JOURNAL OF VIROLOGY, June 2007, p. 6548–6562 Vol. 81, No. 12
0022-538X/07/$08.00+0 doi:10.1128/JVI.02749-06


# Dissecting the Neutralizing Antibody Specificities of Broadly Neutralizing Sera from Human Immunodeficiency Virus Type 1-Infected Donors[▽]

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Received 13 December 2006/Accepted 25 March 2007

**Attempts to elicit broadly neutralizing antibody responses by human immunodeficiency virus type 1 (HIV-1) vaccine antigens have been met with limited success. To better understand the requirements for cross-neutralization of HIV-1, we have characterized the neutralizing antibody specificities present in the sera of three asymptomatic individuals exhibiting broad neutralization. Two individuals were infected with clade B viruses and the third with a clade A virus. The broadly neutralizing activity could be exclusively assigned to the protein A-reactive immunoglobulin G (IgG) fraction of all three donor sera. Neutralization inhibition assays performed with a panel of linear peptides corresponding to the third hypervariable (V3) loop of gp120 failed to inhibit serum neutralization of a panel of HIV-1 viruses. The sera also failed to neutralize chimeric simian immunodeficiency virus (SIV) and HIV-2 viruses displaying highly conserved gp41-neutralizing epitopes, suggesting that antibodies directed against these epitopes likely do not account for the broad neutralizing activity observed. Polyclonal IgG was fractionated on recombinant monomeric clade B gp120, and the neutralization capacities of the gp120-depleted samples were compared to that of the original polyclonal IgG. We found that the gp120-binding antibody population mediated neutralization of some isolates, but not all. Overall, the data suggest that broad neutralization results from more than one specificity in the sera but that the number of these specificities is likely small. The most likely epitope recognized by the monomeric gp120 binding neutralizing fraction is the CD4 binding site, although other epitopes, such as the glycan shield, cannot be excluded.**

An effective human immunodeficiency virus type 1 (HIV-1) vaccine is urgently needed to contain the AIDS pandemic. Such a vaccine will likely be required to induce a virus-specific $CD8^+$ T-cell response and a neutralizing antibody (NAb) response. NAbs have been shown to protect against viral challenge in several animal models (1, 28, 35, 48, 49, 56, 60, 72). However, the enormous sequence diversity of HIV-1 presents a major complication, in that a globally effective vaccine must elicit broadly neutralizing antibodies (bNAbs), i.e., those capable of neutralizing a wide range of primary isolates. To date, the elicitation of such a bNAb response by HIV-1 vaccine candidates remains elusive (13, 84). Antibody responses to conserved regions of the functional envelope spike have been difficult to elicit, due at least in part to the restricted accessibility of these regions. Additional features of the viral spike that appear to contribute to difficulties in eliciting bNAbs include immunodominant loops that are highly variable in sequence, the masking of neutralizing epitopes by these variable loops, an immunologically "silent" glycan shield, differential epitope exposure on functional versus nonfunctional envelopes, and conformational flexibility (15, 37, 38, 40–42, 58, 61, 80, 81). However, two pieces of evidence suggest that a cross-reactive neutralizing response against HIV-1 can be achieved: (i) the discovery of broadly neutralizing monoclonal antibodies (MAbs) (3, 9, 12, 14, 19, 55, 76, 86) and (ii) the identification of broadly neutralizing polyclonal sera from HIV-1-infected individuals (7, 22, 57, 78).

The broadly neutralizing MAbs isolated to date from HIV-1-infected individuals are remarkable because of their ability to recognize and neutralize a diverse range of primary HIV-1 isolates within or across clades. For instance, the bNAb b12 exhibited neutralization of approximately 50% of viruses from a 90-isolate cross-clade panel (9). This broad neutralization of HIV-1 is likely attributable to recognition of a conserved epitope that overlaps with the CD4 receptor-binding site (CD4bs) (3, 12, 14, 66). In the same study, antibodies 2F5 and 4E10, which recognize adjacent sites on the highly conserved membrane proximal external region (MPER) of gp41 (55, 86), showed an even greater neutralization breadth, neutralizing

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▽ Published ahead of print on 4 April 2007.

67% and 100%, respectively, of the cross-clade virus panel. The antibody 2G12, which has a unique dimeric domain-exchanged structure that recognizes a carbohydrate cluster on the so-called silent face of gp120 (16, 67, 69, 76) also neutralized over 40% of the 90-virus panel. Finally, the MAb 447-52D exhibits fairly broad neutralization of sensitive clade B isolates, attributable to its recognition of a conserved motif at the tip of the V3 loop (19, 29, 73, 85). A number of studies suggested that the activity of these bNAbs may correlate with their recognition of functional envelope forms that mediate receptor binding and membrane fusion, whereas non-neutralizing Abs may recognize epitopes on nonfunctional envelope species that are not exposed on functional trimers (54, 62, 66, 68).

Natural infection most often results in a highly isolate-specific NAb response in which efficient responses against heterologous isolates are rare (65, 80). However, a few broadly neutralizing sera have been identified. It has been suggested that in up to 10% of infected patients, the antibody response matures and becomes cross-neutralizing, with prevention of infection by divergent strains having been shown in in vitro peripheral blood mononuclear cell (PBMC)-neutralization assays (7, 22). More specifically, when Beirnaert et al. (7) tested 17 primary isolates belonging to group M (subtypes A to H) and group O in a sensitive PBMC assay, the median titer for limited or noncross-neutralizing samples was smaller than 1:10, while for broadly cross-neutralizing samples, it was 1:160. bNAb responses, such as those seen in a limited number of HIV-1-infected individuals, could contribute significantly to protection in a vaccine situation, potentially making these sera a very valuable resource. Uncovering the NAb specificities in these sera would provide valuable insight into the design of potential HIV-1 vaccines. Moreover, dissecting the NAb responses in these sera may assist in the identification of additional bNAbs, which are important tools in the design and evaluation of vaccine antigens.

Only a few studies have examined the binding profiles of broadly neutralizing sera to different HIV-1 antigens and the envelopes of viral isolates present in these donors (6, 18, 63, 83). Most studies mapping serum NAb specificities have focused generally on immunized animals, whose sera typically exhibit weak neutralization of primary isolates (5, 31, 36, 46, 71). Similarly, sera from naturally infected individuals and vaccine recipients that have been mapped tend to show weak to moderate neutralization (4, 44, 47, 52, 77). These studies have largely approached the problem of mapping polyclonal sera specificities by examining binding titers to a range of HIV-1 antigens and peptides or through the use of neutralization competition assays. A recent study also probed the neutralizing specificities present in immunized animal sera by fractionating sera on linear HIV-1 peptides and recombinant gp120 monomers coupled to Sepharose beads and examining the neutralization capacities of the depleted sera (5). Finally, HIV-2 and simian immunodeficiency virus (SIV) constructs have been used to detect the presence of antibodies reactive with epitopes exposed upon CD4 envelope ligation (CD4i epitopes) and within the MPER of gp41 (8, 21, 82) in polyclonal sera. Here, we have combined and extended these mapping approaches to characterize the nature of the cross-NAb response in the sera of three HIV-1 asymptomatic individuals who have unusually broad neutralizing activities. Our results indicate that the neutralizing specificities in these HIV-1-infected donors are accounted for by the immunoglobulin G (IgG) fraction of their sera. The neutralizing specificities do not seem to map to the third hypervariable loop of gp120 or to the MPER of gp41 but may recognize the CD4bs, conformational epitopes that are not present on monomeric gp120, or carbohydrate epitopes on gp120. Finally, the data presented here suggest that the neutralization breadth in the sera investigated is due to multiple specificities, which may differ in their neutralization potencies.

## MATERIALS AND METHODS

**HIV-1-infected donor sera and polyclonal IgG.** HIV-1-infected donor sera were heat inactivated at 56°C for 30 min prior to use. Polyclonal IgG was purified from the sera by using protein A affinity chromatography (GE Healthcare) according to the manufacturer's instructions. IgG was eluted from the columns in 0.1 M citric acid, pH 3.0. Fractions containing IgG were neutralized, pooled, and dialyzed against phosphate-buffered saline (PBS), pH 7.4. IgG purity was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the concentration was determined by measuring the relative absorbance at 280 nm. To compare the neutralization capacity of purified IgG and column flowthrough, the samples were concentrated using an Amicon Ultra-15 (Millipore) to a volume equal to that of the original volume of serum that was purified. For this comparison and the peptide inhibition neutralization assays described below, FDA2 IgG was purified from a 2003 serum draw. However, due to limited availability of additional FDA2 serum from this sampling time, the gp120 fractionation experiments and large-scale neutralization panels described below were performed using IgG previously purified from serum drawn in 2000. To verify the consistency of results between the different time points, a subset of inhibition neutralization assays was also repeated with FDA2 IgG from 2000.

**MAbs and recombinant proteins.** The MAbs used in this study were 447-52D, kindly provided by S. Zolla-Pazner (19, 29, 30); F425 B4e8, kindly provided by L. Cavacini (17); 8.22.2, kindly provided by A. Pinter (33); 2G12, 2F5, and 4E10 (16, 55, 67, 69, 76, 86), kindly provided by H. Katinger; b12 and Z13, produced in-house (2, 3, 12, 14, 86), and human IgG against HIV-1, provided by John Mascola. IgG from healthy humans was purified as described above from HIV-1-negative blood donations from The Scripps Research Institute (TSRI) normal blood donor service. The sheep antibody D7324, raised against the C5 peptide, APTKAKRRWQREKR, was purchased from Cliniqa (Fallbrook, CA). Linear peptides (>90% pure by high-pressure liquid chromatography) were synthesized by The Scripps Research Institute core facility, and their sequences are listed in Table 2. The L36-V3 polypeptide used for inhibition neutralization assays was synthesized and provided by R. Stanfield and P. Dawson (TSRI). Recombinant $gp120_{JR\text{-}FL}$ was produced in HEK293T cells using recombinant vaccinia viruses designed to express the protein as previously described (25, 71). Wild-type $gp120_{JR\text{-}CSF}$ and core $gp120_{JR\text{-}CSF}$ were produced under contract at Advanced Product Enterprises (APE, Frederick, MD) in stably transfected *Drosophila* cell lines. Recombinant proteins were purified by $Ni_2^+$-nitrilotriacetic acid chromatography. The purity was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie staining. Recombinant $gp120_{JR\text{-}FL}$ ΔN2-mCHO-GDMR was produced as previously described (59). Recombinant $gp41_{HXB2}$ (residues 546 to 682; HXB2 numbering), expressed in *Pichia pastoris*, was purchased from Vybion.

