JOURNAL OF VIROLOGY, Jan. 2005, p. 780–790 Vol. 79, No. 2
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.2.780–790.2005

Antibodies That Are Cross-Reactive for Human Immunodeficiency Virus Type 1 Clade A and Clade B V3 Domains Are Common in Patient Sera from Cameroon, but Their Neutralization Activity Is Usually Restricted by Epitope Masking

Chavdar Krachmarov,[1] Abraham Pinter,[1] William J. Honnen,[1] Miroslaw K. Gorny,[2] Phillipe N. Nyambi,[2,3] Susan Zolla-Pazner,[2,3] and Samuel C. Kayman[1]*

Laboratory of Retroviral Biology, Public Health Research Institute, Newark, New Jersey,[1] and Department of Pathology, New York University School of Medicine,[2] and Research Enhancement Award Program, Veterans Affairs New York Harbor Healthcare System,[3] New York, New York

Received 22 July 2004/Accepted 16 August 2004

Sera from human immunodeficiency virus type 1 (HIV-1)-infected North American patients recognized a fusion protein expressing a V3 loop from a clade B primary isolate virus (JR-CSF) but not from a clade A primary isolate virus (92UG037.8), while most sera from Cameroonian patients recognized both fusion proteins. Competition studies of consensus V3 peptides demonstrated that the majority of the cross-reactive Cameroonian sera contained cross-reactive antibodies that reacted strongly with both V3 sequences. V3-specific antibodies purified from all six cross-reactive sera examined had potent neutralizing activity for virus pseudotyped with envelope proteins (Env) from SF162, a neutralization-sensitive clade B primary isolate. For four of these samples, neutralization of SF162 pseudotypes was blocked by both the clade A and clade B V3 fusion proteins, indicating that this activity was mediated by cross-reactive antibodies. In contrast, the V3-reactive antibodies from only one of these six sera had significant neutralizing activity against viruses pseudotyped with Envs from typically resistant clade B (JR-FL) or clade A (92UG037.8) primary isolates. However, the V3-reactive antibodies from these cross-reactive Cameroonian sera did neutralize virus pseudotyped with chimeric Envs containing the 92UG037.8 or JR-FL V3 sequence in Env backbones that did not express V1/V2 domain masking of V3 epitopes. These data indicated that Cameroonian sera frequently contain cross-clade reactive V3-directed antibodies and indicated that the typical inability of such antibodies to neutralize typical, resistant primary isolate Env pseudotypes was primarily due to indirect masking effects rather than to the absence of the target epitopes.

Infection by human immunodeficiency virus type 1 and consequent disease continues to be a devastating epidemic in many areas of the world (2), while development of a protective vaccine remains elusive (29). One of the challenges in vaccine development is the induction of antibodies against conserved epitopes that mediate potent antibody neutralization of primary isolate viruses (27). The existence of such epitopes is demonstrated by the observation that a small but significant fraction of human HIV-1 patient sera (perhaps as much as 10%) is able to neutralize diverse primary isolates (4, 25). However, the epitopes that mediate this neutralization have not been determined.

Although much attention has been focused on the V3 variable loop of the HIV-1 surface protein gp120, its importance in the neutralization of primary viruses and, thus, its suitability as a vaccine target remain unclear. V3 was identified as the principal neutralizing determinant for viruses adapted for growth in T4 cell lines (TCLA viruses) (22), and it has been estimated that as much as half of the antibody response against HIV-1 Env in patient sera is directed against this region (30). The V3 domain was, nevertheless, not considered to be a useful target for vaccine development for two reasons (27). First, it did not appear to be a major neutralization target for primary virus isolates. Although TCLA viruses were highly sensitive to neutralization by early V3-reactive monoclonal antibodies (MAbs) isolated from immunized rodents, primary virus isolates were highly resistant to neutralization by these MAbs. Consistent with this, removal of V3-reactive antibodies from human patient sera by adsorption with synthetic peptides removed the majority of the neutralizing activity for TCLA viruses but had little effect on the neutralizing activity against primary virus isolates (43, 44, 48). Second, the sequence variation within the V3 loop was believed to preclude cross-reactive antibody responses directed at this region.

The presumption that V3 is not a useful target for vaccine development has been challenged by recent results that suggest that antibodies directed at the V3 loop with neutralizing activity for primary virus isolates are frequently present in patient sera and that in some cases these antibodies are broadly cross-reactive. Serum adsorption studies have shown that type-specific V3-directed antibodies contribute to the neutralization of autologous virus and control of in vivo infection for some patients (6, 41, 42). The existence of V3 epitopes that mediate the neutralization of primary virus isolates and are conserved within clade B was shown by the demonstration that polyclonal

* Corresponding author. Mailing address: Public Health Research Institute, Laboratory of Retroviral Biology, 225 Warren St., Newark, NJ 07103-3535. Phone: (973) 854-3305. Fax: (973) 854-3304. E-mail: skayman@phri.org.

TABLE 1. Sequences of the V3 region relevant to this study

Virus	Sequence[a]
CONA	PVKINCTRPNNNTRKS**IR**IGPG**QA**FY**A**TG**D**IIGDIRQAHCNVSRT
ConCRF02_AG	PVKINCTRPNNNTRKS**VR**IGPG**QT**FY**A**TG**D**IIGDIRQAHCNVSRT
92UG037.8	TVTINCTRPNNNTRKSVRIGPGQTFYATGDIIGDIRQAHCNVSGS
CONB	SVEINCTRPNNNTRKS**IH**IGPG**RA**FY**T**TG**E**IIGDIRQAHCNISRA
JR-CSF	SVKINCTRPSNNTRKSIHIGPGRAFYTTGEIIGDIRQAHCNISRA
SF162	SVEINCTRPNNNTRKSITIGPGRAFYATGDIIGDIRQAHCNISGE
JR-FL	SVEINCTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHCNISRA

[a] The region between the cysteines are the V3 loop. Sequences are from the human retroviruses and AIDS sequence database (http://www.hiv.lanl.gov/content/hiv-db/mainpage.html.) Positions at which the clade A, CRF02_AG and clade B V3 consensus sequences differ are in bold. For each viral sequence, differences within the V3 loop from its clade consensus are underlined.

V3-reactive antibodies isolated from North American patient sera possessed potent neutralizing activity against a number of clade B primary isolates (26). Broader cross-reactivity of V3-directed humoral responses in patient sera was demonstrated by enzyme-linked immunosorbent assay (ELISA) studies that demonstrated that most sera were reactive with peptides matching V3 sequences from more than one clade (3, 10, 37). However, the relative levels of reactivity with different V3 sequences were not clear from these ELISA studies, nor was it clear whether the partial cross-reactivity observed was due to mixtures of antibodies with different clade specificities, a relatively homogeneous population of antibodies with various avidities for different V3 sequences, or the presence of subpopulations of cross-reactive antibodies. In addition, these studies did not address whether the V3-directed antibody responses possessed cross-reactive neutralizing activity. More direct evidence for the existence of cross-clade-conserved V3 epitopes that mediate the neutralization of primary viruses comes from the isolation from human patients of V3-reactive MAbs that are broadly cross-reactive (15, 17, 31, 34, 35, 52) and have neutralizing activity against primary viruses from multiple clades (12, 18).

The existence of V3 epitopes that are conserved across clades is consistent with the accumulating sequence data that show considerable sequence conservation within V3 across group M of HIV-1, as compiled by the human retroviruses and AIDS sequence database (http://www.hiv.lanl.gov/content/hiv-db/mainpage.html); 18 of 33 positions between the cysteines of a canonical length V3 loop have the same consensus residue in all clades of group M. Thus, although these positions show variability among individual isolates within each clade, more than half of the positions in the V3 loop are well conserved across the clades. Indeed, both V3 loop phenetic analysis (grouping on the basis of protein sequence similarities irrespective of phylogenetic relationship) (24) and immunotype analysis based on human V3-reactive MAbs (35, 40, 52) group isolates across clades. Presumably, these relatively conserved residues in V3 represent functional sites within the loop, which might also be expected to be the most potent neutralizing targets if accessible in Env complexes on virions.

Recent studies suggest that a formidable obstacle to V3-based immunogen design is the masking of epitopes in V3 on the functional Env complexes of many primary virus isolates by other regions of gp120. Changes in N-linked glycosylation outside of V3 were found to be responsible for neutralization escape during infection and to confer increased resistance to V3-directed neutralizing MAbs (49), suggesting an "evolving glycan shield" model for immune evasion. Determinants in the V1/V2 domain were shown to be responsible for the difference in neutralization sensitivities between two primary isolate Envs to patient sera and MAbs directed at epitopes in V3 and several other regions of Env (38), suggesting that the V1/V2 domain of many primary isolates masks the accessibility of neutralization targets throughout Env. This effect was seen even for the most cross-reactive and potently neutralizing V3-directed human MAbs available. These observations raise the question of whether V3-directed antibodies exist that are not sensitive to such epitope-masking effects.

In this report, the cross-reactivity of antibodies in human patient sera that are directed against V3 epitopes was explored in more detail. The sera examined were from Cameroon, where viruses with clade A or closely related CRF02_AG Envs predominate (8, 20, 21, 28, 33, 50, 51), and from North America, where viruses with clade B Envs predominate. This analysis used fusion proteins that present fully glycosylated and properly folded V3 domains that closely match the clade A and CRF02_AG or clade B consensus sequences (18, 26) in addition to synthetic peptides corresponding to CRF02_AG and clade B V3 sequences.

