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Alteration of V3 Loop Context within the Envelope of Human Immunodeficiency Virus Type 1 Enhances Neutralization

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Neutralization of a chimeric human immunodeficiency virus (HIV) type 1, containing the V3 loop of the MN isolate substituted within the HXB2 envelope, was enhanced up to 20-fold compared with the HXB2 or MN parental isolates by human HIV-positive sera. MN V3 loop-specific monoclonal antibodies were better able to recognize the chimeric virus compared with MN, staining a greater percentage of infected cells and exhibiting slight increases in relative affinity with a concomitant increase in neutralization titer. Competition analysis revealed that enhanced neutralization by human HIV-positive sera of the chimera was attributable in some cases to better reactivity with the linear V3 loop epitope but in others to conformational loop epitopes or previously cryptic or poorly recognized epitopes outside the loop region. Mice primed with a vaccinia virus-chimeric envelope recombinant and boosted with gp160 developed a spectrum of antibodies different from that of mice similarly immunized with HXB2 or MN recombinants or that of naturally infected humans. The chimeric envelope elicited antibodies with enhanced binding to the native MN V3 loop; however, the sites seen by the BALB/c mice were not neutralizing epitopes. Nevertheless, similar to the observations made with use of human sera, the chimeric virus was more readily neutralized by all of the immune mouse sera, an effect apparently mediated by non-V3 loop epitopes. These studies illustrate that not only the V3 loop sequence and conformation but also its context within the viral envelope influence neutralization.

The V3 loop of human immunodeficiency virus type 1 (HIV-1) consists of approximately 33 amino acids residing between two cysteine residues in the third variable domain of the viral envelope protein. It is a principal type-specific neutralizing determinant of the virus (15, 25, 30, 37) and also elicits virus-specific cytotoxic T lymphocytes and T-helper cell immune responses (2). The loop influences viral tropism (19, 39, 45), perhaps in part by providing a target for digestion by cellular proteases as part of the infection process (16, 24, 42). An attractive hypothesis suggests that cleavage of the V3 loop mediates fusion of virus with target cells following CD4 binding (5). For these reasons, it was of interest to be able to study V3 loops with divergent sequences within a known, well-characterized envelope background. A V3 loop cassette was constructed by using the infectious molecular clone HXB2 (11) as a framework into which a V3 loop corresponding to the sequence of any isolate could be substituted (7). We have used one such chimeric virus, containing the V3 loop of HIV-1_{MN}, to study the effect of the V3 loop on neutralization by sera of naturally infected HIV-seropositive humans. Our approach was to compare the ability of these sera to neutralize the chimera with their ability to neutralize the two parental viruses. The contribution of the linear V3 loop itself compared with regions outside the loop or conformationally dependent V3 loop epitopes was evaluated by competition experiments using V3 loop peptides. Our results show that the exchange of one V3 loop for another has profound effects on the overall conformation of the gp160 framework, modulating V3 loop epitopes and perhaps uncovering cryptic sites outside the V3 loop or altering poorly recognized ones. As a result, the resultant molecule exhibits greater immunologic reactivity with immune sera than does either unmodified parental envelope. Such chimeric immunogens may find applicability in HIV vaccine design.

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MATERIALS AND METHODS

Viruses. HIV-1 isolates used in neutralization assays included the infectious molecular clone of HIV-1_{IIIB} designated HXB2 (11), HIV-1_{MN} (14, 40), and the HXB2/MNLoop construct composed of HXB2 with a chimeric envelope containing the V3 loop of MN substituted for the V3 loop of HXB2. This chimeric virus was constructed by using a V3 loop cassette previously described (7). The cassette, containing the 2.7-kbp *Sal*I-*Bam*HI *env* fragment of HXB2, contains *Mlu*I and *Hpa*I restriction sites at the borders of the V3 loop, which are separated by a short stretch of DNA containing a *Pst*I site (Fig. 1a). The V3 loop of MN was inserted by annealing the four primers illustrated in Fig. 1b and ligating the product into the *Mlu*I and *Hpa*I sites of the cassette. The resultant *Sal*I-*Bam*HI fragment was substituted back into the parental pHXB2 plasmid. Upon transfection into COS-1 cells, this construct yielded chimeric virus which was subsequently transmitted by coculture to H9 cells. HXB2 and MN parental viruses were also propagated on H9 cells.