**ELISA.** Enzyme-linked immunosorbent assay (ELISA) plate wells were coated overnight at 4°C with 50 μl of synthetic peptide at 4 μg/ml in PBS. Recombinant envelope antigens ($gp120_{JR\text{-}FL}$, $gp120_{JR\text{-}CSF}$, core $gp120_{JR\text{-}CSF}$, or gp41) were coated overnight at 2 μg/ml in PBS. Coated plates were washed four times with PBS containing Tween 20 (0.05%) and blocked with bovine serum albumin (BSA) (3% [wt/vol] in water) for 1 h at 37°C. Following aspiration of the BSA, MAbs or IgG samples, diluted in 1% BSA containing 0.02% Tween 20 (dilution buffer), were added to the wells and allowed to incubate for 2 h at 37°C. Subsequent steps were performed at room temperature. The wells were washed four times as before, and goat anti-human IgG $F(ab')_2$-alkaline phosphatase (Pierce), diluted 1:500 in dilution buffer, was added to the wells and allowed to incubate for 45 min. The wells were then washed four times and developed by adding 50 μl alkaline phosphatase substrate, which was prepared by adding one tablet of disodium *p*-nitrophenyl phosphate (Sigma) to 5 ml of alkaline phosphatase staining buffer (10 mM $MgCl_46H_2O$, 80 mM $Na_2CO_3$, 15 mM $NaN_3$ [pH 9.8]). At 20 and 30 min, plates were read at an optical density of 405 nm ($OD_{405}$) on a microplate reader (Molecular Devices). Competition ELISAs with polyclonal IgG and biotinylated 2G12 and b12 were performed as previously de-

scribed by Moore and Sodroski (53). Briefly, saturating amounts of polyclonal IgG were preincubated in triplicate wells with ELISA plates coated as described above. For competition with IgG b12, gp120 proteins were captured on plates coated with the anti-gp120-specific polyclonal antibody D7324. A concentration of biotinylated MAb previously determined to give a half-maximal ELISA signal was then added to the polyclonal IgG, and samples were incubated for 2 h at room temperature. Biotinylated 2G12 was detected using alkaline phosphatase-conjugated streptavidin (1:200 in dilution buffer; Vector Labs), and the plates were developed as described above. Biotinylated b12 was detected using peroxidase-conjugated streptavidin (1:2,000 in dilution buffer; Jackson ImmunoResearch Laboratories), developed with a TMB substrate kit (Pierce) according to the manufacturer's instructions, and read at $OD_{450}$. All peroxidase reactions were stopped with 0.2 M $H_2SO_4$ before an $OD_{450}$ value of 1.5 was reached.

**Viruses.** HIV-1 envelope-pseudotyped virus, capable of single-round replication, was generated for use by Monogram Biosciences as previously described (9). For in-house assays, single-round replication-competent HIV-1-pseudotyped virus was generated by cotransfection of 293T cells with the pNL4-3.luc.$R^-E^-$ vector (NIH AIDS Research and Reference Reagent Program [NIH ARRRP], provided by N. Landau) and the pSVIIIexE7 *env*-expressing vector (kindly provided by J. Sodroski) (34). Similarly, pseudotyped 6535.3 virus was generated by cotransfection of 293T cells with the pSG3Δenv vector (NIH ARRRP, contributed by J. Kappes and X. Wu) and the pSVIIIexE7 *env*-expressing vector.

The neutralization profiles for viruses used in the polyclonal IgG depletion experiments are as follows: JR-FL and 6535.3 are sensitive to neutralization by all four bNAbs, b12, 2G12, 4E10, and 2F5 (9, 45); similarly, ADA displays sensitivity to all four bNAbs, although this virus is somewhat more neutralization-resistant than JR-FL; 92RW020 is sensitive to neutralization by 2G12, 4E10, and 2F5 (9); and 92BR025.9 exhibits neutralization sensitivities to 2G12 and 4E10 similar to those of 92RW020.5 but is resistant to neutralization by b12 and 2F5.

**Neutralization assays.** Several HIV-1 pseudovirus neutralization assays were performed. In all assays, neutralization was measured as the percent reduction in the average amount of luciferase activity in lysates of infected target cells in comparison to antibody or serum-negative control wells.

**(i) Monogram neutralization assay.** Large neutralization panels were performed at Monogram Biosciences, as previously described (65). Briefly, pseudovirions containing a firefly luciferase gene were incubated with sera or antibodies (1 h at 37°C) and infectivity was determined by measuring luciferase activity in lysates of U87.CD4.CXCR4.CCR5 cells.

**(ii) In-house neutralization assays.** Totals of $1.5 \times 10^4$ U87.CD4.CCR5 cells (obtained from NIH ARRRP, contributed by H. Deng and D. Littman) or TZM-bl cells (NIH ARRRP, contributed by J. Kappes, X. Wu, and Tranzyme Inc.) (79) were seeded in 96-well plates (96-well flat bottom; Corning) in 100 μl of medium (Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 300 μg of G418/ml, glutamine, and penicillin-streptomycin) and incubated for 24 h at 37°C in 5% $CO_2$. Sixty microliters of medium containing $2 \times 10^5$ relative light units of pseudovirus was mixed with 60 μl of serially diluted IgG or sera and incubated for 1 h at 37°C. One hundred microliters of the virus mixtures was then added to the U87 cells and incubated for 3 days. On day three, 60 μl of cell culture lysis reagent (Promega, Madison, WI) was added to the cells and lysates were centrifuged at $1,250 \times g$ for 2 min at 4°C. Twenty microliters of lysate was transferred to opaque assay plates (Corning), luciferase reagent was added (Promega), and luciferase activity was measured on a luminometer (Orion; Berthold Detection Systems).

**(iii) Peptide inhibition neutralization assay.** Inhibition assays were performed as described above for in-house neutralization assays, except that serum dilutions were preincubated with peptide (final concentration, 30 or 50 μg/ml) for 30 or 60 min at 37°C before the addition of virus.

**(iv) HIV-2 and SIV MPER-specific neutralization assays.** Neutralization of SIVmac239 engrafted with 2F5 and 4E10 epitopes by donor sera was carried out as previously described by Yuste et al. (82). For neutralization of the HIV-2 chimeric virus, HIV-$2_{7312A}$ was engrafted with the full-length HIV-$1_{YU2}$ MPER sequence (residues 661 to 683, LALDKWASLWNWFDITKWLWYIK) and the assay performed as described by Decker et al. (21, 80).

**Depletion of IgG on gp120-coupled beads.** Recombinant gp120 preparations were coupled to cyanogen bromide-activated Sepharose 4B beads (GE Healthcare), as described previously by Beddows et al. (5). IgG samples were diluted in PBS to 9.6 μg/ml (for depletion on wild-type gp120-coupled beads) or 9.2 μg/ml (for depletion on core gp120-coupled beads). An aliquot (250 μl) of diluted sample was added to microcentrifuge tubes containing 50 μl of a 50% slurry of activated beads, which were then rotated end-over-end at 4°C overnight. The depleted IgG was recovered by centrifugation for 3 min at $3,000 \times g$, passed through a 0.22-μm syringe filter, and concentrated for subsequent ELISA and neutralization assays using Amicon Ultra-15 (Millipore) concentrators. An equal volume of untreated IgG was concentrated in parallel with the depleted samples as a control and appropriately diluted prior to use so as to match the dilution of the depleted IgG.

To confirm that conformational epitopes were preserved postcoupling and establish the amount of IgG that could be efficiently depleted on the beads, known amounts of IgG b12 were slurried with the beads and the depletion efficiency was assessed by ELISA and a JR-FL pseudovirus neutralization assay. The concentration of b12 remaining in depleted fractions was determined from standard curves and used to calculate the efficiency of the depletion. These depletions proved highly reproducible and were performed in parallel with all depletion experiments with polyclonal IgG. The b12 depletion efficiency of all experiments presented here was ≥92%. To assess the degree of nonspecific antibody binding to the antigen-coupled beads, g120-coupled beads were slurried with a solution containing the gp41-specific MAb 2F5. A substantially large shift between the ELISA binding curves of the initial and depleted 2F5 IgG fractions when $gp120_{JR\text{-}FL}$-coupled beads were tested was not observed, although ELISA curves of samples recovered from core $gp120_{JR\text{-}CSF}$-coupled beads revealed that a larger degree of nonspecific depletion occurred on these beads than on the $gp120_{JR\text{-}FL}$-coupled beads. However, as described in detail in Results, all fractions recovered from polyclonal IgG depletion experiments were subjected to careful ELISA analysis, to ensure specific depletion of the desired gp120-reactive antibodies.

## RESULTS

**Identification of broadly neutralizing sera.** We examined the NAb repertoires of three asymptomatic HIV-1-infected individuals, designated FDA2, LT2, and ITM1.

FDA2, identified by Vujcic and Quinnan (78) during their efforts to establish reference reagents for HIV-1-related studies, is an asymptomatic donor from the United States who was infected with a clade B virus more than 10 years ago. FDA2 was chosen for study because serum from this individual was previously reported to contain relatively broad neutralization activity (23, 24, 63, 78). The second HIV-1-infected donor, LT2, a long-term nonprogressor, was identified from a group of 23 HIV-infected individuals, 13 with primary HIV-1 infection and 10 long-term nonprogressors, attending a clinic in San Diego, CA (27, 65). LT2 serum was identified as a potentially broadly neutralizing serum based on the observation that 42 of 81 envelopes from primary isolates from clades A to E were neutralized by LT2 serum, with titers of 1:100 to 1:400, and NL4-3 was neutralized with a titer of 1:3,000. Viral loads for LT2 range from <50 to 3,000 copies/ml, and CD4 T-cell counts range from 800 to 1,100 cells/$mm^3$. LT2 was likely infected in 1985 and has never received antiretroviral therapy.

