one of the strains, a novel glycosylation site was created near a deleted glycosylation site. The affected glycosylation sites were predominantly clustered in regions of gp120 that are not involved in the direct interaction with either CD4, CCR5, CXCR4, or gp41. The mutant viruses containing the deleted glycosylation sites were markedly more infectious in CEM T-cell cultures than wild-type virus.

The entry of human immunodeficiency virus (HIV) into host cells is mediated by the viral envelope glycoproteins gp120 and gp41, which are noncovalently bound to each other. Five variable regions (V1–V5) interspersed by more conserved amino acid sequences have been identified in gp120 (Leonard et al., 1990; Gallaher et al., 1995). The conserved gp120 regions form structures important for interaction with the gp41 ectodomain and the cellular (co-)receptors such as CD4, CXCR4, and CCR5. Several epitopes on gp120 have been identified by different (monoclonal) neutralizing antibodies (Schønning et al., 1996; Trkola et al., 1996; Kwong et al., 1998; Rizzuto et al., 1998; Wyatt et al., 1998; Scanlan et al., 2002). Both conserved and variable regions of gp120 are extensively glycosylated (Geyer et al., 1988; Mizuochi et al., 1988a,b). It is thought that the variability of glycosylation of the gp120 surface is likely to modulate the immunogenicity of gp120, which is the main target for neutralizing antibodies (Nabs) that appear during natural infection (Oloffson and Hansen, 1998; Losman et al., 2001). Wei and colleagues (2003) have shown that Nabs directed toward HIV gp160 were able to force the virus to replace the neutralization-sensitive phenotype by successive populations of Nab-resistant virus strains. Indeed, during the progress of virus infection in persons infected with HIV, gp120 mutates rapidly, primarily at glycosylation sites, resulting in a change of the glycan shield on the surface of the virus, thus affording some immunological escape. This mechanism allows the virus to persist in the presence of an evolving antibody repertoire.

In contrast with Nabs, plant lectins are a class of proteins

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ABBREVIATIONS: Nabs, neutralizing antibodies; HIV, human immunodeficiency virus; SARS, severe acute respiratory syndrome; GNA, *Galanthus nivalis* agglutinin; HHA, *Hippeastrum* hybrid agglutinin; NPA, *Narcissus pseudonarcissus*; CA, *Cymbidium* hybrid agglutinin; LOA, *Listera ovata* agglutinin; UC-781, *N*-[4-chloro-3-(3-methyl-2-butenyloxy)phenyl]-2-methyl-3-furancarbothioamide; AMD-3100, 1,1′-(1,4-phenylenebis(methylene))bis-1,4,8,11-tetraazacyclotetradecane octahydrochloride; PCR, polymerase chain reaction; LTR, long terminal repeat; CS, cell suspension; SN, supernatant.

that bind to carbohydrates and are of nonimmune origin (for an overview, see Van Damme et al., 1998; Sharon and Lis, 2003). They precipitate polysaccharides and glycoproteins and are usually di- or oligovalent (i.e., each lectin molecule has at least two or more carbohydrate binding sites). The large majority of lectins recognize primarily mannose, glucose, galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine, fucose, and *N*-acetylneuraminic (sialic) acid. In addition, they combine specifically with a variety of oligosaccharides composed of these monosaccharides. It has been shown that a number of plant lectins, in particular those with specificity for mannose oligomers, display potent inhibitory activity against several viruses, such as HIV (Lifson et al., 1986; Muller et al., 1988; Matsui et al., 1990; Weiler et al., 1990; Balzarini et al., 1991, 1992; Animashaun et al., 1993), human cytomegalovirus (HCMV) (Balzarini et al., 1991), and coronaviruses [i.e., feline coronavirus (feline infectious peritonitis virus) and human coronavirus (SARS)] (Balzarini et al., 2004c). Moreover, some of these plant lectins [i.e., *Galanthus nivalis* agglutinin (GNA) and *Hippeastrum* hybrid agglutinin (HHA)] were recently demonstrated to represent potential candidate anti-HIV microbicides; they lack mitogenic activity, do not lead to human blood cell agglutination, and show marked stability at relatively low pH and high temperatures for prolonged time periods (Balzarini et al., 2004a). Preincubation of cell-free HIV particles or persistently HIV-infected cells with these plant lectins resulted in a further potentiation of their antiviral efficiency by at least 10- to 20-fold. The plant lectins clearly interact with the entry of HIV into its target cell. We demonstrated recently that HIV-1 exposed to HHA or GNA for prolonged time periods in cell culture acquired mutations in the gp120 envelope protein (Balzarini et al., 2004b). These mutations predominantly appeared at asparagine residues that are part of the *N*-glycosylation motifs. Thus, the mannose-specific plant lectins were selected for a unique drug resistance profile mainly affecting the glycosylation pattern of the HIV envelope glycoprotein. Such a specific drug-resistance profile has not yet been demonstrated for any other type of HIV entry inhibitor.

We have now unraveled the molecular basis of the profound resistance profile of HIV-1(III_B) strains that were exposed for prolonged periods (up to 88 passages) to the plant lectins HHA and GNA under dose-escalating drug regimens in which drug concentrations as high as 1000-fold the original EC_{50} were eventually reached (i.e., 500 μg/ml). The composition of the gp120 and gp41 envelope glycoproteins of such highly drug-exposed virus strains was characterized, their resistance/sensitivity profile against several HIV entry inhibitors was examined, and the infectivity of the drug-resistant virus strains was determined.

Materials and Methods

Test Compounds. The mannose-specific plant lectins from *Galanthus nivalis* (GNA), *Hippeastrum* hybrid (HHA), *Narcissus pseudonarcissus* (NPA), *Cymbidium* hybrid (CA), and *Listera ovata* (LOA) were derived and purified from these plants, as described previously (Van Damme et al., 1987, 1988). UC-781 was obtained from Crompton Ltd. (Middlebury, CT). Tenofovir was from Gilead Sciences (Foster City, CA), AMD-3100 from AnorMed (Langley, BC, Canada), and dextran sulfate-5000 from Sigma-Aldrich (St. Louis, MO). T20 (pentafuside, enfuvirtide) was kindly provided by AIDS Research Alliance (Los Angeles, CA) and cyanovirin by Dr. J.B. McMahon (National Institutes of Health, Bethesda, MD) and Dr. A. Bolmstedt (Göteborg, Sweden). The monoclonal antibody 2G12 was obtained from the Medical Research Council (Potter Bar, Hertfordshire, UK).