MATERIALS AND METHODS

Serum samples. Cameroonian sera were collected after informed consent was obtained from asymptomatic HIV-1-infected patients being followed in Yaounde, Bamenda, and Bafia, Cameroon. The genetic subtypes of the predominant virus in many of these patients has been determined, and in most cases they were clade A or CRF02_AG (33). North American sera were obtained from HIV-1-infected patients being studied at Bellevue Hospital, New York, N.Y. (samples provided by F. Valentine), and from hemophiliac patients that had received contaminated factor VIII being followed at the Pediatric Hematology clinic at the New York Hospital-Cornell Medical Center, New York, N.Y. (samples provided by M. Hildegartner).

V3 fusion glycoproteins and peptides. The HIV-1 sequences incorporated into the V3 fusion proteins, consisting of the V3 loop and a small amount of flanking sequence, are shown in Table 1, where they are aligned with the consensus sequences for clades A, CRF02_AG, and B and the sequences of other viral isolates used in this study. The $V3_{JR\text{-}CSF}$ (clade B) fusion protein and its expression have been described previously (26). The $V3_{92UG037.8}$ (clade A) fusion protein is similar but contains a cleavage site for tobacco etch virus protease (ENLYFQS) (36) and a synthetic T_H epitope (AKFVAAWTLKAA) (1) between the murine leukemia virus-derived carrier domain and the V3 domain. The sequence of the V3 loop of this clade A Env is an exact match to the CRF02_AG consensus for this domain. It was expressed in CHO cells from a derivative of pcDNA3.1zeo(−) (Invitrogen) in which the promoter had been replaced with the intron-containing human cytomegalovirus major immediate-early promoter taken from pEE14 (CellTech). Both fusion proteins were purified on Ni^{2+}-nitrilotriacetic acid resin (NTA Superflow; QIAGEN) as described (26).

The four V3 peptides used in this study were synthesized at the Protein Core Facility at the University of Nebraska. The peptides corresponded to $V3_{92UG037.8}$ or $V3_{JR\text{-}CSF}$. The 92UG037.8 (clade A) peptide contained the complete sequence between the cysteines of the V3 loop (TRPNNNTRKSVRIGPGQTFY ATGDIIGDIRQAH-$CONH_2$). The JR-CSF (clade B) peptide did not include the first five or last three residues of the V3 loop and had two lysines added to the C terminus (NTRKSIHIGPGRAFYTTGEIIGDIR-KK-$CONH_2$).

MAbs. MAbs used in this study are of human origin and were purified by protein A or protein G chromatography. The 447-52D (19), 2557 (16), 2558 (16), 2601 (isolated as previously described [16]), and 4117C (45) MAbs are directed against the V3 loop. MAbs 2557, 2558, and 2601 were isolated from patients infected with clade A or clade CFR02_AG viruses. In ELISAs, 447-52D and 4117C recognized the clade B fusion protein and peptide but not the clade A antigens, 2601 recognized the clade A antigens but not the clade B antigens, and 2557 and 2558 recognized all four of these V3 antigens (data not shown). Immunoglobulin G (IgG) b12 (7), directed against an epitope that overlaps the CD4-binding site, was obtained from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program and was contributed by Dennis Burton and Paul Parren. MAb 2G12 (47), directed against a conformational epitope involving high mannose glycans, and 2F5 (32), directed against an epitope in the ectodomain of gp41, were obtained from the NIH AIDS Research and Reference Reagent Program and were contributed by Hermann Katinger.

Antibody isolation by affinity chromatography. Affinity isolation of V3-reactive antibodies from sera was performed as previously described (26). Briefly, resins containing the V3 fusion proteins were prepared by using cyanogen bromide-activated Sepharose beads (Pharmacia). Human HIV-1 patient sera diluted fivefold with phosphate-buffered saline (PBS) were adsorbed to the resin, and bound antibodies were eluted in 0.2 M glycine-HCl buffer, pH 2.5, and neutralized immediately with Tris buffer, pH 7.4. The buffer was exchanged with PBS by several cycles of concentration with Centricon-50 concentrators (Amicon), and samples were sterilized in 0.2-μm-pore-size microspin filters (Millipore). Bovine serum albumin was added to 0.1% for stabilization.

ELISAs and RIP. ELISAs were performed as previously described (23). For binding inhibition experiments, ELISA plates were coated with V3 fusion protein at a concentration of 2 μg/ml. Serum samples at a dilution of 1:250 and blocking antigens were mixed in dry milk-PBS for 15 min at room temperature before being added to the ELISA plates. Antibody in serum fractions was quantitated by capture ELISA as previously described (46). Radioimmunoprecipitation (RIP) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis were performed as previously described (39). V3 fusion proteins were metabolically labeled with [^{35}S]cysteine (ICN) for 18 h, and culture supernatants were immunoprecipitated with the indicated sera at a 1/100 dilution.

Neutralization assays. Neutralization assays utilized luciferase-encoding HIV-1 virions pseudotyped with the desired HIV-1 Env proteins, as previously described (26). In these assays, luciferase activity postinfection is linear with viral input and neutralization titers are insensitive to the amount of virus used over a broad range. Briefly, viral stocks were prepared by cotransfection of HEK 293 cells with pNL4-3.Luc.R$^-$E$^-$ (13) (obtained from NIH AIDS Research and Reference Reagent Program and contributed by Nathaniel Landau) and an HIV-1 Env expression vector. Appropriate dilutions of the virion-containing culture supernatants were preincubated with antibody for 1 h at 37°C in the presence of 10 μg of polybrene per ml and then added to preplated U87-T4-CCR5 cells (5) (obtained from NIH AIDS Research and Reference Reagent Program and contributed by HongKui Deng and Dan Littman). After 72 to 96 h, luciferase activity was determined on a HARTA MicroLumi 96 luminometer with reagents from Promega. Percent neutralization was defined as the percent decrease in relative light units relative to wells receiving virus that had been incubated in the absence of antibody.

The expression plasmid for SF162 Env has been described (38). The SF162(UG V3) Env was expressed from an analogous plasmid in which two double point mutations (IT->VR and RA->QT; Table 1) were sequentially introduced by PCR overlap mutagenesis to generate an Env with the 92UG037.8 V3 domain in an otherwise SF162 backbone. 92UG037.8 Env was expressed from pSVIIIenv-92UG037.8 (14), which was obtained from the NIH AIDS Research and Reference Reagent Program and contributed by Feng Gao and Beatrice Hahn. The expression plasmids for JR-FL and JR-FL(SF V1/V2) Envs have been described (38). The vesicular stomatitis virus (VSV) G envelope protein was expressed from pHEF-VSVG (9), which was obtained from the NIH AIDS Research and Reference Reagent Program and contributed by Lung-Ji Chang.

RESULTS

A large fraction of Cameroonian sera recognize both clade A-derived and clade B-derived V3 sequences. Fusion proteins expressing the V3 region from the clade A isolate 92UG037.8 or the clade B isolate JR-CSF were used to characterize V3-reactive antibodies present in HIV-1 patient sera. These are both R5 primary isolates, and their V3 loop sequences closely match the consensus sequences of their respective clades (Table 1). This fusion protein system uses the receptor-binding domain of a murine leukemia virus SU protein as a leader sequence and carrier domain and has been shown to express correctly glycosylated and folded V3 loops that are bound by human patient sera at higher titer (26) and by V3-specific human MAbs with higher affinity (18) than are synthetic V3 peptides. The ability of human sera to recognize these fusion proteins was examined by ELISA (Fig. 1) and RIP (Fig. 2). Sera used were from HIV-1-infected patients from Cameroon, where viruses with clade A or very closely related CFR02_AG Envs predominate, and from North America, where viruses with clade B Envs predominate. North American sera showed high-level reactivity with only the clade B fusion protein in both assays. In sharp contrast, approximately 70% (16 of 23 by ELISA) of the sera from Cameroon had significant reactivity with both the clade A and the clade B V3 fusion proteins. A few of the Cameroonian sera were specific for the clade A antigen (4 of 23), and a few were specific for the clade B antigen (3 of 23).

Cross-reactive Cameroonian sera have different titers for the two V3 fusion proteins. Sets of Cameroonian and North American patient sera were selected for more extensive characterization. From Cameroon, three sera specific for clade A V3, three sera specific for clade B V3, and six cross-reactive sera were chosen. Six North American patient sera specific for clade B V3 were also included. The titers of these selected sera for the two V3 fusion proteins and for matching synthetic peptides were determined by using serial dilutions of sera in direct ELISA. The clade A peptide corresponded to the full 33 residues of $V3_{92UG037.8}$, while the clade B peptide corresponded to the central 25 residues of $V3_{JR\text{-}CSF}$. The results are reported as the reciprocal serum dilution giving half-maximal reactivity with each antigen (Table 2). Because with many sera, plateau reactivity was not reached at the highest concentration tested (100-fold dilution), data were analyzed by using the average maximum absorbance of the sera that did reach a distinct plateau. All of the sera that had been scored as clade specific by single point ELISA were also clade specific in this assay, with titers below 100 for the nonreactive V3 fusion protein. All of the sera typed as cross-reactive in the initial screen had titers of at least 400 for both fusion proteins but had a significantly higher titer (at least fivefold) for one of the two V3 fusion proteins, with four sera preferring the clade A antigen and two sera preferring the clade B antigen. Titers for the V3 fusion proteins were at least threefold higher, and frequently more than 10-fold higher, than for the matched synthetic peptides, which is consistent with prior data showing that the V3 fusion proteins are more native antigens than are synthetic peptides (18, 26). The pattern of reactivity with the full-length clade A peptide was similar to that with the shorter clade B peptide, suggesting that the first and last few residues

