

Profile of Resistance of Human Immunodeficiency Virus to Mannose-Specific Plant Lectins

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The mannose-specific plant lectins from the *Amaryllidaceae* family (e.g., *Hippeastrum* sp. hybrid and *Galanthus nivalis*) inhibit human immunodeficiency virus (HIV) infection of human lymphocytic cells in the higher nanogram per milliliter range and suppress syncytium formation between persistently HIV type 1 (HIV-1)-infected cells and uninfected CD4⁺ T cells. These lectins inhibit virus entry. When exposed to escalating concentrations of *G. nivalis* and *Hippeastrum* sp. hybrid agglutinin, a variety of HIV-1(III_B) strains were isolated after 20 to 40 subcultivations which showed a decreased sensitivity to the plant lectins. Several amino acid changes in the envelope glycoprotein gp120, but not in gp41, of the mutant virus isolates were observed. The vast majority of the amino acid changes occurred at the N glycosylation sites and at the S or T residues that are part of the N glycosylation motif. The degree of resistance to the plant lectins was invariably correlated with an increasing number of mutated glycosylation sites in gp120. The nature of these mutations was entirely different from that of mutations that are known to appear in HIV-1 gp120 under the pressure of other viral entry inhibitors such as dextran sulfate, bicyclams (i.e., AMD3100), and chicoric acid, which also explains the lack of cross-resistance of plant lectin-resistant viruses to any other HIV inhibitor including T-20 and the blue-green algae (cyanobacteria)-derived mannose-specific cyanovirin. The plant lectins represent a well-defined class of anti-HIV (microbicidal) drugs with a novel HIV drug resistance profile different from those of other existing anti-HIV drugs.

Plant lectins represent a well-defined class of antiretroviral compounds that differ from other antiviral drugs in many aspects. Lectins are natural, not synthetic, products (proteins) and target the sugar moieties of a wide variety of glycoproteins. They are widespread among higher plants and were recently subdivided into seven families of structurally and evolutionarily related proteins (43). The most prominent anti-human immunodeficiency virus (HIV) activity is found predominantly among the monocot mannose-binding lectins (MBLs) (4, 5, 43). *Galanthus nivalis* agglutinin (GNA) has a specificity for $\alpha(1-3)$ -linked mannose residues, whereas *Hippeastrum* sp. hybrid agglutinin (HHA) recognizes both $\alpha(1-3)$ - and $\alpha(1-6)$ -linked mannose residues (41–43). These lectins occur as tetramers with a molecular mass of 50,000 Da. They suppress HIV infection as well as HIV transmission by preventing the entry of HIV into its target cells, and short preexposure of cell-free HIV type 1 (HIV-1) particles or virus-infected cells markedly potentiates the inhibitory activity of the plant lectins (7a; J. Balzarini, S. Hatse, K. Vermeire, K. Princen, E. De Clercq, E. Van Damme, W. Peumans, and D. Schols, Abstr. Hum. Immunodef. Virus DART 2002, abstr. 024, p. 29, 2002). There is strong evidence that plant lectins with anti-HIV activity predominantly target the heavily glycosylated gp120 envelope glycoprotein. Other compounds known to target HIV

gp120 are soluble CD4 (2), chicoric acid and its tetraacetyl esters (30), polyanions like dextran sulfate derivatives (15, 34), and the peptidic substance cyanovirin, a lectin isolated from the cyanobacterium *Nostoc ellipsosporum* (11). In contrast, other known entry inhibitors such as T-20 interact with gp41 (20), whereas the bicyclams AMD3100 (35) and SCH-C (38) and the quaternary ammonium derivative TAK-779 (3) bind to the chemokine receptors (HIV coreceptors) CXCR4 and CCR5, respectively. The natural agonists of these HIV coreceptors are the chemokines SDF-1 (9, 27) for CXCR4 and RANTES and macrophage inflammatory protein 1 α (MIP-1 α) (especially the isoform LD78 β) and MIP-1 β (12, 14, 24) for CCR5, which were shown to inhibit HIV infection.

There is currently increased interest and attention with regard to the development of antiviral (HIV) microbicides, which are agents that may be used topically to prevent the spread of HIV infection by blocking sexual transmission of HIV. We and others have previously shown that a variety of plant lectins exert a pronounced inhibitory activity against HIV replication in cell cultures (4, 5, 18). Recently, researchers focused on the mannose-specific lectins from snowdrop (GNA) and amaryllis (HHA) lectins and found that they may qualify as potential microbicides to prevent HIV spread (7a; Balzarini et al., Abstr. Hum. Immunodef. Virus DART 2002; D. Schols, S. Hatse, K. Vermeire, K. Princen, W. Peumans, E. Van Damme, E. De Clercq, and J. Balzarini, Abstr. 10th Conf. Retrovir. Opportun. Infect., abstr. 104, p. 94, 2003). These lectins not only inhibit infection of human lymphocytic cell

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cultures with cell-free virus but also prevent virus spread from HIV-infected cells, and their activity is markedly potentiated upon short preincubation of cell-free virus particles or virus-infected cells (7a; Balzarini et al., Abstr. Hum. Immunodef. Virus DART 2002). In sharp contrast with many other lectins, both HHA and GNA proved nonmitogenic to human lymphocytes and they do not agglutinate human red blood cells or show acute toxicity when administered intravenously to mice (7a, 43; Balzarini et al., Abstr. Hum. Immunodef. Virus DART 2002; Schols et al., Abstr. 10th Conf. Retrovir. Opportun. Infect.).

In this study, we selected HIV type 1 (HIV-1) strains with different levels of resistance to two mannose-specific lectins (GNA and HHA) and found that they are endowed with a resistance profile that is strikingly different from that of any other entry inhibitor reported so far. It was shown that glycosylation sites in HIV gp120 were predominantly affected, resulting in different levels of HIV resistance towards plant lectins, depending on the nature and number of glycosylation sites that were affected in the gp120 molecule. These findings also explain the virtual lack of cross-resistance towards other known classes of HIV entry inhibitors.

MATERIALS AND METHODS

Test compounds. The mannose-specific plant lectins GNA, HHA, *Narcissus pseudonarcissus* agglutinin (NPA), *Cambidium* sp. agglutinin (CA) and *Listeria ovata* agglutinin (LOA) were derived and purified from the bulbs of these plants, as described previously (41–43). UC-781 was obtained from Crompton Ltd. (Middlebury, Conn.). Tenofovir was from Gilead Sciences (Foster City, Calif.), AMD3100 was from AnorMed (Langley, British Columbia, Canada), and dextran sulfate 5000 (DS-5000) was from Sigma (St. Louis, Mo.). T-20 (pentafuside, enfuvirtide) was kindly provided by the AIDS Research Alliance (Los Angeles, Calif.), and cyanovirin was kindly provided by J. B. McMahon (National Institutes of Health, Bethesda, Md.).

