

Antibody Specificities Associated with Neutralization Breadth in Plasma from Human Immunodeficiency Virus Type 1 Subtype C-Infected Blood Donors^{∇†}

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Defining the specificities of the anti-human immunodeficiency virus type 1 (HIV-1) envelope antibodies able to mediate broad heterologous neutralization will assist in identifying targets for an HIV-1 vaccine. We screened 70 plasmas from chronically HIV-1-infected individuals for neutralization breadth. Of these, 16 (23%) were found to neutralize 80% or more of the viruses tested. Anti-CD4 binding site (CD4bs) antibodies were found in almost all plasmas independent of their neutralization breadth, but they mainly mediated neutralization of the laboratory strain HxB2 with little effect on the primary virus, Du151. Adsorption with Du151 monomeric gp120 reduced neutralizing activity to some extent in most plasma samples when tested against the matched virus, although these antibodies did not always confer cross-neutralization. For one plasma, this activity was mapped to a site overlapping the CD4-induced (CD4i) epitope and CD4bs. Anti-membrane-proximal external region (MPER) ($r = 0.69$; $P < 0.001$) and anti-CD4i ($r = 0.49$; $P < 0.001$) antibody titers were found to be correlated with the neutralization breadth. These anti-MPER antibodies were not 4E10- or 2F5-like but spanned the 4E10 epitope. Furthermore, we found that anti-cardiolipin antibodies were correlated with the neutralization breadth ($r = 0.67$; $P < 0.001$) and anti-MPER antibodies ($r = 0.6$; $P < 0.001$). Our study suggests that more than one epitope on the envelope glycoprotein is involved in the cross-reactive neutralization elicited during natural HIV-1 infection, many of which are yet to be determined, and that polyreactive antibodies are possibly involved in this phenomenon.

The generation of an antibody response capable of neutralizing a broad range of viruses remains an important goal of human immunodeficiency virus type 1 (HIV-1) vaccine development. Despite multiple efforts in the design of immunogens capable of inducing such humoral responses, little progress has been made (18, 20, 39). The sequence variability of the virus, as well as masking mechanisms exhibited by the envelope glycoprotein, has further hindered this pursuit (6, 22). It is known that while the majority of HIV-infected individuals mount a strong neutralization response against their own virus within the first 6 to 12 months of infection, breadth is observed in only a few individuals years later (5, 10, 15, 26, 33, 40, 41). However, very little is known about the specificities of the antibodies that confer this broad

cross-neutralization. It is plausible that broadly cross-neutralizing (BCN) plasmas contain antibodies that target conserved regions of the envelope glycoprotein, as exemplified by a number of well-characterized broadly neutralizing monoclonal antibodies (MAbs). The b12 MAb recognizes the CD4 binding site (CD4bs), and 2G12 binds to surface glycans (7, 42, 44, 56). The 447-52D MAb recognizes the V3 loop, and 17b, E51, and 412d bind to CD4-induced (CD4i) epitopes that form part of the coreceptor binding site (13, 21, 51, 54). Finally, the MAbs 2F5, 4E10, and Z13e1 recognize distinct linear sequences in the gp41 membrane-proximal external region (MPER) (36, 57). The targets of these neutralizing MAbs provide a rational starting point for examining the complex nature of polyclonal plasma samples.

Several groups have addressed the need to develop methodologies to elucidate the presence of certain neutralizing-antibody specificities (1, 8, 9, 29, 30, 43, 55). A number of these studies reported that the BCN antibodies in plasma can in some cases be adsorbed using gp120 immobilized on beads (1, 9, 29, 30, 43). Furthermore, the activities of some of these anti-gp120 neutralizing antibodies could be mapped to the

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CD4bs, as the D368R mutant gp120 failed to adsorb them (1, 29, 30, 43).

Antibodies to CD4i epitopes are frequently found in HIV-1-infected individuals and are thought to primarily target the coreceptor binding site, which includes the bridging sheet and possibly parts of the V3 region. Decker and colleagues (8) showed that MAbs to HIV-1 CD4i epitopes can neutralize HIV-2 when pretreated with soluble CD4 (sCD4), indicating that the CD4i epitope is highly conserved among different HIV lineages. The poor accessibility of CD4i epitopes, however, has precluded this site from being a major neutralizing-antibody target (24), although a recent study suggested that some of the cross-neutralizing activity in polyclonal sera mapped to a CD4i epitope (30).

Another site that has attracted considerable attention as a target for cross-neutralizing antibodies is the MPER, a linear stretch of 34 amino acids in gp41. Anti-MPER antibodies have been detected in the plasma of HIV-infected individuals by using chimeric viruses with HIV-1 MPER grafted into a simian immunodeficiency virus or an HIV-2 envelope glycoprotein (15, 55). These studies concluded that 2F5- and 4E10-like antibodies were rarely found in HIV-1-infected plasmas; however, other specificities within the MPER were recognized by around one-third of HIV-1-infected individuals (15). More recently, 4E10-like and 2F5-like antibodies (30, 43), as well as antibodies to novel epitopes within the MPER (1), have been shown to be responsible for neutralization breadth in a small number of plasma samples. The anti-MPER MAb 4E10 has been shown to react to autoantigens, leading to the suggestion that their rarity in human infection is due to the selective deletion of B cells with these specificities (17, 35). Furthermore, a recent study found an association between anti-MPER and anti-cardiolipin (CL) antibodies, although an association with neutralization was not examined (31).

A recent study by Binley and coworkers used an array of methodologies to determine the antibody specificities present in subtype B and subtype C plasma samples with neutralization breadth (1). While antibodies to gp120, some of which mapped to the CD4bs, and to MPER were identified, most of the neutralizing activity in the BCN plasma could not be attributed to any of the known conserved envelope epitopes. Furthermore, it is not clear how common these specificities are among HIV-1-positive plasmas and whether they are only associated with BCN activity.

In this study, we investigated a large collection of HIV-1-infected plasmas obtained from the South African National Blood Services. We aimed to determine if there is a relationship between the presence of certain antibody specificities, such as those against CD4i epitopes, MPER, or the CD4bs, and the neutralizing activities present in these plasmas. Furthermore, we evaluated the presence of various autoreactive antibodies and analyzed whether they might be associated with neutralization breadth.

MATERIALS AND METHODS

Plasma samples and viruses. Anonymous plasma aliquots from 107 HIV-1-infected blood units were obtained from the South African National Blood Service in Johannesburg (19). The detection of recent infections was performed using the BED capture enzyme immunoassay (Calypte Biomedical Corporation, MD) (37). This study received ethical approval from the University of the

Witwatersrand. The envelope clones QHO692.42, CAAN5342.A2, AC10.0.29, Du151.2, Du172.17, and Du156.12 have been previously described (27, 28) and are available from the NIH AIDS Research and Reference Reagent Program. The envelope clones CAP45.11, CAP210.H8, CAP239.G3, and COT6.15 were previously cloned in our laboratory (14, 15). HIV-2 7312A and derived MPER chimeras were obtained from George Shaw (University of Alabama, Birmingham).