Three vaccinia virus constructs were used for immunizing mice. vPE16 contained the entire envelope of HIV-1_{BH10} (9, 10). VVTG 6193, called MN/Lai, contained a hybrid envelope consisting of gp120 from HIV-$1_{MN\ ST1}$ and gp41 from HIV-1_{Lai}. In this construct, the cleavage site between gp120 and

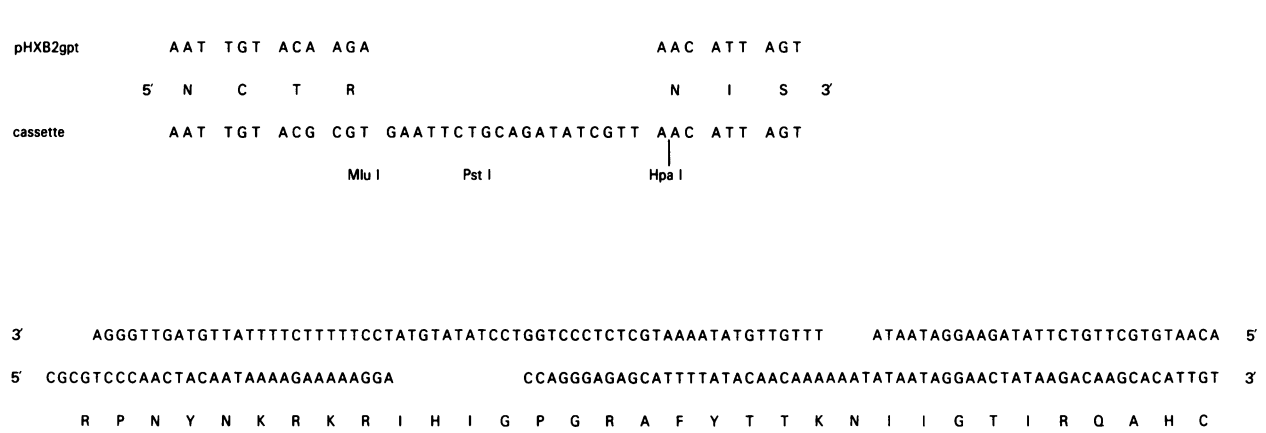

FIG. 1. Insertion of the MN V3 loop into HXB2. (a) The unique *Mlu*I and *Hpa*I sites at the borders of the V3 loop region in the pHXB2 loop cassette; (b) primers used to construct HXB2 with the V3 loop of MN substituted in the envelope. The amino acid sequence of the MN V3 loop is shown at the bottom.

gp41 was deleted, as was the transmembrane region, and the cytoplasmic tail was truncated with only 29 amino acids remaining (22). VVTG 6192, called HXB2/MNLoop/Lai, contained the V3 loop of MN within the gp120 of HXB2 and the gp41 of Lai, engineered similarly to VVTG 6193.

Sera. Sera were obtained from healthy HIV-seropositive donors from the United States and Europe, from AIDS patients from Kinshasa, Zaire, participating in an immune therapy trial, and from healthy adult seropositive volunteers from a brigade of paratroopers based in Ndjili, Zaire. (The blood samples used in this work were obtained according to French and Zairian medical codes and ethics as documented by an Attestation of the French Embassy in Zaire. However, they were not obtained in accordance with Department of Health and Human Services human subject regulations, as the study did not receive institutional review board review prior to its initiation. The research reported involved minimal risk to the volunteer blood donors. The original codes of the samples have been changed in this publication to ensure confidentiality, so that the publication adds no additional risk for subjects.) Mouse monoclonal antibodies (MAbs) 50.1 and 83.1, specific for the MN V3 loop (46), were used as purified immunoglobulin G (IgG) preparations. A pool of human sera was composed of three sera from U.S. donors with good envelope reactivity for both the IIIB and MN isolates.