Finally, we desired a broadly neutralizing serum from an individual infected with a non-clade B isolate for study. ITM1 was selected from a cohort of 1,100 HIV-1-positive patients attending a clinic at the Institute of Tropical Medicine (ITM) in Antwerp, Belgium. Initial selection of 100 subjects was based on infection with non-clade B isolates, an antiretroviral therapy-naïve status, and regular attendance at the ITM clinic. Of the 100 individuals from whom informed consent was received, approximately 45 individuals donated plasma for screening in a PBMC neutralization assay (20). Neutralization of two to four isolates of each subtype (A, B, C, D, and E) was examined. Of the 10 patients whose plasma exhibited neutralization of at least 75% of isolates within a particular subtype, ITM1 showed the highest degree of cross-neutralization. ITM1 was infected by mother-to-child transmission in 1986. This HIV-1-infected donor has viral loads ranging from 1,000 to

60,000 HIV RNA copies/ml and CD4 T-cell counts ranging from 200 to 600 cells/mm$^3$.

**Cross-neutralization is mediated by IgG.** To confirm that the three sera contained cross-neutralizing activity, pseudovirus-based neutralization assays were performed. Neutralization activity was tested against a panel of 40 well-characterized isolates representing Env subtypes A ($n$ = 3), B ($n$ = 15), C ($n$ = 11), D ($n$ = 6), and AE ($n$ = 3). Control pseudoviruses included subtype B isolates JR-CSF and NL4-3 as positive controls and amphotropic murine leukemia virus (aMLV) as a control for nonspecific neutralization. Sera were tested in parallel with an internal reference serum, N16 (9). Due to limited availability of FDA2 serum from an appropriate sampling time point, previously purified FDA2 IgG was concentrated to 10 mg/ml for analysis, as an approximation of expected serum IgG levels.

The neutralization breadth of the sera is shown in Table 1. The LT2 and ITM1 sera exhibited very similar and extensive cross-clade neutralizing activities, positively neutralizing 40 and 39, respectively, of the 40 isolates tested (100% and 98%), with arithmetic mean 50% inhibitory concentration ($IC_{50}$) titers of 1:231 and 1:244, respectively (control viruses excluded). FDA2 IgG exhibited weaker cross-neutralization, positively neutralizing 11 of the 39 isolates tested (28%), with an arithmetic mean $IC_{50}$ titer of 1:63, and neutralized a larger number of clade B isolates than clade A or C isolates. Interestingly, a large difference in neutralization potency was observed between JR-FL ($IC_{50}$ of 1:2,821) and JR-CSF ($IC_{50}$ of 1:204) with LT2 serum. These viruses were originally isolated from different compartments of a single HIV-1-infected individual and share more than 90% sequence homology (Los Alamos HIV sequence database, http://www.hiv.lanl.gov; accession no. U63632 and M38429). The greatest degree of amino acid variation between the two gp120 envelope sequences maps to the V4 and V5 loops. Additionally, each virus contains three N-linked glycosylation sequences that are not present in the other envelope. When this unusually high neutralization titer for JR-FL is excluded, the mean cross-clade $IC_{50}$ titer for LT2 is 1:165.

To determine the component of the HIV-1-infected donor sera responsible for the cross-neutralizing activity, polyclonal IgG from each serum was purified on protein A-Sepharose columns. The neutralization capacity of each column fraction was compared to the degree of neutralization in the original sera. A total of four *env*-pseudotyped viruses were used in an in-house neutralization assay: JR-FL, ADA (subtype B), 92RW020.5 (subtype A), and 92BR025.9 (subtype C). In each case, we observed comparable neutralizing activity in the original sera and purified IgG, whereas little to no neutralization activity was present in the IgG-depleted fraction (Fig. 1).

**V3 peptides do not inhibit neutralization.** As a first approach to dissect the neutralizing activity in the three sera, clade B V3 peptides were tested as inhibitors in neutralization assays to determine the contribution of antibodies to linear V3 epitopes to the cross-neutralizing serum activity. The V3 peptides (Table 2) were 24 residues in length and corresponded to portions of the V3 loops of clade B isolates JR-FL, R2, and YU2. In addition to the peptides, a chimeric protein termed L36-V3 was used. This protein molecule was generated by Robyn Stanfield (TSRI) following a structural search of the Protein Data Base for proteins containing loops with conformations similar to those of the V3 peptide bound in the 447-52D crystal structure. The sequence of a loop of L36 (Protein Data Base accession no. 1DFE) was then changed to reflect that of a V3 loop from a clade B isolate, in hopes of obtaining a structural mimic of the V3 loop. The resulting protein, designated L36-V3, bound MAb 447-52D with only a twofold reduction in the half-maximal ELISA binding signal relative to that of wild-type $gp120_{JR\text{-}CSF}$. The peptides and the L36-V3 protein were tested for their ability to inhibit serum neutralization of the clade B isolates JR-FL, ADA, R2, and SF162. Representative results of these neutralization inhibition exper-

TABLE 1. Neutralization breadth of HIV-1-infected donor sera

| Virus | Clade | Serum $IC_{50}$ (1/diln) for:[a] | | | | |
|---|---|---|---|---|---|---|
| | | LT-2 | ITM1 | FDA2[b] | N16 1[c] | N16 2[c] |
| HIV-1 isolates | | | | | | |
| 92UG031 | A | 126 | 166 | <20 | 45 | 74 |
| 93RW029 | A | 139 | 234 | <20 | 37 | 75 |
| 93UG077 | A | 132 | 388 | 54 | 58 | 64 |
| 94UG103 | A | 215 | 184 | <20 | 60 | 72 |
| 92TH006 | AE | 129 | 275 | 25 | 74 | 68 |
| 92TH022 | AE | 126 | 406 | 34 | 50 | 35 |
| CMU02 | AE | 230 | 188 | 20 | 63 | 85 |
| CMU06 | AE | 192 | 193 | <20 | 75 | 115 |
| 302056 (=91US056) | B | 202 | 365 | 26 | 89 | 94 |
| 692 | B | 133 | 104 | | 43 | |
| 1168 | B | 56 | <20 | 64 | <20 | 22 |
| 1196 | B | 121 | 230 | <20 | 60 | 45 |
| 5768p27 | B | 197 | 146 | 34 | 77 | 47 |
| 6101p15 | B | 140 | 246 | 22 | 32 | 35 |
| 92BR020 | B | 298 | 290 | 91 | 67 | 29 |
| 92BR021 | B | 202 | 113 | <20 | 70 | 86 |
| 92HT593 | B | 62 | 115 | | 59 | |
| 92HT594 | B | 424 | 594 | 88 | 48 | 103 |
| 92US712 | B | 116 | 118 | 32 | 46 | 30 |
| 93TH305 | B | 298 | 468 | 92 | 76 | 74 |
| JRFL | B | 2821 | 277 | 104 | 69 | 65 |
| ME1 | B | 121 | 117 | 41 | 58 | 86 |
| QHO515 | B | 199 | 179 | <20 | 34 | 33 |
| QHO692 | B | | | 35 | | 80 |
| 21068 | C | 134 | 240 | <20 | 105 | 111 |
| 93MW959 | C | 170 | 420 | <20 | 63 | 88 |
| 93MW960 | C | 180 | 232 | 39 | 128 | 183 |
| 97ZA012 | C | 181 | 166 | <20 | 32 | 58 |
| 98BR004 | C | 210 | 276 | <20 | 90 | 76 |
| 98CN006 | C | 186 | 314 | 33 | 93 | 90 |
| 98CN009 | C | 154 | 297 | 43 | 74 | 77 |
| 98IN022 | C | 129 | 258 | 29 | 60 | 89 |
| 93IN905 | C | 173 | 297 | 30 | 62 | 79 |
| 93IN999 | C | 110 | 100 | <20 | 51 | 54 |
| 94IN11246-3 | C | 225 | 291 | 22 | 97 | 81 |
| 92UG001 | D | 124 | 87 | <20 | 44 | 65 |
| 92UG005 | D | 164 | 170 | 33 | 34 | 47 |
| 92UG024 | D | 81 | 130 | 42 | 55 | 109 |
| 92UG035 | D | 103 | 224 | 28 | 40 | 31 |
| 92UG046 | D | 115 | 249 | <20 | 61 | 86 |
| 94UG114 | D | 130 | 381 | 25 | 57 | 50 |
| *Control Viruses:* | | LT-2 | ITM-1 | FDA2 | N16 1 | N16 2 |
| JRCSF | B | 172 | 228 | 44 | 236 | 224 |
| JRCSF | B | 256 | 304 | 45 | 228 | 200 |
| JRCSF | B | 184 | 232 | 38 | 161 | 209 |
| NL43 | B | 2787 | 661 | 741 | 1273 | 1497 |
| NL43 | B | 3515 | 843 | 748 | 1313 | 1096 |
| NL43 | B | 2511 | 683 | 799 | 1456 | 1132 |
| aMLV | - | 22 | 32 | <20 | <20 | <20 |
| aMLV | - | 30 | 22 | <20 | <20 | <20 |
| aMLV | - | 22 | 34 | <20 | <20 | <20 |

[a] Serum neutralization was assessed for a panel of isolates representing Env subtypes A, B, C, D, and AE, and the reciprocal of the dilution (1/diln) at which virus infectivity is inhibited by 50% is reported. Nonspecific neutralization was assessed using aMLV. White boxes represent samples that did not meet the criteria for a positive neutralization result, based on the neutralization titer against aMLV (9). Positive neutralization is color-coded as follows: orange, 1,000 to 5,000; yellow, 300 to 999; green, 50 to 299; blue, <50; pink, not tested.

[b] Due to limited availability of serum from the appropriate time, FDA2 IgG (concentrated to 10 mg/ml) was examined for cross-neutralization.

[c] N16 serum was run in parallel with HIV-infected donor sera (N16 1 with LT2 and ITM1, N16 2 with FDA2) as an internal reference.