Neutralization assays. Neutralization was measured as a reduction in luciferase gene expression after a single round of infection of JC53bl-13 cells, also known as TZM-bl cells (NIH AIDS Research and Reference Reagent Program; catalog no. 8129), with Env-pseudotyped viruses (32). Titers were calculated as the 50% inhibitory concentration or the reciprocal plasma/serum dilution causing 50% reduction of relative light units (ID_{50}). Anti-MPER specific activity was measured using HIV-2 7312A and HIV-2/HIV-1 MPER chimeric constructs (15). To evaluate anti-CD4i antibodies, HIV-2 7312A was preincubated with 9 nM of sCD4 (R&D Systems, Minneapolis, MN) before the diluted plasma/serum was added.

gp120 production and isolation. The gp120-encoding region of the envelope clone Du151.2 was inserted into the pPPI4 expression vector (Progenics Pharmaceuticals, Inc., Tarrytown, NY) (2). The D368R, E370A, and I420R mutations were introduced into gp120 using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The resulting constructs were transfected into 293T cells seeded in a HyperFlask (Corning Inc., Lowell, MA) using Fugene (Roche Applied Science, Indianapolis, IN). The cell supernatant was collected after 48 h and every second day thereafter for another three harvests. gp120 was isolated using *Galanthus nivalis* lectin agarose matrix (Sigma-Aldrich, St. Louis, MO) and eluted with 1 M methyl- α -D-mannopyranoside (Sigma). The remaining protein contaminants were eliminated through ion-exchange chromatography using Fast-Flow Q-Sepharose (GE Healthcare Life Science, Piscataway, NJ), equilibrated in phosphate-buffered saline (PBS), and reconstituted in 2 M NaCl/PBS. The pure protein was collected in the flowthrough, washed in PBS, and concentrated to 5 mg/ml. The purity of the final gp120 preparation was tested by running 10 μ g of protein in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein preparations with purity higher than 99% were used in subsequent experiments.

Serum adsorption of anti-gp120 antibodies and antibody elution. Adsorption and elution of anti-gp120 antibodies were done as previously described (30), using Du151 gp120 covalently coupled to tosyl-activated magnetic beads. The antibody concentrations in the eluates were determined using the immunoglobulin G (IgG) quantification enzyme-linked immunosorbent assay (ELISA) protocol described below. Anti-gp120 activity was measured by ELISA as previously described (30).

IgG quantification ELISA. Goat anti-human IgG antibody (Sigma-Aldrich) was immobilized in a 96-well high-binding ELISA plate (Corning) in carbonate-bicarbonate buffer (Sigma-Aldrich) overnight at 4 μ g/ml. The plates were washed four times in PBS-0.05% Tween 20 and blocked with 5% goat serum-5% skim milk in PBS-0.05% Tween 20 (dilution buffer). The eluted antibodies were serially diluted and added to the plate for 1 h at 37°C. The bound IgG was detected using a total anti-human IgG-horseradish peroxidase conjugated at 1:1,000 (Sigma-Aldrich). The ELISA was developed with TMB substrate (Thermo Fisher Scientific, Rockford, IL) and stopped with 1 M H_2SO_4 . The plates were read at 450 nm on a microplate reader (Molecular Devices, Sunnyvale, CA). A standard curve was run on each plate with serial dilutions of a commercial human IgG (Sigma-Aldrich).

Autoantibody assays. An anti-CL ELISA was used as previously described (16, 49). Autoantibodies (to SSA/Ro, SS-B/La, Sm, ribonucleoprotein [RNP], Jo-1, double-stranded DNA [dsDNA], centromere B, and histone) were measured by the FDA-approved AtheNA Multi-Lyte ANA II Test Kit from Zeus Scientific, Inc., according to the manufacturer's instructions and as described previously (17). Assays to measure IgM rheumatoid factor (RF) using IgG antigen were performed as previously described (52).

Statistical analysis. Statistical analysis was performed using the software GraphPad Prism 4.0 and R 2.7.0. Medians of viruses neutralized by the positive and negative groups of each of the antibody specificities were compared using a Mann-Whitney U test. A Kruskal-Wallis rank sum test was used to evaluate the differences in the median numbers of viruses neutralized between groups of plasmas categorized by their antibody specificities. Spearman rank tests were used to determine the correlation between two variables. Univariate and multivariate linear regression models were used to study the factors associated with neutralization breadth. First, a univariate model was run using the positivity calls for each of the five antibody specificities as a predictor. To pick a multivariate model, an exhaustive search was performed on all models, including the five predictors. The Bayesian information criterion (47) was used to choose the best-fitting model. Additionally, the same exhaustive search was used to select

the best model from the set of all models, including the five main effects and their second-order interactions.

RESULTS

Identifying samples with BCN activity. Plasma samples from 107 HIV-1-seropositive blood donor units were obtained from the South African National Blood Services in 2005. A BED capture enzyme immunoassay identified 37 of these samples (35%) as having a low titer of anti-HIV specific IgG relative to total IgG, indicating that seroconversion had probably occurred within the previous 180 days. Since broadly neutralizing antibodies are unlikely to be present in samples from recent infections, we excluded these from further analysis.

The 70 samples identified as chronic HIV-1 infections were screened for neutralization breadth against 10 HIV-1 pseudoviruses, including 7 subtype C (Du151.2, Du172.17, Du156.12, CAP45.11, CAP210.H8, CAP239.G3, and COT6.15) and 3 subtype B (QHO692.42, CAAN5342.A2, and AC10.0.29) viruses (see Table S1 in the supplemental material). Except for Du151 and COT6, all of the envelope clones are part of the subtype C or subtype B tier 2 standard reference panels (27, 28). Du151 and COT6 are primary viruses with a tier 2 phenotype (14). Sixteen plasmas were able to neutralize 8 or more of these viruses, two of which (BB70 and BB105) neutralized all 10 viruses. These 16 samples are designated BCN plasmas throughout the study. The frequency of neutralization breadth in this blood donor cohort was therefore 16/70 (23%).

A heat map of the log neutralization titers of all 70 plasma samples against all 10 pseudoviruses is shown in Fig. 1. The dendrogram at the top of the figure shows two clusters of five viruses, with those on the right (COT6, Du156, Du172, Du151, and AC10) being more sensitive to neutralization, as titers against these viruses were considerably higher. All of these envelopes were obtained from peripheral blood mononuclear cell-cultured virus isolates, while the remaining viruses, except for QHO692, were cloned from plasma RNA (27, 28). There was no difference in neutralization sensitivity between subtype B and C viruses, as they were distributed in both the resistant and less resistant groups. The dendrogram on the left split the plasmas into three groups. Group 1 contained 20 plasma samples with no neutralizing activity. The second group of plasmas ($n = 27$) showed weak to intermediate neutralizing-antibody activity, characterized mostly by low titers and sporadic strain-specific high titers, such as that of BB89 against AC10. Plasma BB105 fell into this category; while the sample was able to neutralize all the viruses, it did so at very low titers. Group 3 plasmas ($n = 23$) had the highest titers covering multiple viruses and included 14 of the 16 samples categorized as BCN.

Contribution of anti-CD4bs antibodies to neutralization. Recent studies using antibody adsorption and elution from gp120-coated beads have mapped the activities of some BCN sera to the CD4bs (1, 29, 30, 43). Following a similar approach, we used a subtype-matched recombinant gp120 to assess the presence of CD4bs antibodies in plasma samples that showed a range of neutralization breadths. Characterization of wild-type and CD4bs mutant Du151 gp120s demonstrated that the mutation D368R eliminated recognition by MAbs b12 and b6 (see Fig. S1 in the supplemental material). Plasmas were di-

luted 1:20 and adsorbed onto magnetic beads coated with wild-type or mutant gp120. Blank beads were used as a negative control. The removal of anti-gp120 antibodies was assessed by ELISA against the wild-type and D368R mutant gp120s. The plasmas adsorbed with wild-type gp120 lost considerable binding activity as measured by ELISA against both wild-type and mutant gp120s (see Fig. S2a and b in the supplemental material). However, samples adsorbed with D368R gp120-coated beads retained considerable activity against the wild-type gp120 in an ELISA (see Fig. S2a in the supplemental material) while they lost their reactivity to the mutant gp120 (see Fig. S2b in the supplemental material). This disparity between the material adsorbed with wild-type and mutant gp120s, when tested for binding to the wild-type protein, suggested the presence of binding antibodies to the CD4bs in all plasmas.