Synthetic peptides and proteins. Synthetic peptides were obtained from Multiple Peptide Systems, Inc, San Diego, Calif. The sequences of the peptides used in competition studies were YNKRKRIHIGPGRAFYTTKNIIGC (MN), NNTRKS IRIQRGPGRAFVTIGKIGC (BH10), and NNTRKRIRIQR GPGRAFVTIGKIGC (HXB2). The carboxy-terminal cysteines were added to facilitate conjugation to protein carriers. Envelope proteins used for immunizations and binding studies included gp160 and gp120 of the IIIB strain produced in a baculovirus system (38), native gp160 (IIIB) purified from tissue culture fluid of infected cells (20), and truncated gp160 of the MN/Lai hybrid envelope purified from tissue culture fluid of chicken embryo cells infected with the MN/Lai vaccinia virus construct.

Animal experiments. BALB/c mice were divided into three groups of five mice each. Group I was immunized with VVTG 6192 (HXB2/MNLoop/Lai insert), group II was immunized with VVTG 6193 (MN/Lai insert), and group III was immunized with vPE16 (BH10 insert). Primary immunizations at day 0 consisted of 10^6 PFU given intravenously. A secondary immunization of 10^7 PFU was given intraperitoneally at day 30. At day 60, each mouse received the same tertiary immunization, consisting of 20 μg of a mixture of equal amounts of native gp160 from the IIIB strain and truncated gp160 of MN/Lai emulsified in incomplete Freund's adjuvant. Mice were bled on day 74, and sera were assessed for neutralizing and binding antibodies.

Immunologic assays. Neutralizing antibody assays were carried out as previously described on H9 target cells (35), using fresh tissue culture supernatant as a source of virus. Neutralization titers are defined as the reciprocal of the serum dilution at which virus expression is 60% of control values following normalization of infectivity levels to that obtained in the presence of normal human serum. The ability of peptides to inhibit neutralizing antibody activity was determined by first incubating peptide (20 μl of 12.5 μg/ml) with an equal volume of test serum for 30 min at room temperature. Virus was added, and the neutralization assay was carried out as described previously (35) except that 20 μl rather than 15 μl was plated into duplicate microtiter wells.

Peptide binding assays were carried out as previously described (36). Binding to envelope proteins was similarly assessed by enzyme-linked immunosorbent assay; however, the proteins were directly bound to the microtiter plate (0.2 μg per well) without polylysine or glutaraldehyde treatment. Binding titers were defined as the reciprocal of the serum dilution at which absorbance was twice that of preimmune or control serum diluted 1:100.

The ability of a pooled human serum (PHS; see above) or MN V3 loop-specific MAb to recognize H9 cells producing MN or the HXB2/MNLoop chimera was assessed by FACScan analysis. One million cells were washed in phosphate-buffered saline (PBS) containing 0.1% sodium azide (PBS-azide). The PHS (1:10 dilution) or MAb 50.1 or 83.1 (10 μg/ml) was added and incubated for 45 min at 4°C. Normal human serum (1:10 dilution) or ascites fluid (1:200 dilution) from P3 × 63 cells served as control. The cells were washed with PBS-azide and stained with fluorescein isothiocyanate-conjugated goat anti-human or goat anti-mouse IgG for 45 min at 4°C. Following washing, the cells were suspended in PBS-azide and examined with a Becton Dickinson FACScan. Analysis was performed with the Lysis II program.