Cells. Human T lymphocytic CEM and SupT1 cells were obtained from the American Type Culture Collection (Manassas, VA) and persistently infected CEM/HIV-1 cells were obtained by exposing CEM cell cultures to wild-type HIV-1(III_B) or mutant (GNA- or HHA-resistant) HIV-1 for 3 to 4 weeks. All cell lines mentioned were cultivated in RPMI 1640 medium supplemented with 10% fetal bovine serum (fetal bovine serum) (Cambrex Bio Science Verviers S.p.r.l., Verviers, Belgium), 2 mM L-glutamine, and 0.075 M $NaHCO_3$.

Human astroglioma U87 cells transfected with CD4 and either CCR5 (U87.CD4.CCR5 cells) or CXCR4 (U87.CD4.CXCR4 cells), chemokine receptors/HIV-1 coreceptors, were kindly provided by Dr. D. R. Littman (Skirball Institute of Biomolecular Medicine, New York, NY) and were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum, 0.01 M HEPES buffer (Invitrogen), 0.2 mg/ml Geneticin (G-418 sulfate) (Invitrogen), and 1 μg/ml puromycin (Sigma-Aldrich). Subcultivations were done every 2 to 3 days by digestion of the monolayers with trypsin/EDTA (Invitrogen).

Viruses. HIV-1(III_B) was provided by Dr. R. C. Gallo and Dr. M. Popovic (Institute of Human Virology, University of Maryland, Baltimore, MD).

Selection and Isolation of GNA- and HHA-Resistant HIV-1 Strains. The procedure followed for the selection of drug-resistant virus mutants was essentially as described previously (26). In brief, HIV-1(III_B) was added to CEM cell cultures in 48-well plates in the presence of 0.5 μg/ml GNA or 0.25 μg/ml HHA. For the generation of drug-resistant virus mutants, an increased drug concentration was administered when full cytopathicity was obtained in the previous cell culture. For the clinical isolate HIV-1/HE, isolated from a Belgian patient and belonging to HIV-1 clade B, p24 production in the presence of the different concentrations of the test compounds (instead of virus-induced cytopathicity) was measured for each passage, before subculturing the virus-exposed cells and deciding on the dilution of the supernatants that had to be transferred.

Antiretrovirus Assays. The methodology of the anti-HIV assays has been described previously (Balzarini et al., 2004a,b). In brief, CEM cells (4.5×10^5 cells per ml) were suspended in fresh culture medium and infected with HIV-1 at 100 $CCID_{50}$ per ml of cell suspension. Then, 100 μl of the infected cell suspension was transferred to microplate wells, mixed with 100 μl of the appropriate dilutions of the test compounds, and further incubated at 37°C. After 4 to 5 days, giant cell formation was recorded microscopically in the CEM cell cultures. The 50% effective concentration (EC_{50}) corresponds to the compound concentrations required to prevent syncytium formation by 50% in the virus-infected CEM cell cultures.

Genotyping of the HIV-1 *env* Region. Proviral DNA was extracted from cell pellets using the QIAamp Blood Mini Kit (QIAGEN, Hilden, Germany) as described previously (Van Laethem et al., 2005). Both the gp120 and gp41 genes were included in this assay.

Viral Load Determination. The RNA viral load of virus supernatant samples was determined using VERSANT HIV-1 RNA 3.0 Assay (bDNA; Bayer HealthCare, Brussels, Belgium) as described by the manufacturer. The p24 antigen was determined using the commercial assay kit from PerkinElmer Life and Analytical Sciences (Zaventem, Belgium).

HIV Entry PCR. The U87.CD4.CXCR4 and U87.CD4.CCR5 cells were seeded in 24-well plates at 5×10^4 cells per well and incubated overnight. Virus stocks (diluted to a p24 titer of 10,000 pg/ml) were treated with 500 U/ml of RNase-free DNase (Roche Molecular Biochemicals, Mannheim, Germany) for 1 h at room temperature. Then, the cells in each well were infected with 1000, 500, or 250 pg/ml p24. To control for possible residual contamination of the viral inoculum with viral DNA, a parallel infection was carried out on the CCR5-

transfected (CXCR4-negative) U87.CD4 cells. After incubation at 37°C for 2 h, the medium was aspirated, the cells were washed once with PBS, and total DNA was extracted from the infected cells using the QIAamp DNA Mini Kit (QIAGEN). The DNA was eluted from the QIAamp spin columns in a final volume of 250 μl of elution buffer. Then, 10 μl of each DNA sample was subjected to 36 cycles of HIV-1 LTR R/U5-specific and 33 cycles of β-actin-specific PCR on a Biometra T3 Thermocycler. Each cycle comprised a 45-s denaturation step at 95°C, a 45-s annealing step at 61°C, and a 45-s extension step at 72°C. The primers used were: LTR R/U5 sense primer, 5′-GGCTACTAGGGAACCCACTG-3′ (nucleotides 496 to 516, according to the HIV-1 HXB-2 DNA sequence); LTR R/U5 antisense primer, 5′-CTGCTAGAGATTTTCCACACTGAC-3′ (nucleotides 612 to 635); β-actin sense primer, 5′-TCTGGCGGCACCACCATGTACC-3′ (nucleotides 2658 to 2679); and β-actin antisense primer, 5′-CGATGGAGGGGCCGGACTCG-3′ (nucleotides 2961 to 2980). The reaction mixtures contained PCR buffer (supplied with the enzyme), 200 μM concentrations of dATP, dGTP, dCTP, and dTTP (Invitrogen, Carlsbad, CA), 0.4 μM concentrations of the sense and antisense primers, and 0.5 U of SuperTaq DNA polymerase (HT Biotechnology, Cambridge, England) in a total volume of 25 μl. After gel electrophoresis through a 2% agarose gel, the amplified DNA fragments were visualized by ethidium bromide. In preliminary experiments, the exponential range of the PCR amplification curve was determined for both the HIV-1 LTR R/U5 and the β-actin PCRs by varying the amount of input DNA and the number of PCR cycles. Based on these experiments, appropriate conditions were chosen to perform the PCRs.