We next explored the neutralization capacities of these samples using the pseudotyped viruses HxB2 and Du151. The ID₅₀ neutralization titer for each sample adsorbed with wild-type and D368R mutant gp120s, as well as blank beads, is shown in Table 1. Neutralizing activity against HxB2 could be adsorbed in all 16 samples by gp120-coated beads (Table 1). In 14 of 16 samples, this was mainly mediated by CD4bs antibodies, as shown by the less effective reduction in plasma neutralization titers upon adsorption with D368R than with wild-type gp120-coated beads. This was because the CD4bs antibodies were not removed from the plasma by the mutant protein and were therefore still available to mediate neutralization. The amount of CD4bs antibody was calculated as the difference between wild-type- and mutant-adsorbed gp120 relative to blank beads (Table 1). Only plasmas BB47 and BB54 lost their neutralizing activities after adsorption with both wild-type and mutant gp120-coated beads, suggesting that in these cases, HxB2 neutralization was mainly via antibodies to gp120 that did not include the CD4bs.

A different scenario was evident when the adsorbed plasmas were evaluated for neutralization of the primary virus, Du151. Seven of the 16 plasmas tested contained anti-gp120 antibodies that neutralized Du151 above 50% (Table 1). Two of these samples, BB10 and BB54, showed that >30% of the anti-gp120 antibodies were directed to the CD4bs (Table 1). For the remaining samples, no considerable differences in ID₅₀ titers were observed between adsorption with wild-type and mutant gp120s and blank beads.

Anti-gp120 antibodies can mediate heterologous neutralization. We examined two samples with breadths that contained anti-gp120 neutralizing antibodies against Du151 to determine if these antibodies could neutralize heterologous viruses. BB55 and BB70 were adsorbed with beads coated with wild-type Du151, D368R, or a double CD4bs mutant D368R/E370A gp120. The adsorbed plasmas were tested for neutralization using viruses against which they showed activity (Du151, Du156, COT6, and either AC10 or CAAN5342). For both plasmas, adsorption with wild-type gp120 reduced their neutralization activity against Du151, as well as against the other four heterologous viruses, indicating that these anti-gp120 antibodies were BCN (Fig. 2A). The D368R CD4bs mutant adsorbed the neutralizing activity almost as efficiently as the wild-type gp120 (Fig. 2A), suggesting that the neutralizing antibodies in these samples were not affected by the D368R

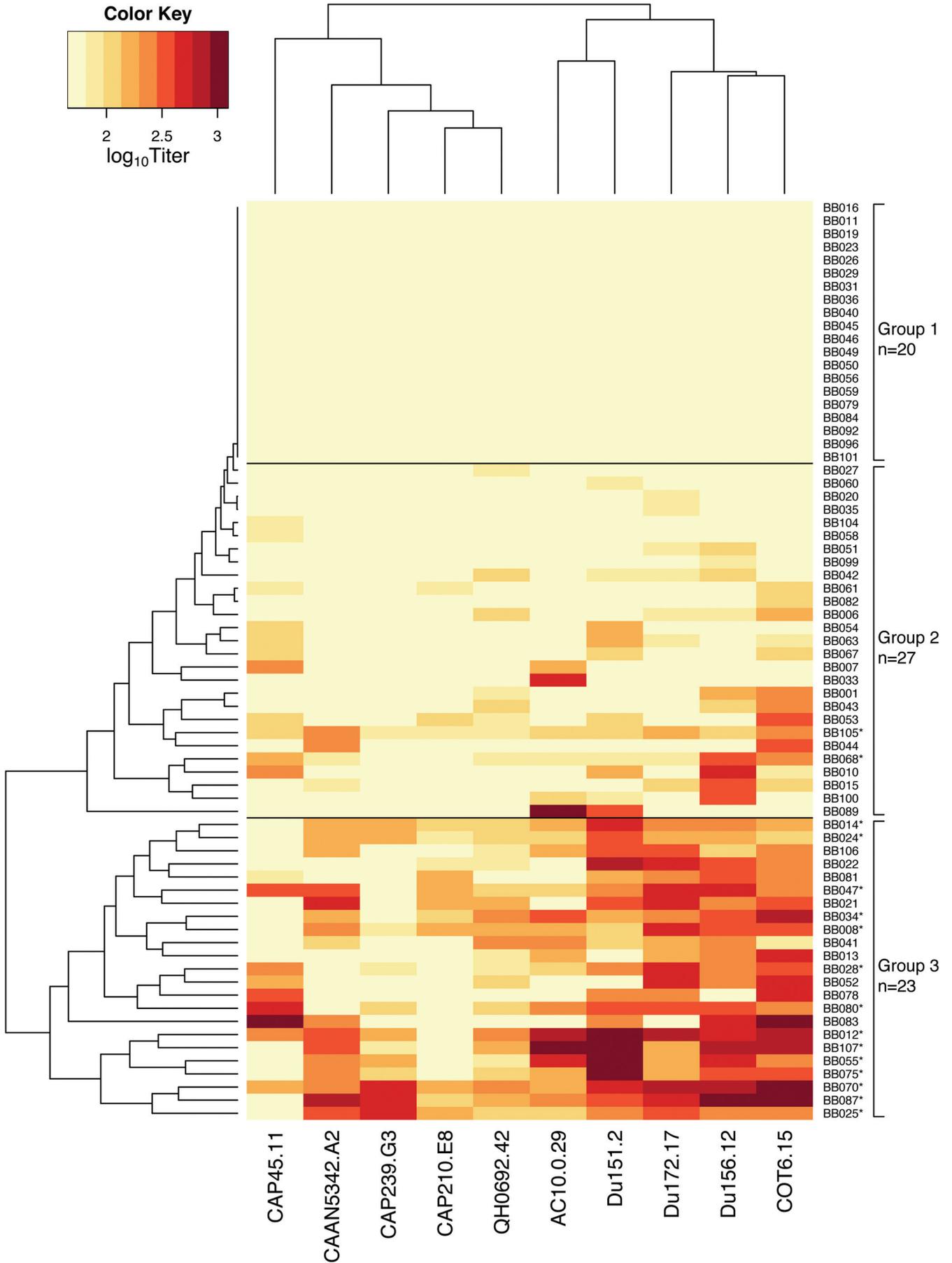
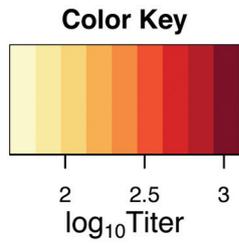


TABLE 1. Summary of neutralization activities of plasmas after adsorption with gp120-coated beads

Plasma sample	% Viruses neutralized	HxB2					Du151					
		ID ₅₀ ^a			% gp120 directed ^b	% CD4bs directed ^c	ID ₅₀			% gp120 directed ^b	% CD4bs directed ^c	% gp120 neut CD4bs directed ^d
		Blank	gp120 wild-type	gp120 D368R			Blank	gp120 wild-type	gp120 D368R			
BB70	100	1,391	509	1,241	63	53	914	416	487	54	8	14
BB12	90	546	50	606	91	100	1,848	757	963	59	11	20
BB14	90	8,108	863	5,772	89	61	1,110	599	713	46		
BB24	90	304	50	207	84	52	278	303	291	0		
BB47	90	222	50	50	77	0	675	435	539	36		
BB55	80	660	156	434	76	42	283	50	50	82	0	0
BB34	80	7,681	3,793	8,467	51	61	544	420	310	23		
BB75	80	376	50	325	87	73	11,676	6,410	6,851	45		
BB80	80	383	50	405	87	93	377	281	428	25		
BB53	50	487	155	425	68	55	365	223	291	39		
BB83	50	2,029	692	2,455	66	87	463	330	415	29		
BB10	40	176	50	139	72	51	410	50	247	87	48	55
BB78	40	345	50	184	86	39	163	129	121	21		
BB67	30	694	228	547	67	46	124	50	50	60	0	0
BB54	20	162	50	50	69	0	488	50	211	90	33	37
BB89	20	738	369	721	50	48	264	50	50	81	0	0

^a ID₅₀ using samples adsorbed with Du151 wild-type gp120, Du151 D368R gp120, or blank beads. In cases where no neutralization was observed at the lowest dilution (1:100), a value of 50 was given.

