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Broad Neutralization of Human Immunodeficiency Virus Type 1 Mediated by Plasma Antibodies against the gp41 Membrane Proximal External Region▽†

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We identified three cross-neutralizing plasma samples with high-titer anti-membrane proximal external region (MPER) peptide binding antibodies from among 156 chronically human immunodeficiency virus type 1-infected individuals. In order to establish if these antibodies were directly responsible for the observed neutralization breadth, we used MPER-coated magnetic beads to deplete plasmas of these specific antibodies. Depletion of anti-MPER antibodies from BB34, CAP206, and SAC21 resulted in 77%, 68%, and 46% decreases, respectively, in the number of viruses neutralized. Antibodies eluted from the beads showed neutralization profiles similar to those of the original plasmas, with potencies comparable to those of the known anti-MPER monoclonal antibodies (MAbs), 4E10, 2F5, and Z13e1. The anti-MPER neutralizing antibodies in BB34 were present in the immunoglobulin G3 subclass-enriched fraction. Alanine scanning of the MPER showed that the antibodies from these three plasmas had specificities distinct from those of the known MAbs, requiring one to three crucial residues at positions 670, 673, and 674. These data demonstrate the existence of MPER-specific cross-neutralizing antibodies in plasma, although the ability to elicit such potent antiviral antibodies during natural infection appears to be rare. Nevertheless, the identification of three novel antibody specificities within the MPER supports its further study as a promising target for vaccine design.

The induction of broadly neutralizing antibodies has been one of the most pursued outcomes in the development of a preventive vaccine against human immunodeficiency virus type 1 (HIV-1). In spite of the substantial effort invested in the design of an immunogen capable of inducing such antibodies, little success has been achieved. However, it is known that some individuals develop broadly cross-neutralizing antibodies during natural HIV-1 infection (5, 6, 18, 25, 26). The nature of these antibodies and the epitopes that they recognize in the envelope glycoprotein have been under scrutiny in several recent studies (3, 12, 16, 28; reviewed in references 1 and 32). In some cases, broadly cross-neutralizing antibodies have been mapped to the CD4 binding site, the coreceptor binding site (CD4i), and other undefined epitopes within gp120. The inability to adsorb cross-neutralizing antibodies with recombinant gp120 suggests that some of these antibodies recognize epitopes only apparent in the context of the trimeric glycoprotein or on the gp41 molecule (3, 12, 16, 28). Indeed, a few of these recent studies have reported cross-neutralizing antibodies that target the membrane proximal external region (MPER) in gp41 (16, 28, 30).

The MPER has attracted considerable attention as a potential target for vaccine-induced broadly neutralizing antibodies (20, 23, 24). This linear stretch of around 24 amino acids proximal to the transmembrane region is highly conserved among HIV isolates (27, 36). Furthermore, three of the very few cross-neutralizing antibodies against HIV-1 (2F5, 4E10, and Z13e1) recognize epitopes within this region (19, 38). 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 (11, 35). These studies concluded that 2F5- and 4E10-like antibodies were rarely found in HIV-1-infected plasmas; however, other epitopes within the MPER were recognized by around one-third of HIV-1-infected individuals, although their neutralizing potential was not explored. We have previously reported a significant association between neutralization breadth and the presence of anti-MPER antibodies among 50 HIV-1 subtype C plasmas from chronically infected blood donors (12). However, that study did not unambiguously demonstrate that these antibodies were directly responsible for neutralization breadth. In the present study, we addressed this question by assessing the impact of depleting anti-MPER antibodies from broadly cross-reactive plasmas on their neutralizing activities.