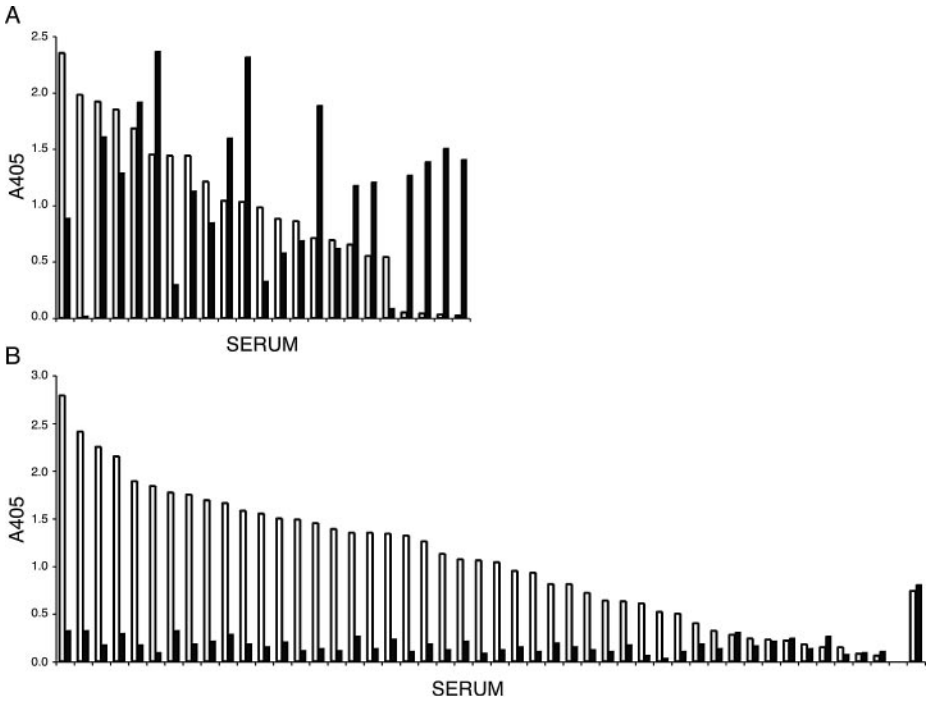

FIG. 1. Serum reactivity with V3 fusion proteins by ELISA. (A) Sera (1/150 dilution) from 23 HIV-1-infected patients from Cameroon were assayed by ELISA against the clade A (filled bars) and clade B (open bars) fusion proteins. (B) Sera (1/100 dilution) from 47 HIV-1-infected patients from North America were assayed by ELISA against the clade A (filled bars) and clade B (open bars) fusion proteins. The right-most serum was a Cameroon serum previously shown to have similar reactivity with the two V3 fusion proteins.

of the V3 loop did not contribute significantly to the epitopes seen by these sera.

Serum cross-reactivity was often due to antibodies with high affinity for both V3 sequences. The cross-reactivity of Cameroonian patient sera might have been due either to the presence of cross-reactive antibodies or to mixtures of antibodies specific for each of the V3 fusion proteins. To distinguish between these possibilities, the ability of the V3 peptides to block the binding of serum antibodies to each of the V3 fusion proteins in ELISA was determined. If the binding to a V3 fusion protein were by truly cross-reactive antibodies, it should be blocked by both peptides, while if it were by clade-specific antibodies, it should only be blocked by the autologous peptide. Peptide concentrations giving 50% inhibition of binding derived from such plots are presented in Table 3. As expected, the clade B peptide efficiently competed with serum antibody binding to the clade B fusion protein, and the clade A peptide efficiently competed with binding to the clade A fusion protein (50% inhibition at concentrations less than 1 μg/ml; usually less than 0.2 μg/ml) in all cases. Binding by the clade-specific sera to the fusion protein they recognized was not blocked by the other clade peptide at the highest concentration tested, 30 μg/ml. These results demonstrated that the competition assay was able to identify clade-specific antibodies.

For the four cross-reactive Cameroon sera with higher titer for the clade A fusion protein (sera 5, 56, 65, and 68), reactivity with this fusion protein was efficiently competed by the clade A peptide but not by the clade B peptide, indicating that the bulk of the clade A V3-reactive antibody in these sera was not reactive with the clade B V3 sequence and was, therefore, clade specific. In sharp contrast, the reactivity of sera 5, 56, and 65 with the clade B fusion protein was efficiently competed by

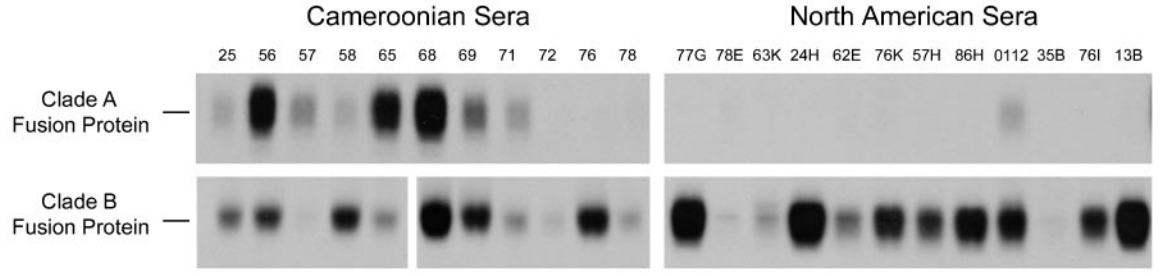

FIG. 2. Serum reactivity with V3 fusion proteins by RIP. [^{35}S]cysteine labeled culture supernatant from cells producing the indicated V3 fusion protein was immunoprecipitated with the indicated serum at a 1/100 dilution and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography.

TABLE 2. Serum titers for V3 fusion proteins and peptides

Serum	Specificity[a]	Titer[b]			
		Clade A (92UG037.8)		Clade B (JR-CSF)	
		Fusion protein	Peptide	Fusion protein	Peptide
Cameroonian 39	Clade A	2600	450	–	–
Cameroonian 39b	Clade A	2900	400	–	–
Cameroonian 49	Clade A	3900	300	–	–
Cameroonian 58	Clade B	–	–	900	100
Cameroonian 76	Clade B	–	–	800	–
Cameroonian 78	Clade B	–	–	200	–
Cameroonian 5	Both	5400	900	400	–
Cameroonian 56	Both	42000	5000	5300	250
Cameroonian 65	Both	13000	2000	1500[c]	–
Cameroonian 68	Both	4600	500	700	–
Cameroonian 25	Both	600	150	3300	150
Cameroonian 69	Both	400	150	2100	100
N. American 2323	Clade B	–	–	17000	1500
N. American 1914	Clade B	–	–	18000	900
N. American 0112	Clade B	–	–	4600	300
N. American 1301	Clade B	–	–	6800	550
N. American 1303	Clade B	–	–	5800	300
N. American 1314	Clade B	–	–	4800	250

[a] As determined by single point ELISA against the two V3 fusion proteins.
[b] Reported as reciprocal dilution giving 50% maximal ELISA reactivity for each antigen, determined for serial threefold dilutions of sera beginning at 1/100. Sera that did not reach 50% binding at 1/100 dilution are indicated with a dash. Maximum binding for each antigen was determined as either the average plateau value for sera reaching plateaus (for fusion protein antigens) or the average maximal value for sera clustering at a maximal value at the highest concentration tested (for peptide antigens).
[c] This serum behaved anomalously on this antigen, reaching a clear plateau at somewhat less than half the absorbence of other sera. Its titer is based on its own maximal binding value.

TABLE 3. Inhibition of serum binding to V3 fusion proteins by V3 peptides

Serum	Specificity[a]	Binding to Clade A fusion protein[b]		Binding to Clade B fusion protein[b]	
		Blocking by clade A peptide	Blocking by clade B peptide	Blocking by clade A peptide	Blocking by clade B peptide
39b	Clade A	<0.2	>30		
49	Clade A	<0.2	>30		
5	Clade A>B	<0.2	>30	<0.2	<0.2
56	Clade A>B	<0.2	>30	<0.2	<0.2
65	Clade A>B	<0.2	>30	0.8	0.3
68	Clade A>B	0.4	>30	>30	<0.2
25	Clade B>A	<0.2	>30	>30	0.3
69	Clade B>A	<0.2	<0.2	>30	<0.2
58	Clade B			>30	0.3
76	Clade B			>30	0.7
78	Clade B			>30	<0.2
2323	Clade B			>30	<0.2
1303	Clade B			>30	0.3
1314	Clade B			>30	<0.2
1914	Clade B			>30	0.3

[a] Specificity was determined by ELISA titers for V3 fusion proteins.
[b] For cross-reactive sera, A > B indicates higher avidity for the clade A fusion protein, while B > A indicates higher avidity for the clade B fusion protein. Peptide concentrations (μg/ml) yielding a 50% reduction in ELISA binding are given. Fusion protein antigens were coated at a concentration of 2 μg/ml; sera were diluted 300-fold.

both peptides. Thus, the antibodies in these sera that bound to the clade B fusion protein were highly reactive with both clade A and clade B V3 sequences. Serum 68 gave a different result. For this serum, reactivity with both the clade A and clade B V3 fusion proteins was competed only by the matched peptide, indicating that the cross-reactivity of serum 68 was due to a mixture of antibodies that were clade specific.