^b Percent neutralization directed to gp120, calculated as the reduction of the ID₅₀ by gp120 wild-type adsorption relative to blank beads [(blank – gp120 wild-type)/blank]. Values equal to or greater than 50% are shown in boldface.

^c Percentage of total neutralization directed to the CD4bs, calculated as the reduction of the ID₅₀ between wild-type and D368R gp120 adsorptions relative to blank beads [(gp120 D368R – gp120 wild-type)/blank]. It indicates the percentage of the total gp120 neutralization that targets the CD4bs. Values equal to or greater than 30% are shown in boldface.

^d Percentage of anti-gp120 neutralization directed to the CD4bs (% CD4bs directed/% gp120 directed). Values equal to or greater than 30% are shown in boldface.

mutation. However, BB55 retained some activity when adsorbed with the D368R/E370A mutant gp120, suggesting the presence of low levels of cross-neutralizing anti-CD4bs antibodies in this sample.

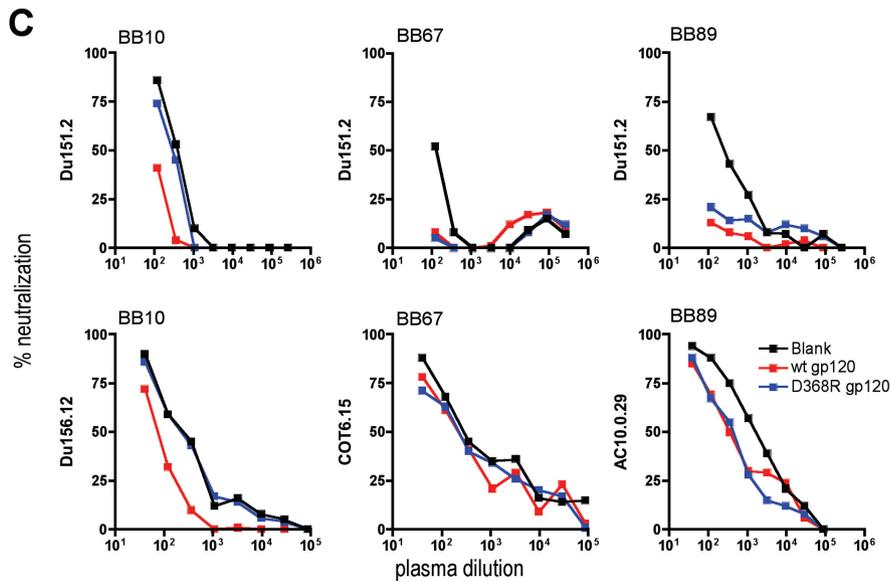
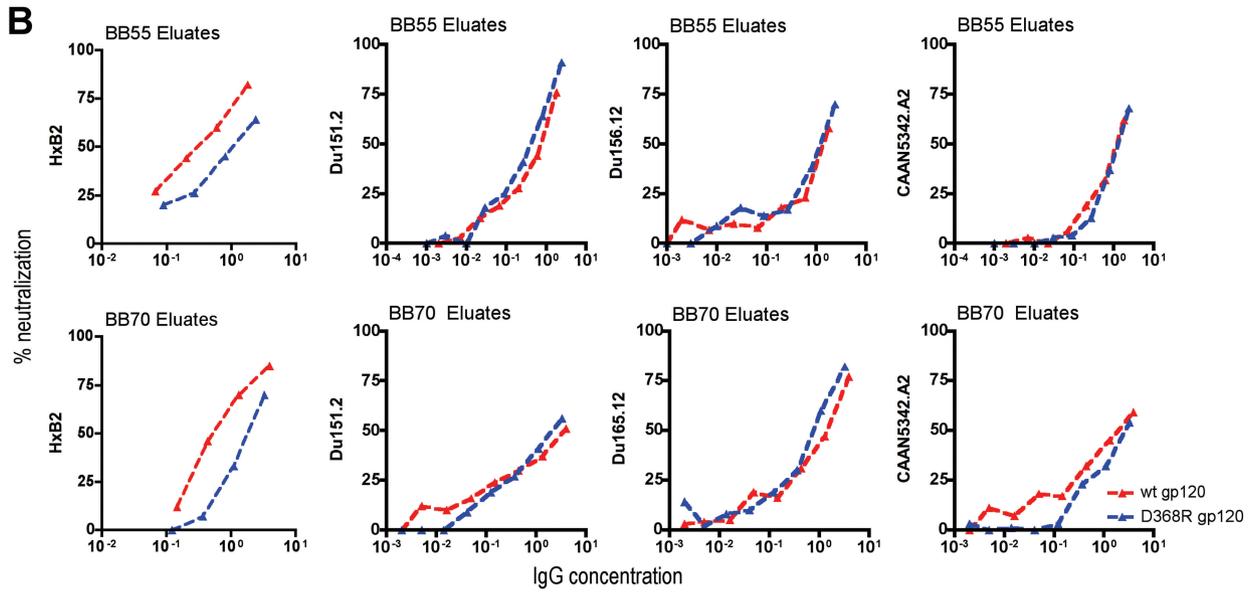
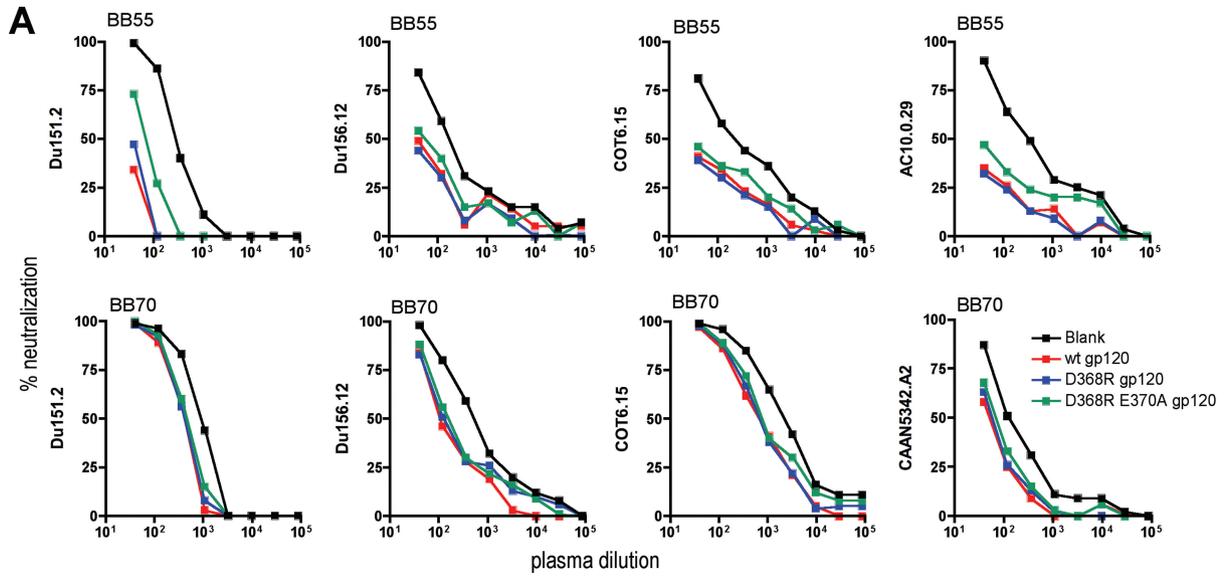
BB55 and BB70 antibodies eluted from both wild-type and D368R mutant proteins neutralized Du151, Du156, and CAAN5342 at high potency, with 50% inhibitory concentrations of 1 µg/ml (Fig. 2B). Consistent with the adsorption data, the eluates from the wild-type gp120 neutralized HxB2 better than the eluates from the D368R mutant gp120. This suggested that some antibodies present in these samples were able to access only the CD4bs on the sensitive laboratory strain and were not involved in the cross-neutralization of primary viruses. Eluates from the double-mutant gp120 were not tested, as they showed adsorption profiles similar to that of the single mutant.