MATERIALS AND METHODS

Plasma samples and viruses. Plasmas BB34, BB81, BB105, and SAC21 were from HIV-1-infected blood donors identified by the South African National

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Blood Service in Johannesburg. The BB samples were collected between 2002 and 2003 and have been described previously (3, 12). The SAC plasma samples are from a second blood donor cohort that was assembled using a similar approach. Briefly, aliquots from 105 HIV-1-infected blood donations made between 2005 and 2007 were screened in the BED assay to eliminate 29 incident infections. Eight samples neutralized the vesicular stomatitis virus G control pseudovirus and were excluded. SAC21 was among the remaining 68 aliquots that were tested against three subtype B and three subtype C primary viruses to identify those with neutralization breadth. The plasma sample CAP206 corresponded to the 3-year visit of an individual in the Centre for the AIDS Programme of Research in South Africa (CAPRISA) cohort (11, 34). The envelope genes were either previously cloned in our laboratory (11) or obtained from the NIH AIDS Research and Reference Reagent Program or the Programme EVA Centre for AIDS Reagents, National Institute for Biological Standards and Control, United Kingdom. The 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 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 (17). Titers were calculated as the 50% inhibitory concentration (IC_{50}) or the reciprocal plasma/serum dilution causing 50% reduction of relative light units with respect to the virus control wells (untreated virus) (ID_{50}). Anti-MPER specific activity was measured using the HIV-2 7312A and the HIV-2/HIV-1 MPER chimeric constructs (11). Titers threefold above background (i.e., the titer against 7312A) were considered positive.

Serum adsorption and elution of anti-MPER antibodies. Streptavidin-coated magnetic beads (Dynal MyOne Streptavidin C1; Invitrogen) were incubated with the biotinylated peptide MPR.03 (KKKNEQELLELDKWASLWNWFDITNW LWYIRKKK-biotin-NH_2) (NMI, Reutlingen, Germany) at a ratio of 1 mg of beads per 20 μg peptide at room temperature for 30 min. Plasmas were diluted 1:20 in Dulbecco's modified Eagle's medium (DMEM)-10% fetal bovine serum and incubated with the coated beads for 1 h at a ratio of 2.5 mg of coated beads per ml of diluted plasma. This was followed by a second adsorption at a ratio of 1.25 mg of coated beads per ml of diluted sample. After each adsorption, the beads were removed with a magnet, followed by centrifugation, and were stored at 4°C. The antibodies bound to the beads were eluted by incubation with 100 mM glycine-HCl elution buffer (pH 2.7) for 30 s with shaking and then pelleted by centrifugation and held in place with a magnet. The separated immunoglobulin G (IgG) was removed and placed into a separate tube, where the pH was adjusted to between 7.0 and 7.4 with 1 M Tris (pH 9.0) buffer. The same beads were acid eluted twice more. The pooled eluates were then diluted in DMEM, washed over a 10-kDa Centricon plus filter, and resuspended in DMEM. Antibody concentrations were determined using an in-house total-IgG quantification enzyme-linked immunosorbent assay (ELISA) as described below. The adsorbed sera were then used in ELISAs and neutralization assays.

MPER-peptide ELISA. Synthetic MPR.03 peptide or V3 peptide (TRPGNN TRKSIRIGPGQTFFATGDIIGDIREAH) was immobilized at 4 μg/ml in a 96-well high-binding ELISA plate in phosphate-buffered saline (PBS) overnight at 4°C. The plates were washed four times in PBS-0.05% Tween 20 and blocked with 5% skim milk in PBS-0.05% Tween 20 (dilution buffer). Adsorbed plasmas, as well as control samples, were serially diluted in dilution buffer and added to the plate for 1 h at 37°C. Bound antibodies were detected using a total anti-human IgG-horseradish peroxidase conjugate (Sigma-Aldrich, St. Louis, MO) and developed using TMB substrate (Thermo, Rockford, IL). The plates were read at 450 nm on a microplate reader.

IgG quantification ELISA. Goat anti-human IgG antibody was immobilized in a 96-well high-binding plate in carbonate-bicarbonate buffer overnight at 4 μg/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. 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 conjugate (Sigma-Aldrich) as described above.

IgG subclass fractionation. Total IgG was extracted from plasma samples using a protein G column (NAb Protein G Spin Kit; Thermo). The IgG3 fraction was separated from the other IgG subclasses using a protein A column (NAb Protein A Spin Kit; Thermo). Protein G and protein A flowthrough fractions and eluted IgGs were tested using a Human IgG Subclass Profile ELISA Kit (Invitrogen Corporation, Carlsbad, CA). The concentration of each IgG subclass was calculated relative to a subclass-specific standard curve provided by the manufacturer.