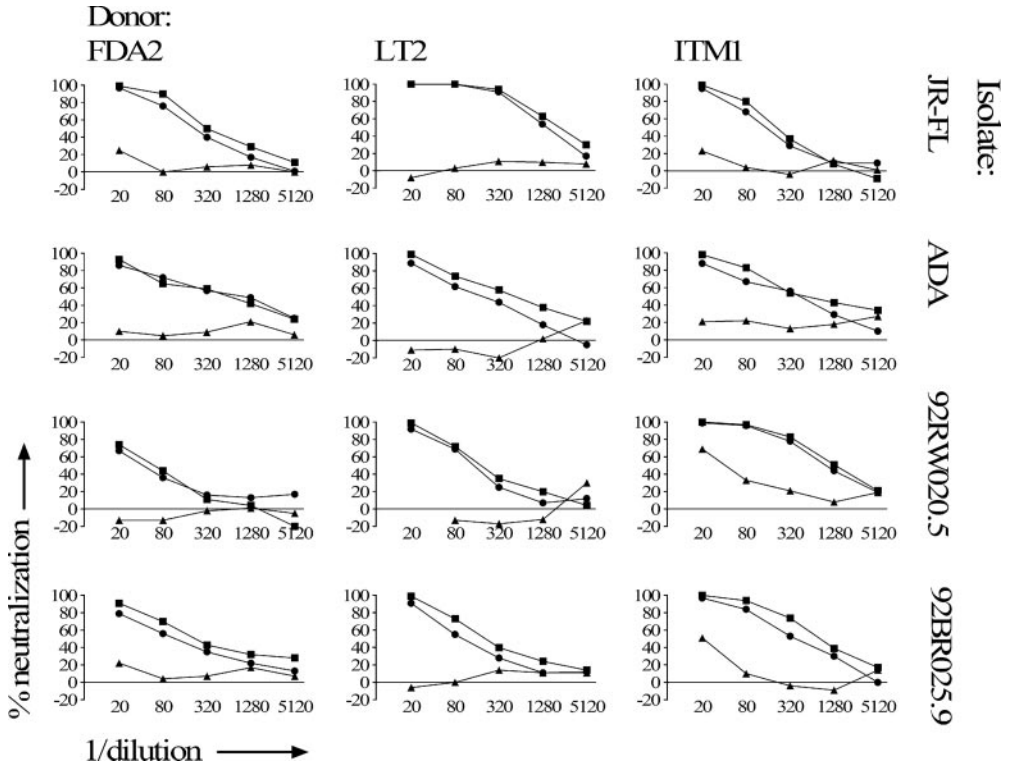


FIG. 1. Neutralization curves of IgG fractions purified from sera on protein A. The neutralization capacity of purified polyclonal IgG (●) is compared with those of the column flowthrough (▲) and original serum (■). To allow comparison of all samples, the purified IgG and column flowthrough were concentrated to volumes equal to that of the original volume of serum run over the column. Neutralization of FDA2, LT2, and ITM1 column fractions was compared for the subtype B isolates JR-FL and ADA, the subtype A isolate 92RW020.5, and the subtype C isolate 92BR025.9.

iments are shown in Fig. 2. Despite strong serum reactivity in the ELISA, no inhibition of serum neutralization was observed. In comparison, pseudovirus neutralization by the V3 MAbs 447-52D or F425 B4e8 was efficiently inhibited by the V3 peptides, thus demonstrating the ability of the peptides to inhibit V3-directed neutralizing activity.

Peptides corresponding to the V1 and V2 loops and the C-terminal portion of the V3 loop of JR-FL were also tested for their ability to inhibit serum neutralization of the corresponding isolate, JR-FL. However, as above, no inhibition was observed.

TABLE 2. Sequences of linear peptides used for inhibition neutralization assays

| Peptide | Sequence | Length |
|---|---|---|
| JR-FL V3 | CTRPNNNTRKSIHIGPGRAFYTTG | 24-mer |
| YU2 V3 | CTRPNNNTRKSINIGPGRALYTTG | 24-mer |
| R2 V3 | CSRPNNNTRKSIPMGPGRAFYTTG | 24-mer |
| L36 V3[a] | . . .RHVIGPGR. . . | 38-mer |
| ITM-1 V3 | TRTSVHIGPGQAFYATG | 17-mer |
| C-terminal portion JR-FL V3 | GRAFYTTGEIIGDIRQAH | 18-mer |
| JR-FL V2 | NITTSIRDEVQKEYALFYKLDVVPIDNNT | 30-mer |
| JR-FL V1 | KDVNATNTTNDSEGTMERGEIKN | 24-mer |

[a] The sequence of the portion of L36 that was mutated to reflect a V3 loop sequence is shown; the flanking bacterial protein sequence is not included.

Finally, a linear peptide corresponding to the V3 sequence of a clade A virus isolated from ITM1 was tested for its ability to inhibit ITM1 plasma neutralization of isolates JR-FL, SF162 (clade B), ITM1, 92UG037.8, 92RW020.5 (clade A), and 92BR025.9 (clade C). Again, no inhibition of neutralization was observed in the presence of the peptide. Of note, pseudovirus R2 and the corresponding R2 peptide included in the V3 neutralization panel represented an autologous FDA2 isolate.

**MPER-specific neutralization activity is not detected in broadly neutralizing sera.** Although traditional antibody binding assays, such as the ELISA, allow detection of antibodies specific for a given epitope in polyclonal sera, the neutralization activities of these antibody populations cannot be as readily determined. The use of SIV and HIV-2 chimeras displaying defined HIV-1 epitopes to detect NAbs specific for the given epitope in polyclonal HIV-1-infected donor sera permitted the assessment of whether 2F5- and 4E10-like NAbs, or other MPER-reactive antibodies, are present in the three sera (8, 82).

The presence of MPER-specific NAbs was first examined using SIVmac239 chimeras containing the 2F5 and 4E10 epitopes, which have been shown to be specifically neutralized by MAbs 2F5 and 4E10, respectively. The sera were assayed for their ability to neutralize both these chimeric SIV viruses and the parental SIVmac239 virus. No significant neutralization of any of the viruses was observed for any of the three sera.

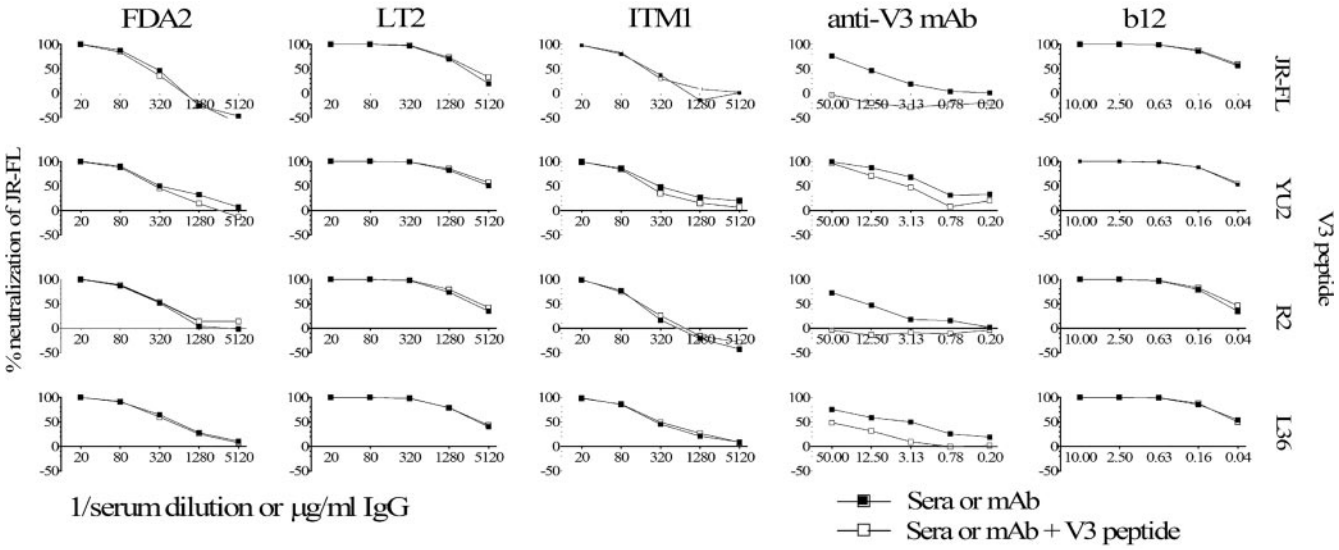


FIG. 2. Neutralization of JR-FL by HIV-infected donor sera and MAb controls in the presence or absence of competing V3 peptides. Peptides representing portions of immunodominant variable loops of clade B isolates (Table 2) were tested for their ability to inhibit serum neutralization of four primary clade B isolates. Representative neutralization curves are shown for JR-FL, YU2, R2, and L36 V3 peptides tested for their ability to inhibit serum neutralization of JR-FL. Serum or IgG neutralization of JR-FL is shown by the closed symbols, and neutralization in the presence of the competitor peptides is shown by the open symbols. Anti-V3 MAb 447-52D (tested with L36-V3) or F425 B4e8 (tested with JR-FL, YU2, and R2 V3) was used as a positive control for neutralization inhibition in the presence of the V3 peptides, and MAb b12 was tested as a negative control.

The results of the analysis of our sera in this SIV system were recently reported as part of a study by Yuste et al. (82).