These assays also indicated differences in the V3 specificities of the antibodies present in the two cross-reactive Cameroonian sera with higher titer for the clade B fusion protein. For serum 69, both peptides competed efficiently for binding to the clade A fusion protein, indicating that its clade A reactivity was due to cross-reactive antibodies. For serum 25, the clade B peptide did not compete for binding to the clade A fusion protein, indicating that its clade A reactivity was due to clade-specific antibodies. For both of these sera, the clade A peptide did not compete for binding to the clade B fusion protein, indicating that their clade B-reactive antibodies were largely clade specific.

In summary, the reactivity of Cameroonian sera 5, 56, and 65 for the clade B fusion protein and serum 69 for the clade A fusion protein was due to cross-reactive antibodies that bound strongly to both the clade A and clade B V3 sequences. In addition to these cross-reactive antibodies, these four sera had higher levels of antibodies that were clade-specific for the sequences for which they had higher titers. In contrast, the dual reactivity of sera 25 and 68 was due to mixtures of antibodies that were specific for each of the V3 sequences.

Isolation of V3-reactive antibodies from patient sera for characterization of neutralization activity. In order to determine whether the V3-reactive antibodies present in these Cameroonian patient sera possessed neutralizing activity for HIV-1, antibodies were affinity purified on resins containing each of the V3 fusion proteins. Depleted sera were collected, and bound antibodies were recovered by elution with pH 2.5 buffer. Adsorption and recovery were monitored by ELISA against each of the V3 fusion proteins; representative data are presented in Fig. 3. In control adsorptions that used affinity resin bearing only the fusion protein carrier domain, a small degree of nonspecific loss of V3 reactivity was seen, while only small amounts of antibody with insignificant ELISA reactivity against the fusion partner or either of the V3 fusion proteins were recovered in the eluates (data not shown), indicating that adsorption to and isolation of V3-reactive antibodies from these affinity resins were dependent on the presence of a V3 antigen.

In these experiments, adsorption of serum ELISA reactivity for the fusion protein present on the affinity resin was usually in excess of 90%. While autologous V3 reactivity was always recovered in the eluate, yields were usually low, on the order of 10 to 20%. The amount of antibody recovered ranged from 40 to 200 μg per ml of serum. Surprisingly, V3-directed antibodies in sera that were clade specific by ELISA were usually at least partially adsorbed by the "nonreactive" V3 fusion protein and were often recovered in the eluate from this resin (Fig. 3, sera 49, 58, and 1301). This suggested that much of the V3-directed antibody in these sera had a low affinity for the nonreactive fusion protein that allowed adsorption in the presence of excess antigen on the affinity resin but was insufficient for detec-

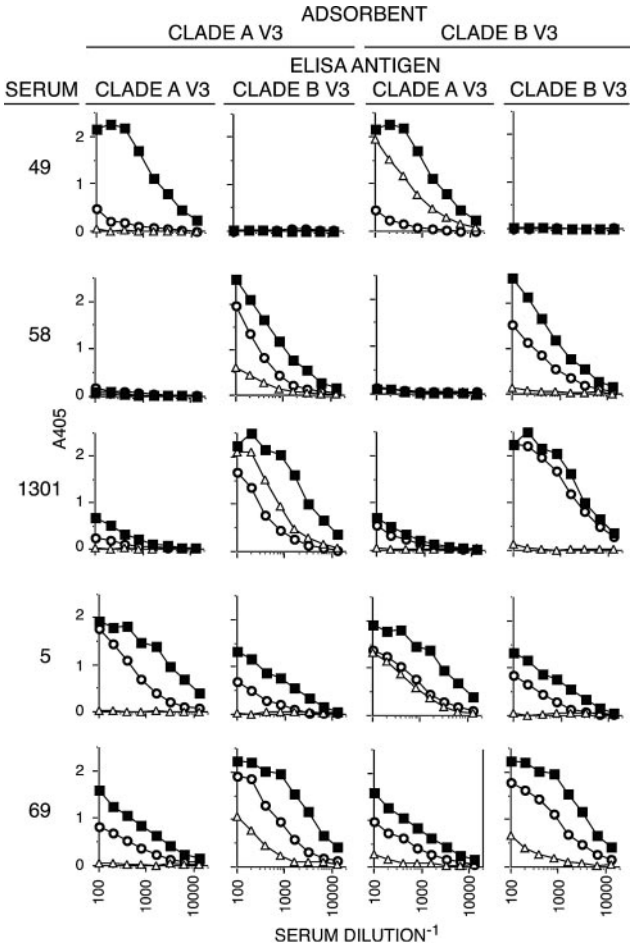

FIG. 3. Serum adsorption on V3 fusion proteins. Sera were fractionated by adsorption to the indicated V3 fusion protein affinity resin, followed by elution in pH 2.5 buffer. Whole sera (■), depleted column flowthrough samples (△), and pH 2.5 eluates (○) were assayed for ELISA reactivity against each of the V3 fusion proteins as indicated. A_{405} is plotted versus reciprocal serum dilution.

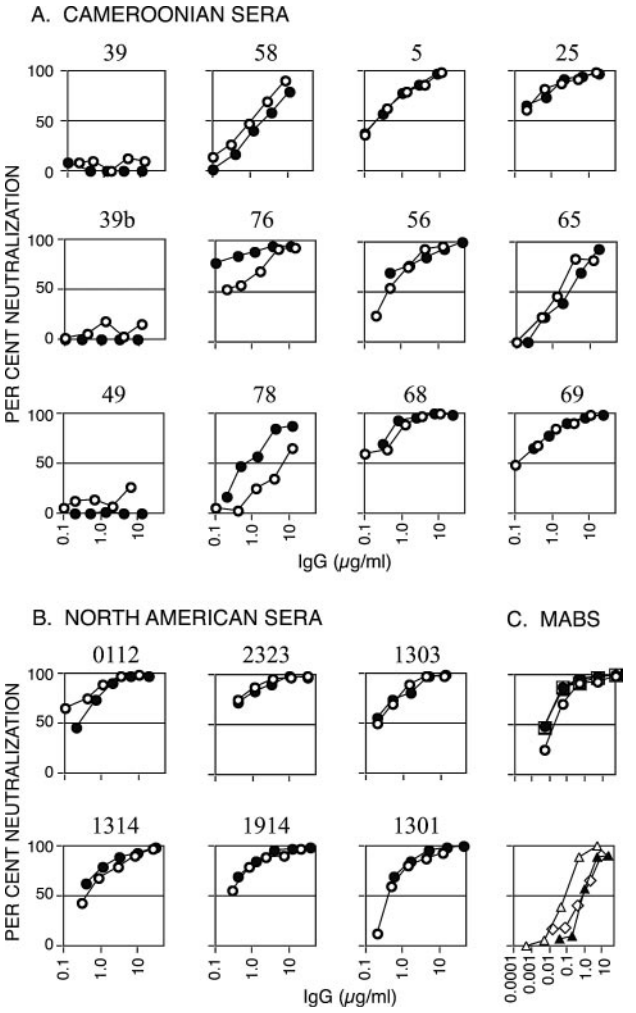

FIG. 4. Neutralization of SF162 by V3-reactive antibodies. Virions derived from pNL4-3. Luc.R$^-$E$^-$ were pseudotyped with SF162 Env and used in luciferase neutralization assays. Percent neutralization is plotted as a function of antibody concentration. (A and B) Antibodies purified from sera on clade B fusion protein (●) and antibodies purified from sera on clade A fusion protein (○). (C) MAbs IgG b12 (△), 2G12 (▲), 2F5 (◇) (bottom panel), 447-52D (○), 4117C (●), 2557 (□), and 2558 (■) (top panel).

tion by ELISA under the conditions used. For the Cameroonian sera that were reactive with both the clade A and clade B antigens (Fig. 3, sera 5 and 69), significant reactivity against both antigens was recovered from both adsorbents. In some cases reactivity against the other clade V3 antigen was only partially removed by one of the adsorbents (e.g., serum 5).

V3-reactive antibodies neutralize primary isolate SF162 Env pseudotypes. The ability of immunoaffinity-purified V3-reactive antibodies from these 18 sera to neutralize primary isolate HIV-1 viruses was determined by using luciferase-encoding pseudotyped virions. SF162 is a clade B primary isolate that is relatively sensitive to neutralization by V3-reactive MAbs (16, 18, 38); it was neutralized by most Cameroonian patient sera tested (data not shown). Antibody recovered from selected sera by using control affinity columns containing only the fusion protein carrier domain had no neutralizing activity against SF162 pseudotypes (data not shown), demonstrating that the recovery of neutralizing activity by affinity purification was dependent on the use of a V3 immunoadsorbent. Also, V3-reactive antibodies purified from selected sera (including Cameroonian serum 56; see below) had no neutralization activity against HIV-1 particles pseudotyped with the VSV-G protein (data not shown), demonstrating that the observed neutralization was specific for HIV-1 Env. As expected, no neutralizing activity for SF162 Env pseudotypes was found in the V3-reactive antibodies recovered from Cameroonian sera that were specific for the clade A fusion protein (Fig. 4A, first column), further demonstrating the specificity of the observed neutralization.