Site-directed mutagenesis. Specific amino acid changes in the MPER of the envelope clone COT6.15 (9) were introduced using the QuikChange Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Mutations were confirmed by sequence analysis.

RESULTS

Adsorption of anti-MPER antibodies. To examine the contribution of anti-MPER antibodies to heterologous neutralization, we devised a method to specifically adsorb these antibodies with magnetic beads coated with a peptide containing the MPER sequence. We first tested three plasma samples from the BB cohort, BB34, BB81, and BB105, which were previously found to have anti-MPER antibody titers of 1:4,527, 1:264, and 1:80, respectively (12). The monoclonal antibody (MAb) 4E10 was used as a positive control. The effective depletion of the anti-MPER antibodies was demonstrated by the loss of binding in an MPER-peptide ELISA, as well as a reduction in neutralization of the HIV-2–HIV-1 MPER chimeric virus C1C for all three plasmas and MAb 4E10 (Fig. 1A). There was no change in ELISA reactivity to a V3 peptide after treatment of samples with the blank or MPER-peptide-coated beads, demonstrating that the anti-MPER antibodies were specifically depleted from the plasma (Fig. 1A).

We tested the adsorbed plasmas and their corresponding controls for neutralization of three heterologous subtype C viruses, COT6.15, CAP206.8, and Du156.12. The depletion of anti-MPER antibodies affected the heterologous neutralizing activity of only plasma BB34. The other two plasmas retained their neutralizing activities despite the efficient removal of anti-MPER antibodies (Fig. 1B). This indicated that anti-MPER antibodies in BB81 and BB105 were not involved in the neutralization of these viruses. Since the anti-MPER titers of these two plasmas were substantially lower than that of BB34, this suggested that high anti-MPER titers may be required to mediate the neutralization of primary viruses. This notion is supported by the observation that the HIV-2–HIV-1 MPER chimeras were 1 to 2 log units more sensitive to the MAbs 4E10 and Z13e1 than HIV-1 primary viruses (3). We therefore decided to identify additional samples with high anti-MPER antibody titers for further experiments.

Screening for broadly cross-neutralizing plasma samples containing anti-MPER antibodies. Three plasma samples with broadly cross-neutralizing activities and high titers of anti-MPER antibodies were identified following a comprehensive screening of three cohorts of chronically infected individuals (Table 1). BB34, described above, was one of 70 plasmas collected from HIV-infected blood donors, 16 of which were found to be broadly neutralizing (12). Of these, 11 had anti-MPER antibodies; however, only BB34 had anti-C1C titers above 1:1,000. We also tested plasmas from 18 participants in the CAPRISA cohort, corresponding to 3 years postinfection. Four of these were able to neutralize 50% or more of the subtype C primary viruses, two of which had anti-MPER antibodies. Of these, only CAP206 had titers above 1:1,000 and bound the linear peptide in an ELISA. Plasma SAC21 was selected from a second group of 68 blood donors (the SAC cohort), 4 of which had neutralization breadth and anti-MPER antibody titers above 1:1,000. However, only SAC21 bound the MPER peptide in an ELISA.

The levels of anti-MPER antibodies in these three plasma samples were high when tested against the HIV-2–HIV-1