Virologic assays. Reverse transcriptase activity of the paren-

TABLE 1. Neutralization of standard and chimeric HIV-1 isolates by U.S./European and Zairian human sera

Serum	Neutralization of:					
	HXB2		MN		HXB2/MNLoop	
	Range of titer	Geometric mean titer	Range of titer	Geometric mean titer	Range of titer	Geometric mean titer
U.S./European	0–590	70	25–1,750	250	150–7,425	835
Zairian	25–340	95	0–290	25	140–>2,025	490

tal and chimeric viruses was determined on clarified culture supernatants precipitated with polyethylene glycol (32), using poly(rA)$_n$-oligo(dT)$_{12-18}$ as the template-primer with Mg^{2+} as the cation (31). The expression of p24 and gp120 was similarly determined on clarified culture supernatants, using the Coulter p24 antigen capture kit and the ABT gp120 antigen capture kit, respectively. The latter was modified by using as the detecting antibody a mixture of mouse MAb specific for the V3 loop of the IIIB (M77 [7]) or MN (83.1) isolates. Subsequently, incubation with horseradish peroxidase-conjugated goat anti-mouse IgG followed by a substrate solution containing *o*-phenylenediamine was carried out as previously described (34). Envelope protein associated with intact virions was quantified on viral pellets following centrifugation at 30,000 rpm for 1 h in a Beckman 70 Ti rotor. Infectious titers on H9 cells were determined on serial dilutions of viral supernatants. Assays were carried out in triplicate wells of 96-well microtiter plates, and viral expression was monitored at 2 weeks by the Coulter p24 antigen capture assay.

RESULTS

Enhanced neutralization of chimeric virus by human sera. The ability of 11 HIV antibody-positive sera from the United States and Europe and 10 from Zaire to inhibit the infectivity of standard HXB2 and MN isolates and the HXB2/MNLoop chimera was assessed in a cell-free neutralization assay. As shown in Table 1, the sera were in general able to neutralize all three viruses, although the U.S./European sera tended to neutralize MN better than HXB2, while the opposite was true for the Zairian sera. In both cases, enhanced neutralization of the HXB2/MNLoop chimera was observed. Mean neutralizing titers of U.S./European sera were 12-fold greater against the chimera compared with HXB2 and 3-fold greater compared with MN. The enhanced neutralization of the chimeric virus compared with MN by Zairian sera was even more pronounced, with a 20-fold difference observed. These Zairian sera neutralized the chimera with fivefold-higher titers compared with HXB2.

Characterization of parental and chimeric viruses. To determine if the enhanced neutralization of the chimeric virus was due to altered growth characteristics or viral properties leading to greater ease of neutralization, several virological characteristics of the parental and chimeric viruses were compared and are summarized in Table 2. Viral protein expression, including reverse transcriptase activity and p24 and gp120 levels, in supernatant media from infected H9 cultures was comparable for the three viruses. Furthermore, the percentages of gp120 in the viral pellet fraction compared with that found free in the culture supernatant were similar for the parental and chimeric viruses, indicating that one virus did not shed its envelope more rapidly than another. With regard to infectivity, the infectious titers of the viruses were all in the same range. In addition, the kinetics of infection of the three viruses were similar when cell-free supernatant medium was used to infect H9 cells (not shown). Thus, the enhanced neutralization of the chimeric virus appeared to be based on immunologic recognition rather than altered replicability or infectivity.

Recognition of HXB2/MNLoop chimera and the MN isolate by MAbs. H9 cells productively infected with either the MN or HXB2/MNLoop virus were stained in an indirect immunofluorescence assay using either the PHS or two MAbs specific for an overlapping region of the MN V3 loop and analyzed by FACScan. The epitope of MAb 50.1 is RIHIG, and that of MAb 83.1 is IHIGPGR. As shown in Table 3, MN- and HXB2/MNLoop-infected cells had similar amounts of envelope protein on their surfaces, as staining of both with the PHS was equivalent. However, the H9 cells expressing the chimeric virus exhibited a slightly higher percentage of cells stained by the MN V3 loop-specific MAb. When normalized to the percentage of cells expressing HIV envelope protein, represented by the cells stained with the PHS, approximately one-third of the MN-producing cells were stained by MAb 50.1, compared with more than half of the cells producing the chimeric virus (Table 3). Similarly, half of the MN-producing cells were stained by MAb 83.1, compared with two-thirds of the HXB2/MNLoop-producing cells. Moreover, the relative affinity of the MAb was slightly greater for cells expressing the HXB2/MNLoop chimera compared with the parental MN (Table 3). Taken together, these results suggest that the MN V3 loop within the chimeric virus is presented with a slightly altered conformation and/or has become more accessible to neutralizing antibody compared with the V3 loop within the parental MN isolate. This supposition was substantiated by a functional assay. MAb 50.1 neutralized the HXB2/MNLoop chimera with sixfold less IgG than necessary to neutralize the