Sera were also tested for their ability to neutralize an HIV-2 chimera displaying the complete HIV-$1_{YU2}$ MPER sequence. The neutralization specificity of the chimeric virus was confirmed with MAbs 2F5 and 4E10, which neutralized the corresponding chimera with $IC_{50}$ values of 0.16 and 0.13 μg/ml but had no effect ($IC_{50} \gg 10$ μg/ml) on the control virus lacking the cognate epitope (Fig. 3, bottom panels). A comparison of serum neutralization of wild-type HIV-$2_{7312A}$ with this HIV-$2_{7312A}$ chimera confirmed that MPER-specific NAbs were not present at detectable levels (Fig. 3, top panels) despite the extraordinary sensitivity of the chimeric virus to control MPER-specific human MAbs. Interestingly, FDA2 serum exhibited weak neutralization of wild-type HIV-$2_{7312A}$ that is not attributable to reactivity against the MPER epitope. This activity has been observed in about 1% of HIV-1 sera tested for their ability to neutralize the HIV-2 strain used in this study

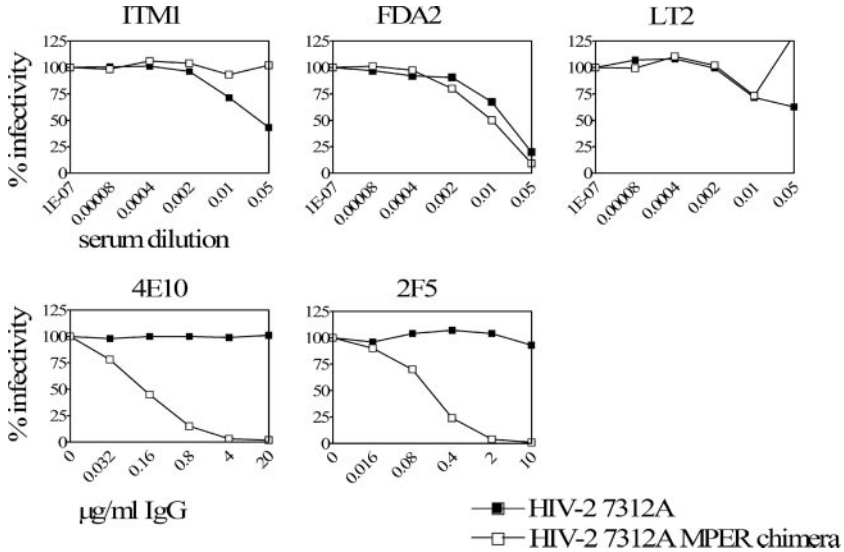


FIG. 3. Neutralization of HIV-$2_{7312A}$ and chimeric HIV-$2_{7312A}$ containing the complete HIV-$1_{YU2}$ MPER. The presence of NAbs specific for the MPER region of gp41 was evaluated by comparing the abilities of sera (top panels) to neutralize a chimeric HIV-$2_{7312A}$ virus with the full-length HIV-$1_{YU2}$ MPER grafted into an appropriate location and the parental HIV-$2_{7312A}$ virus. The neutralization specificities were assessed using MAbs 4E10 and 2F5 (bottom panels).

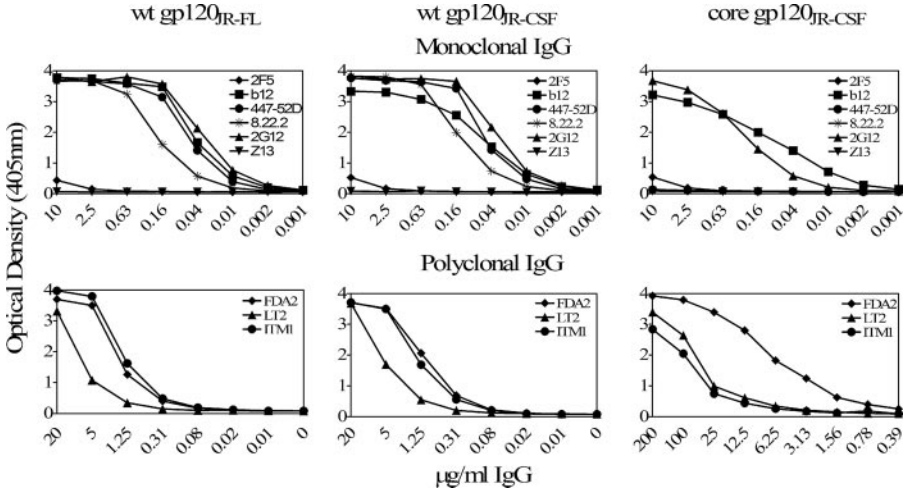


FIG. 4. Antigenic profiles of recombinant gp120 monomers used in serum depletion experiments. MAb and polyclonal IgG binding to $gp120_{JR\text{-}FL}$, $gp120_{JR\text{-}CSF}$, and core $gp120_{JR\text{-}CSF}$ were analyzed.

(G. Shaw, unpublished data). It has been reported that certain residues within the bridging sheet of gp120, some of which are involved in the formation of the chemokine receptor binding site, are conserved across certain HIV-1 and HIV-2 isolates (21). The serum neutralization activity exhibited against HIV-$2_{7312A}$, as well as the efficient recognition of core gp120 by FDA2 IgG (Fig. 4), could potentially be due to recognition of these conserved residues.

**Antigenic profiles of recombinant $gp120_{JR\text{-}FL}$, $gp120_{JR\text{-}CSF}$, and core $gp120_{JR\text{-}CSF}$ monomers to be used in serum depletion studies.** A polyclonal IgG depletion assay was established to investigate the contribution of antibodies specific for epitopes on gp120 to cross-neutralization. Three recombinant clade B monomers were chosen for these studies, $gp120_{JR\text{-}FL}$, $gp120_{JR\text{-}CSF}$, and core $gp120_{JR\text{-}CSF}$ monomer. The $gp120_{JR\text{-}FL}$ protein was produced using the vaccinia virus system in 293T cells, whereas the $gp120_{JR\text{-}CSF}$ was expressed using a *Drosophila* expression system (described in Materials and Methods). The core $gp120_{JR\text{-}CSF}$, in which the N and C termini and V1, V2, and V3 loops are truncated, was also expressed using a *Drosophila* expression system. These proteins were chosen because they correspond to well-characterized isolates and have representative clade B sequences. The core $gp120_{JR\text{-}CSF}$ was also selected for these studies so as to further assess the contribution of variable loop-reactive antibodies to cross-neutralization.

The results of the ELISA binding studies showed that both wild-type gp120 proteins reacted very similarly to gp120-specific MAbs, as well as to polyclonal IgG (Fig. 4). Antibodies specific for the CD4bs (b12), the V2 loop (8.22.2), the V3 loop (447-52D), and gp120 glycans (2G12) bound with very similar affinities to both envelopes. The core $gp120_{JR\text{-}CSF}$ monomer did not react with V2 and V3 loop-specific antibodies, but retained binding to b12 and 2G12, although with a somewhat lower affinity relative to that of wild-type gp120. The anti-gp41 antibodies 2F5 and Z13 did not bind any of the gp120s, as would be expected. The three HIV-infected donor IgGs bound similarly to both $gp120_{JR\text{-}FL}$ and $gp120_{JR\text{-}CSF}$, but bound more weakly to the core $gp120_{JR\text{-}CSF}$. Notably, FDA2 IgG reacted more strongly than ITM1 or LT2 IgG with core $gp120_{JR\text{-}CSF}$.

**Depletion of polyclonal HIV-infected donor IgG on gp120-coupled beads.** To examine the role of clade B gp120 monomer-reactive antibodies in our broadly neutralizing samples, polyclonal IgG was fractionated on the gp120-coupled beads. The initial and depleted IgG fractions were first tested by ELISA for specific removal of the desired antibodies, and then the neutralization capacities of the fractions were compared. ELISA binding of the initial and depleted samples to the gp120 antigen of interest demonstrated that antibodies reactive with the antigen coupled to the Sepharose beads were no longer significantly present in the depleted fractions (Fig. 5, left panels). In contrast, the binding titers to gp41 remained very similar (Fig. 5, right panels), thus demonstrating specific removal of gp120-reactive antibodies. In some cases, a small shift in gp41 binding titers between the initial and depleted IgG fractions was observed, likely representing a small degree of nonspecific binding to the beads during the depletion experiments.

The reduction of the neutralization capacity of LT2 IgG depleted on the gp120-coupled beads is shown in Fig. 6. A notable decrease in the neutralization capacity of fractionated LT2 IgG for the clade B isolate JR-FL was observed with $gp120_{JR\text{-}FL}$. Similarly, a striking decrease in the neutralization capacity was observed for the clade B isolate ADA when IgG was fractionated against $gp120_{JR\text{-}FL}$, indicating that the antibody specificities predominantly responsible for neutralizing both of these isolates are reactive with epitopes present on the clade B gp120. A similar decrease in the neutralization capacity for ADA was also observed when IgG was depleted of $gp120_{JR\text{-}CSF}$-reactive antibodies. In contrast, a much smaller decrease in the neutralization capacity for clade B isolate 6535.3 was observed when polyclonal IgG was depleted of antibodies reactive with $gp120_{JR\text{-}FL}$, suggesting that a different set of NAb specificities is dominant in the neutralization of this third clade B isolate.

At the highest concentrations tested, the results of the LT2 depletion experiments look quite similar. However, when lower concentrations are considered, differences in neutralization can be seen for the different isolates. Specifically, as men-