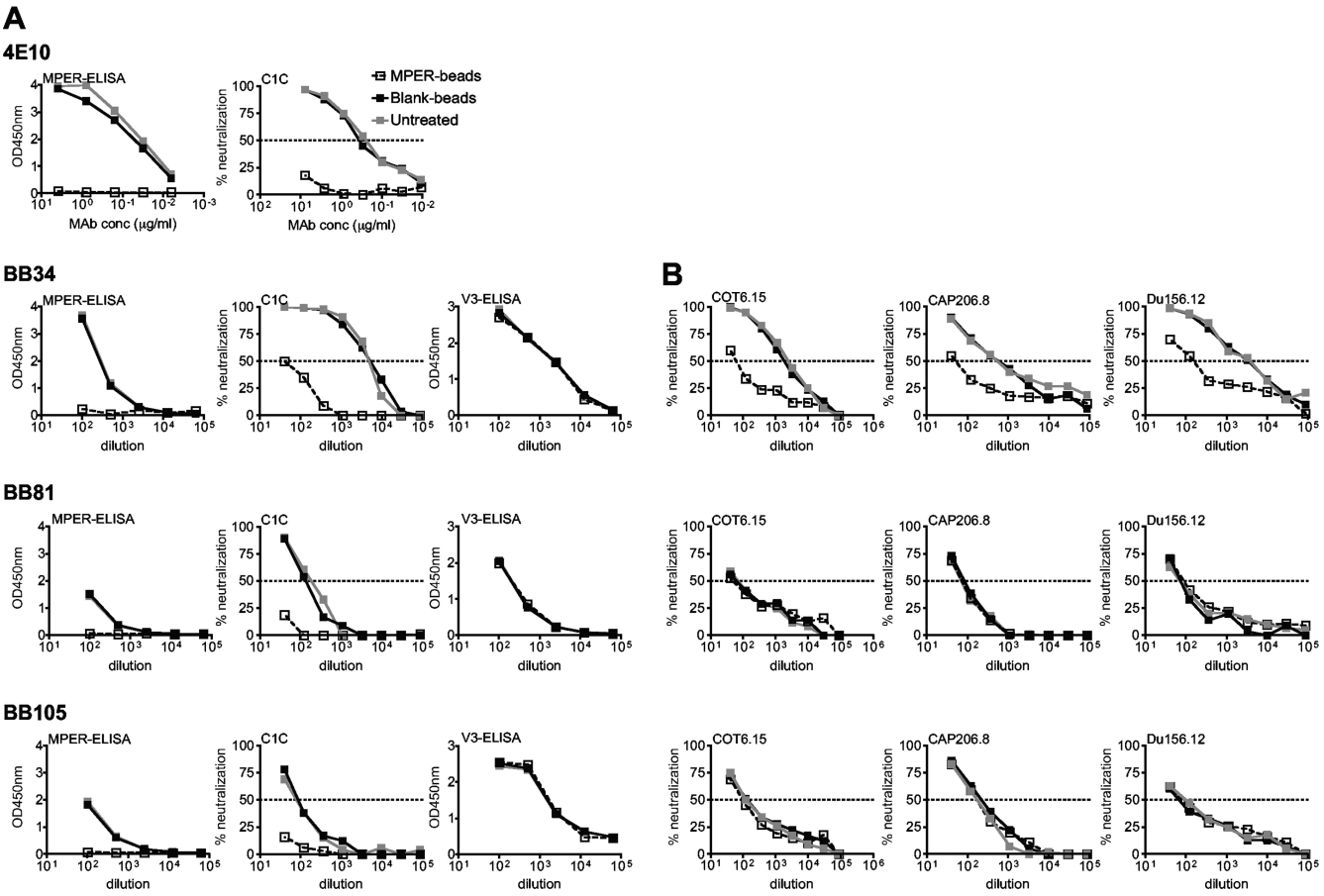


FIG. 1. Adsorption of anti-MPER antibodies from plasmas BB34, BB81, and BB105. MAb 4E10 and plasma samples were adsorbed with MPER-peptide-coated beads or blank beads or left untreated. (A) All samples were assayed by ELISA for binding to the MPER or V3 peptide and tested for neutralization of the HIV-2–HIV-1 MPER chimera C1C. OD, optical density; conc, concentration. (B) Adsorbed plasmas were tested for neutralization of the HIV-1 envelope-pseudotyped viruses COT6.15, CAP206.8, and Du156.12.

MPER chimera C1C, with ID_{50} titers of 1:4,802 for BB34, 1:4,527 for CAP206, and 1:3,157 for SAC21. The extent of neutralization breadth of these plasmas was determined using a large panel of envelope-pseudotyped viruses of subtype A (n = 5), B (n = 13), C (n = 24), and D (n = 1). Plasma BB34 was able to neutralize 60% of all the viruses tested, while CAP206 neutralized 50% and SAC21 neutralized 47% of the panel (see Table S1 in the supplemental material).