V3-reactive antibodies with similar neutralization potencies were recovered from each of the V3 fusion protein immunoadsorbents from most Cameroonian sera that were either specific for the clade B fusion protein (Fig. 4A, second column) or cross-reactive for the two V3 antigens (Fig. 4A, third and fourth columns.) The North American sera also yielded V3-reactive antibodies with similar neutralization potencies for

TABLE 4. Blocking of SF162 neutralization by V3-reactive antibodies purified from cross-reactive sera

Antibody	% Neutralization[a]	% Blocking by:	
		Clade A	Clade B
Cameroonian sera			
5	79	99	116
56	90	14	56
65	78	119	118
68	100	110	108
25	95	14	111
69	100	62	110
MAbs to V3 epitopes			
447-52D	94	12	103
4117C	98	−2.0	101
MAbs to non-V3 epitopes			
2F5	86	2.2	1.6
2G12	70	32	−6.9
b12	100	−0.4	−0.4

[a] Antibodies were used at a concentration of 5 μg/ml (3×10^{-8} M) in the standard luciferase neutralization assay. Soluble V3 fusion proteins were added at 5×10^{-5} M for blocking.

SF162 pseudotypes from both the clade A and clade B immunoadsorbents (Fig. 4B). Both North American and Cameroonian sera frequently yielded V3-reactive antibodies with 90% neutralizing doses (ND_{90}s) against SF162 pseudotypes of approximately 5 μg/ml and 50% neutralizing doses (ND_{50}s) often well below 1 μg/ml. This was not as potent as many V3-reactive human MAbs, which typically had ND_{90}s of approximately 1 μg/ml (Fig. 4C). The neutralizing antibodies in these polyclonal samples may have had lower potency than did the available MAbs, or only a subset of the antibodies present in these samples may have contributed to the neutralization of SF162. The latter explanation is highly likely, particularly for the antibodies fractionated from those sera (the cross-reactive Cameroonian sera 5, 56, 65, and 68) in which only a small fraction of the titer against the V3 domain was reactive with the clade B sequence.

Neutralization of SF162 pseudotypes was often mediated by cross-reactive V3-directed antibodies. Neutralization of SF162 pseudotypes by the V3-reactive antibodies purified from cross-reactive Cameroonian sera might have been due to clade B-specific antibodies or to antibodies cross-reactive for clade A and clade B V3 sequences. This question was resolved by examining the ability of soluble V3 fusion proteins to block neutralization (Table 4). As expected, neutralization by MAbs having clade B-specific V3 reactivity (447-52D and 4117C) was fully blocked by the clade B fusion protein but not by the clade A fusion protein, while neutralization by MAbs reactive with non-V3 epitopes was insensitive to the presence of either fusion protein, confirming that the blocking of neutralization by soluble V3 fusion protein resulted from epitope-specific interaction with the neutralizing antibody.

Similar blocking experiments showed that four of the six cross-reactive Cameroonian sera contained cross-reactive antibodies that recognized both clade A and clade B V3 sequences and neutralized SF162 pseudotypes. Neutralization of SF162 pseudotypes by V3-directed antibodies was fully blocked by both V3 fusion proteins for two of the three Cameroon sera whose bulk reactivity with the clade B fusion protein was due to cross-reactive antibodies (sera 5 and 65), as well as for serum 68, which had a higher titer for the clade A fusion protein but whose bulk reactivity with the clade B fusion protein was due to clade-specific antibodies. For serum 68, neutralization therefore appeared to be mediated by a subset of the clade B-reactive antibodies present. For serum 69, which had a higher titer for the clade B fusion protein and whose reactivity with the clade A fusion protein was due to cross-reactive antibodies, neutralization of SF162 pseudotypes completely blocked by the clade B fusion protein and was partially blocked by the clade A fusion protein. This suggested that both clade-specific and cross-reactive antibodies contributed to SF162 neutralization by this sample. For serum 25, which had a higher titer for the clade B fusion protein and whose reactivity with the clade A fusion protein was due to clade-specific antibodies, neutralization of SF162 pseudotypes was blocked by the clade B fusion protein but not by the clade A fusion protein. Thus, for this serum, the V3-reactive antibodies that neutralized SF162 pseudotypes were clade B specific.

The neutralization blocking experiment did not yield a definitive result for the third Cameroonian serum, whose bulk reactivity with the clade B fusion protein was due to cross-reactive antibodies (Table 3, serum 56). Neutralization of SF162 pseudotypes was only partially blocked by the clade B fusion protein and was not blocked by the clade A fusion protein. Failure of the V3 fusion proteins to effectively block neutralization of SF162 pseudotypes by the V3-reactive antibodies from serum 56 may have been due to the presence of antibodies preferentially reactive with the unusual sequence features of the SF162 V3 domain or with unusual, conserved epitopes only poorly presented by the fusion proteins. In this regard, it may be significant that this patient was infected by a CRF01_AE virus (33), since the consensus for this clade contains a Thr corresponding to the otherwise unusual Thr preceding the crown of the loop in SF162.

V3-reactive antibodies from only one cross-reactive Cameroonian serum were active against neutralization-resistant primary isolates from clades A and B. Since SF162 is unusually sensitive to V3-mediated neutralization, each of the purified V3-reactive antibody fractions was assayed for neutralization against two additional viruses that have neutralization sensitivity more typical of primary isolates. JR-FL is an R5-tropic clade B primary isolate that is more than three orders of magnitude less susceptible than SF162 to neutralization by many V3-reactive human MAbs (16, 18, 38). Pseudotypes with clade A primary isolate 92UG037.8 were also resistant to V3 MAbs that are highly reactive with its V3 sequence (Fig. 5B, MAbs 2557, 2558, and 2601) and, like JR-FL pseudotypes, were at best poorly neutralized by Cameroonian sera (data not shown). V3-reactive antibodies isolated from most sera did not neutralize either of these pseudotypes (data not shown). However, Cameroonian serum 56 yielded V3-reactive antibodies with significant neutralizing activity against both of these pseudotypes, with ND_{50}s of approximately 10 μg/ml (Fig. 5A and B). Similar titers of neutralizing activity were recovered from each of the two adsorbents.

The potency of the V3-reactive antibodies isolated from serum 56 for JR-FL pseudotypes was similar to that of the best

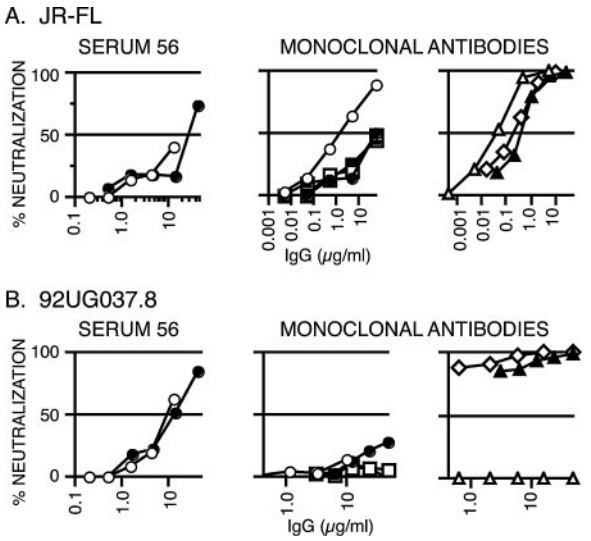

FIG. 5. Neutralization of JR-FL and 92UG037.8 by V3-reactive antibodies from Cameroonian serum 56. Virions derived from pNL4-3/Luc were pseudotyped with JR-FL Env (A) or 92UG037.8 Env (B) and used in luciferase neutralization assays. Percent neutralization is plotted as a function of antibody concentration. V3-reactive antibodies isolated on clade B fusion protein (○) and V3-reactive antibodies isolated on clade A fusion protein (●) are shown. In panel A, the MAbs are as follows: IgG b12 (△), 2G12 (▲), 2F5 (◇), 447-52D (○), 4117C (●), 2557 (□), and 2558 (■). In panel B the MAbs are as follows: IgG b12 (△), 2G12 (▲), 2F5 (◇), 447-52D (○), 2601 (●), 2557 (□), and 2558 (■).

available V3-reactive MAbs. Only a small fraction of the V3-specific titer in serum 56 was reactive with the clade B sequence, suggesting that most of the antibody present in this sample was not involved in neutralization of the JR-FL Env pseudotypes and, thus, that the potency of the neutralizing antibodies in this sample for the resistant Env pseudotypes was actually higher than those of the available MAbs. The potency of the V3-reactive antibodies from serum 56 against SF162 pseudotypes was not unusually high, suggesting that neutralization of the 92UG037.8 and JR-FL Env pseudotypes by this sample was not due to a higher concentration of antibodies similar to those present in the other sera but, rather, to an unusual V3 reactivity. Failure of either V3 fusion protein to completely block neutralization of SF162 pseudotypes by the V3-reactive antibodies from serum 56 also suggested that the antibodies involved had an unusual epitope specificity. Unfortunately, there was insufficient material to allow further characterization of the V3-reactive antibodies from serum 56 that were responsible for neutralization of JR-FL and 92UG037.8 pseudotypes by neutralization blocking experiments.