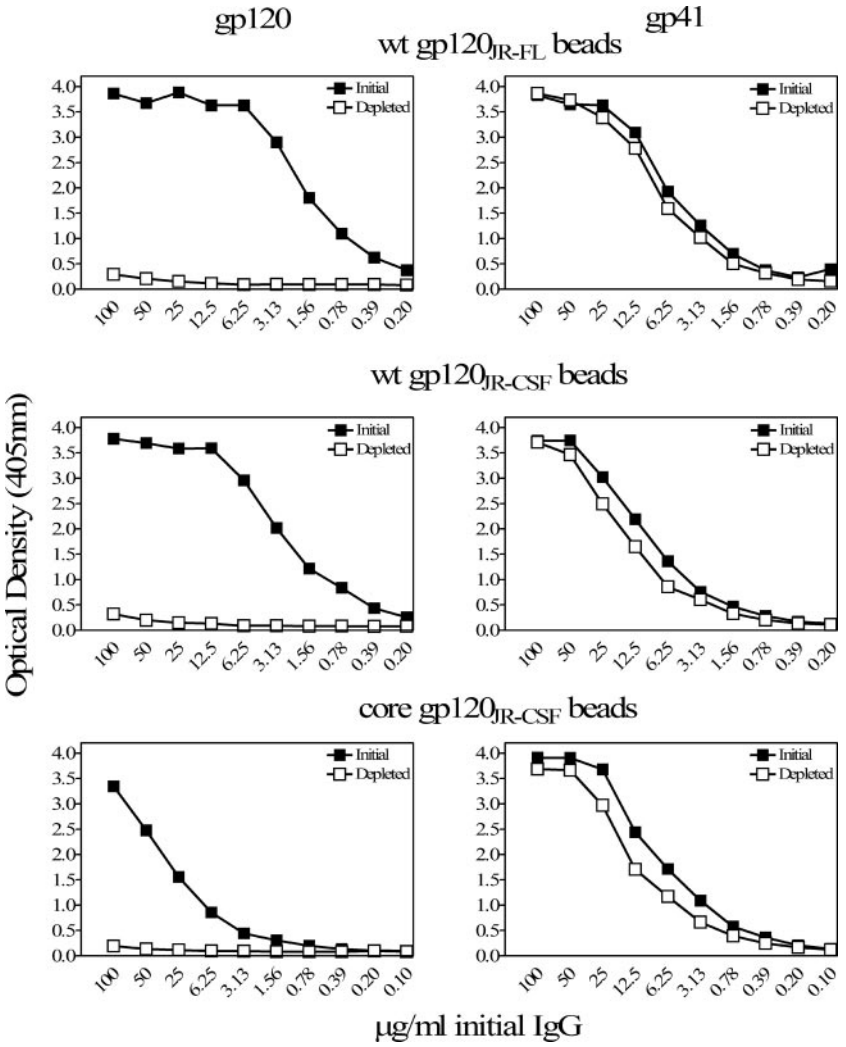


FIG. 5. ELISA binding curves of polyclonal ITM1 IgG depleted on gp120-coupled beads. Polyclonal ITM1 IgG was incubated with $gp120_{JR\text{-}FL}$-, $gp120_{JR\text{-}CSF}$-, and core $gp120_{JR\text{-}CSF}$-coupled beads. The initial and depleted IgG fractions were titered on wild-type or core gp120 and gp41 to assess the removal of antigen-specific antibodies in each depletion experiment. wt, wild-type.

tioned above, we observed that the neutralization of JR-FL and ADA by the depleted fraction differed from the neutralization of 6535.3 at lower concentrations. At the highest concentration point, the depleted fractions may still reach an $IC_{50}$ against JR-FL and ADA, but at the two lowest concentrations, no significant neutralization of these isolates was observed. In contrast, the depleted IgG retained its activity against 6535.3 at all dilutions. This concentration-dependent neutralization activity could be due to the presence of several specificities in the HIV-1-infected donor IgG. At higher concentrations, specificities with lower affinities for the depletion antigens or gp41-specific antibodies that were not depleted may exhibit some degree of neutralization activity. However, as the antibody concentration decreased, the neutralization activity attributed to this lower-affinity population would also diminish accordingly.

Although a large degree of the clade B neutralization activity present in LT2 IgG could be mapped to epitopes present on $gp120_{JR\text{-}FL}$, depletion of this antigen had a smaller effect on the neutralization of the clade A and C isolates tested. Interestingly, little or none of the broad neutralization activity was removed using the core $gp120_{JR\text{-}CSF}$-coupled beads.

The results of depletion experiments carried out with polyclonal ITM1 IgG are shown in Fig. 7. Here, we observed that $gp120_{JR\text{-}FL}$ depletion had a different effect on the neutralization of isolate 6535.3 than on the neutralization of all other isolates tested. Again, at the highest IgG concentration, the results of all depletion experiments were quite similar, while at the lower concentrations, differences became clear. As discussed above, this difference may be explained by the presence of antibodies with lower affinities for the depletion antigen or gp41-reactive antibodies that exhibit neutralization at higher concentrations. As seen above for LT2 IgG depletion experiments, the core $gp120_{JR\text{-}CSF}$ failed to remove most of the cross-clade neutralization activity.

Due to the weaker potency of polyclonal FDA2 IgG in comparison to our other two polyclonal IgGs, a more limited set of depletion experiments was carried out (Fig. 8). As seen

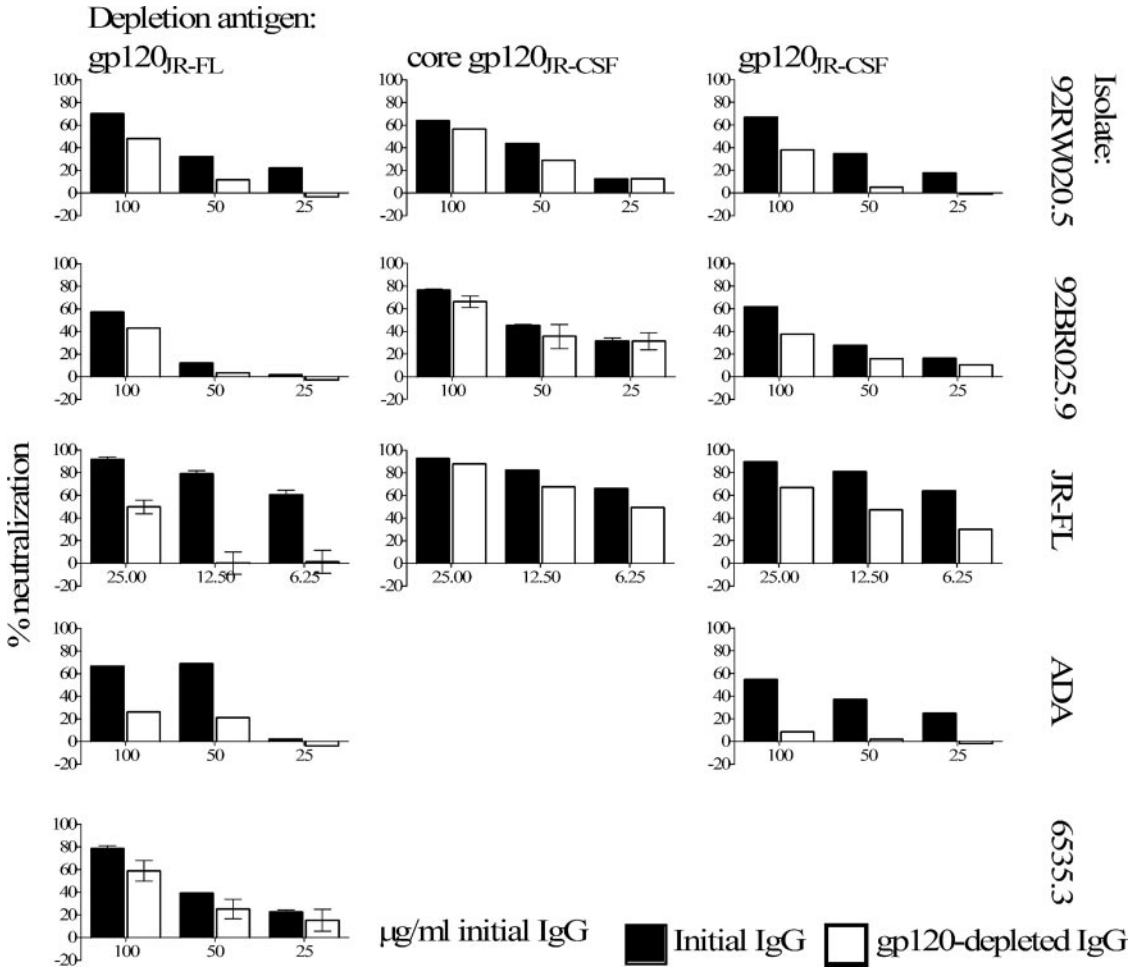


FIG. 6. Neutralization of polyclonal LT2 IgG depleted on gp120-coupled beads. Polyclonal LT2 IgG was incubated with beads coupled with $gp120_{JR-FL}$, core $gp120_{JR-CSF}$, or $gp120_{JR-CSF}$. The neutralization capacities of the initial and depleted fractions were tested against clade A isolate 92RW020.5, clade C isolate 92BR025.9, and clade B isolates JR-FL, ADA, and 6535.3. Error bars represent results from two independent experiments.

with LT2, a striking decrease of the neutralization capacity for JR-FL was observed when the FDA2 IgG was depleted of $gp120_{JR-FL}$ monomer-reactive antibodies and, again, a smaller proportion of the cross-clade neutralization activity was removed with this antigen. The core $gp120_{JR-CSF}$-coupled beads once again failed to reduce the neutralization capacities of HIV-1-infected-donor IgGs.

In light of the failure of the core $gp120_{JR-CSF}$-coupled beads to remove any substantial cross-neutralizing serum activity, an additional set of neutralization inhibition assays was performed to further investigate the contribution of antibodies specific for the V1, V2, and V3 loops. As previously described, peptides corresponding to the V1 and V2 loops and the C-terminal portion of the V3 loop of JR-FL (Table 2) were tested for their ability to inhibit serum neutralization of the homologous isolate JR-FL. However, no inhibition of this isolate was observed. As detailed below, the results of the depletion experiments carried out with the core gp120 monomer, combined with the results of peptide inhibition neutralization assays, could potentially be explained by the presence of antibodies sensitive to conformational changes, which therefore exhibit differential binding to the wild-type and core gp120 monomers.