TABLE 1. Screening for broadly cross-neutralizing plasma samples containing anti-MPER antibodies

Parameter[a]	Value in:		
	BB cohort	CAPRISA	SAC cohort
Total no. of plasmas	70	18	68
No. (%) BCN	16 (23)[b]	4 (22)[c]	17 (25)[d]
No. of BCN anti-MPER antibodies positive	11	2	6
No. of BCN anti-MPER titers > 1:1,000	1	1	4
No. MPER peptide binding	1	1	1
Sample analyzed	BB34	CAP206	SAC21

[a] BCN, broadly cross-neutralizing. Anti-MPER activity was defined as neutralization of the HIV-2–HIV-1 MPER chimeric virus C1C.
[b] BCN plasmas were defined as able to neutralize at least 8 of 10 viruses tested (12).
[c] BCN plasmas were defined as able to neutralize at least 8 of 12 viruses from the tier 2 subtype C virus panel.
[d] BCN plasmas were defined as able to neutralize at least four of six viruses tested.

Anti-MPER antibodies mediate heterologous neutralization. To determine how much of the breadth in these three plasma samples was MPER mediated, we depleted this antibody specificity using peptide-coated beads and tested the adsorbed plasmas against viruses that were neutralized at titers above 1:80. The percentage reduction in the ID_{50} after adsorption on MPER-peptide-coated beads relative to the blank beads was calculated for each virus. Reductions of more than 50% were considered significant. Neutralization of C1C was considerably diminished by the removal of anti-MPER in all three plasmas (Table 2). Similarly, there was a substantial decrease in the neutralization of the majority of primary viruses tested. For BB34, 77% (17/22) of the viruses tested with the adsorbed plasma showed evidence that neutralization was mediated by anti-MPER antibodies, while for CAP206 and SAC21, it was 68% (13/19) and 46% (6/13), respectively. None of the subtype A and D viruses were neutralized significantly (<50%) by the anti-MPER antibodies in these plasmas, although only a few clones were available to test. Neutralization

TABLE 2. Effect of anti-MPER antibody adsorbtions on neutralization breadth

Subtype	Virus	ID_{50}		% Reduction[c]
		Blank[a]	MPER[b]	
Adsorbed BB34 plasma				
HIV-2/HIV-1 MPER	C1C	4,802	41	**99**
Subtype C	COT6.15	1,350	65	**95**
	CAP85 9	7,134	1,140	**84**
	CAP88 B5	258	<40	**84**
	CAP206 8	1,350	86	**94**
	CAP210 B8	148	102	31
	CAP228 51	245	73	**70**
	CAP255 16	164	<40	**76**
	Du151.2	484	636	0
	Du422.1	155	<40	**74**
	Du156.12	3,869	151	**96**
	ZM197M.PB7	1,068	<40	**96**
	ZM233M.PB6	219	66	**70**
	ZM135M.PL10a	1,651	250	**85**
Subtype B	6535.3	549	102	**81**
	QHO692.42	179	42	**77**
	CAAN5342.A2	139	129	7
	TRO.11	646	<40	**94**
	SC422661.8	758	175	**77**
	REJO4541.67	331	80	**76**
	JR-FL	129	<40	**69**
Subtype A	92RW009	1,296	827	32
Subtype D	92UG024	1,480	1,006	32
Adsorbed CAP206 plasma				
HIV-2/HIV-1 MPER	C1C	4,527	222	**95**
Subtype C	COT6.15	1,236	109	**91**
	CAP45 G3	4,720	193	**96**
	CAP63 A9	180	132	27
	CAP85 9	2,856	352	**88**
	CAP88 B5	223	<40	**82**
	CAP206 8	1,870	1,555	17
	Du151.2	105	<40	**62**
	Du422.1	165	47	**72**
	Du156.12	692	57	**92**
	Du172.17	234	<40	**83**
	ZM197M.PB7	309	82	**73**
	ZM135M.PL10a	248	91	**63**
Subtype B	QHO692.42	383	66	**83**
	AC10.0.29	111	47	**58**
	WITO4160.33	144	99	31
	TRO.11	491	<40	**92**
Subtype A	92RW009	915	793	13
	Q23.17	320	340	0
Subtype D	92UG024	1,556	1,268	19
Adsorbed SAC21 plasma				
HIV-2/HIV-1 MPER	C1C	3,157	246	**92**
Subtype C	COT6.15	183	<40	**78**
	CAP85 9	447	276	38
	CAP88 B5	88	42	**52**
	CAP206 8	361	140	**61**
	CAP255 16	109	115	0
	Du151.2	117	69	41
	ZM197M.PB7	117	85	27
	ZM233M.PB6	100	79	21
	ZM135M.PL10a	1,114	301	**73**
Subtype B	TRO.11	147	47	**68**
	SC422661.8	88	<40	**55**
Subtype A	92RW009	1,665	1,045	37
Subtype D	92UG024	1,889	1,491	21