Results

Selection of HHA- and GNA-Resistant HIV-1(III_B) Strains. The plant lectins HHA and GNA were exposed to HIV-1(III_B)-infected 1 ml-CEM cell cultures in 48-well microtiter plates at 1- to 2-fold their EC_{50}. Every 3 to 4 days, the drug-exposed cultures were subcultured by transferring 100 μl of supernatant or cell suspension to freshly prepared 900-μl CEM cell cultures containing ~ 3 to 4 × 10^5 cells. As soon as the full cytopathic effect (giant cell formation) was recorded microscopically, a higher drug concentration was added at the next passage of the cell cultures. To reach a concentration of 500 μg/ml GNA or HHA to be added to the virus-infected cell cultures after the dose-escalating procedure (i.e., a drug concentration that is 3 orders of magnitude higher than the EC_{50}), at least 65 to 90 subcultivations were required (Fig. 1). GNA and HHA resistance was also selected against a clinical HIV-1 isolate (designated HIV-1/HE) derived from a Belgian AIDS patient (29). The treatment modalities are depicted in Table 1. Instead of recording virus-induced cytopathicity for each subcultivation, p24 levels in the cell culture supernatants were determined to monitor virus breakthrough (Table 1).

Mutational Analysis of the HIV-1 Strains Isolated upon Escalating HHA and GNA Drug Exposure. The gp120 and gp41 genes of two HIV-1(III_B) strains and one HIV-1(III_B) strain isolated after HHA- and GNA-escalating drug administration to the HIV-1-infected cell cultures, respectively, were sequenced and analyzed for the presence of mutations in comparison with the envelope gene of the original wild-type HIV-1(III_B) that had also been subcultured for a similar number of passages in the absence of the plant lectins. A variety of mutations at *N*-glycosylation sites in gp120 were detected in the three drug-exposed virus strains, whereas none of the *N*-glycosylation sites in gp41 was affected. At least eight glycosylation sites were mutated in the two HHA-exposed and independently selected virus strains, and seven glycosylation sites were affected in the GNA-exposed virus strain (Table 2). The mutations occurred either directly at Asn sites of gp120 (two of eight mutations in HIV-1/HHA-500SN; three of eight mutations in HIV-1/HHA-500CS; three of seven mutations in HIV-1/GNA-500CS) or, predominantly, at Thr or Ser sites of gp120 that are part of the *N*-glycosylation motifs (Table 2). The mutations were preferentially targeted at the asparagine residues that carried a high-mannose type glycan (74%), rather than at those

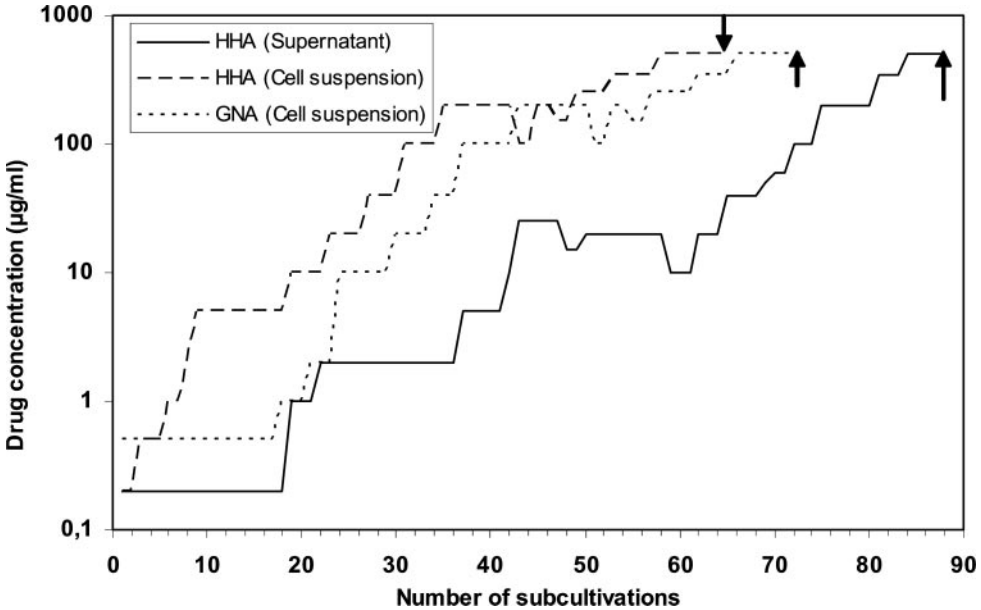

Fig. 1. Schedule of selection of mutant HIV-1(III_B) strains that are highly resistant against the plant lectins HHA and GNA. Arrows indicate the time points where the virus isolates were made (dotted line, HIV-1/GNA-500CS; broken line, HIV-1/HHA-500CS; full line, HIV-1/HHA-500SN]. Dotted line represents the subcultivation schedule in which for each passage cell suspensions (CS) of GNA-exposed CEM cell cultures were transferred. Broken and full lines represent the subcultivation schedule in which each passage, cell suspensions (CS) and supernatants (SN), of HHA-exposed CEM cell cultures were transferred.

asparagines that carried a complex-type glycan (26%). It is interesting that in the HIV-1/HHA-500SN strain, one mutation from Thr to Asn at amino acid position 188 resulted in the annihilation of the Asn186 glycosylation site but created a new glycosylation site at Asn188: because the amino acid at 190 consists of Ser, a new glycosylation motif, 188NTS160, was thus created. Given the fact that gp120 of our HIV-1(III_B) strain contains 22 potential *N*-glycosylation sites, the three virus strains isolated in the presence of HHA or GNA contained a markedly lower number glycans in their envelope glycoprotein gp120 than the wild-type virus (i.e., 64–68% of the wild-type glycans).