When placed into neutralization-sensitive Env backbones, JR-FL and 92UG037.8 V3 domains were neutralized by the V3-directed antibodies commonly present in Cameroonian sera. Primary isolate virus resistance to neutralization by V3-directed antibodies is often due to masking effects of sequences outside V3 rather than to variation within the epitopes themselves. Envs that present either the JR-FL or 92UG038.7 V3 domain in a neutralization-sensitive background allowed determination of whether the failure of the V3-directed antibodies frequently found in Cameroonian patient sera to neutralize these isolates was due to epitope masking. For SF162 and JR-FL, the V1/V2 domain is the major determinant of neutralization phenotype, particularly for V3-directed antibodies (38). For JR-FL V3, a neutralization-sensitive chimeric Env in which the V1/V2 domain of JR-FL has been replaced with that of SF162 [the JR-FL(SF V1/V2) Env] was used. Because pseudotypes bearing a similar construct for 92UG037.8 Env were not infectious (data not shown), an Env containing the 92UG037.8 V3 domain was generated by mutating the V3 domain of SF162 Env to match that of 92UG037.8 [the SF162(UG V3) Env, containing IT→VR and QT→RA changes; Table 1]. SF162(UG V3) Env pseudotypes were sensitive to neutralization by V3-directed MAbs that recognize clade A V3 antigens (Table 5, MAbs 2557, 2558, and 2601), indicating that the insensitivity of 92UG037.8 Env pseudotypes to these MAbs was due to epitope masking effects.

V3-directed antibody samples from cross-reactive Cameroonian patient sera with higher titer for clade A V3 (serum 65) or clade B V3 (sera 25 and 69) neutralized both the JR-FL(SF V1/V2) and SF162(UG V3) Env pseudotypes (Table 5). Antibodies from sera with higher titers for the clade B V3 sequence had higher potency against JR-FL(SF V1/V2) than did the antibodies from the serum with a higher titer for the clade A V3 (ND_{50}s of 0.019 and 0.029 versus 0.19 μg/ml), which is consistent with the clade B-specific antibodies in the

TABLE 5. Neutralization of JR-FL and 92UG037.8 V3 domains in sensitive Envs

Antibody	Neutralization (ND_{50}) of pseudotype (μg/ml)[a]			
	JR-FL	JR-FL(SF V1/V2)	92UG037.8	SF162(UG V3)
MAbs				
447-52D	15	0.0056	≫20	3.7
4117c	8.4	0.0074	≫20	≫20
2557	>20	0.011	≫20	0.21
2558	>20	0.0032	≫20	0.26
2601	≫20	≫20	≫20	0.050
Serum fractions				
25	>10	0.019	≫10	0.80
65	≫10	0.19	>10	0.95
69	≫15	0.029	≫15	0.95

[a] Determined in luciferase neutralization assays of the indicated pseudotype. > indicates that ND_{50} was not reached at the maximal concentration used; ≫ indicates that no neutralization was seen at that concentration.

former sera contributing to neutralization. Surprisingly, antibodies from all three sera neutralized SF162(UG V3) with similar potency (ND_{50}s of approximately 1 μg/ml), suggesting that the clade A-specific antibodies in the serum 65 sample did not contribute significantly to the neutralization. The potencies of all three sera were significantly lower against SF162(UG V3) than against JR-FL(SF V1/V2) or SF162, as were those of the cross-reactive MAbs 2557 and 2558. This result suggested that the cross-reactive neutralizing antibodies in these sera had higher avidity for the clade B than for the clade A V3 sequence in Env complexes. Overall, these results show that the resistance of JR-FL and 92UG037.8 pseudotypes to neutralization by the V3-directed antibodies in these patient sera was due to epitope masking effects, not to the absence of their epitopes.

DISCUSSION

This study characterized the V3-specific antibodies present in HIV-infected patients from Cameroon, most of whom were infected by viruses with clade A or CRF02_AG Envs, and from North America, where infections by viruses with clade B Envs predominate. The consensus sequence for the V3 domain differs at six positions between clades A, CFR02_AG, and B. At the crown of the loop, clades A and DRF02_AG have GPGQ, while clade B has GPGR. GPGR is rarely found among clade A and CRF02_AG isolates, while GPGQ is rarely found among clade B isolates (http://www.hiv.lanl.gov/content/hiv-db/mainpage.html). At the other five positions, the distinction between these clades is less pronounced, with each of the consensus residues being moderately common in isolates of all three clades, although the R (in bold) at position 12 [S(I/V)**RI**] of the clade A and CRF02_AG V3 consensus sequences is poorly represented among clade B sequences. Despite this similarity in consensus sequence and the finding that in many cases V3 domains from clade A and clade B isolates group together by phenetic (24) and immunotype (35, 52) analysis, clade A and clade B serotypes were clearly distinguished by using peptide-binding ELISA under carefully defined conditions (11).

By using fusion proteins that present native V3 domains that closely match the clade A and CRF02_AG or clade B consensus sequences, the North American sera were found to be specific for the clade B (JR-CSF) V3 domain, having at most weak reactivity with the clade A (92UG037.8) V3 domain. This appears to differ from a report that most sera from France, which is expected to have a largely clade B-infected population, recognize both clade A and clade B consensus V3 peptides in direct binding ELISAs (3). However, the very low level of reactivity frequently seen here for the clade A fusion protein might have been sufficient to have been scored as reactive in the peptide studies where the criterion used was reactivity above a cutoff defined by negative control sera, rather than the ratio between reactivity with clade A and clade B sequences as was used here (3, 10, 37).

Strikingly different results were obtained with the Cameroonian patient sera: 70% of these sera showed significant reactivity with both V3 fusion proteins. The major virus Env subtype infecting many of these patients has been determined by a combination of heteroduplex mobility assay and C2V3 sequencing (33). Of the 17 cross-reactive sera, 7 came from patients with clade A or CFR02_AG Env infections, 1 from clade CRF01_AE, 1 from clade F2, and 8 from patients that failed in the subtype analysis. Three of the four clade A-specific sera came from patients with clade A Env infections; the fourth patient failed in the subtype analysis. Of the sera reactive with the clade B but not the clade A fusion protein, one came from a patient with a clade A Env infection, one with clade D, and one with clade F2. These data suggested that infection by clade A Env viruses in Cameroon frequently induced a cross-reactive V3 humoral response; based on the small numbers available, cross-reactive responses also appear to be induced in this population by infections with viruses having Envs of other clades.

What accounts for the different frequency of the cross-reactive humoral responses to V3 in these two populations? The most straightforward explanation is that clade-specific V3 epitopes were more immunodominant in clade B Envs than in the non-clade B Envs that gave rise to cross-reactive responses in Cameroonian patients. This might be due to higher immunogenicity of the restricted epitopes or lower immunogenicity of the conserved epitopes. Such differential immunogenicity might be due to residues within the V3 domain or might result from differences elsewhere in Env. If this were a property of the V3 loop itself, it would suggest that immunization with non-clade B V3 sequences might induce more cross-reactive antibody responses than with clade B V3 sequences. If the differential induction of cross-reactive V3-directed antibodies by clade B and non-clade B viruses were a global property of Env, it would suggest that non-clade B envelope immunogens might induce more cross-reactive responses, at least within V3. These questions might be addressed by animal studies with V3 fusion proteins and intact Envs as immunogens.

Other explanations are possible. The viruses circulating in North America are overwhelmingly of clade B and the incidence of infection is low, while the viruses circulating in Cameroon are highly diverse and the incidence of infection is high. It is possible that Cameroonian patients are frequently exposed to multiple viruses and therefore develop mixtures of clade-specific V3-reactive antibodies. Two of six cross-reactive sera examined in detail did prove to contain mixtures of antibodies specific for each of the V3 fusion proteins. However, the other four sera contained truly cross-reactive antibodies that had similar affinities for the clade A and clade B V3 sequences. These cross-reactive antibodies were present together with higher titers of V3-reactive antibodies that were clade specific for one of the two fusion proteins. It is possible that the initial response to the virus that established infection included low levels of cross-reactive antibodies in both North American and Cameroonian patients and that subsequent exposure of Cameroonian patients to other viruses then selectively amplified the cross-reactive component. Alternatively, prior exposures to different virus isolates that failed to establish infection might have primed the immune system to respond to conserved V3 epitopes upon productive infection of the Cameroonian patients.

An important measure of the significance of the cross-reactive V3 antibodies present in the Cameroonian sera is whether these antibodies had cross-neutralizing activity. Neutralization activity was examined by using V3-specific antibodies that were affinity purified from patient sera. Only one of six cross-reac-

tive sera yielded V3-reactive antibodies that neutralized virus pseudotyped with typically neutralization-resistant primary isolate Envs from clade A (92UG037.8) and clade B (JR-FL). However, all six of these V3-reactive antibody samples neutralized virus pseudotyped with the Env of the unusually neutralization-sensitive clade B primary isolate SF162. For four of the six cross-reactive Cameroonian sera, neutralization of SF162 pseudotypes was blocked by both the clade A and clade B fusion protein, demonstrating that this neutralization was mediated by antibodies cross-reactive with the clade A and clade B sequences.

The resistance of 92UG037.8 and JR-FL pseudotypes to neutralization by the V3-reactive antibodies isolated from most of the cross-reactive Camerooninan sera might have been due to the absence in these Envs of the epitopes for the neutralizing antibodies or to masking effects that prevent binding of V3-directed antibodies to their epitopes in the functional Env complexes on the virion surface (38, 49). The ability of these antibodies to neutralize virus pseudotyped with recombinant Envs in which the 92UG037.8 and JR-FL V3 domains were present in backbones that did not exhibit V1/V2 domain masking of V3 epitopes showed that these primary isolate Envs did contain the epitopes for the cross-reactive V3-directed neutralizing antibodies frequently present in Cameroonian patient sera. Thus, the inability of these V3-directed antibodies to neutralize 92UG037.8 and JR-FL pseudotypes was due to blocking effects by sequences outside V3. These observations strongly support the idea that the major problem facing development of V3-based immunogens is not sequence variation within V3 but, rather, that access of most V3-directed antibodies to their epitopes in functional Env complexes is blocked, often by the V1/V2 domain (38). The ability of V3-directed antibodies from one Cameroonian serum to neutralize virus pseudotyped with Envs from neutralization-resistant clade A and clade B primary isolates was an encouraging indication that epitopes in the V3 domain can mediate antibody neutralization that is not highly sensitive to such epitope-blocking effects. Confirming this observation and defining the determinants of such neutralization may contribute to the development of V3-based vaccines.