TABLE 2. Characteristics of the parental HXB2 and MN isolates and the HXB2/MNLoop chimeric virus[a]

Virus	Reverse transcriptase activity (cpm/ml)	p24 expression (ng/ml)	Total gp120 expression (ng/ml)	gp120 in viral pellet (ng/ml)	Pellet gp120/ total gp120	Infectious titer/ml
H9/MN	8.5×10^5	850	93	5.0	5.4	1.6×10^4
H9/HXB2/MNLoop	1.1×10^6	720	140	9.5	6.8	1.6×10^4
H9/HXB2	5.7×10^5	410	50	4.5	9.0	6.8×10^3

[a] Results reflect the mean of two to three experiments for each parameter measured.

TABLE 3. Expression of V3 loop epitopes by H9 cells producing the MN isolate and the HXB2/MNLoop chimera

Cells infected with:	% Fluorescent cells[a]			% MAb 50.1$^+$/ % PHS$^+$	% MAb 83.1$^+$/ % PHS$^+$	Relative affinity[b]	
	PHS	MAb 50.1	MAb 83.1			MAb 50.1	MAb 83.1
MN	74	27	38	36	51	1.7	1.8
HXB2/MNLoop	69	39	47	57	68	1.1	1.4

[a] Normalized to the percentage of cells in the culture expressing p24, determined by indirect immunofluorescence assay using a p24-specific MAb.
[b] Defined as the antibody concentration (micrograms per milliliter) at which a 50% reduction in median fluorescent intensity of cells stained by a dilution series of each MAb was observed.

MN isolate (not shown). Comparative neutralization by MAb 83.1 was not assessed.

Basis for the enhanced neutralization of the HXB2/MNLoop chimera by human sera. To determine if the enhanced neutralization of the chimeric virus by HIV-positive human sera was due to better recognition of the V3 loop, competition experiments using peptides representative of the V3 loops of MN and IIIB (both HXB2 and BH10) were carried out. The results for three different HIV-positive sera are shown in Fig. 2A, with an interpretation of the data shown schematically in Fig. 2B.

In the case of serum L, the MN V3 loop peptide is able to inhibit completely the neutralizing activity against the MN isolate, indicating that anti-MN V3 loop reactivity is the principal neutralizing component of this serum. The serum also recognizes nonlinear V3 loop epitopes or epitopes outside the loop in the HXB2 isolate, however, as neither the MN nor the IIIB V3 loop peptide is able to inhibit neutralization of HXB2. Competition of the chimeric virus, HXB2/MNLoop, with the MN V3 loop peptide abolishes the enhanced neutralization and results in a neutralizing titer similar to that obtained against HXB2 itself. Serum L, therefore, exhibits enhanced neutralization of the HXB2/MNLoop chimera by virtue of better recognition of the MN V3 loop.

Serum P represents a different situation in which recognition of linear V3 loop epitopes by neutralizing antibody is lacking. This was shown by the failure of the V3 loop peptide of either MN or IIIB to inhibit neutralization of any of the three viruses. Therefore, the enhanced neutralization of the HXB2/MNLoop chimera by serum P, five- and ninefold over that for HXB2 and MN, respectively, is likely due to better recognition of epitopes outside the V3 loop or to conformationally dependent V3 loop epitopes.

Serum C represents yet a third situation in which the V3 loop of MN, but not that of HXB2, is recognized by neutralizing antibodies, yet this does not account for the enhanced neutralization of the chimeric virus. This was shown by the incomplete inhibition of neutralization of MN by the MN V3 loop peptide and the failure of the MN V3 peptide to completely inhibit the enhanced level of neutralization of the chimeric virus. Serum C must recognize a linear V3 loop epitope which is not better recognized in the chimera. Therefore, a conformational alteration affecting a second V3 loop epitope and/or regions outside the loop appears to be respon-

Competition of Serum Neutralizing Activity by V3 Loop Peptides

A.