Cells. Human T-lymphocytic CEM and Sup-T1 cells were obtained from the American Type Culture Collection (Rockville, Md.), and persistently infected HUT-78/HIV-1 or CEM/HIV-1 cells were obtained by exposing HUT-78 or CEM cell cultures to wild-type HIV-1(III_B) or mutant (GNA-resistant) HIV-1/GNA for 3 to 4 weeks. All cell lines mentioned were cultivated in RPMI 1640 medium supplemented with 10% fetal bovine serum (BioWittaker Europe, Verriers, Belgium), 2 mM L-glutamine, and 0.075 M NaHCO₃.

Viruses. HIV-1(III_B) was provided by R. C. Gallo and M. Popovic (at the National Cancer Institute, National Institutes of Health, Bethesda, Md.).

Selection and isolation of GNA- and HHA-resistant HIV-1 strains. HIV-1(III_B) was subjected to subcultivations in HIV-1-infected CEM cell cultures (3×10^5 to 4×10^5 cells/ml) in 48-well plates in the presence of 0.5 µg of GNA/ml or 0.25 µg of HHA/ml. The multiplicity of the initial infection was ~200 times the cell culture 50% infective dose (CCID₅₀). Passages were performed every 3 to 4 days by the addition of 100 µl of the infected culture supernatant or 100 µl of the infected cell suspension to 900 µl of a suspension containing 3×10^5 to 4×10^5 uninfected CEM cells/ml. At each subcultivation, the same concentration of test compound was administered; an increased drug concentration was given when full cytopathicity was obtained in the previous cell cultures. The escalation of drug concentrations as well as the time points and drug concentrations at which the virus isolates were recovered are shown in Fig. 1.

Nucleic acid isolation, amplification, and HIV-1 population sequencing. Proviral DNA was extracted from cell pellets with a QIAamp DNA blood mini kit (QIAGEN, Hilden, Germany). A 3,876-bp nucleotide fragment, encompassing the *gp120* and the *gp41* genes, was amplified in an outer PCR by using an Expand High Fidelity PCR system (Roche Diagnostics, Mannheim, Germany). A 2,346-bp nucleotide fragment of *gp120* and a 2,048-bp nucleotide fragment of *gp41* were separately amplified in two inner PCRs with an Expand High Fidelity PCR system. After gel purification (Qiaquick gel extraction kit; QIAGEN), virus population sequencing was performed by using an ABI PRISM BigDye Terminator version 3.0 ready reaction cycle sequencing kit and an ABI3100 genetic analyzer (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). The sequences were analyzed by using SeqScape Software version 1.0 (Applied Biosystems).

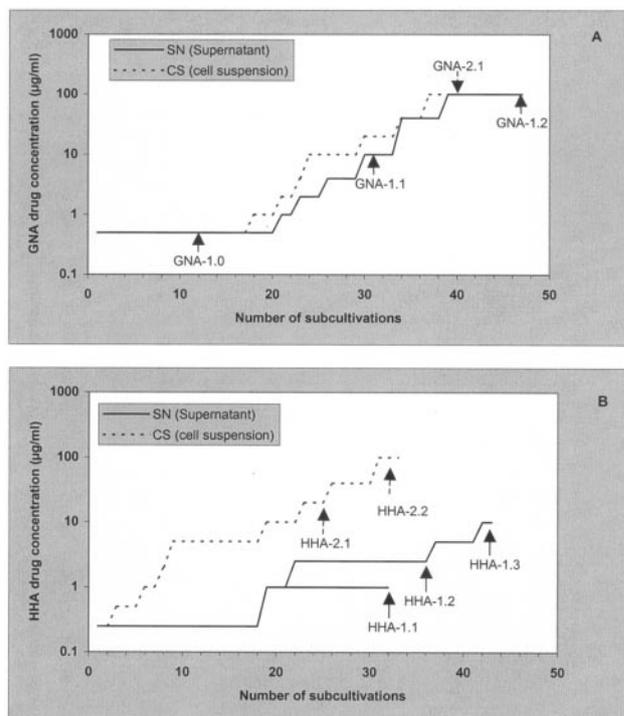


FIG. 1. Schedule of drug-escalating selection of plant lectin (GNA [A] and HHA [B])–resistant HIV-1 strains as a function of time. Arrows indicate the time points when the virus isolates were made. Unbroken lines represent the subcultivation schedule in which supernatants were transferred with each passage, whereas broken lines represent the subcultivation schedule in which cell suspensions were transferred with each passage.

Antiretrovirus assays. The methodology of the anti-HIV assays has been described previously (6, 7). Briefly, CEM cells (4.5×10^5 cells per ml) were suspended in fresh culture medium and infected with HIV-1 at 100 times the CCID₅₀ per ml of cell suspension. Next, 100 µl of the infected cell suspension was transferred to microplate wells, mixed with 100 µl of the appropriate dilutions of the test compounds (i.e., final concentrations of 200, 40, 8, 1.6, 0.32, and 0.062 µg/ml), 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 (EC₅₀) corresponded to the compound concentrations required to prevent syncytium formation by 50% in the virus-infected CEM cell cultures.

Cocultivation assays. Persistently (wild-type- and GNA-resistant) HIV-1-infected HUT-78 or CEM cells (designated HUT-78/HIV-1, HUT-78/HIV-1 GNA, CEM/HIV-1, and CEM/HIV-1 GNA) were washed to remove free virus from the cell culture medium, and 5×10^4 cells (50 µl) were transferred to 96-well microtiter plates (Sterilin). Next, 5×10^4 Sup-T1 cells (50 µl) and an appropriate concentration of test compound (100 µl) were added to each well. The mixed cell cultures were cultured at 37°C in a CO₂-controlled atmosphere. The first syncytia arose after about 6 h of cocultivation. After 16 to 20 h, marked syncytium formation was noted, and the number of syncytia was examined and quantified under a microscope.

Examination of coreceptor usage by wild-type and GNA-resistant HIV-1(III_B). CXCR4- and CCR5-transfected U87.CD4 cells were seeded in 24-well plates at 2×10^4 cells per well and infected with 5,000 pg of p24/well of the wild-type or the GNA-resistant virus. As a positive control, we also included the dual-tropic R5/X4 HIV-1 strain HE, which is able to infect both cell lines. After 5 days, the virus-induced cytopathic effect (syncytium formation) on the different cell cultures was evaluated microscopically, and virus production was measured in the cell culture supernatants by p24 core antigen (Ag) enzyme-linked immunosorbent assay (Perkin-Elmer, Boston, Mass.).