ACKNOWLEDGMENTS

These studies were supported by U.S. Public Health Service grants AI51987 to S.C.K., AI50452 to A.P., and AI36085, AI47053, and HL59725 and research funds from the Department of Veterans Affairs to S. Z.-P.

We thank Fred Valentine and Margaret Hildegartner for providing serum samples and Karl Drlica for critical comments on the manuscript.

REFERENCES

1. **Alexander, J., J. Sidney, S. Southwood, J. Ruppert, C. Oseroff, A. Maewal, K. Snoke, H. M. Serra, R. T. Kubo, and A. Sette.** 1994. Development of high potency universal DR-restricted helper epitopes by modification of high affinity DR-blocking peptides. Immunity **1:**751–761.
2. **Anonymous.** 2004. 2004 Report on the global HIV/AIDS epidemic: 4th global report. Joint United Nations Program on HIV/AIDS, Geneva, Switzerland.
3. **Barin, F., Y. Lahbabi, L. Buzelay, B. Lejeune, A. Baillou-Beaufils, F. Denis, C. Mathiot, S. M'Boup, V. Vithayasai, U. Dietrich, and A. Goudeau.** 1996. Diversity of antibody binding to V3 peptides representing consensus sequences of HIV type 1 genotypes A to E: an approach for HIV type 1 serological subtyping. AIDS Res. Hum. Retrovir. **12:**1279–1289.
4. **Beirnaert, E., P. Nyambi, B. Willems, L. Heyndrickx, R. Colebunders, W. Janssens, and G. van der Groen.** 2000. Identification and characterization of sera from HIV-infected individuals with broad cross-neutralizing activity against group M (*env* clade A-H) and group O primary HIV-1 isolates. J. Med. Virol. **62:**14–24.
5. **Bjorndal, A., H. Deng, M. Jansson, J. R. Fiore, C. Colognesi, A. Karlsson, J. Albert, G. Scarlatti, D. R. Littman, and E. M. Fenyo.** 1997. Coreceptor usage of primary human immunodeficiency virus type 1 isolates varies according to biological phenotype. J. Virol. **71:**7478–7487.
6. **Bures, R., L. Morris, C. Williamson, G. Ramjee, M. Deers, S. A. Fiscus, S. Abdool-Karim, and D. C. Montefiori.** 2002. Regional clustering of shared neutralization determinants on primary isolates of clade C human immunodeficiency virus type 1 from South Africa. J. Virol. **76:**2233–2244.
7. **Burton, D. R., J. Pyati, R. Koduri, S. J. Sharp, G. B. Thornton, W. H. Parren, L. S. Sawyer, R. M. Hendry, N. Dunlop, and P. L. Nara.** 1994. Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody. Science **266:**1024–1027.
8. **Carr, J. K., J. N. Torimiro, N. D. Wolfe, M. N. Eitel, B. Kim, E. Sanders-Buell, L. L. Jagodzinski, D. Gotte, D. S. Burke, D. L. Birx, and F. E. McCutchan.** 2001. The AG recombinant IbNG and novel strains of group M HIV-1 are common in Cameroon. Virology **286:**168–181.
9. **Chang, L. J., V. Urlacher, T. Iwakuma, Y. Cui, and J. Zucali.** 1999. Efficacy and safety analyses of a recombinant human immunodeficiency virus type 1 derived vector system. Gene Ther. **6:**715–728.
10. **Cheingsong-Popov, R., S. Lister, D. Callow, P. Kaleebu, S. Beddows, J. Weber, and the WHO Network for HIV Isolation and Characterization.** 1994. Serotyping HIV type 1 by antibody binding to the V3 loop: relationship to viral genotype. AIDS Res. Hum. Retrovir. **10:**1379–1386.
11. **Cheingsong-Popov, R., S. Osmanov, C. P. Pau, G. Schochetman, F. Barin, H. Holmes, G. Francis, H. Ruppach, U. Dietrich, S. Lister, J. Weber, and the UNAIDS Network for HIV-1 Isolation and Characterization.** 1998. Serotyping of HIV type 1 infections: definition, relationship to viral genetic subtypes, and assay evaluation. AIDS Res. Hum. Retrovir. **14:**311–318.
12. **Conley, A. J., M. K. Gorny, J. A. Kessler II, L. J. Boots, M. Ossorio-Castro, S. Koenig, D. W. Lineberger, E. A. Emini, C. Williams, and S. Zolla-Pazner.** 1994. Neutralization of primary human immunodeficiency virus type 1 isolates by the broadly reactive anti-V3 monoclonal antibody, 447-52D. J. Virol. **68:**6994–7000.
13. **Connor, R., B. K. Chen, S. Choe, and N. R. Landau.** 1995. Vpr is required for efficient replication of human immunodeficiency virus type 1 in mononuclear phagocytes. Virology **206:**935–944.
14. **Gao, F., S. G. Morrison, D. L. Robertson, C. L. Thornton, S. Craig, G. Karlsson, J. Sodroski, M. Morgado, B. Galvao-Castro, H. v. Briesen, S. Beddows, J. Weber, P. M. Sharp, G. M. Shaw, B. H. Hahn, and the WHO and NIAID Networks for Isolation and Characterization.** 1996. Molecular cloning and analysis of functional envelope genes from human immunodeficiency virus type 1 sequence subtypes A through G. J. Virol. **70:**1651–1667.
15. **Gorny, M. K., J. R. Mascola, Z. R. Israel, T. C. VanCott, C. Williams, P. Balfe, C. Hioe, S. Brodine, S. Burda, and S. Zolla-Pazner.** 1998. A human monoclonal antibody specific for the V3 loop of HIV type 1 clade E cross-reacts with other HIV type 1 clades. AIDS Res. Hum. Retrovir. **14:**213–221.
16. **Gorny, M. K., K. Revesz, C. Williams, B. Volsky, M. K. Louder, C. A. Anyangwe, C. Krachmarov, S. C. Kayman, A. Pinter, A. Nadas, P. Nyambi, J. R. Mascola, and S. Zolla-Pazner.** 2004. The V3 loop is accessible on the surface of most HIV-1 primary isolates and serves as a neutralization epitope. J. Virol. **78:**2394–2404.
17. **Gorny, M. K., T. C. VanCott, C. Hioe, Z. R. Israel, N. L. Michael, A. J. Conley, C. Williams, J. A. Kessler II, P. Chigurupati, S. Burda, and S. Zolla-Pazner.** 1997. Human monoclonal antibodies to the V3 loop of HIV-1 with intra- and interclade cross-reactivity. J. Immunol. **159:**5114–5122.
18. **Gorny, M. K., C. Williams, B. Volsky, K. Revesz, S. Cohen, V. R. Polonis, W. J. Honnen, S. C. Kayman, C. Krachmarov, A. Pinter, and S. Zolla-Pazner.** 2002. Human monoclonal antibodies specific for conformation-sensitive epitopes of V3 neutralize human immunodeficiency virus type 1 primary isolates from various clades. J. Virol. **76:**9035–9045.
19. **Gorny, M. K., J.-Y. Xu, S. Karwowska, A. Buchbinder, and S. Zolla-Pazner.** 1993. Repertoire of neutralizing human monoclonal antibodies specific for the V3 domain of HIV-1 gp120. J. Immunol. **150:**635–643.
20. **Heyndrickx, L., W. Janssens, P. M. Ndumbe, K. Vereecken, S. Coppens, K. De Houwer, K. Fransen, G. Van der Auwera, and G. van der Groen.** 2000. HIV-1 genetic variability in Cameroon. AIDS **14:**1862–1864.
21. **Heyndrickx, L., W. Janssens, L. Zekeng, R. Musonda, S. Anagonou, G. Van der Auwera, S. Coppens, K. Vereecken, K. De Witte, R. Van Rampelbergh, M. Kahindo, L. Morison, F. E. McCutchan, J. K. Carr, J. Albert, M. Essex, J. Goudsmit, B. Asjo, M. Salminen, A. Buve, Study Group on Heterogeneity of HIV Epidemics in African Cities, and G. van Der Groen.** 2000. Simplified strategy for detection of recombinant human immunodeficiency virus type 1 group M isolates by *gag*/*env* heteroduplex mobility assay. J. Virol. **74:**363–370.
22. **Javaherian, K., A. J. Langlois, C. McDanal, K. L. Ross, L. I. Eckler, C. L. Jellis, A. T. Profy, J. R. Rusche, D. P. Bolognesi, S. D. Putney, and T. J. Matthews.** 1989. Principal neutralizing domain of the human immunodefi-