Competing Peptide	Serum Neutralizing Antibody Titers on Indicated Viruses								
	Serum L			Serum P			Serum C		
	HXB2	MN	HXB2/MN Loop	HXB2	MN	HXB2/MN Loop	HXB2	MN	HXB2/MN Loop
None	405	175	1350	245	125	1175	545	945	3850
MN V3	805	–	475	265	90	1350	545	315	2485
IIIB V3	370	125	980	420	90	790	1190	1155	3220

Schematic Representation of Basis for Enhanced Neutralization of Chimera

B.

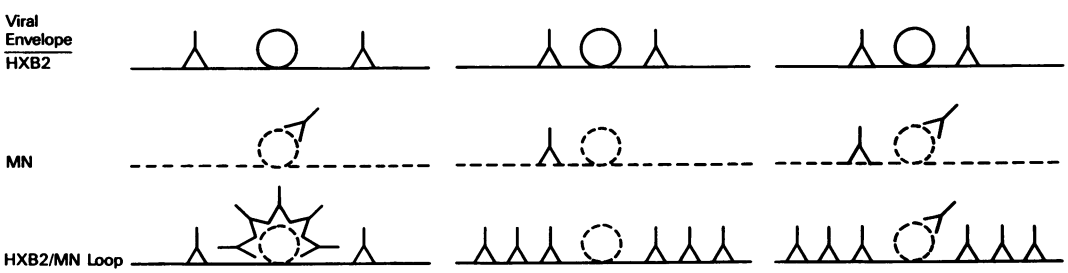

FIG. 2. Competition of serum neutralizing activity by V3 loop peptides. (A) Results of competition studies using V3 loop peptides; (B) interpretation of the results. Values represent the means of at least three or four determinations.

TABLE 4. Analysis of neutralizing antibodies of mice immunized with conventional and chimeric envelopes

Mouse group	No. positive/5 tested (mean titer)		
	HXB2/MNLoop	MN	HXB2
I (HXB2/MNLoop/Lai)	3 (50)	0	2 (40)
II (MN/Lai)	5 (100)	3 (50)	2 (75)
III (IIIB)	4 (150)	1 (75)	1 (40)

sible for the enhanced neutralization of the chimeric virus by serum C.

Immunogenicity of the chimeric envelope in vivo. The enhanced neutralization of the HXB2/MNLoop chimera by human sera seropositive for HIV-1 suggested that the envelope of this virus might provide an immunogen capable of eliciting broadly neutralizing antibodies of high titer and/or affinity. This hypothesis was tested by immunizing mice in a prime-boost protocol, chosen because purified envelope protein of the chimeric virus was not available. The mice were primed with two immunizations of a vaccinia virus recombinant containing either the HXB2, MN, or HXB2/MNLoop gp120. All constructs contained all or part of the IIIB/Lai gp41 as outlined in Materials and Methods. Our previous results have suggested that a protein subunit booster is necessary for eliciting good neutralizing antibody activity following priming with vaccinia virus–HIV-1 recombinants (1). Therefore, a mixed booster inoculation consisting of native IIIB and MN/Lai envelopes was given to all mice. The results of this experiment are shown in Tables 4 to 6.

Contrary to our expectation, immunization with chimeric envelope did not result in enhanced neutralization of any of the three viruses (Table 4). In fact, mice immunized with either the MN/Lai or IIIB construct exhibited greater ability to neutralize the three isolates than did mice immunized with the chimera, both in number of neutralizing sera per group and in overall titer. Sera of mice immunized with the HXB2/MNLoop chimera exhibited poor reactivity to the linear MN V3 loop, as shown by their failure to neutralize the MN virus and their minimal binding to the linear MN V3 loop peptide (Table 5). Thus, they were precluded from showing enhanced neutralization due to a linear MN V3 loop epitope.