To reveal whether the plant lectins also select for deletions in gp120 *N*-glycosylation sites of a clinical HIV-1 strain isolated from a Belgian patient (designated HIV-1/HE), GNA and HHA were administered to the virus in a dose-escalating manner as performed for the laboratory HIV-1(III_B) strain and shown in Table 1. Instead of scoring virus breakthrough at each subcultivation by estimating microscopically visible giant cell formation, the p24 levels produced in the CEM cell cultures were measured after each passage as a parameter for virus production in the presence of the different compound concentrations. The treatment schedule and p24 measurements are depicted in Table 1. The clinical HIV-1/HE isolate is intrinsically less sensitive to both plant lectins. When the *N*-glycosylation sites of gp120 of HIV-1/HE were determined and compared with those on gp120 of HIV-1(III_B), five *N*-glycosylation sites were absent (i.e., a 142*SS*-GHMIME150 deletion, resulting in loss of the 141NSS143 glycosylation motif; a N230D mutation; a Asn234 deletion; an S291P mutation, resulting in the loss of the 289*NQS*291 glycosylation motif, and a Asn356 deletion) but three new glycosylation sites were created [i.e., K130*NCT*132, T413*NET*415, and a glycosylation motif created by the insertion of the dipeptide E*T* after Asn432]. (Italic amino acids are part of a glycosylation motif.) However, at 17 passages upon the administration of HHA- and GNA-escalating concentrations, three *N*-glycosylation sites in HIV-1/HE were affected (i.e., 130NCT132 was converted to 130DCT132; 276NFT278

TABLE 1

Drug resistance selection against a clinical HIV-1/HE strain in the presence of escalating HHA and GNA concentrations

Subcultivations were performed every 4 or 5 days.

Passage Number	Compound Concentration			p24 Levels in CEM Cell Cultures in the Presence of		
	HHA	GNA	None	HHA	GNA	None
	μg/ml			*ng/ml*		
1	5	20	0	1837	2044	1665
2	10	40	0	328	901	1830
3	10	40	0	1351	1219	397
4	10	40	0	1645	1442	1370
5	25	100	0	2193	2348	1753
6	25	100	0	1414	1800	1408
7	50	200	0	1158	2002	1111
8	50	200	0	395	1827	1074
9	50	200	0	242	1412	1486
10	100	200	0	32	331	1220
11	100	200[a]	0	61	2041	1028
12	100		0	42		1148
13	100		0	25		1394
14	100		0	1617		1084
15	100		0	2619		1006
16	200		0	188		1481
17	200		0	6		1254
18	200		0	3		707
19	200[a]		0[b]	2		1514

[a]Virus stock prepared for gp120 gene sequencing analysis.

TABLE 2

Glycosylation sites affected in HIV-1(III_B) gp120 under HHA or GNA pressure in CEM cell cultures

Amino acid sequence numbering according to Kwong et al. (1998), and assignment of complex- or highly mannose-type glycosylation sites according to Leonard et al. (1990). Mutant HIV-1(III_B) strains emerged under stepwise escalating HHA or GNA concentrations, starting from 0.25 up to 500 μg/ml. Underlined mutated amino acids represent mutations at the asparagine residues that are part of a glycosylation motif; those not underlined represent mutations at threonine/serine residues that are part of a glycosylation motif. The T188N mutation leads to destruction of the *N*-glycosylation site at N186 but creates a novel potential *N*-glycosylation site at N188 as part of the newly created glycosylation motif 188NTS190. The 397NST399 glycosylation site is deleted in HIV-1(III_B). The 463NES465 glycosylation site does not exist in the HIV-1(III_B) strain used in our experiments because of the presence of a 463NGP465 sequence.

Glycosylated Asparagines in HIV-1(III_B) gp120	Glycosylation Sites	Mutations in gp120 That Appeared in Drug-Resistant Virus Strains		
		HIV-1/GNA-500CS	HIV-1/HHA-500SN	HIV-1/HHA-500CS
88NVT90	C	Thr/Ile90	Ile90	
136NDT138	C			
141NSS143	C			
156NCS158	C			
160NIS162	C			
186NDT188	C		Asn188	
197NTS199	C			
230NKT232	M	Met232		Met232
234NGT236	M	Lys234	Ala236	Ile236
241NVS243	M			
262NGS264	M			
276NFT278	C			Thr/Ile278
289NQS291	M	Asn/Asp289; Ser/Phe291	Lys289	
295NCT297	M		Ile297	Ile297
301NNT303	C	Tyr301		Tyr301
332NIS334	M		Asn334	Asn334
339NNT341	M	Ile341	Ile341	Tyr339
356NKT358	C			
386NST388	M		Asp386	
392NST394	M	Ile394		Asp392; Ile394
397NST399	C			
406NNT408	C			
448NIT450	M			
463NES465	C			

C, complex; M, highly mannose.

was converted to 276NFM278, and 397NST399 was converted to a mixture of 397*N*/DS*T*399). Thus, plant lectins exposed to a clinical HIV strain in a dose-escalating manner select for mutations in the glycosylation motifs of gp120 as also found for the laboratory HIV-1(III_B) strain.

Sensitivity of Mutant Virus Isolates against Plant Lectins and Other HIV Entry Inhibitors. The mutant HIV-1 isolates that were isolated after dose-escalating exposure to HHA and GNA were evaluated for their sensitivity against several mannose-specific plant lectins, the mannose-specific blue-green alga (cyanobacterium)-derived lectin cyanovirin, and other entry inhibitors, including the adsorption inhibitor dextran sulfate, the gp41-targeting T-20, and the CXCR4 antagonist AMD-3100. The three virus isolates proved to be highly resistant (100- to 1000-fold) to the inhibitory effects of HHA and GNA. They also showed cross-resistance to other mannose-specific plant lectins such as those derived from LOA, CA, and NPA (Table 2). The three mutant virus strains were only modestly resistant (~5- to 30-fold) to cyanovirin and showed no cross-resistance against any of the other viral entry inhibitors (i.e., dextran sulfate, AMD-3100, T-20) (Table 2). In addition, the reverse transcriptase inhibitors UC-781 and tenofovir retained their full inhibitory activity against the mutant virus strains. Finally, the monoclonal antibody 2G12 that binds to an HIV-1 oligomannose epitope of gp120 completely lost its inhibitory potential against the mutant virus strains (Table 3).

Degree of Infectivity of Plant Lectin-Resistant HIV-1 Strains Versus Wild-Type Virus. The infectivity potential of the three mutant virus strains was determined in comparison with that of the wild-type parental HIV-1(III_B) strain (Table 4). To this end, the amount of p24 and viral RNA and the $CCID_{50}$ was determined for each virus. The HIV-1/GNA-500CS strain proved approximately 30-fold more infectious than wild-type when the infectivity ($CCID_{50}$) was calculated relative to the RNA content. The increased infectivity normalized to the p24 content confirms our findings of the increased infectivity normalized to virus RNA content (Table 4). The HIV-1/HHA-500SN and HIV-1/HHA-500CS virus strains were also more infectious than wild-type virus (11- to 13-fold and 7- to 11-fold, respectively) (Table 4).