**Serum IgGs compete with b12 for binding to wild-type gp120 but do not compete for b12 binding to a mutant having greatly limited access to the CD4bs.** The presence of CD4bs antibodies in the three polyclonal IgGs was next examined by competition ELISA. The ability of b12 to compete with HIV-1-infected donor IgG for binding to $gp120_{JR-FL}$ and a hyperglycosylated $gp120_{JR-FL}$ mutant was compared. The hyperglycosylated mutant, designated $gp120_{JR-FL}$ ΔN2-mCHO-GDMR, does not bind conventional nonneutralizing CD4bs antibodies but retains binding of the broadly neutralizing CD4bs antibody b12 (59). As shown in Table 3, 100 μg/ml of FDA2, LT2, and ITM1 IgGs was able to efficiently inhibit binding of biotinylated b12 to the wild-type $gp120_{JR-FL}$, indicating that each of the three polyclonal IgGs contains a CD4bs-reactive antibody population. In contrast, however, the three polyclonal IgG samples did not inhibit binding of b12 to the $gp120_{JR-FL}$ mutant at 100 μg/ml. This suggests that any CD4bs antibodies present in the three sera and responsible for broad

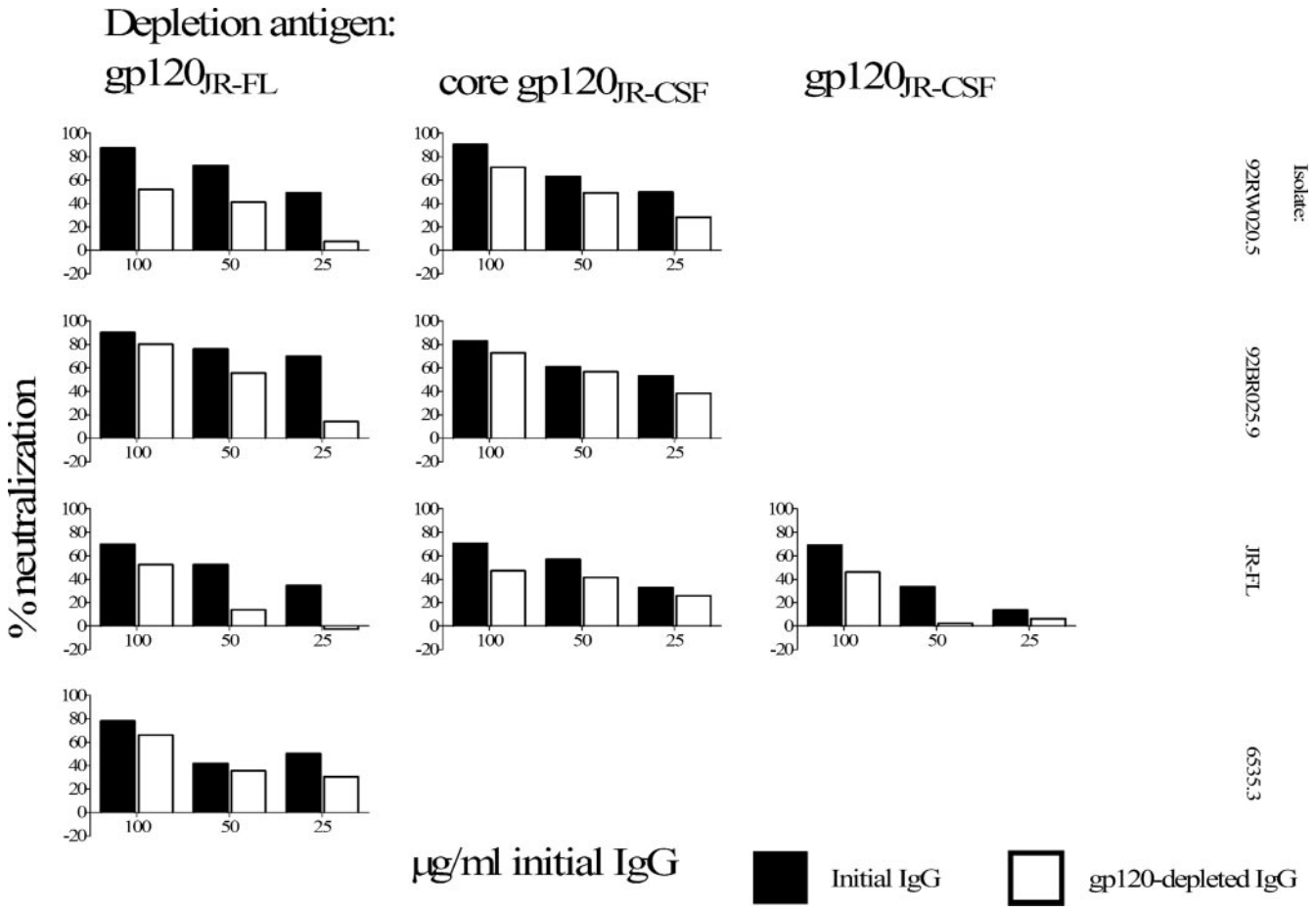


FIG. 7. Neutralization results of polyclonal ITM1 IgG depleted on gp120-coupled beads. Polyclonal ITM1 IgG was incubated with $gp120_{JR\text{-}FL}$-, core $gp120_{JR\text{-}CSF}$-, and $gp120_{JR\text{-}CSF}$-coupled beads. The neutralization capacities of the initial and depleted fractions were tested against clade A isolate 92RW020.5, clade C isolate 92BR025.9, and clade B isolates JRFL and 6535.3. Representative results from two independent experiments are shown for IgG depleted against core $gp120_{JR\text{-}CSF}$-coupled beads and tested for neutralization against 92BR025.9.

neutralization must recognize the CD4bs at least somewhat differently than b12.

## DISCUSSION

In this study, we addressed the basis of the broad neutralizing activity in the sera of three selected HIV-1-infected, asymptomatic donors. Our results show that the neutralization activity is mediated by the protein A-purified IgG component in the sera. Linear peptides representing the third hypervariable loop (V3) of gp120 did not inhibit neutralization in competition assays, and the broadly neutralizing sera also failed to react significantly with chimeric HIV-2 and SIV viruses displaying highly conserved gp41-neutralizing epitopes on their envelopes. To more thoroughly address the nature of the neutralizing specificities, polyclonal IgG was fractionated on recombinant monomeric gp120. Interestingly, the neutralization activity was completely depleted by gp120 for some viruses, yet for other isolates, the removal of a large population of monomeric gp120-reactive antibodies translated into a limited effect on neutralization. These observations are considered, along with other aspects of this study, in more detail below.

The ELISA results clearly demonstrated, in the three cases studied, the complete removal of a high concentration of gp120-specific IgG from the HIV-1-infected donor samples by gp120 affinity chromatography. Strikingly, however, the removal of this population of antibodies had various effects on the neutralization activities, dependent on the serum and test virus. For example, neutralization of isolates JR-FL and ADA by LT2 IgG is predominantly mediated by the $gp120_{JR\text{-}FL}$-reactive antibody population. However, this population of antibodies was responsible for only a small fraction of the serum IgG neutralization of isolate 6535.3. In addition, fractionation of LT2 IgG on $gp120_{JR\text{-}FL}$ and $gp120_{JR\text{-}CSF}$ resulted in a similar reduction in the neutralization of ADA, whereas fractionation on the two gp120 proteins had a different effect on the neutralization of JR-FL. This observation suggests that the antibody population mediating neutralization of ADA is likely cross-reactive with both gp120 proteins, whereas the unusually potent neutralization of JR-FL is likely due to a population of predominantly JR-FL-specific antibodies. Clearly, more than one specificity is responsible for the broad neutralizing activity of LT2 serum.

Further complexities in the nature of the neutralizing species became evident even within the gp120-reactive fractions

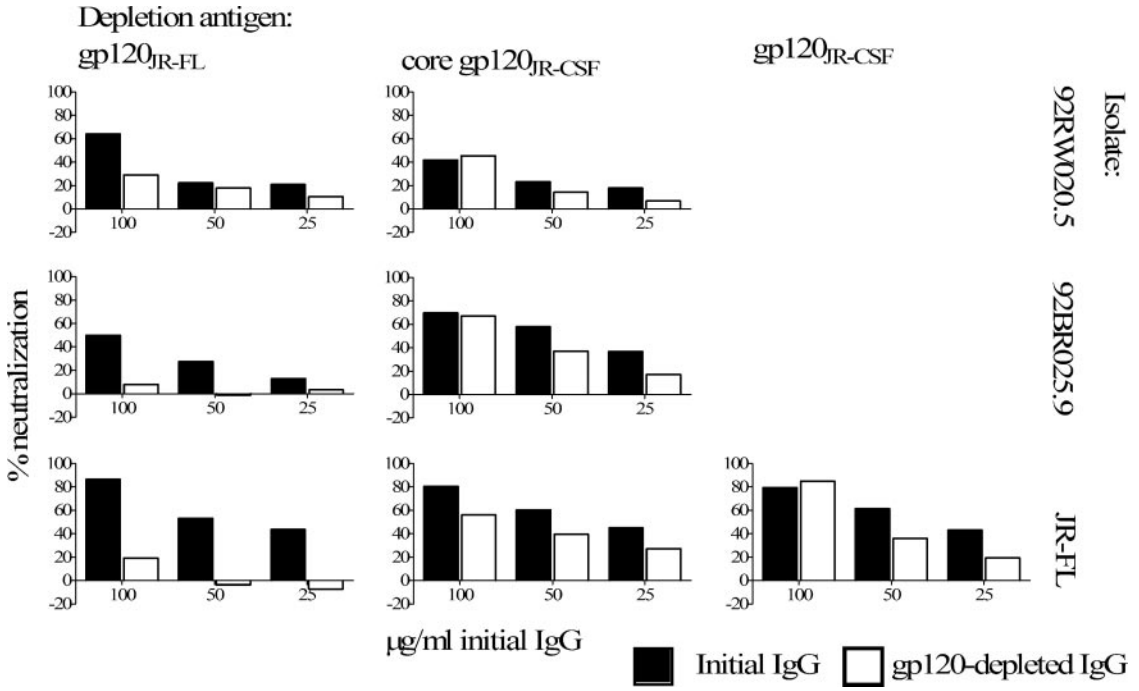


FIG. 8. Neutralization results of polyclonal FDA2 IgG depleted on gp120-coupled beads. Polyclonal FDA2 IgG was incubated with $gp120_{JR\text{-}FL}$-, core $gp120_{JR\text{-}CSF}$-, and $gp120_{JR\text{-}CSF}$-coupled beads. The neutralization capacities of the initial and depleted fractions were tested against clade A isolate 92RW020.5, clade C isolate 92BR025.9, and clade B isolate JR-FL. Representative results from two independent experiments are shown for IgG depleted against $gp120_{JR\text{-}FL}$-coupled beads and tested for neutralization against JR-FL and for IgG depleted against core $gp120_{JR\text{-}CSF}$-coupled beads and tested for neutralization against 92BR025.9.

from all three sera. The gp120-depleted samples often retained a certain degree of neutralizing activity at the highest IgG concentration, suggesting that more than one neutralizing population is present in the sera. The activity in the gp120-depleted samples may be due to a lower-affinity or gp41-reactive population that exhibits weak neutralizing activity at the highest IgG concentration. As the antibody concentration is diluted out, the neutralization activity of this lower-affinity population is also likely to decrease.