Site-directed mutagenesis and expression of viral clones. A proviral vector, pBRU-2 (obtained from R. Axel through the MRC AIDS Reagent Repository, Potters Bar, United Kingdom), containing the entire genome of HIV-1(BRU) (LAV strain) and some of the pBR322 sequences vector, was used for construc-

tion of a mutant HIV-1(BRU) clone containing the N200Q, N204Q, and N211Q mutations in the LA loop of gp120 as described previously (23). The HIV-1(BRU) *env* gene was subjected to nucleotide-directed mutagenesis by using a USE mutagenesis kit (Pharmacia Upjohn, Stockholm, Sweden) according to the manufacturer's instructions. Briefly, a 2,701-bp BamHI-SalI fragment containing almost the entire envelope gene was excised from the proviral vector pBRU-2 and cloned into the pUC18 plasmid. The resulting plasmid was designated pUCO. The nucleotide changes were introduced into this plasmid by using an oligonucleotide (Scandinavian Gene Synthesis AB, Köping, Sweden), one forming primers containing the desired mutations in C2 (5'GCGATTCTAAAATG TAATCAGAAGACGTTCCAAGGAACAGGACCATGTACACAAGTCCAG CACAGTACAA3'; the mutated nucleotides are underlined) and the other forming a selection primer (5'TTCGATAGTGAACAGATCTCCGGGTACCG AG3'), changing the unique restriction enzyme site BamHI to a BglII site. The fragments DraIII-StuI (249 bp) and StuI-NheI (426 bp) containing the introduced mutation were excised from pUCO and cloned back into pBRU-2 via pUCO with the unchanged BamHI site. Correct mutations were verified by sequencing with the Sequenase version 2.0 sequencing kit (U.S. Biochemical, Cleveland, Ohio).

A procedure to obtain the expression of viral clones was performed as described previously (23). Briefly, wild-type and mutated proviral plasmids were transfected into COS-1 (ATCC CRL-1650) cells by using the Fugene reagent (Roche). At 48 h posttransfection, the COS-1 cells were mixed with uninfected H9 cells, and the cells were cocultured for 5 days. The culture supernatant was transferred to fresh H9 cells and cultured for 10 days. Thereafter, the cultures were centrifuged at 11,000 × *g* for 15 min and the supernatant was aliquoted and stored at -80°C until use.

RESULTS

Long-term subcultivation of HIV-1(III_B)-infected CEM cell cultures in the presence of escalating concentrations of GNA and HHA. HIV-1(III_B)-infected CEM cells were cultured in the presence of GNA and HHA at an initial concentration that was about one- to twofold the EC₅₀ of the particular test compounds (Fig. 1). Subcultivations were performed by transferring either culture supernatants or cell suspensions to fresh uninfected CEM cell cultures. Generally, at least 18 to 20 subcultivations of the HIV-1-infected CEM cells in the presence of fresh noninfected CEM cells were required to afford abundant syncytium formation in the presence of plant lectin concentrations that exceeded their EC₅₀ by more than fivefold (EC₅₀ of GNA and HHA for HIV-1 infection of CEM cells, ~0.3 µg/ml; EC₅₀ of GNA and HHA for syncytium formation in cocultures of persistently HIV-1-infected cells and uninfected cells, ~1.2 µg/ml). Virus samples were then stored for further analysis (i.e., sequencing of gp120/gp41 and drug sensitivity testing), while the concentrations of the plant lectins were further increased in a stepwise manner as soon as full cytopathicity was evident, according to the schedule shown in Fig. 1. To obtain virus isolates that could grow in the presence of GNA concentrations that were at least 100-fold higher than the EC₅₀, a minimum of 30 to 40 subcultivations was required. The different time points at which virus samples were taken for sequencing and drug sensitivity testing are shown by the arrows in Fig. 1.

Determination of amino acid changes in the gp120 of the virus isolates. The gp120 and gp41 envelope genes have been sequenced from eight virus isolates. A wide variety of amino acid changes were found in gp120 but not gp41 of these virus isolates that were grown in the presence of different concentrations of either GNA or HHA (Table 1 and Fig. 2). At least 20 different amino acid sites were affected. In a number of cases, mixtures of the wild type and the mutated amino acid were noticed. However, there was a trend toward the presence

of pure mutations in those virus strains that were selected in the presence of the higher drug concentrations (e.g., mutated amino acids at positions 162 [M], 204 [K], 206 [I], 271 [Y], and 369 [I]) (Table 1). There was also a trend towards a higher number of amino acid changes in the virus isolates that were grown in the presence of the higher drug concentrations (2 amino acid changes in the presence of 0.5 µg of GNA/ml versus 8 or 9 changes in the presence of 100 µg of GNA/ml and 5 to 7 amino acid changes in the presence of 1 to 10 µg of HHA/ml versus 11 changes in the presence of 100 µg of HHA/ml) (Table 1).

When all amino acid changes were considered, four mutations occurred at the N-glycosylated asparagine (e.g., N200S, N204K, N259K, and N271Y), seven mutations occurred in the N glycosylation motif converting S or T to another amino acid (e.g., T60I, T202M, T206A/I, T248I, S261F/P, T311I, and T369I), one mutation occurred at a potential O glycosylation site (e.g., T162M), and seven mutations occurred at an amino acid position not directly related to a glycosylation site (e.g., R136K, A144T, N270Y, N272Y, R305K, N430D, G434E, and K470R).

A number of mutations that directly (mutation of N) or indirectly (mutation of T or S in the glycosylation motif) affected glycosylation of the gp120 N glycosylation motifs (e.g., N204K, N271Y, T311I, and N430D) were invariably found in the majority of the virus isolates, whereas the mutations that were not related to the glycosylation of gp120 were often found in only one of the seven virus isolates (e.g., R136K, A144T, N270Y, R305K, G434E, and K470R). In addition, they were never present as pure mutations but were always present as a mixture with the wild type.

The gp120 amino acid sequence of the HIV-1(III_B) strain used at the start of the selection experiment and the gp120 sequence of the virus that was passaged during 42 subcultivations in the absence of the plant lectins were determined. No mutations at any of the N glycosylation sites in gp120 occurred within this time period, except at amino acid position N259. At passage 42, a mixture of N259 and 259F/S/C was found.

In conclusion, the mutations found in the different virus isolates that appeared under GNA or HHA pressure were predominantly related to nine N glycosylation sites and one potential O glycosylation site in the HIV-1 gp120 envelope glycoprotein.

Sensitivity spectrum of virus isolates recovered in the presence of different GNA and HHA concentrations to plant lectins and other HIV entry inhibitors. The different virus isolates recovered in the presence of different concentrations of GNA and HHA were investigated for their sensitivity-resistance profile against a variety of mannose-specific plant lectins and also against several other types of HIV entry inhibitors (e.g., DS-5000, AMD3100, and T-20) and the reverse transcriptase inhibitors UC-781 and tenofovir that have been previously shown to qualify as potential microbicide drugs. HIV-1/GNA-1.0 and HIV-1/HHA-1.1 that were isolated in the presence of low plant lectin concentrations (0.5 µg of GNA/ml and 1 µg of HHA/ml) showed a relatively low (2.5- to 10-fold) level of resistance to a variety of five different mannose-specific plant lectins, including GNA and HHA. Neither the adsorption inhibitor DS-5000, the gp41 inhibitor T-20, the CXCR4 antagonist AMD3100, nor the reverse transcriptase (RT) inhibitors UC-781 and tenofovir