We next examined three plasmas with limited breadths that contained anti-gp120 neutralizing antibodies to determine if they were capable of some cross-reactivity. Plasma BB89 neutralized Du151 and AC10, and this was shown to be via anti-gp120 antibodies that were not CD4bs directed (Fig. 2C). However, these anti-gp120 antibodies were unable to neutralize any of the other viruses in the panel (see Table S1 in the supplemental material). In the case of BB67, the adsorption of

anti-gp120 antibodies by Du151 gp120 affected the neutralization of Du151, but not of COT6, suggesting the presence of strain-specific antibodies. For plasma BB10, the adsorption of anti-gp120 antibodies reduced the neutralization activity of Du151, as well as Du156, and in both cases the D368R gp120 failed to adsorb this activity, mapping this neutralizing antibody specificity to the CD4bs (Fig. 2C). However, these anti-CD4bs antibodies had limited breadth, as the plasma neutralized only 4 of the 10 viruses tested in this study (see Table S1 in the supplemental material).

Association of anti-CD4i antibodies with neutralization breadth. The presence of antibodies that target CD4i epitopes was assayed in a subset of 50 plasmas that included all 16 BCN samples. These samples were tested for neutralization of HIV-2 7312A with and without sCD4. Titers threefold above background (i.e., the titer against 7312A without sCD4) were considered positive. Anti-CD4i antibodies were detected in 21 of the 50 samples tested (see Table S1 in the supplemental material). Those samples that contained anti-CD4i antibodies neutralized a significantly higher number of viruses (median = 8) than plasma samples that had no anti-CD4i antibodies (median = 1) ($P = 0.0007$). Furthermore, there was a significant correlation between anti-CD4i antibody titers and the number of viruses neutralized (Fig. 3A).

FIG. 1. Hierarchical clustering of sera and viruses according to the ID₅₀ neutralization titers. The neutralization titer of each of 70 plasma samples against 10 HIV-1 strains is shown in a heat map with the most potent plasmas shown in dark red and those with no activity in light yellow. The remaining values are binned into eight distinct colors from a palette ranging from pale yellow to dark red. Rows and columns are clustered using a complete linkage-clustering algorithm based on Euclidean distance between log₁₀ titer values (12). The resulting rows and columns are reordered, within the constraints of the respective dendrogram, using row and column means. The 16 samples with BCN antibodies are indicated with asterisks.



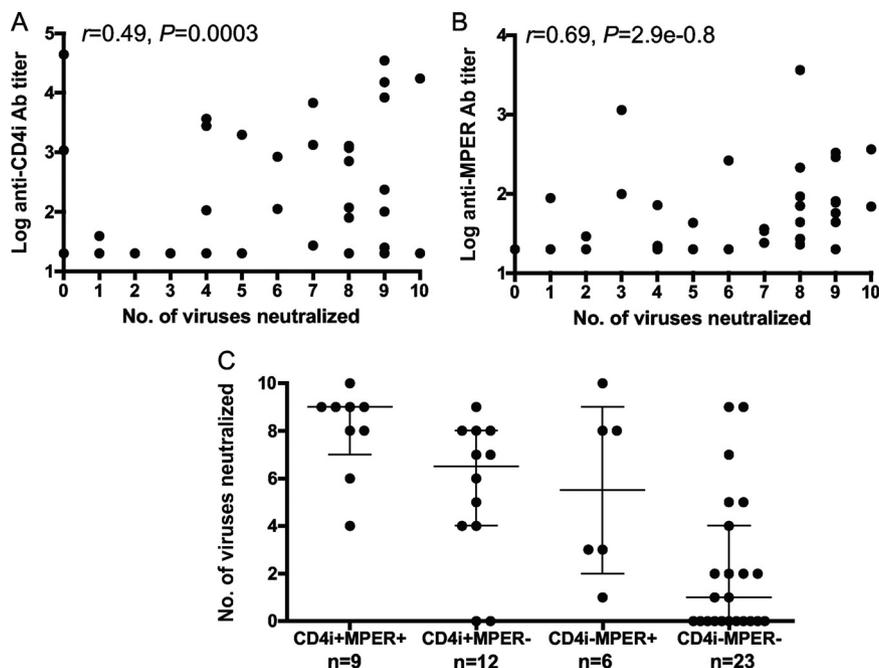


FIG. 3. Analysis of anti-CD4i epitope and anti-MPER antibodies in comparison to neutralization breadth. Shown are correlations of the number of viruses neutralized with anti-CD4i (A) and anti-MPER (B) antibody (Ab) titers. Spearman rank coefficients and P values are shown for each correlation. (C) A Kruskal-Wallis test indicated a significant difference between the median numbers of viruses neutralized by plasma segregated into four groups based on their antibody specificities ($P = 0.0003$). The median and interquartile range are indicated.

Given that BB55 and BB70 contained high titers of anti-CD4i antibodies (see Table S1 in the supplemental material) and also contained undefined anti-gp120 neutralizing activity (Table 1), we performed adsorptions using gp120 containing the I420R mutation (27). This protein failed to bind the CD4i MAb E51, as expected (see Fig. S1 in the supplemental material). Control experiments using HIV-2 7312A in the presence of sCD4 confirmed that this mutation reduced binding of antibodies to the CD4i epitope (Fig. 4A). When tested against Du151 and Du156, the adsorption of BB55 with I420R gp120 failed to remove all the neutralizing activity, unlike the wild type, suggesting the presence of antibodies against the CD4i epitope in this sample (Fig. 4B and data not shown). Since a similar pattern was seen with the CD4bs double mutant (Fig. 2A), we performed sequential adsorptions with both CD4bs and CD4i mutant proteins to determine if these activities overlapped or were additive. Adsorption with the D368R/E370A mutant protein using I420R gp120-depleted plasma showed no additional reduction in neutralization, suggesting that these activities were the result of the same antibody (Fig. 4B). Reverse sequential adsorptions showed similar results, confirming the above conclusion (Fig. 4C). Furthermore, BB55 eluates from wild-type and D368R mutant gp120s had CD4i activity

while those from I420R did not (Fig. 4D). A similar pattern was evident when eluates were tested against Du151 (Fig. 4E and 2B). A surprising finding was that the CD4bs double mutant behaved like the CD4i mutant rather than the CD4bs single mutant (Fig. 4D and E). This suggests that the CD4bs residue E370 (or the combination of D368 and E370) is involved in the CD4i epitope, which was supported by the finding that this mutant protein was not recognized by the CD4i MAb E51 (see Fig. S1 in the supplemental material). No difference in CD4i activity was noted for plasma BB70 after adsorption with gp120 (data not shown). It is possible that the CD4i antibodies in BB70 could not be depleted using monomeric gp120 in the unbound conformation. Experiments using stabilized gp120 in a CD4-bound conformation may be required for further analysis of this sample.

Association of anti-MPER neutralizing antibodies with neutralization breadth. The presence of anti-MPER antibodies was tested using an HIV-2 strain containing an HIV-1 MPER region optimized for HIV-1 subtype C sequences (7312A C1C). As for the anti-CD4i activity, antibody titers threefold above background were considered positive. Our analysis revealed anti-MPER antibodies in 15 of the 50 plasmas tested (see Table S1 in the supplemental material). Anti-MPER-

FIG. 2. Neutralization analysis of plasmas adsorbed onto gp120-coated beads. The plasmas were adsorbed onto magnetic beads coated with Du151 wild-type (wt) gp120, D368R gp120, D368R/E370A gp120, or blank beads. The residual and eluted antibodies were tested for neutralization of various envelope-pseudotyped viruses. (A) Neutralization curves of adsorbed plasmas, BB55 (top row) and BB70 (bottom row), against four primary viruses. (B) Antibodies eluted from beads were tested for neutralization and standardized by their IgG concentrations. Shown are BB55 (top row) and BB70 (bottom row) against HxB2, Du151, Du156, and CAA5342. (C) Neutralization curves of adsorbed plasma samples BB10 (left column), BB67 (middle column), and BB89 (right column) using viruses against which these plasmas were active.