ciency virus type 1 envelope protein. Proc. Natl. Acad. Sci. USA **86:**6768–6772.
23. **Kayman, S. C., Z. Wu, K. Revesz, H.-C. Chen, R. Kopelman, and A. Pinter.** 1994. Presentation of native epitopes in the V1/V2 and V3 regions of human immunodeficiency virus type 1 gp120 by fusion glycoproteins containing isolated gp120 domains. J. Virol. **68:**400–410.
24. **Korber, B. T., K. MacInnes, R. F. Smith, and G. Myers.** 1994. Mutational trends in V3 loop protein sequences observed in different genetic lineages of human immunodeficiency virus type 1. J. Virol. **68:**6730–6744.
25. **Kostrikis, L. G., Y. Cao, H. Ngai, J. P. Moore, and D. D. Ho.** 1996. Quantitative analysis of serum neutralization of human immunodeficiency virus type 1 from subtypes A, B, C, D, E, F and I: lack of direct correlation between neutralization serotypes and genetic subtypes and evidence for prevalent serum-dependent infectivity enhancement. J. Virol. **70:**445–458.
26. **Krachmarov, C. P., S. C. Kayman, W. J. Honnen, O. Trochev, and A. Pinter.** 2001. V3-specific polyclonal antibodies affinity purified from sera of infected humans effectively neutralize primary isolates of human immunodeficiency virus type 1. AIDS Res. Hum. Retrovir. **17:**1737–1748.
27. **Labrijn, A. F., and W. H. Parren.** 1999. Neutralizing epitopes of HIV-1, p. IV-18-34. *In* B. Korber, C. Brander, B. Haynes, J. Moore, R. Koup, B. Walker, and D. Watkins (ed.), HIV molecular immunology database 1999. Los Alamos National Laboratory, Los Alamos, N.M.
28. **Mboudjeka, I., L. Zekeng, J. Takehisa, T. Miura, E. Ido, M. Yamashita, L. Kaptue, and M. Hayami.** 1999. HIV type 1 genetic variability in the northern part of Cameroon. AIDS Res. Hum. Retrovir. **15:**951–956.
29. **McMichael, A. J., and T. Hanke.** 2003. HIV vaccines 1983–2003. Nat. Med. **9:**874–880.
30. **Moore, J. P., and D. D. Ho.** 1993. Antibodies to discontinuous or conformationally sensitive epitopes on the gp120 glycoprotein of human immunodeficiency virus type 1 are highly prevalent in sera of infected humans. J. Virol. **67:**863–975.
31. **Moore, J. P., A. Trkola, B. Korber, L. J. Boots, J. A. K. II, F. E. McCutchan, J. Mascola, D. D. Ho, J. Robinson, and A. J. Conley.** 1995. A human monoclonal antibody to a complex epitope in the V3 region of gp120 of human immunodeficiency virus type 1 has broad reactivity within and outside clade B. J. Virol. **69:**122–130.
32. **Muster, T., F. Steindl, M. Purtscher, A. Trkola, A. Klima, G. Himmler, F. Ruker, and H. Katinger.** 1993. A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. J. Virol. **67:**6642–6647.
33. **Nyambi, P., L. Heyndrickx, K. Vereecken, S. Burda, K. De Houwer, S. Coppens, M. Urbanski, C. Williams, P. Ndumbe, and W. Janssens.** 2002. Predominance of infection with HIV-1 circulating recombinant form CRF02_AG in major Cameroonian cities and towns. AIDS **16:**295–296.
34. **Nyambi, P. N., H. A. Mbah, S. Burda, C. Williams, M. K. Gorny, A. Nadas, and S. Zolla-Pazner.** 2000. Conserved and exposed epitopes on intact, native, primary human immunodeficiency virus type 1 virions of group M. J. Virol. **74:**7096–7107.
35. **Nyambi, P. N., A. Nadas, H. A. Mbah, S. Burda, C. Williams, M. K. Gorny, and S. Zolla-Pazner.** 2000. Immunoreactivity of intact virions of human immunodeficiency virus type 1 (HIV-1) reveals the existence of fewer HIV-1 immunotypes than genotypes. J. Virol. **74:**10670–10680.
36. **Parks, T. D., K. K. Leuther, E. D. Howard, S. A. Johnston, and W. G. Dougherty.** 1994. Release of proteins and peptides from fusion proteins using a recombinant plant virus proteinase. Anal. Biochem. **216:**413–417. 8179197.
37. **Pau, C. P., M. Kai, D. L. Holloman-Candal, C. C. Luo, M. L. Kalish, G. Schochetman, B. Byers, J. R. George, and the WHO Network for HIV Isolation and Characterization.** 1994. Antigenic variation and serotyping of HIV type 1 from four World Health Organization-sponsored HIV vaccine sites. AIDS Res. Hum. Retrovir. **10:**1369–1377.
38. **Pinter, A., W. Honnen, Y. He, M. Gorny, S. Zolla-Pazner, and S. Kayman.** 2004. The V1/V2 domain of gp120 is a global regulator of sensitivity of primary human immunodeficiency virus type 1 isolates to neutralization by antibodies commonly induced upon infection. J. Virol. **78:**5205–5215.
39. **Pinter, A., W. J. Honnen, S. Tilley, C. Bona, H. Zaghouani, M. K. Gorny, and S. Zolla-Pazner.** 1989. Oligomeric structure of gp41, the transmembrane protein of human immunodeficiency virus type 1. J. Virol. **63:**2674–2679.
40. **Plantier, J. C., S. Le Pogam, F. Poisson, L. Buzelay, B. Lejeune, and F. Barin.** 1998. Extent of antigenic diversity in the V3 region of the surface glycoprotein, gp120, of human immunodeficiency virus type 1 group M and consequences for serotyping. J. Virol. **72:**677–683.
41. **Schreiber, M., H. Müller, C. Wachsmuth, T. Laue, F. T. Hufert, M. D. V. Laer, and H. Schmitz.** 1997. Escape of HIV-1 is associated with lack of V3 domain-specific antibodies in vivo. Clin. Exp. Immunol. **107:**15–20.
42. **Schreiber, M., C. Wachsmuth, H. Müller, S. Odemuyiwa, H. Schmitz, S. Meyer, B. Meyer, and J. Schneider-Mergener.** 1997. The V3-directed immune response in natural human immunodeficiency virus type 1 infection is predominantly directed against a variable, discontinuous epitope presented by the gp120 V3 domain. J. Virol. **71:**9198–9205.
43. **Spenlehauer, C., S. Saragosti, H. J. A. Fleury, A. Kirn, A.-M. Aubertin, and C. Moog.** 1998. Study of the V3 loop as a target epitope for antibodies involved in the neutralization of primary isolates versus T-cell-line-adapted strains of human immunodeficiency virus type 1. J. Virol. **72:**9855–9864.
44. **Stamatatos, N. M., J. R. Mascola, V. S. Kalyanaraman, M. K. Louder, L. M. Frampton, D. L. Birx, and T. C. VanCott.** 1998. Neutralizing antibodies from sera of human immunodeficiency virus type 1-infected individuals bind to monomeric gp120 and oligomeric gp140. J. Virol. **72:**9656–9667.
45. **Tilley, S. A., W. J. Honnen, M. E. Racho, T. C. Chou, and A. Pinter.** 1992. Synergistic neutralization of HIV-1 by human monoclonal antibodies against the V3 loop and the CD4 binding site of gp120. AIDS Res. Hum. Retrovir. **8:**461–467.
46. **Tilley, S. A., W. J. Honnen, M. E. Racho, M. Hilgartner, and A. Pinter.** 1991. A human monoclonal antibody against the CD4 binding site of HIV-1 gp120 exhibits potent, broadly neutralizing activity. Res. Virol. **142:**247–259.
47. **Trkola, M. Purtscher, T. Muster, C. Ballaun, A. Buchacher, N. Sullivan, K. Srinivasan, J. Sodroski, J. P. Moore, and H. Katinger.** 1996. Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. J. Virol. **70:**1100–1108.
48. **VanCott, T. C., V. R. Polonis, L. D. Loomis, N. L. Michael, P. L. Nara, and D. L. Birx.** 1995. Differential role of V3-specific antibodies in neutralization assays involving primary and laboratory-adapted isolates of HIV type 1. AIDS Res. Hum. Retrovir. **11:**1379–1391.
49. **Wei, X., J. M. Decker, S. Wang, H. Hui, J. C. Kappes, X. Wu, J. F. Salazar-Gonzalez, M. G. Salazar, J. M. Kilby, M. S. Saag, N. L. Komarova, M. A. Nowak, B. H. Hahn, P. D. Kwong, and G. M. Shaw.** 2003. Antibody neutralization and escape by HIV-1. Nature **422:**307–312.
50. **Zhong, P., S. Burda, F. Konings, M. Urbanski, L. Ma, L. Zekeng, L. Ewane, L. Agyingi, M. Agwara, Saa, A. Z. E., T. Kinge, S. Zolla-Pazner, and P. Nyambi.** 2003. Genetic and biological properties of HIV type 1 isolates prevalent in villagers of the Cameroon equatorial rain forests and grass fields: further evidence of broad HIV type 1 genetic diversity. AIDS Res. Hum. Retrovir. **19:**1167–1178.
51. **Zhong, P., S. Burda, M. Urbanski, H. Kenfack, M. Tongo, L. Heyndrickx, A. Nanfack, J. Shang, L. Agyingi, S. Zolla-Pazner, L. Zekeng, and P. Nyambi.** 2002. HIV type 1 group M clades infecting subjects from rural villages in equatorial rain forests of Cameroon. J. Acquir. Immune Defic. Syndr. **31:**495–505.
52. **Zolla-Pazner, S., M. K. Gorny, P. N. Nyambi, T. C. VanCott, and A. Nadas.** 1999. Immunotyping of human immunodeficiency virus type 1 (HIV): an approach to immunologic classification of HIV. J. Virol. **73:**4042–4051.