Because some mutations in gp120 have been reported to enable coreceptor switch of the mutant viruses, we performed infection experiments of wild-type and the mutant virus strains in U 87 cell lines expressing both CXCR4 and CCR5. The different mutant virus strains and the parental wild-type virus were exposed to the U 87 cell cultures at virus inocula that contained equal amounts of p24 (i.e., 1000, 500, and 250 pg of p24). A semiquantitative HIV-1 LTR R/U5-specific PCR on total DNA was then performed at 2 h after infection of U87.CD4.CCR5 cells (lanes 13–16) or U87.CD4.CXCR4 cells (lanes 1–12) with wild-type HIV-1 (lanes 1–3 and 13), HIV-1/GNA-500CS (lanes 4–6 and 14), HIV-1/HHA-500CS (lanes 7–9 and 15), and HIV-1/HHA-500SN (lanes 10–12 and 16) (Fig. 2). No DNA signal could be measured in lanes 13 to 16, where the wild-type and the three mutant HIV-1(III_B) strains had been exposed to CD4- and CCR5-expressing (CXCR4-negative) U87 cell cultures. However, appearance of viral DNA occurred when the wild-type virus and the three mutant virus strains were exposed to CD4- and CXCR4-expressing U87 cell cultures. The most infectious mutant virus strain (HIV-1/GNA-500CS) (Table 4) gave also the strongest DNA signal in the cell cultures 2 h after infection (lanes 4–6). The least infectious (wild-type)

TABLE 3

Sensitivity/resistance profile (EC_{50} values) of mutant HIV-1 strains against plant lectins and other viral entry or reverse transcriptase inhibitors

Values in brackets represent the degree (-fold) of resistance of the test compounds compared with wild-type virus.

Compound	Wild-type HIV-1(III_B)	HIV-1/HHA-500CS	Fold Resistance	HIV-1/HHA-500SN	Fold Resistance	HIV-1/GNA-500CS	Fold Resistance
	μg/ml	*μg/ml*		*μg/ml*		*μg/ml*	
GNA	0.27 ± 0.058	26.7 ± 5.77	[99]	250 ± 87	[926]	157 ± 98	[581]
HHA	0.21 ± 0.12	55 ± 34	[262]	193 ± 93	[919]	103 ± 45	[490]
LOA	0.057 ± 0.021	6.2 ± 3.4	[109]	13 ± 6.1	[228]	14 ± 6.0	[246]
CA	0.43 ± 0.058	60.0 ± 34.6	[140]	≥100	[≥233]	57 ± 38	[133]
NPA	0.37 ± 0.058	71 ± 38	[192]	≥100	[≥270]	≥100	[≥270]
CV-N	0.015 ± 0.0058	0.077 ± 0.021	[5.1]	0.43 ± 0.32	[29]	0.17 ± 0.20	[11]
DS-5000	0.60 ± 0.17	0.50 ± 0.26	[0.8]	0.60 ± 0.35	[1]	0.21 ± 0.17	[0.35]
AMD3100	0.031 ± 0.020	0.057 ± 0.006	[1.8]	0.025 ± 0.013	[0.8]	0.10 ± 0.09	[3.2]
T-20	0.038 ± 0.010	0.047 ± 0.014	[1.2]	0.036 ± 0.026	[1.0]	0.053 ± 0.015	[1.4]
UC-781	0.0015 ± 0.0004	0.002 ± 0.0003	[1.3]	0.003 ± 0.0004	[2.0]	0.003 ± 0.0007	[2.0]
(*R*)-PMPA	1.0 ± 0.25	1.3 ± 1.2	[1.3]	4.1 ± 2.8	[4.1]	2.8 ± 0.73	[2.8]
Mab 2G12	1.4 ± 0.4	>25	[>15]	>25	[>15]	>25	[>15]

TABLE 4

Characterization of mutant HIV-1 strains

p24 indicates amount of HIV-1 p24 antigen in the virus stocks prepared from the supernatants of (mutant) virus-infected CEM cell cultures. Viral load is the number of RNA copies present in the virus stocks as determined by a signal amplification nucleic acid probe assay (VERSANT HIV-1 RNA 3.0 Assay). Infectivity factor that is required to dilute the virus stocks to obtain equal (100%) infectivity of the cell cultures as quantified by the number of $CCID_{50}$. Relative infectivity is calculated relative to wild-type virus.

Virus strain	p24	Viral load	Ratio viral load/p24	Infectivity factor	Relative infectivity	
					Normalized to Equal p24 Amounts	Normalized to Equal RNA Amounts
	pg/ml × 10^{-3}	*copies/ml* × 10^{9}	*copies/pg p24* × 10^{6}			
HIV-1 (WT)	2040	8152	4.0	16	1	1
HIV-1/GNA-500CS	1330	5025	3.8	300	29	30
HIV-1/HHA-500CS	1100	2794	2.5	60	7.0	11
HIV-1/HHA-500SN	4780	15,878	3.3	400	11	13

virus strain gave the poorest DNA signal (lanes 1–3), and the intermediate infectious virus strains (HIV-1/HHA-500CS and HIV-1/HHA-500SN) (lanes 7–9 and 10–12) led to an intermediate DNA signal (Fig. 2). For each individual virus strain, there was also a dose-dependent decrease in DNA signal, depending the amount of initial p24 used in the infection experiment (i.e., 1000, 500, or 250 pg of p24) (Fig. 2). The corresponding PCR signal of the nonrelated β-actin gene was comparable in all conditions (Fig. 2). However, although there seemed to be a correlation between the degree of infectivity of the virus strains and the intensity of PCR signal of viral DNA synthesis, it should be kept in mind that accurate quantitation of virus entry and synthesis of the very first viral DNA products can only be achieved by real-time PCR analysis.

Discussion

In marked contrast with the glycosylation sites of the gp120 envelope glycoprotein of the mutant virus strains, the four glycosylated asparagines of the gp41 fusion glycoprotein were not affected in any of the three HHA- and GNA-resistant virus isolates. Because these glycans are complex types of unknown nature (2), the highly mannose-specific plant lectins used in this study may not be able to efficiently recognize these glycans on gp41. These findings demonstrate that plant lectins are targeting gp120, but not gp41, to exert their antiviral activity.