TABLE 1. Mutations at the amino acid positions of gp120

Virus ^a	Lectin (dose [μ g/ml]) ^c	Codons and amino acid assignment(s) at position ^b :																											
		60	136	162	200	202	204	206	248	259	261	270	271	272	305	311	322	369	430	434	470								
Wild type	Wild type	ACA T	AGA R	ACG T	AAT N	ACG T	ACA T	ACA T	ACA T	AAC N	TCT S	AAC N	WAC N/Y	AAT N	AGA R	ACT T	CAA Q	ACT T	AAT N	GGG G	AAG K								
GNA-1.0	GNA (0.5)				AAK N/K								TAC Y																
GNA-1.1a	GNA (10) (SN)	AYA T/I			AAK N/K	ART N/S					TYT F/S		TAC Y			ATT I			RAT D/N										
GNA-1.1b	GNA (10) (SN)				AAK N/K	ART N/S					TYT F/S		TAC Y			ATT I			RAT D/N										
GNA-1.2	GNA (100) (SN)			ATC M						AYG T/M		WAC N/Y				ATT I		AYT T/I	RAT D/N										
GNA-2.1a	GNA (100) (CS)	AYA T/I		ATG M							TYT F/S		TAC Y	WAT N/Y		ATT I			RAT D/N										
GNA-2.1b	GNA (100) (CS)	AYA T/I		ATG M						AAK K				WAT N/Y		ATT I		AYT T/I	RAT D/N										
HHA-1.1	HHA (1) (SN)		ARA R/K					RCA T/A			F/S		AAK N	WAT N/Y	ARA R/K	ATT I													
HHA-1.2	HHA (2.5) (SN)		ARA R/K					GCA A					AAK N			ATT I													
HHA-1.3	HHA (10) (SN)		ARA R/K					GCA A					AAK N	TAT Y		ATT I													
HHA-2.1	HHA (20) (CS)			AYG T/M				ATA I	AYA T/I		YCT S/P		TAC Y			ATT I	CWA O/L	ATT I											
HHA-2.2	HHA (100) (CS) ^d			AYG T/M				ATA I	AYA T/I		YCT S/P		TAC Y			ATT I		ATT I	GRG G/E	ARG K/R									

^a GNA-1.1b and GNA-2.1b represent the virus isolates GNA-1.1a and GNA-2.1a that were subcultured for an additional three passages without drug before they were prepared for DNA sequencing.

^b Nucleotide mixtures in the sequencing reaction were designated as follows: M, C and A; Y, C and T; R, G and A; W, A and T; K, T and G. The wild-type sequence has been determined by sequencing of the wild-type HIV-1(III_B) strain used in the experiments. Only amino acid changes compared to the wild-type virus are indicated. The amino acid and position numbering have been designated according to the numbering shown in Fig. 2.

^c SN, virus isolates obtained after serial subcultivations of virus-infected cell cultures, transferring culture supernatant at each subcultivation. CS, virus isolates obtained after serial subcultivations of virus-infected cell cultures, transferring cell-containing culture fluids at each subcultivation.

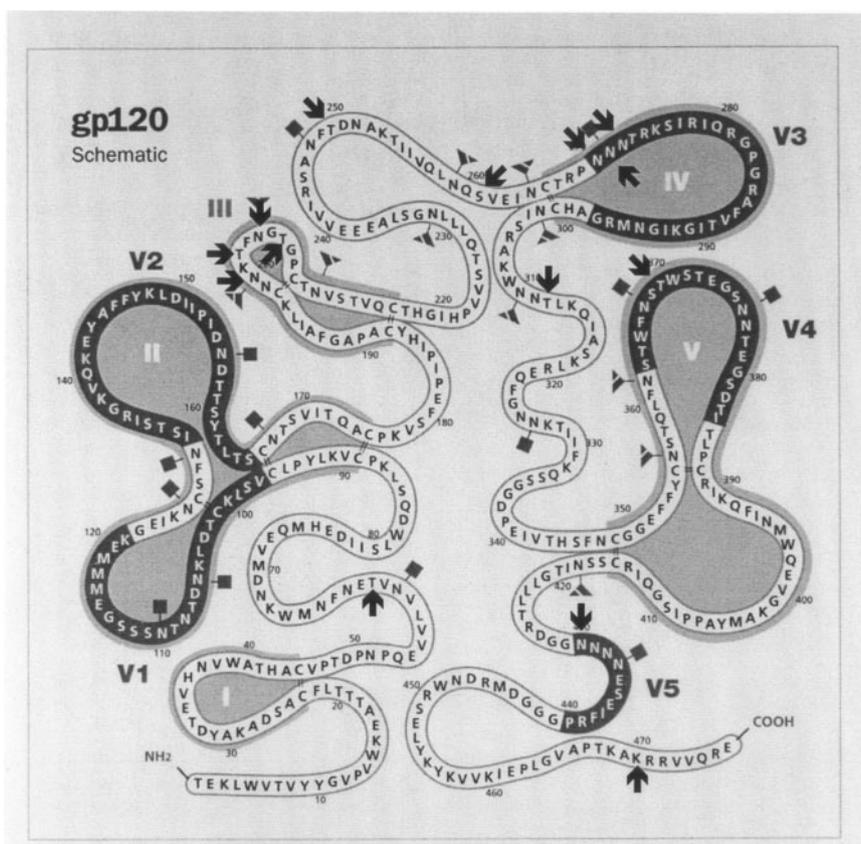


FIG. 2. Alignment of the amino acids of HIV-1 gp120 according to Leonard et al. (22). Arrows represent amino acid mutations found in the plant lectin-resistant virus strains. Triangles represent glycosylation sites bearing high-mannose types of sugars. Squares represent glycosylation sites bearing complex types of sugars.

lost any activity against these virus strains (Table 2). The virus isolates that were recovered from cell cultures in the presence of moderately high plant lectin concentrations (10 to 20 $\mu\text{g/ml}$) (HIV-1/GNA-1.1, HIV-1/HHA-1.3, and HIV-1/HHA-2.1) showed a more pronounced cross-resistance (30- to 50-fold for GNA and 10- to 50-fold for HHA) to the five different mannose-specific plant lectins, whereas again no markedly decreased sensitivity was found for the entry and RT inhibitors (except for AMD3100, which was less active against HIV-1/GNA-1.1 by 10-fold) (Table 2). The highest resistance levels for the plant lectins (70- to >100-fold) were obtained against those virus strains that were isolated in the presence of the highest concentrations of the drugs. LOA was only 20- to 40-fold resistant to HIV-1/GNA-2.1, HIV-1/HHA-1.3, and HIV-1/HHA-2.2. Interestingly, the mannose-specific lectin from the blue-green algae (cyanobacteria) cyanovirin inhibited the highly plant lectin-resistant virus strains HIV-1/GNA-2.1 and HIV-1/HHA-2.2 to an extent similar to that of its inhibition of wild-type virus. In contrast, the neutralizing antibody (NAb) 2G12 directed against HIV-1 gp120 (39) completely lost its inhibitory potential against these virus strains (Table 2). As noted for the other virus mutants selected in the presence of GNA and HHA, the sensitivity of the mutant virus strains to the inhibitory effect of DS-5000, T-20, AMD3100, UC-781, and tenofovir was preserved (Table 2).