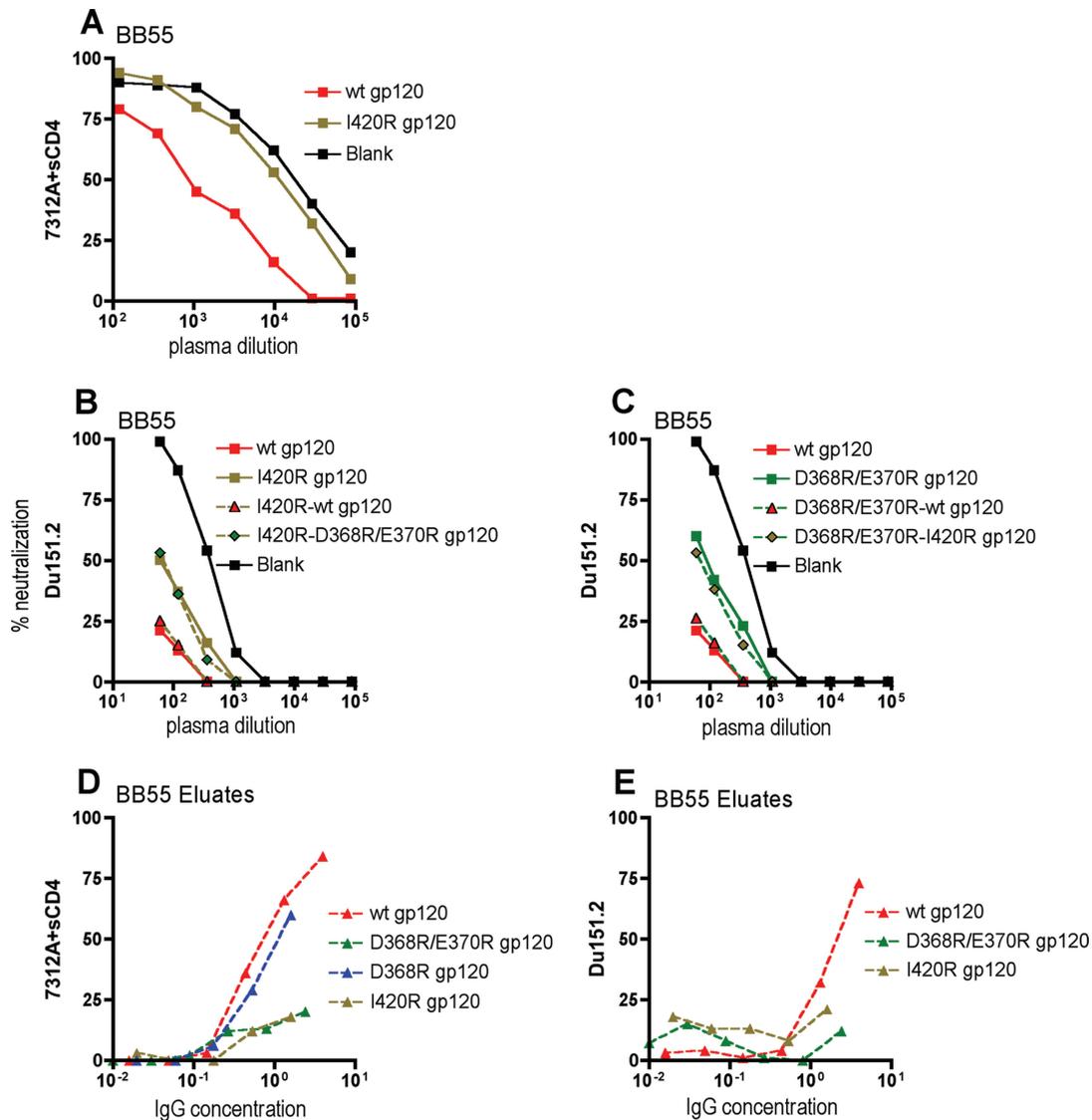


FIG. 4. Contributions of anti-CD4i and anti-CD4bs antibodies to cross-neutralizing activity in BB55. (A) Control experiments showing neutralization of 7312A plus 9 nM sCD4 by BB55 plasma previously adsorbed with either wild-type or I420R gp120. No neutralization of 7312A was evident in the absence of sCD4. (B) Neutralization of Du151 by BB55 plasma after absorption with I420R gp120 alone or I420R gp120 followed by wild-type or D368R/E370A gp120. (C) Neutralization of Du151 by BB55 plasma after absorption with D368R/E370A gp120 alone or D368R/E370A gp120 followed by wild-type or I420R gp120. Wild-type gp120 and blank beads were included as controls. (D and E) BB55 eluates from wild-type, D368R, D368R/E370A, and I420R gp120s were assessed for neutralization of 7312A plus 9 nM sCD4 (D) and Du151 (E). (Figure 2B shows the D368R gp120 curve).

positive samples neutralized a significantly higher number of viruses (median = 7) than their negative counterparts (median = 3.2) ($P = 0.0005$). The strong correlation of these parameters confirmed the association between anti-MPER antibody titers and neutralization breadth (Fig. 3B).

Cumulative effect of anti-CD4i and anti-MPER responses. In order to evaluate the cumulative effect of anti-CD4i and anti-MPER antibodies on neutralization breadth, we stratified all 50 samples into four groups based on the presence or absence of these specificities. The number of viruses neutralized for each sample was plotted against the corresponding group (Fig. 3C). A Kruskal-Wallis test indicated a significant difference in the number of viruses neutralized between these

four groups ($P = 0.0002$). Furthermore, concurrency of CD4i and MPER antibodies tended to yield samples with higher overall neutralization titers than those with single specificities. At the other extreme, double-negative samples had little to no heterologous neutralization. A regression analysis was unable to detect an interaction between these two parameters, suggesting that the cumulative effect of anti-MPER and anti-CD4i specificities was simply additive.

Fine mapping of anti-MPER activity. Six of the plasma samples with the highest anti-MPER titers were tested against eight HIV-2 chimeric viruses containing HIV-1 MPER fragments to determine which region of the MPER was being recognized (Table 2). None of the samples tested showed 2F5-

TABLE 2. Mapping of anti-MPER neutralizing antibodies^a

Chimera	MPER sequence	2F5	4E10	Z13e1	BB25	BB28	BB34	BB47	BB68	BB70
7312A	NMYELQKLNSWDFGNWFDLASWVKYIQYGVYIV	-	-	-	<20	<20	<20	48	<20	35
C1	NMYELLALDKWASLWNWFDITKWLWYIKYGVYIV	++	++	++	195	216	5,560	210	74	206
C1C	NMYELLALDSWKNLWNWFDITKWLWYIKYGVYIV	-	++	++	207	209	3,945	249	223	311
C1C F/L	NMYELLALDSWKNLWNWLDITKWLWYIKYGVYIV	-	-	+	38	122	1,779	156	112	136
C3	NMYELLALDKWASLWNWFDLASWVKYIQYGVYIV	++	-	-	<20	<20	<20	42	<20	<20
C3C	NMYELLALDSWASLWNWFDLASWVKYIQYGVYIV	-	-	-	<20	<20	<20	29	<20	<20
C7	NMYELQALDKWAVFGNWFNFDLASWVKYIQYGVYIV	++	-	-	<20	<20	<20	56	<20	<20
C6	NMYELQKLNSWDFGNWFDITSWIKYIQYGVYIV	-	++	-	<20	<20	<20	42	<20	22
C4	NMYELQKLNSWDFGNWFDITKWLWYIKYGVYIV	-	++	-/+	47	<20	<20	176	<20	27
C4GW	NMYELQKLNSWDFWNNWFDITKWLWYIKYGVYIV	-	++	++	145	198	7,482	228	176	254
C8	NMYELQKLNSWDSLWNWFDITKWLWYIKYGVYIV	-	++	+	217	121	3,351	208	47	82