Although the complex sugar type glycosylation sites in HIV-1 gp120 [11 of 22 sites in the HIV-1(III_B) strain used in this study] are slightly more abundant than the high-mannose glycosylation sites (Leonard et al., 1990; Gallaher et al., 1995), it is striking that the vast majority of mutations (~75%) that appear under plant lectin pressure predominantly occur at *N*-glycosylation sites that bear high-mannose type sugar oligomers and not the complex type sugar moieties. However, because 90% of the complex oligosaccharides in gp120 are assumed to be fucosylated and 94% of them are reported to be sialylated (Leonard et al., 1990; Hart et al., 2002), it is likely that efficient recognition of these complex oligomer sugar structures by the mannose-specific plant lectins is hampered by the presence of additional non–mannose-like sugars. Although cross-resistance to other mannose-specific plant lectins has not been surprising (resistance degree ranging from 100 to 1000), it was interesting to notice that the blue-green alga-derived mannose-specific cyanovirin (Boyd et al., 1997; Bolmstedt et al., 2001) still retained marked antiviral activity (EC_{50}, 0.077–0.43 μg/ml; resistance degree ranging from 5 to 30) (Table 2) against the mutant virus strains. These findings point to subtle differences in mannose oligomer specificities between cyanovirin and the mannose-specific plant lectins. In fact, we also selected for drug resistance of HIV-1(III_B) against the cyanobacterium-derived mannose-specific cyanovirin. A virus isolate recovered after 35 subcultivations (in the presence of stepwise drug-escalating concentrations using 0.025, 0.05, 0.10, 0.4, 0.60, 0.75, and eventually 1 μg/ml cyanovirin) contained a mixture of Y/N301N and S334N, N339S, and N448S mutations in its gp120. This virus strain displayed 12-fold resistance to cyanovirin in CEM cell cultures. Thus, another mannose-specific lectin that was not derived from a plant, but from a procaryotic microorganism, selected for mutations in gp120 at *N*-glycosylation sites or at NXS glycosylation motifs, as also observed for mannose-specific plant lectins. These observations are in agreement with recent findings of Witvrouw et al. (2005) that cyanovirin and also the mannose-specific concanavalin plant lectin select for deletions of glycosylation sites in HIV-1 gp120.

The selection of plant lectin-resistant HIV-1(III_B) strains has been performed by monitoring giant cell formation as a parameter to decide whether the compound concentration should be increased or maintained during the next subcultivation. Because changes in the glycosylation pattern of gp120 can modify the capacity of HIV-1 strains to form syncytia independently from its infectivity characteristics, it cannot be excluded that some bias occurred in the representativeness of the mutant virus isolates during the selection process. However, when selecting for plant lectin resistance against the clinical HIV-1/HE isolate, p24 production in the cell culture medium was determined before each subcultivation instead of syncytia formation in the cell cultures. As found for the HIV-1(III_B) resistance selection experiments, the glycosylation of gp120 of the drug-exposed clinical virus isolate was also clearly affected. One of the mutations resulted in the annihilation of the *N*-glycosylation site at amino acid position 276, which was also observed in the plant lectin-resistant HIV-1/HHA 500CS strain (Table 2).

The removal of several glycosylation sites in gp120 resulted in more infectious virus compared with the parental wild-type HIV-1. The markedly increased infectivity of the mutant viruses was observed relative to the RNA content of the virus particles, and it was shown that the more infectious the mutant virus strains, the more abundant (and faster) the very first DNA transcripts appear in the virus-exposed cells. It can be hypothesized that the increased infectivity of the mutant virus strains relative to the wild-type may be related to a higher affinity of mutant gp120 for its (co)receptor and

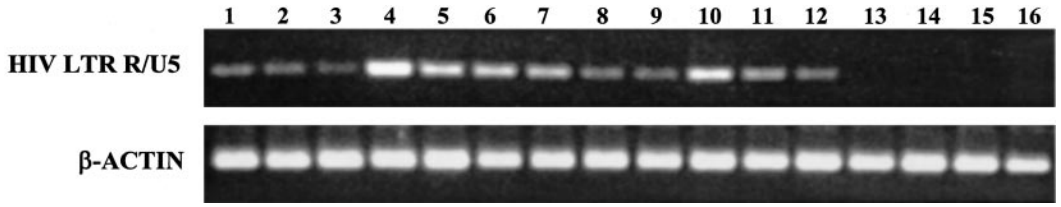

Fig. 2. PCR-based detection of virus entry into U87.CD4.CXCR4 cells. Two hours after infection with 1000, 500, or 250 pg/ml p24 antigen of the wild-type HIV-1 III_B strain (lanes 1, 2, and 3, respectively) or the plant lectin-resistant strains HIV-1/GNA-500CS (lanes 4–6), HIV-1/HHA-500CS (lanes 7–9) or HIV-1/HHA-500SN (lanes 10–12), total DNA was extracted from the cells and analyzed by semiquantitative PCR using HIV-1-specific primers from the LTR R/U5 region. As a control, DNA samples from virus-inoculated (1000 pg/ml p24 antigen) nonsusceptible U87.CD4.CCR5 cells were also included and produced no signal with the HIV-specific PCR (lane 13, wild type; lane 14, HIV-1/GNA-500CS; lane 15, HIV-1/HHA-500CS; lane 16, HIV-1/HHA-500SN). DNA recovery was controlled by PCR with β-actin-specific primers.

thus a likely increase in efficiency of the virus to enter the target cell. It has indeed been shown previously that a mutation in the *N*-glycosylation site of the V3 loop can lead to coreceptor switch because of increased affinity for the receptor CXCR4 (Pollakis et al., 2001; Polzer et al., 2003). This increased affinity has been ascribed to a markedly increased exposure of the positive charges of the gp120 V3 loop (previously largely covered by the glycan on amino acid position 301) to the negatively charged CXCR4 receptor. The particular mutation(s) in the gp120 of the mutant viruses responsible for this phenomenon remains unclear. However, the infection experiments with the mutant virus strains aimed at detecting the very first DNA transcription products revealed that no proviral DNA could be detected when these virus strains were exposed to CD4- and CCR5-expressing U87 cells, revealing that no coreceptor switch from CXCR4 to CCR5 had occurred for the mutant viruses.