[a] ID_{50} of plasmas adsorbed on blank beads. These titers were similar to the ID_{50} obtained with the untreated sera.
[b] ID_{50} of plasmas adsorbed on beads coated with the MPER peptide.
[c] Percentage reduction in ID_{50} due to adsorption on MPER-coated beads (1 − MPER/blank). Cases where the percent reduction was >50% are in boldface.

of the subtype B viruses appeared to be as effective as subtype C virus neutralization. Overall, these results suggested that the anti-MPER antibodies found in these HIV-1 subtype C plasma samples were largely responsible for the observed heterologous neutralization.

Potencies of eluted anti-MPER antibodies. We confirmed that the adsorbed antibodies had heterologous neutralizing activity by assaying antibodies eluted from the MPER-peptide-coated beads. The eluates from all three plasmas neutralized C1C efficiently (Fig. 2A). BB34 was the most potent, with an IC_{50} of 0.18 μg/ml, while CAP206 and SAC21 were similar at 0.39 and 0.31 μg/ml, respectively. The eluates were also tested against four subtype C and one subtype B primary viruses that were sensitive to all three plasmas, and BB34 was also tested against JR-FL (Fig. 2B). The BB34 eluate was able to neutralize all six viruses with potency comparable to or greater than those of the MPER MAbs. Thus, the virus CAP206.8 was neutralized over 10-fold more efficiently by BB34 eluates than by MAb 4E10. For JR-FL, the BB34 MPER eluate was even more effective than MAbs 2F5, 4E10, and Z13e1. The eluate from CAP206 was less potent and more comparable to the activity of MAb Z13e1. Interestingly, it was most potent against the CAP206.8 virus, suggesting a role for these anti-MPER antibodies in autologous neutralization. Despite multiple attempts, the antibody concentration of the SAC21 eluates was too low, and neutralization of viruses other than C1C was not observed. Similarly, the BB34 and CAP206 eluates did not have activity against viruses that the plasma neutralized at a low ID_{50}, such as CAP88.B5 and Du151.2 (data not shown). Eluates from blank beads, used as negative controls, did not show activity against any of the viruses tested (data not shown).

IgG subclasses in plasma and eluates. To establish the nature of these anti-MPER antibodies, we determined the IgG subclass profiles of the antibodies eluted from the beads and compared them to those of the parent plasmas. All three plasma samples displayed the classical profile of IgG1 > IgG2 > IgG3 > IgG4, although each had a different subclass distribution (Fig. 3). The eluates from the MPER beads were enriched in some subclasses. The BB34 eluate was enriched in IgG1 and IgG3 antibodies, while IgG2 and IgG4 were below detection. The CAP206 eluate was enriched in IgG1 and IgG4, while SAC21 was enriched in IgG1, IgG3, and IgG4 compared to whole plasma.

IgG3 anti-MPER antibodies mediate neutralization in plasma BB34. Given that the eluates from BB34 were enriched in IgG3 antibodies, we decided to explore the contribution of this IgG subclass to anti-MPER neutralization. We extracted total IgG from the plasmas using a protein G column. This was followed by fractionation through a protein A column, which specifically excludes IgG3 antibodies. The fractions were tested for their IgG subclass profiles to corroborate that IgG3 antibodies were enriched in the protein A column flowthrough (FTpA) and excluded in the eluate (EpA) (Fig. 4A). Binding to the MPER peptide and the neutralizing activities of the fractions were compared after their total IgG concentrations were standardized. Interestingly, while no differences in binding were observed between the fractions, the FTpA fraction showed a 100-fold increase in neutralization of C1C compared to the EpA fraction (Fig. 4C). This suggested that most of the anti-MPER activity resided within the IgG3 fraction. Similar