Inhibitory activity of plant lectins, entry inhibitors, and RT inhibitors against syncytium formation in cocultures of uninfected Sup-T1 cells and HUT-78/HIV-1 GNA-2.1 or CEM/HIV-1 GNA-2.1 cells that were persistently infected with the GNA-resistant HIV-1/GNA-2.1 strain. The mannose-specific plant lectins were able to prevent syncytium formation between uninfected Sup-T1 cells and persistently HIV-1-infected HUT-78/HIV-1 or CEM/HIV-1 cells at concentrations that were only 5- to 10-fold higher than those required to inhibit CEM cell infection by cell-free virus (Table 3). Interestingly, syncytium formation in cocultures of Sup-T1 cells and HUT-78/HIV-1 GNA-2.1 or CEM/HIV-1 GNA-2.1 cells persistently infected with the GNA-resistant HIV-1/GNA-2.1 strain was inhibited at plant lectin concentrations that were \sim 4- to 5-fold or \sim 15- to 25-fold higher than those required when wild-type HUT-78/HIV-1 cells or wild-type CEM/HIV-1 cells, respectively, were used in the cocultivation assays. Cyanovirin, which prevented syncytium formation at an EC_{50} of 0.12 to 0.20 $\mu\text{g/ml}$, kept full inhibitory potential against syncytium formation between the uninfected Sup-T1 cells and the cells persistently infected with the plant lectin-resistant virus strains (Table 3). In contrast, the other virus entry inhibitors, DS-5000, T-20, and AMD3100, retained their full inhibitory activity against syncytium formation with these viral mutants. As ex-

TABLE 2. Antiviral activity of plant lectins against HIV-1 strains selected for resistance to GNA and HHA

Test compound	EC ₅₀ (µg/ml) for virus ^a :									
	HIV-1(III _B)	GNA-1.0 (0.5)	GNA-1.1a (10)	GNA-1.2 (100)	GNA-2.1 (100)	GNA-2.1 (100)	HHA-1.1 (1)	HHA-1.3 (10)	HHA-2.1 (20)	HHA-2.2 (100)
GNA	0.40 ± 0.14	1.0 ± 0.0	12.2 ± 5.2	48.8 ± 8.5	28.8 ± 2.5	28.8 ± 2.5	2.8 ± 1.8	20 ± 0.0	5.5 ± 2.1	31.0 ± 12.3
HHA	0.28 ± 0.17	1.2 ± 0.5	10.0 ± 3.2	37.5 ± 9.6	21.0 ± 10.5	21.0 ± 10.5	2.0 ± 0.7	11 ± 1.4	6.1 ± 5.5	18.8 ± 10.4
NPA	0.40 ± 0.14	0.90 ± 0.14	30.0 ± 14.1	43.3 ± 5.8	36.7 ± 5.8	36.7 ± 5.8	6.0 ± 2.8	19 ± 16	10.0 ± 2.8	26.7 ± 5.8
CA	0.65 ± 0.21	6.0 ± 0.0	35.0 ± 7.1	70.0 ± 42.4	55.0 ± 7.1	55.0 ± 7.1	8.0 ± 2.8	20 ± 0.0	17.5 ± 10.6	37.5 ± 3.5
LOA	0.08 ± 0.0	0.2 ± 0.0	1.7 ± 0.7	6.5 ± 0.7	3.4 ± 0.9	3.4 ± 0.9	0.80 ± 0.28	3.3 ± 1.1	0.75 ± 0.35	1.3 ± 0.35
Cyanovirin 2G12MAb	0.019 ± 0.012				0.056 ± 0.034	0.056 ± 0.034				0.12 ± 0.06
DS-5000	1.6 ± 0.57				>50	>50				>50
T-20	0.60 ± 0.20	0.90 ± 0.85	1.1 ± 0.45	0.90 ± 0.42	0.95 ± 0.10	0.95 ± 0.10	0.70 ± 0.42	0.55 ± 0.35	0.45 ± 0.07	0.85 ± 0.51
AMD3100	0.087 ± 0.060	0.035 ± 0.021	0.12 ± 0.10	0.038 ± 0.004	0.13 ± 0.11	0.13 ± 0.11	0.06 ± 0.01	0.55 ± 0.35	0.075 ± 0.035	0.029 ± 0.019
UC-781	0.050 ± 0.014	0.075 ± 0.035	0.49 ± 0.57	0.20 ± 0.07	0.15 ± 0.11	0.15 ± 0.11	0.13 ± 0.10	0.05 ± 0.0	0.11 ± 0.07	0.12 ± 0.07
Tenofovir	0.0016 ± 0.0005	0.0021 ± 0.0012	0.0080 ± 0.0024	0.0043 ± 0.0025	0.0030 ± 0.0	0.0030 ± 0.0	0.002 ± 0.0	0.003 ± 0.0	0.0035 ± 0.0007	0.0028 ± 0.0011
	3.3 ± 1.1	1.6 ± 0.6	7.9 ± 2.9	6.3 ± 3.5	4.7 ± 1.2	4.7 ± 1.2	2.3 ± 0.8	4.9 ± 1.2	2.3 ± 0.0	4.0 ± 3.2

^a EC₅₀ compound concentration required to inhibit virus-induced syncytium formation in CEM cell cultures by 50%. With the exception of HIV-1(III_B), HIV-1 strains were isolated in the presence of different concentrations of GNA and HHA. The code numbers of the various virus isolates correspond to the gp120 sequencing data shown in Table 1 (first column).

TABLE 3. Inhibitory effect of plant lectins against syncytium formation between persistently (wild-type or mutant) HIV-1-infected cells and uninfected Sup-T1 cells

Test compound	EC ₅₀ (µg/ml) for cell culture ^a :			
	HUT-78/HIV-1	HUT-78/GNA-2.1	CEM/HIV-1	CEM/GNA-2.1
GNA	5.6 ± 2.9	29.0 ± 8.9	1.0 ± 0.20	25.4 ± 12.5
HHA	4.1 ± 1.9	15.0 ± 6.0	0.85 ± 0.10	19.0 ± 12.4
NPA	8.0 ± 2.0	34.0 ± 19.7	1.2 ± 0.25	26.7 ± 5.8
CA	27.5 ± 3.5	70.0 ± 42.4	2.1 ± 0.51	30.0 ± 0.0
LOA	0.8 ± 0.0	3.3 ± 1.1	0.63 ± 0.75	2.0 ± 0.71
Cyanovirin	0.2 ± 0.0	0.29 ± 0.18	0.12 ± 0.06	0.2 ± 0.0
DS-5000	2.5 ± 0.0	2.2 ± 0.47	1.5 ± 0.0	0.75 ± 0.29
T-20	1.7 ± 1.5	0.67 ± 0.63	0.35 ± 0.56	0.27 ± 0.36
AMD3100	6.3 ± 5.5	3.3 ± 1.8	1.2 ± 0.87	2.7 ± 2.3
UC-781	>20	>20	>20	>20
Tenofovir	>25	≥25	>25	≥25

^a EC₅₀, compound concentration required to prevent syncytium formation between persistently HIV-1- or HIV-1 GNA-2.1-infected HUT-78 or CEM cells and uninfected Sup-T1 cells by 50%.

pected, the RT inhibitors were completely inactive in these assays (Table 3).