	<20
	20-100 less than 2-fold over background
	20-100 more than 2-fold over background
	100-200
	200-1,000
	>1,000

^a Plasma ID₅₀s were determined against MPER grafted HIV-2 viruses. The sequence carried by the MPER of each of the constructs is indicated in the second column. Grafted or mutated amino acids are indicated in blue. Further mutations on the chimeras are indicated in red. The neutralization by MAbs 2F5, 4E10, and Z13e1 are qualitatively indicated relative to the titers obtained with the C1 chimera.

or 4E10-like antibodies, as they did not neutralize C3 and C6, which contained the minimum number of mutations needed to reconstitute these epitopes, respectively (Mab-positive controls in Table 2). However, all six recognized an epitope in the C-terminal region of the MPER overlapping the 4E10 and Z13e1 epitopes, demonstrated by their neutralization of C4GW and C8. Four of these were unable to neutralize C4, suggesting they required a tryptophan (W) residue at position 670 for recognition. Two samples (BB25 and BB47) neutralized C4. However, for BB25 the ID₅₀ titers dropped considerably in the absence of W670 (compare C4GW and C4), and only BB47 recognition was shown to be relatively independent of this residue.

We modified the C3 chimera to represent a more common subtype C sequence by mutating residue 665, within the 2F5 epitope, from a lysine to a serine. This modification eliminates recognition by 2F5. No neutralization was detected by this mutant, supporting the observation that these samples did not recognize an epitope over the N-terminal region of the MPER that contained the 2F5 epitope.

The mutation of F673L, which affects sensitivity to 4E10 and, to a lesser extent, Z13e1, had some effect on the neutralization of all plasmas, reducing the ID₅₀ by half, except for BB25, which suffered almost a log unit drop in neutralization titer. Overall, these data suggested that the plasmas recognized distinct epitopes within the MPER.

Analysis of autoantigen reactivity and its association with neutralization breadth. Given the suggestion that some anti-HIV-1 broadly neutralizing MAbs react with autoantigens, we tested all 16 BCN plasmas plus a subset of non-BCN plasmas for reactivity with various autoantigens. None of the samples showed reactivity with SSA, SSB, Sm, RNP, Scl, Jo1, Cent B, and histone, except BB40, which reacted with Sm (see Table S2 in the supplemental material). However, a number of plasma

samples were positive for anti-dsDNA antibodies (15 out of 45), RF (15 out of 43), and anti-CL antibodies (16 out of 45). Anti-CL antibodies were predominantly IgG, and the titers were generally low. A Spearman rank analysis demonstrated that of these three specificities only anti-CL antibodies correlated positively with the number of viruses neutralized (Fig. 5A to C). Interestingly, RF showed a negative correlation with the number of viruses neutralized (Fig. 5C). The median number of viruses neutralized in the anti-CL-positive group was significantly higher at 7.5 than a median of 1 virus neutralized by the anti-CL-negative group ($P = 0.00008$).

Multivariate analysis of all specificities. We selected a group of 40 samples in which all five antibodies, i.e., anti-CD4i, anti-MPER, anti-CL, anti-dsDNA, and RF, were measured to investigate the prediction capacity of the neutralization breadths of these specificities. The univariate and multivariate linear regression analyses were performed using the number of viruses neutralized as the outcome. The presence of anti-CD4i, anti-MPER, and anti-CL antibodies was a strong predictor of neutralization breadth (Table 3). The presence of RF was associated with a lack of neutralization breadth in the univariate analysis. Interestingly, interaction analyses between these specificities revealed that anti-CL and anti-MPER antibodies had a positive interaction with RF, with an increased number of viruses neutralized when both anti-CL and RF were present ($P = 0.006$) or when both anti-MPER and RF ($P = 0.006$) were present. However, when both anti-CD4i and RF were positive, their interaction resulted in a lower number of viruses neutralized, suggesting a negative interaction ($P < 0.001$). The concomitant presence of anti-CL and anti-MPER did not show interaction in this model. However, we found that anti-CL antibodies were correlated with anti-MPER antibody titers (Fig. 5D).

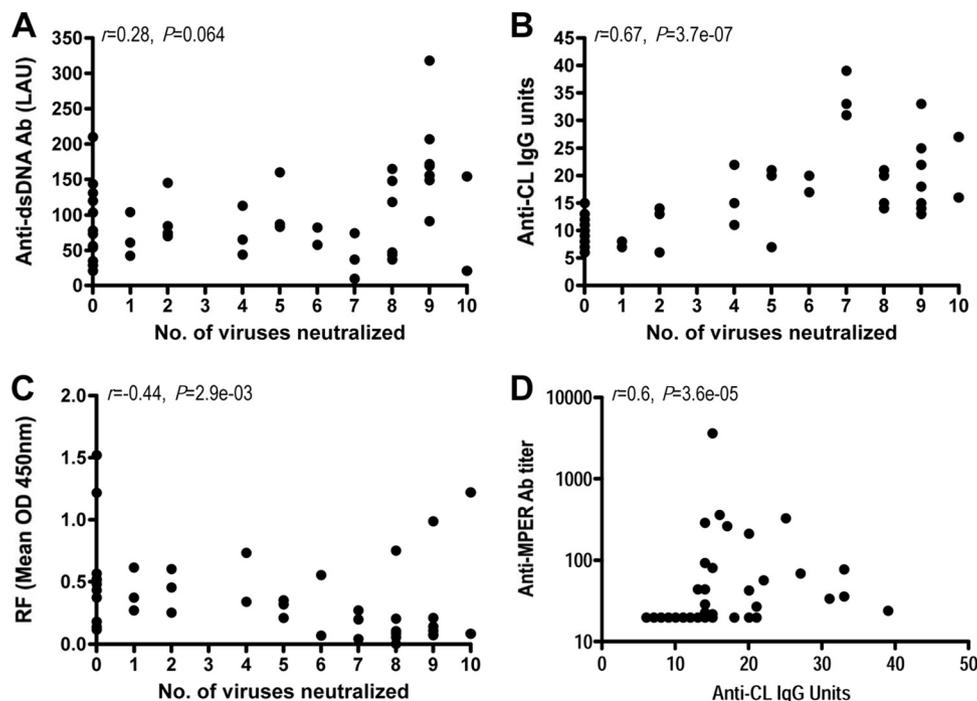


FIG. 5. Analysis of reactivity to autoantigens in relation to neutralization breadth. (A to C) Correlations between antibody (Ab) titers to anti-dsDNA (A), anti-CL (B), and RF (C) and the number of viruses neutralized. (D) Correlation of anti-CL and anti-MPER antibody titers. Spearman rank coefficients and *P* values are shown for each correlation. OD, optical density; LAU, Luminex Athena units.

DISCUSSION

The present study identified antibody specificities associated with neutralization breadth. We found that both anti-MPER and anti-CD4i antibody titers were correlated with the number of viruses neutralized. Furthermore, antibodies to CL were found to be correlated with neutralization breadth and with anti-MPER antibodies. We did not find anti-gp120 or anti-CD4bs antibodies more frequently in samples with breadth. However, a number of samples had neutralizing antibodies that bound monomeric gp120, some of which were shown to mediate cross-neutralization.