Although the patterns of neutralization described here suggest that more than one neutralizing specificity is present in each of the sera studied, the number of specificities is likely relatively limited. Fractionation on a gp120 from a single isolate does deplete the neutralization activity for multiple isolates within a single HIV-1-infected donor sample. For example, $gp120_{JR\text{-}FL}$ fractionation of ITM1 IgG led to a similar reduction in neutralization for isolates 92RW020.5 (clade A), 92BR025.9 (clade C), and JR-FL (clade B). Fractionation of LT2 IgG on $gp120_{JR\text{-}FL}$ also led to a similar reduction in neutralization for isolates JR-FL and ADA. If the broad neutralizing activity of the sera were due to the accumulation of a very large number of specificities against variable parts of the viral envelope, it would not be possible to fractionate activity on a single gp120.

TABLE 3. Serum IgGs compete with b12 for binding to wild-type gp120 but do not compete for b12 binding to a mutant having greatly limited access to the CD4bs

| Competitor IgG (µg/ml) | % Binding of biotinylated b12 to $gp120_{JR\text{-}FL}$[a] for | |
|---|---|---|
| | Wild type | ΔN2-mCHO-GDMR |
| FDA2 (100) | 13 | 101 |
| LT2 (100) | 24 | 99 |
| ITM1 (100) | 56 | 97 |
| Human IgG against HIV-1 (100) | 22 | 103 |
| Healthy human IgG[b] (100) | 98 | 102 |
| b12 (1) | 2 | 6 |
| b12 (0.1) | 71 | 53 |
| b12 (0.01) | 97 | 101 |
| Biotinylated b12 alone[c] | 100 | 100 |

[a] Results are reported as the percent binding signal of biotinylated b12 in the presence of a competitor relative to the binding signal of biotinylated b12 in the absence of the competitor.
[b] Healthy human IgG was purified from HIV-1-negative samples as a control.
[c] Biotinylated b12 binding was measured in the absence of competing IgG.

The inability of core $gp120_{JR\text{-}CSF}$ to deplete neutralization activity against the isolates tested could be due to the presence of antibodies specific for the truncated variable loops, as these antibodies would not be absorbed by core gp120. This is not likely to be the case for a number of reasons. Linear peptides corresponding to the V3 loop were unable to inhibit neutralization of the isolates tested in a competition format. Studies have shown that, in solution, linear peptides can efficiently inhibit binding of V3 MAbs to native gp120 proteins (50, 51). Our inhibition neutralization assays were performed in solution, and therefore, it is likely that all of the antibodies specific for the presented V3 sequences were absorbed. Although the contribution of V1- or V2-specific neutralization was not tested for all the isolates used in our depletion experiments, peptides representing the V1 and V2 loops of $gp120_{JR\text{-}FL}$ did not inhibit serum neutralization of the corresponding isolate JR-FL. The

failure of core gp120 to remove the neutralization activity could be explained by the presence of antibodies specific for conformational epitopes that are perturbed by truncation of variable loops and termini. In this case, NAbs with certain conformational epitopes may bind core gp120 with lower affinity and thus deplete inefficiently.

A broadly neutralizing response is most likely to arise from recognition of exposed conserved elements of the envelope spike, such as the receptor and coreceptor binding sites on gp120, and regions of gp41, such as the MPER. We sought to investigate whether the MPER could explain the neutralization breadth in our three sera using SIV and HIV-2 viruses engineered to display a single HIV-1 MPER epitope on their envelopes. Because serum from HIV-1-infected patients does not normally exhibit significant neutralization of SIV and HIV-2 viruses, the chimeric viruses allow detection of NAbs specific for the HIV-1 MPER displayed on the envelope (82). The three sera were tested for their ability to neutralize SIVmac293 chimeras displaying the 4E10 and 2F5 epitopes and an HIV-$2_{7312A}$ chimera displaying the full-length HIV-$1_{YU2}$ MPER on their envelopes. None of the sera displayed significant neutralization of any of these chimeric viruses, even though these viruses are readily neutralized by HIV-1-specific anti-MPER NAbs. LT2 serum, however, did exhibit weak reactivity with the SIVmac239-4E10 chimera, but this activity did not map to the purified IgG fraction. This weak activity may be due to nonantibody factors in the serum, such as chemokines and cytokines, as previous work has shown that these factors can contribute to in vitro neutralization (11). Our results are in agreement with those recently published by Yuste et al. (82) which suggest that 4E10- and 2F5-like neutralizing specificities are rare in HIV-1-infected patients. Of note, 4E10 and 2F5 are both of the IgG3 subclass, which reacts poorly with protein A (39, 70). However, in our three sera, IgG3 subtype antibodies are not responsible for the broad neutralizing activity, as we have demonstrated that the neutralizing specificities of interest are those reactive with protein A.

A recent study reported higher levels of 2G12-like antibodies present in broadly neutralizing sera from long-term nonprogressors (10). Our analysis by competition ELISA did not show a significant decrease in the binding of biotinylated 2G12 in the presence of 100 μg/ml of polyclonal IgG, with a detection sensitivity of 2 μg/ml of 2G12-like antibodies. These results indicate that 2G12-like antibodies are not present at high concentrations in the three HIV-1-infected donors studied here. However, we cannot exclude the possibility that other carbohydrate-specific antibodies are present in the sera. Future experiments, such as fractionation of sera on peptide-*N*-glycosidase F-treated gp120, will be required in order to address this issue in more detail.

Perhaps the best candidate epitope to explain gp120-reactive neutralization is the CD4bs. Recognition of this highly conserved gp120 epitope can translate into broad neutralization of HIV-1 isolates, as seen for IgG b12 (2, 9). In all of the experimental conditions used in our depletion experiments, the gp120-coupled beads efficiently depleted IgG b12. If b12-like antibodies specific for conserved epitopes overlapping with the CD4bs were present in the sera, it should be possible to absorb and deplete the neutralization activity of more than one isolate on a single gp120, as we observed for LT2 and ITM1 IgG fractionated on $gp120_{JR-FL}$. However, the case for b12-like CD4bs antibodies is complicated when gp120-reactivity cannot explain the neutralizing activity, as we observed for isolate 6535.3. Moreover, although the capacity of core $gp120_{JR-CSF}$-coupled beads was lower than that of the wild-type gp120-coupled beads, under the experimental conditions used, IgG b12 was also depleted on the core-coupled beads. The core $gp120_{JR-CSF}$, however, failed to significantly deplete the neutralization activity against the isolates we tested. As suggested earlier, it is possible that certain CD4bs epitopes, distinct from the b12 epitope, are perturbed by the truncation of variable loops and termini, and as a result, these CD4bs antibodies are not depleted on core gp120. The ability of the three polyclonal IgGs to compete with b12 for binding to wild-type $gp120_{JR-FL}$, but their inability to block b12 binding to a b12-specific $gp120_{JR-FL}$ mutant, also suggest, that the CD4bs antibodies present in the three sera recognize epitopes distinct from the precise b12 epitope. It is also possible that certain neutralizing CD4bs epitopes exist that are not well presented on monomeric gp120 in comparison to their presentation on native envelope trimers, and hence, fractionation on monomeric gp120 fails to remove the neutralizing activity in these cases. We also cannot exclude the possibility that antibodies specific for oligomeric forms of envelope contribute toward neutralization in cases where monomeric gp120 fails to remove the neutralizing activity.

The serum neutralization profiles of these HIV-infected donors make them ideal candidates for the selection of HIV-1-neutralizing MAbs. Identification of bNAbs would help unambiguously determine the fine specificities that give rise to the cross-neutralizing activity in the serum and would help guide the design of new vaccine antigens. Screening of antibody display libraries, such as phage and yeast libraries (2, 12, 26), and the improved rescue of human memory B cells (75) may prove valuable in identifying the monoclonal neutralizing specificities present in the antibody repertoires of these HIV-1-infected donors.

The question also arises as to how these broadly neutralizing responses evolved during the course of natural infection. Individuals with broad responses have often been infected over long periods, and their neutralizing repertoire may be shaped gradually over time by extensive somatic hypermutation and affinity maturation of B-cell clones specific for neutralizing epitopes. For instance, one study suggested that somatic hypermutation and class-switching can account for up to a 100-fold increase in neutralization potency (74). Interestingly, we have observed a loss of neutralizing breadth for FDA2. A recent neutralization panel performed with FDA2 serum drawn in 2005 indicates a reduction in the cross-neutralizing activity compared to that of a serum sample collected in 2000. Only five of the isolates in the cross-clade panel shown in Table 1 were neutralized by 2005 FDA2 serum, whereas thirteen of these isolates were neutralized by the 2000 FDA2 serum. This loss could result from continual evolution of the antibody repertoire and eventual displacement of clones specific for broadly neutralizing epitopes, presented at a lower frequency, from bone marrow survival niches by new plasma cells (32, 43, 64). As suggested by others (43), these observations imply that multiple boosts may be required in a vaccine setting to promote the required maturation of the antibody response and

generate memory B cells at a frequency sufficient to achieve long-lived protection.

## ACKNOWLEDGMENTS

We acknowledge support from grants from the National Institutes of Health (AI33292 to D.R.B. and AI057039 to W.E.J.), the International AIDS Vaccine Initiative (IAVI) Neutralizing Antibody Consortium, the Bill & Melinda Gates Foundation grand challenges program (to G.M.S.), the UCSD Center for AIDS Research (AI36214 to D.D.R.), the Natural Sciences and Engineering Research Council (NSERC) of Canada (to A.K.D.), Funds for Scientific Research, Flanders, Belgium (FWO) (travel grants to H.D. and grant G.0302.01), and the Belgian American Educational Foundation (BAEF) (to H.D.).

We are grateful to H. Katinger, L. Cavacini, J. Mascola, A. Pinter, and S. Zolla-Pazner for kindly providing gp120- and gp41-specific MAbs. We thank R. Stanfield and P. Dawson for providing the L36-V3 protein, T. Wrin at Monogram Biosciences for large-scale neutralization data, and A. Hessell for assistance with IgG purification.

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