Comparisons of the three mouse groups overall revealed that regardless of immunogen, the chimeric virus was more readily neutralized than either parental strain, with regard to both number of sera able to neutralize (12 of 15) and overall mean titer (100 for HXB2/MNLoop, 40 for MN, and 50 for HXB2). This ready neutralization of the chimeric virus appears to be mediated primarily by epitopes outside the V3 loop or by conformationally dependent V3 loop epitopes, a conclusion supported by results of the V3 binding studies (Table 5). The linear MN V3 loop was poorly recognized by sera of any of the mouse groups relative to the V3 loop of IIIB (either BH10 or HXB2).

TABLE 5. Analysis of V3 loop-binding antibodies of mice immunized with conventional and chimeric envelopes

Mouse group	Geometric mean titer against:		
	MN V3	BH10 V3	HXB2 V3
I (HXB2/MNLoop/Lai)	1,960	4,965	4,045
II (MN/Lai)	6,040	13,390	13,140
III (IIIB)	3,465	11,035	7,975

TABLE 6. Analysis of envelope-binding antibodies of mice immunized with conventional and chimeric envelopes[a]

Mouse group	Geometric mean titer against:			
	MN/Lai gp160	IIIB $gp160_B$	IIIB $gp120_B$	IIIB $gp160_N$
I (HXB2/MNLoop/Lai)	363,245	1,370	2,200	4,995
II (MN/Lai)	519,995	3,260	1,820	7,025
III (IIIB)	78,215	414,000	175,000	269,275

[a] $gp160_B$ and $gp120_B$ were produced in a baculovirus expression system; $gp160_N$ represents native envelope purified from tissue culture supernatant.

While the chimera did not elicit enhanced neutralizing antibody in BALB/c mice, it did elicit high-titered binding antibodies to the MN V3 loop within the context of native protein. The high degree of binding (titer of >360,000) to the MN/Lai gp160 by sera of mice immunized with the chimeric virus (Table 6) can in part be attributed to recognition of IIIB/Lai gp41 epitopes present in all three constructs and protein immunogens. The level of such gp41-specific reactivity can be approximated either by the binding of group III mouse sera (immunized with the BH10 recombinant) to MN/Lai envelope (titer > 78,000) or by the difference in binding between IIIB gp160 and gp120 (titer difference = 239,000). The remaining binding activity of group I sera (immunized with the HXB2/MNLoop/Lai recombinant) to MN/Lai gp160 (titer of approximately 124,000 to 285,000) must be due either to recognition of the V3 loop in a native conformation or to common epitopes outside the loop shared between IIIB and MN. The latter possibility is unlikely, given the poor binding to the native or baculovirus-produced IIIB envelopes by all but group III mice (Table 6). Thus, the high level of binding to MN/Lai envelope by HXB2/MNLoop/Lai-immunized mice must result from antibody production to an altered native conformation of the MN V3 loop with or without contribution from other envelope epitopes, resulting from presentation within the HXB2 envelope. This conformational epitope recognized by the mice does not, however, constitute a neutralizing epitope (Table 4).

DISCUSSION

Neutralization of HIV-1 can be mediated by both linear and conformational envelope epitopes. While the V3 loop was initially identified as the principal neutralizing determinant of the virus (15, 25, 30, 37), other neutralizing epitopes exist across the viral envelope, including regions in the C2 (17) and V2 (12) domains of gp120 and in gp41 (4). In addition to these linear epitopes, conformational neutralization sites suggested by earlier studies (26, 34, 41) have been substantiated by studies of animal derived and human MAb (6, 18, 21, 33, 43). Recent work, in fact, suggests that antibodies which recognize conformational epitopes represent a much larger proportion of the neutralizing antibody repertoire in infected humans than those which bind to linear ones (28). Clearly, a spectrum of neutralizing epitopes subject to modulation by conformational changes exists, as has been readily demonstrated by studies of immune-selected neutralization-resistant HIV escape mutants (23, 29, 44, 47). However, the results reported here are the first to demonstrate enhanced, rather than reduced, neutralization resulting from a conformational change in the viral envelope,

leading to modification of readily available sites or to exposure of otherwise cryptic epitopes.