The interaction of gp120 with CD4 is based upon contacts with one amino acid of the V1/V2 stem, loop *L*D (279–282), the β15–α3 excursion (365–368 and 369–372), the β20–β21 hairpin (422–427 and 430–435), the β23 strand (451–457), and the β24–α5 connection (464–470 and 475–484) and the interaction of gp120 with CXCR4/CCR5 involves the amino acids of the V3 loop (296–330), the bridging sheet containing β2 and β3, the β20 and β21 strands, and the amino acids Arg419, Lys421, and Gln422 at the end of β19 (Kwong et al., 1998; Wyatt et al., 1998) (Fig. 3). Except for the glycosylated Asn301 amino acid found in the V3 domain of gp120, no mutations were found in the areas that interact with the (co)receptors. It also explains why the mannose-specific plant lectins are unable to prevent gp120/CD4 binding, but instead inhibit the virus fusion process at a stage that follows binding of the virus to the target cells (Matsui et al., 1990; Weiler et al., 1990).

Several investigators have studied the effect of mutations in gp120 on viral infectivity and (co)receptor interaction. Lee

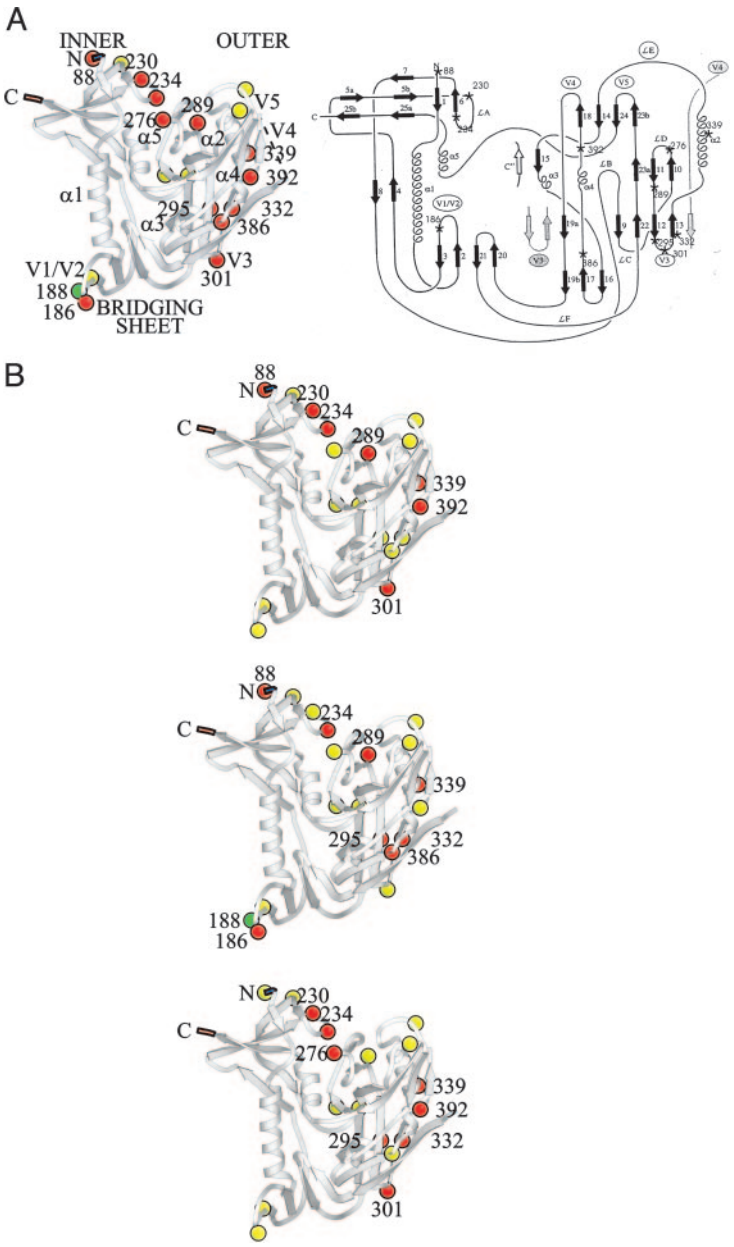

Fig. 3. The gp120 core structure according to Kwong et al. (1998). A, a ribbon diagram (left) and topology diagram (right) are shown. Loops are labeled *L*A–*L*F and V1–V5. The 5-helixes are labeled α1–α5. The 25 β-strands are shown as arrows. The viral membrane is oriented above and the target cell membrane below. The approximate location of the 12 glycosylation sites that were mutated in the plant lectin-resistant virus strains are shown as black asterixes [topology diagram (right)] or as red balls [ribbon diagram (left)]. The other glycosylation sites that are not mutated in the plant lectin-resistant virus strains are indicated as yellow balls. The open circle on the V1/V2 loop at amino acid 188 represents the newly created *N*-glycosylation site that appeared by mutating T188 to 188N. B, ribbon diagrams showing the mutations at the glycosylation sites (red balls) in gp120 of HIV-1/GNA-500CS (top), HIV-1/HHA-500SN (middle), and HIV-1/HHA-500CS (bottom). Structures were drawn according to MOLSCRIPT (Kraulis, 1991).

and colleagues (1992) mutated each of the 24 asparagines in HIV-1 gp120 that are part of the glycosylation motif N-X-T/S. They found that all these single mutants kept their ability to infect SupT1 cells and form giant cells, although the infectivity was somewhat impaired by mutations at amino acid positions 88, 141, 160, 197, 262, and 276. The authors showed that this impaired infectivity was caused by the lack of the glycans in gp120, except for amino acid 160, where it was shown that the impaired infectivity was caused by the amino acid change (Asn→Gln) itself rather than to the deletion of the glycan moiety. The amino acids 88 and 276, but not the other asparagine mutations that showed impaired infectivity, were mutated in one or two of the plant lectin-resistant mutant virus strains. However, given the increased infectivity of our mutant virus strains, this phenomenon can not be ascribed to amino acid mutations 88 or 276.

Hemming et al. (1994) performed site-directed mutagenesis on the C-terminal Asn406, Asn448, and Asn463 that are located in the CD4 binding area of gp120. When abolished all together, the absence of these three glycosylation sites had no effect on syncytia induction and they are thus dispensable for an efficient CD4 binding and subsequent viral fusion. The C-terminal *N*-glycosylation sites were not affected in the plant lectin-resistant virus strains.

Lack of the *N*-linked glycan at Asn186 in the V1 loop had also no effect on infectivity in cell culture (Gram et al., 1994). However, because this virus mutant was more resistant to neutralization by monoclonal antibodies to the V3 loop and by sCD4, it was concluded that the *N*-linked glycan in V1 may modulate the three-dimensional conformation of gp120 without changing the overall functional integrity of the molecule.