Coreceptor use of mutant HIV-1/GNA in comparison with wild-type HIV-1(III_B). The coreceptor phenotype of the wild-type and GNA-resistant HIV-1/GNA-2.1 strains was examined by the use of U87.CD4 cells transfected with either of the HIV coreceptors CXCR4 and CCR5. At day 7 after infection of the U87.CD4.CXCR4 cells, strong cytopathicity was observed with both the mutant and the wild-type virus strain, and both viruses yielded comparably high p24 Ag concentrations in the cell culture supernatants (i.e., 151,788 and 159,520 pg/ml for the wild-type and GNA-resistant viruses, respectively). In contrast, like wild-type HIV-1(III_B), the GNA-resistant mutant virus completely failed to infect U87.CD4.CCR5 cells; no p24 Ag could be detected in the cell culture supernatants of these cells at day 7 after inoculation with either of the viruses. As a control, we included the dual-tropic R5/X4 HIV-1 strain HE, which readily infected both cell lines with p24 Ag productions of 155,952 and 81,610 pg/ml in U87.CD4.CXCR4 and U87.CD4.CCR5 cells, respectively. Thus, the accumulation of gp120 mutations associated with the GNA-resistant phenotype apparently did not change its coreceptor use.

Inhibitory potential of plant lectins against a mutant HIV-1 clone. An HIV-1 clone has been constructed in which three mutations at the glycosylation sites N200Q, N204Q, and N211Q of the LA loop of the gp120 have been introduced. We have chosen these amino acid mutations since the amino acid sites at the N200 and N204 positions were mutated in many of our HIV-1 strains that were selected for plant lectin resistance (Table 1). Abolishment of the oligosaccharides in the LA loop of gp120 resulted in pronounced HHA and GNA resistance (≥20- to 25-fold) but not LOA resistance. The entry inhibitors AMD3100 and DS-5000 showed only four- to fivefold resistance as also observed for cyanovirin (Table 4).

DISCUSSION

The mannose-specific plant lectins GNA and HHA from the monocot *Amaryllidaceae* family target virus strains that show different levels of resistance to GNA and HHA. The mutant virus strains are cross resistant to other mannose-specific lectins from other monocot families (e.g., CA and LOA

TABLE 4. Antiviral activity of test compounds against the wild type and an HIV-1 clone mutated in the LA loop of gp120 at several N glycosylation sites

Test compound	EC ₅₀ (μg/ml) for ^a :	
	Wild-type HIV-1 ^b	Mutant HIV-1 ^c (N200Q, N204Q, N211Q)
HHA	0.23 ± 0.10	≥4
GNA	0.15 ± 0.07	≥4
LOA	0.28 ± 0.32	0.35 ± 0.07
Cyanovirin	0.013 ± 0.007	0.070 ± 0.014
AMD3100	0.011 ± 0.006	0.045 ± 0.35
DS-5000	0.45 ± 0.07	1.75 ± 0.35
UC781	0.002 ± 0.001	0.003 ± 0.0
Tenofovir	0.49 ± 0.20	2.0 ± 1.22

^a EC₅₀, compound concentration required to inhibit HIV-1-induced cytopathicity in CEM cell cultures by 50%. Data are the means of two to three independent experiments (± standard deviations). The wild-type and mutant (N200Q, N204Q, and N211Q) HIV-1 stocks contained 1,899 and 1,847 of p24/ml, respectively. The wild-type HIV-1 stocks contained 5,000 CCID₅₀, whereas the mutant (N200Q, N204Q, and N211Q) stocks contained 350 CCID₅₀. Therefore, the mutant HIV-1 clone has a ~14-fold decreased infectivity compared to wild-type HIV-1 in CEM cell cultures.

^b Wild-type HIV-1(BRU) clone.

^c Mutant HIV-1(BRU) clone containing the N200Q, N204Q, and N211Q mutations in the hypervariable LA loop of gp120.

from the *Orchidaceae* family and NPA from the *Amaryllidaceae* family). The levels of decreased activity against the mutant virus isolates are similar for the different lectins included in the study and depend on the number and nature of the mutations present in the HIV gp120. As a rule, the higher the number of amino acid mutations present in the HIV envelope glycoprotein, the higher the resistance level of the virus isolates to the mannose-specific plant lectins. Low-level resistance was afforded by the presence of either N204K (present in HIV-1/GNA-1.0) or T206A and/or T311I (present in HIV-1/HHA-1.1). Several other amino acid mutations were eventually added to this genetic background

during the drug-escalating selection process, resulting in high-level (>100-fold) resistance to the plant lectins (e.g., N271Y, T162M, N430D, and S261F/P). The vast majority of amino acid changes found in gp120 are related to specific N glycosylation sites. Either the glycosylated N was mutated or the T or S from the N glycosylation N-X-S/T motif was mutated so as to incapacitate the N glycosylation site (Table 1 and Fig. 2). In four cases, the affected glycosylation site carries a high-mannose-type carbohydrate moiety (Fig. 2, triangles), whereas in four other cases, the mutations affect glycosylation sites bearing complex types of sugars (Fig. 2, squares). A ninth amino acid change (T162M mutation) lies in the middle of a T- and S-rich amino acid region in the absence of surrounding lipophilic amino acids, which is indicative of a potential O glycosylation site that can also be of the high-mannose type (44). However, it is not presently known whether this potential gp120 O glycosylation site is indeed glycosylated in the virus strains used in our studies. In fact, Leonard et al. (22) and Geyer et al. (17) did not observe O-glycosylated amino acids in gp120 of HIV-1/CL44 and HIV-1(III_B), respectively. Of note, four mutations at amino acid positions 200, 202, 204, and 206 appeared to be clustered in the LA region of gp120, a highly conserved loop located between the variable V2 and V3 regions, whereas four other amino acid mutations were located in other highly conserved regions (Fig. 2 and 3). Only one single glycosylation site in V2 (T162), V3 (N271), and V4 (T269 in the N-X-T motif) was also affected. It should be mentioned that for all the virus strains that were made to be resistant to the plant lectins, the HIV-1 transmembrane fusion glycoprotein gp41 was also examined for amino acid mutations, but in no case was an N glycosylation site or a T or S site that is part of the N-X-S/T glycosylation motif found to be mutated in gp41 of the plant lectin-resistant strains.