For this study, we used samples collected from South African HIV-1-infected blood donors who were assumed to be infected with HIV-1 subtype C, since this is the predominant subtype in the country. Indeed, gp160 sequence analyses of 15 samples confirmed these to be HIV-1 subtype C (not shown). After exclusion of the incident infections, we found 16 out of

70 (23%) plasmas from chronically HIV-1-infected individuals to have BCN antibodies. However, this frequency may be confounded by the fact that these samples were obtained from blood bank donors and therefore may be enriched for healthy individuals who were unaware of their HIV infections. While it is not clear that disease progression is related to the presence or lack of neutralizing antibodies, it is fair to presume that a healthy immune system may be more likely to generate these antibodies. Nevertheless, our data are in line with other recent studies that report similarly high proportions of chronically infected individuals with antibodies able to mediate cross-neutralization (11, 43).

Fourteen of the 16 BCN samples included here have been previously investigated (1). Our study builds on this by including non-BCN samples to ascertain which specificities were associated with breadth. In addition, we used the subtype C strain Du151 for both adsorption and neutralization experiments to maximize the opportunities for detecting relevant antibodies. Similar to the Binley study, we found antibodies targeting the CD4bs using gp120-adsorbed antibody preparations. However, these antibodies were found in plasmas with a range of neutralizing activities. They mediated gp120 binding, as well as neutralization of the laboratory-adapted strain HxB2, but had limited ability to neutralize the primary virus, Du151. In one sample (BB10), CD4bs antibodies were shown to mediate heterologous neutralization. However, these antibodies were not BCN, since this plasma inhibited only 40% of the panel. While recent studies have identified rare individuals with broadly neutralizing anti-CD4bs antibodies, in most cases anti-CD4bs antibodies have been shown to have only limited breadth, similar to what we have described (1, 30, 43). This is

TABLE 3. Univariate and multivariate analyses of antibody specificities correlated with neutralization breadth

Antibody specificity	Univariate model		Multivariate model	
	Estimate (95% CI) ^a	<i>P</i> value	Estimate (95% CI)	<i>P</i> value
Anti-CD4i	4.5 (2.5, 6.5)	<0.001	2.8 (1.1, 4.4)	0.0014
Anti-MPER	5.2 (2.9, 7.5)	<0.001	2.9 (1.0, 4.8)	0.0038
Anti-CL	4.7 (2.7, 6.7)	<0.001	3.4 (1.8, 4.9)	0.0001
Anti-dsDNA	1.6 (-0.9, 4.1)	0.2	NA ^b	NA
RF	-3.2 (-5.5, -0.8)	0.009	NA	NA

^a CI, confidence interval.

^b NA, not applicable.

analogous to the reactivities of existing CD4bs MAbs, such as b6, b13, 15e, and F105, which have little activity against primary isolates (3, 23, 38). Overall, our data suggest that while anti-CD4bs antibodies are readily produced during HIV-1 infection, they vary both qualitatively and quantitatively among different polyclonal plasma samples.

In our study, monomeric Du151 gp120 was able to adsorb >50% of neutralizing antibodies against the Du151 virus in 7/16 (44%) plasmas tested. In two cases (BB55 and BB70), these anti-gp120 antibodies were shown to be responsible for the heterologous neutralizing activity. The finding that a significant number of BCN plasmas can be adsorbed with monomeric gp120 may be somewhat surprising given the prevailing notion that neutralizing antibodies bind trimeric proteins (34). It is perhaps also intriguing that monomeric gp120 vaccines have been unable to induce equivalent antibodies *in vivo*, given that the epitopes are at hand. It suggests either that these antibodies are not produced at sufficiently high titer in vaccinees to mediate neutralization or that these epitopes cannot be induced by a protein mimic, similar to the failed attempts to induce neutralizing antibodies to other HIV epitopes (25). Another possibility is that BCN antibodies require repeated antigenic stimulation for an extended period, similar to natural HIV infection.

Interestingly, we found a good association between neutralization breadth and the presence of anti-CD4i and anti-MPER antibodies. CD4i antibodies develop in natural HIV-1 infection (8, 15), although their role in virus neutralization remains unclear given the poor accessibility of the site (24). Nevertheless, CD4i antibodies have been found in samples with neutralization breadth (30), in support of what we found here. Moreover, our data suggested that anti-CD4i antibodies in BB55 contributed to heterologous neutralization, although its epitope may overlap with the CD4bs, as was evident from the data using the D368R/E370A mutant. Further studies to assess the contribution of anti-CD4i antibodies to virus neutralization may shed light on new targets for vaccine design.

A number of groups have recently shown that anti-MPER antibodies are found in samples with neutralization breadth that in some cases were identified as 4E10-like, Z13-like (1, 29, 43), or 2F5-like (50). While we also found anti-MPER antibodies, they did not appear to correspond to either 4E10 or 2F5 specificities. Based on the chimeric mapping data of six samples, we speculate that we have at least three distinct specificities targeting the MPER. BB25 and BB47 may contain Z13-like antibodies based on their neutralization of C4. BB68 and BB70 showed a similar pattern of neutralization that was distinct from that of BB28 and BB34. Antibodies in BB34 have been extensively analyzed and shown to map to a novel epitope (E. S. Gray, unpublished data). These data suggest that the MPER is immunogenic and is recognized in diverse manners by the immune system. While these observations suggest that both anti-MPER and anti-CD4i antibodies may be involved in the neutralization breadth displayed by these samples this remains to be fully demonstrated, as it is possible that these antibodies are simply a surrogate for other specificities that confer cross-neutralization.

HIV infection is associated with hypergammaglobulinemia and with elevated serum titers to a variety of self-antigens, including anti-phospholipids, anti-nuclear antibodies, and RF

(4, 53). Here, we found anti-CL antibodies in 36% of the plasma samples tested, consistent with reports by others (48). While anti-CL antibodies are frequently found in HIV-1 infection, they are not significantly associated with thrombotic events or other manifestations of anti-phospholipid syndrome (4, 48). In this study, we found anti-CL antibodies to be significantly associated with neutralization breadth. In addition, we found that anti-CL antibodies were correlated with anti-MPER titers. Similar observations have been reported recently by another group (31), although they measured anti-MPER peptide-binding antibodies in sera while we measured anti-MPER neutralization titers. Nevertheless, these results strongly suggest that anti-MPER and anti-CL antibodies are linked and related to the development of broad neutralization. However, our results do not explain if this autoreactivity is a property of the same antibody mediating neutralization, as has been suggested by MAbs 4E10 and 2F5 (17, 46). Alternatively, these autoantibodies represent a parallel humoral response, perhaps indicative of a particular genetic background that supports the elicitation of neutralizing antibodies. Thus, it will be of interest to determine if these anti-CL antibodies cross-react with HIV proteins or, even more importantly, if they have neutralizing activity.

Overall, our data extend recent studies examining the antibody specificities associated with neutralization breadth and suggest additional insights into their properties. While antibodies targeting specific sites could be identified in some cases, the majority are still unknown. It is likely that cross-reactive antibodies target epitopes on the trimeric structure of the functional envelope glycoprotein or on gp41. Although rare cases of cross-neutralization due to a single antibody specificity, such as anti-CD4bs or anti-MPER, have been reported (30, 43), it is highly likely that more than one antibody confers neutralization breadth. In support of this, we and others have shown that multiple known specificities can be found in an individual plasma sample (such as BB70, which contained anti-gp120 and anti-MPER antibodies). A recent study examining the repertoire of anti-gp140 antibodies did not find a single specificity that accounted for the neutralization breadth exhibited by the plasma, suggesting that multiple antibodies with more limited activities contributed to breadth (45). Major questions that remain are how neutralization breadth develops and why it develops only in some individuals (are host genetic factors involved?). Longitudinal studies are needed in which the evolution of various antibody specificities is dissected and their contributions to overall neutralization breadth are determined. The identification of the precise epitopes of these BCN antibodies remains a top priority for vaccine research.

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