It is interesting that Trkola et al. (1996) and Scanlan et al. (2002) investigated mutations on gp120 at Asn295, Asn332, Asn339, Asn386, and Asn392 by Asn-to-Ala substitutions, resulting in markedly decreased affinity for monoclonal antibody 2G12 binding. This neutralizing anti-HIV-1 antibody recognizes a cluster of $\alpha 1 \rightarrow 2$ mannose residues of oligomannose glycans on the outer phase of gp120. Whereas Asn339 and Asn386 proved less critical for 2G12 binding, and may instead play an indirect role in maintaining the epitope conformation, Asn295 and Asn392 were crucial for 2G12 epitope binding. It is interesting that all five Asn positions in gp120 were also found to be mutated in one or several of our plant lectin-resistant mutant virus strains. Given that it was also shown that cyanovirin competed with 2G12 for these interaction sites and prevented 2G12 binding to this epitope on gp120, it is likely that the plant lectins might recognize this region of the gp120 molecule as well. These conclusions are also in line with the resistance of our mutant virus strains against the monoclonal antibody 2G12. Moreover, because it has been suggested that 2G12 antibodies may have a high potential to prevent natural HIV-1 infection in vivo (Wolbank et al., 2003), it would be interesting to further explore plant lectins in this respect.

In the HIV-1/HHA-500SN strain, there was one glycosylation site (at position 186) in the V1/V2 stem of gp120 deleted but replaced by a novel glycosylation site (at position 188); thus, only 2 amino acids downstream of the original (deleted) glycosylation site. The significance of this observation is currently unclear (Fig. 3A, green ball in ribbon structure).

The annihilation of clustered glycosylation sites under plant lectin pressure predominantly occurred for all three mutant virus strains at the top of the inner domain (near the gp41 binding site) and, except for the mutation in the V3 loop, the left upper half and the right side of the outer domain (Fig. 3). Given the multivalent nature of the plant lectins (GNA is a tetramer with 12 mannose-binding sites), it would now be interesting to cocrystallize gp120 with the plant lectins to reveal the interaction points of these proteins with the gp120 glycans. It may be assumed that the conformational changes in gp120 that need to occur sequentially during the fusion process may be inhibited by complexation of gp120 with the plant lectins.

The presence of the sugar moieties in gp120 may not seem necessary to afford efficient virus-cell fusion, because the mutant virus strains were more infectious than the wild-type virus. Therefore, it can be hypothesized that as more glycosylation sites are removed from gp120, the fusion process proceeds more easily and the virus displays more resistance to the plant lectins. However, additional studies should address the conformational changes induced by the plant lectins in the viral envelope glycoproteins (particularly gp120) and any potential conformational changes in the gp120 envelope that may occur upon mutation of the glycosylated asparagine sites.

The pressure that plant lectins exert on the virus to mutate its gp120 envelope in cell culture, resulting in changes of its glycan shield, may be somewhat comparable with the pressure neutralizing antibodies exert during the progress of virus infection in persons infected with HIV (Wei et al., 2003). Indeed, it has been shown that in vivo, the envelope gene of HIV-1 rapidly mutates, primarily at glycosylation sites of gp120. This phenomenon, observed in persons infected with HIV, results in a change of the glycan shield on the surface of the virus and creates a physical block to the binding of neutralizing antibody. This mechanism of immunological escape markedly contributes to the HIV persistence in the face of an evolving antibody repertoire. It can be speculated, therefore, that plant lectins, if ever used to treat persons infected with HIV, might markedly hamper the potential of HIV to escape the immune surveillance.

Whereas the virus seems to need to continuously change its envelope glycan shield in response to the immune system, lectins may force the virus to a very limited pattern of successful escape mutations. Plant lectin-exposed HIV may get caught by two noncompatible escape pathways by which the virus may run out of valuable options to escape drug pressure and immunological pressure at the same time. Therefore, one interesting possibility would be to immunize persons infected with HIV with gp120 epitopes that are likely to appear under plant lectin pressure (thus lacking several parts of the glycan shield of gp120), after which drug pressure should be put on the virus. Then, in an attempt to escape from the plant lectin pressure, the virus may try to abolish part of its sugar content on gp120, allowing the immune system to more efficiently tackle these mutant gp120-containing virus strains. It would seem justified to further explore this hypothesis. Indeed, Bolmstedt and colleagues reported that immunization of mice with HIV-1 gp120 lacking the Asn336 glycosylation signal (mutant 306NNA308) in the V3 loop (the gp120 amino acid numbering of the authors corresponds to 339NNA341 according to our numbering in Table 2 and Fig. 3) resulted in higher serum IgG-binding titers to HIV-1

gp120 (immunization with DNA-encoding mutant HIV-1 env) than when wild-type HIV-1 gp120 was used (immunization through DNA-encoding wild-type HIV-1 env) (Bolmstedt et al., 2002). These authors could also demonstrate that no differences in neutralization titers were obtained in mice immunized against wild-type or mutant HIV-1 env DNA, and they concluded that the Asn336 mutant gp120 resulted in immunization against a non-neutralization epitope. However, it was clear that by changing the glycosylation pattern of HIV-1 gp120, more efficient antibody responses as well as T cell responses were directed to wild-type gp120 (Bolmstedt et al., 2002). These observations are also in agreement with earlier reports that HIV-1 gp120 protein glycosylation can be a primary modifier of virus antigenicity that is susceptible to the host immune response (Schønning et al., 1996).

In conclusion, plant lectins with absolute preference for mannose oligomers efficiently inhibit HIV replication in cell culture by preventing entry of the virus into its target cells. From this perspective, the plant lectins may qualify as potential anti-HIV microbicide drug candidates. The plant lectins select drug-resistant virus strains that retain full sensitivity to other viral entry inhibitors and have accumulated mutations in the gp120 that result in a dramatically decreased degree of *N*-glycosylation and a concomitantly increased infectivity. In this respect, these compounds can be considered rather unique in terms of specificity for their molecular target compared with other reported viral entry inhibitors. Given the importance of a rapidly evolving glycan shield at the HIV envelope to continuously escape the immune surveillance, plant lectins may play an important role to force the virus to convert to a gp120 phenotype that cannot properly escape from the immune system, and this may in turn result in a more efficient elimination of infectious HIV from the body.

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