Thus, the virus strains selected for resistance to the man-

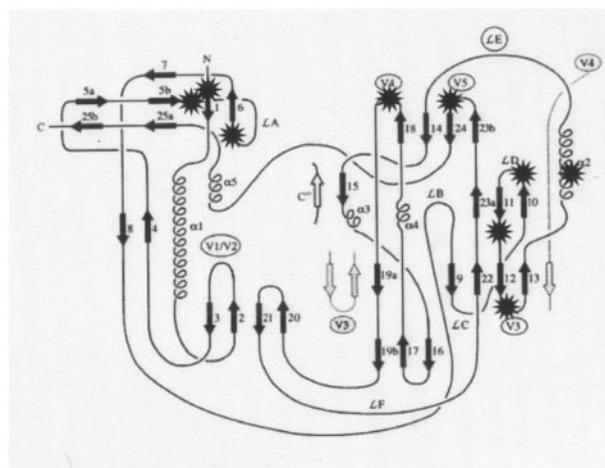
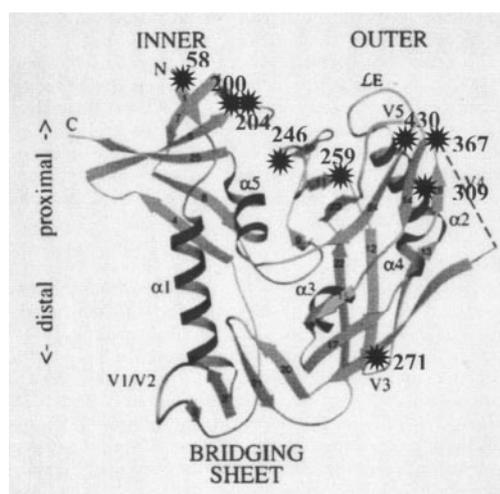


FIG. 3. Core structure of gp120 according to the data of Kwong et al. (21). A ribbon diagram (left) and a topology diagram (right) are shown. The viral membranes are oriented above the core structure, and the target cell membrane is oriented below the core structure. The left portion of the core gp120 is indicated as the inner domain, the right portion is indicated as the outer domain, and the four-stranded sheet at the bottom left of gp120 is indicated as the bridging sheet. Loops are labeled LA to LF and V1 to V5. The five helices are labeled $\alpha 1$ to $\alpha 5$. The 25 β -strands are shown as arrows. The approximate locations of the nine glycosylation sites that were mutated in the mutant virus strains are shown with asterisks.

nose-specific plant lectins predominantly contain specific mutations at the potential glycosylation sites of gp120. In fact, there are a total of 24 potential N glycosylation motifs (N-X-S/T) in gp120 bearing 11 high-mannose-type and 13 complex-type sugar moieties on the asparagines (16, 22). About half of these asparagines are highly conserved among all clades of HIV-1. The other asparagines show conservation within several but not all HIV clades. However, all 24 potential glycosylation sites reported by Leonard et al. (22) were invariably also found in the HIV-1(III_B) strain used in our studies. It should be noticed that the HIV-1/GNA-2.1 and HIV-1/HHA-2.2 strains essentially keep the mutations in the gp120 envelope molecule after 10 additional subcultivations in the absence of the plant lectins, pointing to a considerable amount of stability of these amino acid mutations in gp120.

The mutational specificity observed for the mannose-specific plant lectins is different from the resistance mutations described and characterized for all other entry inhibitors. Indeed, dextran sulfate as well as chicoric acid and the bicyclam AMD3100 have been shown to target mutations in gp120 that are different from the mannosylated asparagines or the potential O glycosylation site found in the plant lectin-resistant virus strains (13, 15, 30). These findings explain why mannose-specific plant lectins belonging to five different plant species and two different plant families show a highly comparable cross-resistance spectrum among each other, whereas there is a virtual lack of cross-resistance for these mutant virus strains with any other entry inhibitor tested, including the *N*-acetylglucosamine-specific lectin of *Urtica dioica* (data not shown). However, given the fact that cyanovirin, another selective entry inhibitor of HIV that was recently shown to prevent rectal transmission of simian-human immunodeficiency virus in macaques as a topical microbicide (40), has been characterized as a mannose-specific lectin derived from a primitive blue-green algae (10, 11, 28), its activity profile against several plant lectin-resistant HIV-1 strains derived in our study was also determined. No cross-resistance was observed for those mutant HIV-1 strains that showed the highest degree of resistance (i.e., ≥ 100 -fold) against HHA and GNA. Likewise, it would be of particular interest to uncover the nature of the amino acid mutations emerging during selection of cyanovirin-resistant viruses and to reveal the binding site of cyanovirin to gp120. Moreover, whereas cyanovirin has also been reported to be active against other lentiviruses (simian and feline immunodeficiency viruses), as is also the case for GNA and HHA (Balzarini et al., unpublished data), cyanovirin lacks inhibitory activity against cytomegalovirus (10). This finding is strikingly different from those for GNA and HHA, which were shown to be potent inhibitors of cytomegalovirus (4, 5). Thus, although cyanovirin and the plant lectins described in this study both seem to be endowed with a (mannose) sugar specificity, they must still be different in some aspects of their sugar conformation preference to explain the differences in virus selectivity and resistance-sensitivity profiles against the plant lectin-resistant virus strains. One contributing factor to explain the lack of cross-resistance of cyanovirin to the plant lectin-resistant virus strains may be the different sugar specificity of cyanovirin (specificity for α -1,2-mannose glycans) and the plant lectins (specificity for α -1,3- and/or α -1,6-mannose oligomers). In this respect, it will also be of interest to investigate the activity of

the C-type MBL of the innate immune system against the plant lectin-resistant viruses (19). MBL is a serum protein of hepatic origin that binds to carbohydrates of microorganisms and has been shown to bind to gp120 and to recognize high-mannose complexes and/or hybrid N-linked glycans for binding to its target glycoproteins (19).