Enhanced neutralization of the chimeric virus by HIV-positive human sera was shown to result in some cases from better recognition of the linear V3 loop and in other cases from better recognition of either epitopes outside the loop or altered conformationally dependent V3 loop epitopes. With regard to the linear V3 loop, our studies with MAbs showed that this better recognition was manifested by increases in neutralization titer and by slight increases in relative affinity and in the percentage of cells with exposed V3 loop epitopes. These results indicate that substitution of the MN V3 loop into the HXB2 envelope resulted in conformational changes affecting the overall accessibility of the V3 loop region and its interaction with antibody. Amino acid substitutions within the V3 loop have previously been shown to influence neutralization by virtue of altering an epitope (27) or loop conformation (7). Here we demonstrate that the context of the unaltered V3 loop within different viral envelopes can have profound effects on conformation, as judged by resultant virus neutralization mediated by V3 loop-specific antibodies, either monoclonal or present in sera of naturally infected individuals.

Previously reported neutralization of the MN isolate by sera of HIV-positive Zairians was attributed to linear V3 loop-specific antibodies (3). Therefore, it was surprising to find that in some cases enhanced neutralization by sera of our Zairian cohort was dependent upon non-V3 loop or conformationally dependent V3 loop epitopes. We considered the possibility that the recognition of these sites by Zairian sera obtained from Kinshasa AIDS patients may have resulted from their participation in an immune therapy trial in which they were immunized with fixed autologous cells expressing the envelope protein of the IIIB strain (48). We cannot rule out that this therapy may have had an effect on neutralizing antibody titers obtained. However, sera of asymptomatic U.S. and European donors and Zairian volunteers who had not participated in the trial exhibited similar enhanced neutralization of the chimeric virus. Moreover, serum C (Fig. 2) represents a European donor in whom enhancement was not attributable to linear V3 loop epitopes. Therefore, recognition of altered envelope context resulting from V3 loop substitution is not only a property of sera from AIDS patients immunized with envelope preparations but also a phenomenon attributable to HIV-positive sera in general.

The viral envelope exhibits a great plasticity with resultant changes in viral functions and immune reactivity. Amino acid sequence substitutions affect not only the folding of the gp120 and gp41 proteins but also interactions between the two envelope proteins (13) and functionally important regions such as the conformationally dependent CD4 binding site (23). Oligomerization of the envelope protein (8) provides yet another level at which primary sequence changes can influence envelope quaternary structure with resultant alteration in epitope presentation. All of these changes may lead to uncovering of previously cryptic or partially hidden linear or conformational epitopes which are poorly immunogenic in parental viruses yet become more so in a chimeric version. For example, it has been reported that a neutralizing epitope in the C2 domain of the HIV envelope is weakly immunogenic in the native protein yet exhibits increased immunogenicity as a linear peptide (17). By analogy, if sufficient linearization and exposure of this epitope occurred in a chimeric envelope, enhanced immunogenicity would result. Thus, the use of chimeric viruses such as HXB2/MNLoop may elicit antibodies with increased affinity or avidity for linear or noncontiguous viral epitopes in standard isolates and may also provide a means for eliciting immune responses to a larger number of epitopes with a single immunogen.

Attempts to demonstrate increased immunogenicity of the chimeric virus in mice were not completely successful. Although the chimeric virus primed for enhanced recognition of the native MN V3 loop, the particular V3 loop epitopes recognized by the BALB/c mice were not neutralizing. Nevertheless, the experiments confirmed our observations obtained by using human sera of enhanced neutralizability of the chimera. In mice, this enhancement appeared to be primarily due to alteration of non-V3 loop epitopes. Use of the chimeric envelope as immunogen should be further evaluated in a nonhuman primate model, in which functional immune responses would be expected to more closely represent those of humans. We believe that the V3 loop chimera approach has great utility for elucidation of multiple envelope-neutralizing determinants as well as a potential utility for vaccine development.

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