The gp120 molecule contains five relatively conserved domains interspersed with five hypervariable disulfide-bonded loop structures (22). There are also 24 potential sites for N glycosylation that are all utilized. For the HIV-1/CL44 gp120, 13 sites that contain complex-type oligosaccharides as the predominant structure and 11 sites that contain primarily high-mannose-type and/or hybrid-type oligosaccharide structures were found (25, 26). Of the complex oligosaccharides, 90% were found to be fucosylated and 94% were sialylated. No O-linked oligosaccharides were found. Gp120 of HIV-1(III_B) even contains approximately 50% of the high-mannose-type oligosaccharides (17). One of the functions of these oligosaccharides is to fend off potential (conserved) epitopes on HIV gp120 for NAbs (1, 29, 31, 45). Interestingly, the binding site of gp120 with the cellular CD4 receptor is devoid of carbohydrates (46). Indeed, the gp120 area of interaction with CD4 consists of one amino acid residue in the V1/V2 stem, the loop LD (positions 249 to 252), the β 15- α 3 excursion (positions 335 to 338 and 339 to 442), the β 20- β 21 hairpin (positions 392 to 397 and 400 to 405), the β 23 strand (positions 421 to 427), and the β 24- α 5 connection (positions 434 to 440 and 445 to 454) (21, 45) (Fig. 3). None of these residues were mutated in any of the plant lectin-resistant mutant virus strains. Also, the interaction domain(s) of gp120 with the coreceptors CCR5 and CXCR4 have been determined and were found to be predominantly located in the positively charged V3 loop (positions 266 to 300), the basic surface of the bridging sheet containing the β 2 and β 3 and the β 20 and β 21 strands, and the amino acids R389, K391, and Q392 at the end of the β 19 strand (21, 45) (Fig. 3). Again, none of the positively charged amino acids of the V3 loop nor any of the other amino acids of gp120 interacting with the coreceptor were altered in the mutant HIV-1 strains that emerged in the presence of escalating plant lectin concentrations. The N glycosylation site 271 in the V3 loop was abolished only in the higher plant lectin-resistant HIV-1 strains, allowing a more pronounced exposure of the positively charged amino acids of the gp120 V3 loop to the negatively charged coreceptor. Thus, the plant lectins clearly seem not to interfere with coreceptor binding areas on gp120. Indeed, when the N glycosylation sites that were mutated in the gp120 of the plant lectin-resistant mutant virus were located on the ribbon and topology diagrams in Fig. 3, they clearly clustered (except for the V3 mutation) at the top of the inner domain (near the gp41 binding site) and in the upper half of the outer domain but not at the coreceptor binding domains.

Interestingly, Scanlan et al. (33) and Trkola et al. (39) revealed that the broadly neutralizing anti-HIV-1 antibody 2G12 recognizes a cluster of α 1 \rightarrow 2 mannose residues of oligomannose glycans on the outer face of gp120. Mutations at N265, N302, N309, N356, and N362 resulted in a significant decrease in 2G12 binding affinity to gp120. However, further studies showed that the glycans at N309 and N356 were shown not to be critical for 2G12 binding to gp120. Thus, the most likely epitope for 2G12 is formed from mannose residues contrib-

uted by the glycans that are attached to N265, N302, and N362, with the other glycans (N309 and N356) play an indirect role in maintaining the epitope conformation (33). In a number of our plant lectin-resistant HIV-1 strains, the N309 glycosylation site was abolished. Interestingly, the 2G12 antibody fully lost its inhibitory potential to these virus strains as well (Table 2). These findings are in agreement with the indirect role of N309 in maintaining the gp120 2G12 epitope conformation, although it cannot be excluded that any of the other mutated amino acids in these 2G12-resistant mutant virus strains may play a similar role. It has been shown recently (37) that the MBL of the innate immune system can also bind gp120 of HIV-1, thereby preventing DC-SIGN to bind to HIV and thus exerting an antiviral effect. Further studies should reveal whether plant lectin binding to gp120 can also prevent HIV binding to DC-SIGN and, more importantly, whether the plant lectin-resistant virus strains show a lower efficiency of binding to DC-SIGN.

Given the fact that the location of the plant lectin-induced amino acid mutations in gp120 is not involved in the very first steps of virus adsorption and coreceptor binding, it is unclear what the role of the plant lectins is in the inhibition of the HIV entry-fusion process. It is rather unlikely that the oligosaccharides that are abolished in the mutant virus strains play a significant role in the fusion process, since the mutated viruses do not seem to have a compromised efficiency of infectivity in CEM cell cultures. Therefore, it may be more likely that attachment of the plant lectins to these parts of the gp120 molecule may seriously compromise the fusion process after CD4 and/or CXCR4/CCR5 interaction by steric hindrance or conformational restriction. The more glycosylation sites that are abolished, the less the lectins are bound to gp120; thus, it is easier for the fusion process to occur. Since the gp41 attachment to gp120 occurs at the C- and N-terminal amino acid domain of gp120 (Fig. 3) (8), which is rather close to some of the mutations found in the plant lectin-resistant viruses, the presence of the lectins that are tightly bound to gp120 may indeed interfere with the further conformational changes and interactions with gp120 where gp41 is involved. It will be interesting to reveal the localization and the number of the binding sites of gp120 for the mannose-specific plant lectins and to determine their affinity constants for gp120 in comparison with those for the coreceptors to better understand the mechanism of antiviral action of the plant lectins at the molecular level. In this respect, construction of a variety of mutant virus strains that contain one or several of the observed mutations in gp120 may already provide useful information. Therefore, we have deleted the glycosylated amino acid sites in the LA loop of gp120 to reveal the role of these amino acids in the susceptibility of the lectins to such mutant virus. The deglycosylated LA loop resulted in pronounced resistance to GNA and HHA and partial resistance to cyanovirin but no resistance to LOA (Table 4). These observations clearly point to subtle differences in the individual lectin interaction with the sugar part of gp120 and are in agreement with our findings that LOA shows a lesser degree of resistance than the other plant lectins (GNA, HHA, and CA) in several mutant virus strains. These findings also justify a profound exploration of the activity of a wide variety of mannose-specific plant lectins against HIV.

It has been reported previously that glycans shield antibody

epitopes (29, 32, 36, 45). Very recently, Wei et al. (45) showed that NABs directed towards HIV gp160 exert sufficient viral inhibitory activity to force the virus to replace the neutralization-sensitive virus by successive populations of resistant virus. Interestingly, during the progress of virus infection, the envelope gene of HIV-1 mutates rapidly, primarily at glycosylation sites, resulting in a change of the glycan shield on the surface of the virus, thereby creating a physical block to NAb binding. This mechanism of immunological escape contributes to HIV-1 persistence in the face of an evolving antibody repertoire. It will therefore be of particular interest to combine plant lectins with neutralizing antibodies of HIV to investigate whether lectin drug pressure in the presence of NAB compromises the escape of HIV-1 to NABs and thus keeps NABs more effective during a longer time period of drug or NAB exposure.

Given that the concomitant presence of multiple amino acid mutations is required to afford a high level of resistance of virus strains to the plant lectins, it is not surprising that significant drug resistance development against plant lectins is a long-term event. Our observations that fixed plant lectin concentrations that are only 5- to 10-fold higher than their EC_{50} values are able to completely knock out the virus in long-term HIV-1-infected cell cultures (Balzarini et al., unpublished) are in agreement with the mutational findings (the need for multiple mutations in gp120 to afford significant levels of drug resistance) and may represent an additional beneficial property for a drug to qualify as an efficient microbicide. A drug concentration ratio of 5 to 10 is markedly lower than the ratio of the knockout concentration to the EC_{50} (≥ 100) of several nonnucleoside reverse transcriptase inhibitors and some nucleoside reverse transcriptase inhibitors such as lamivudine (6, 7).

In conclusion, we have observed specific plant lectin-related HIV resistance mutations that are different from the currently approved and investigational anti-HIV drugs and result in a lack of cross-resistance to any other reported viral entry inhibitor that selects for mutations in the same target, viz., the viral envelope glycoprotein gp120.

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ERRATUM

Profile of Resistance of Human Immunodeficiency Virus to Mannose-Specific Plant Lectins

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