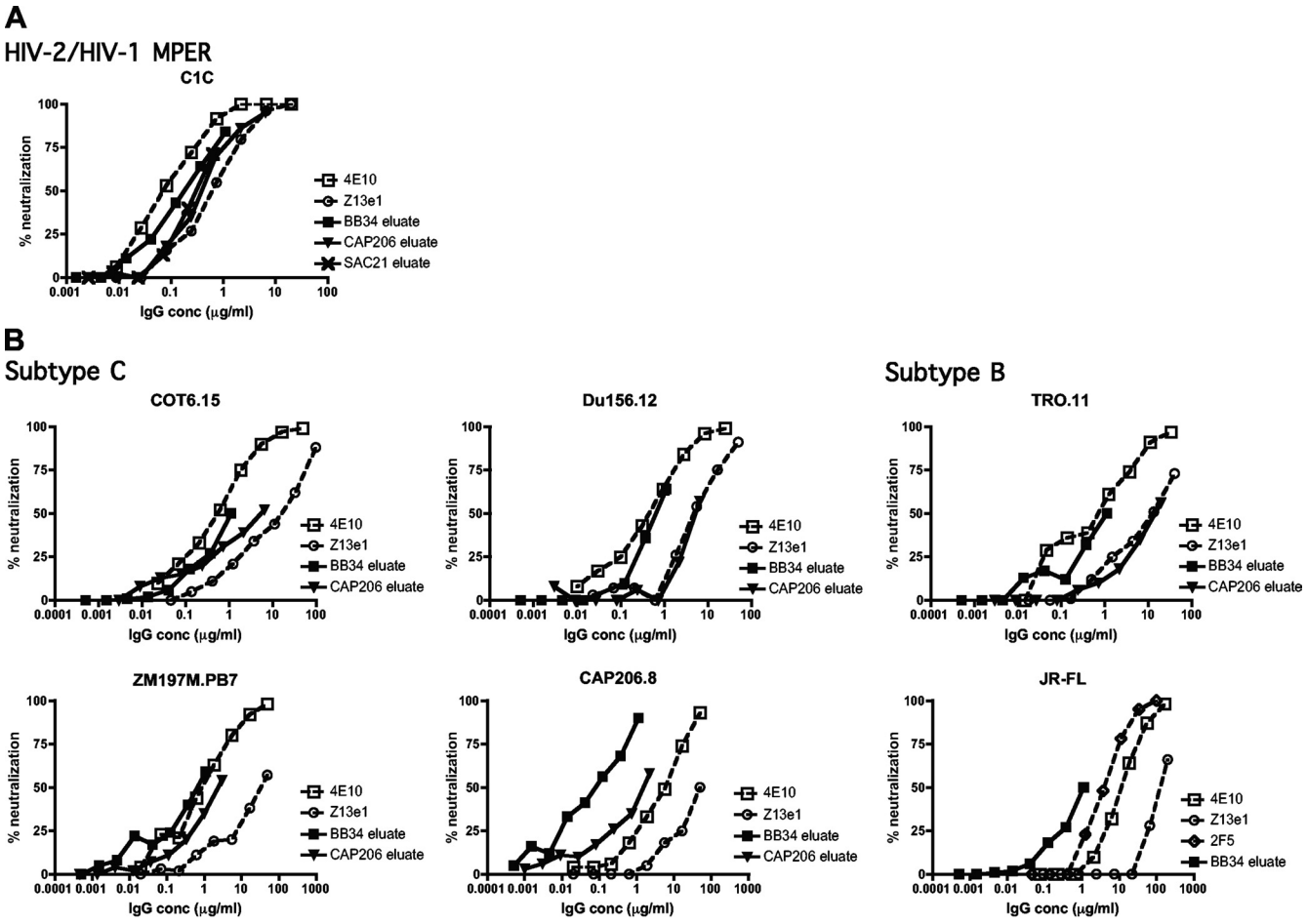


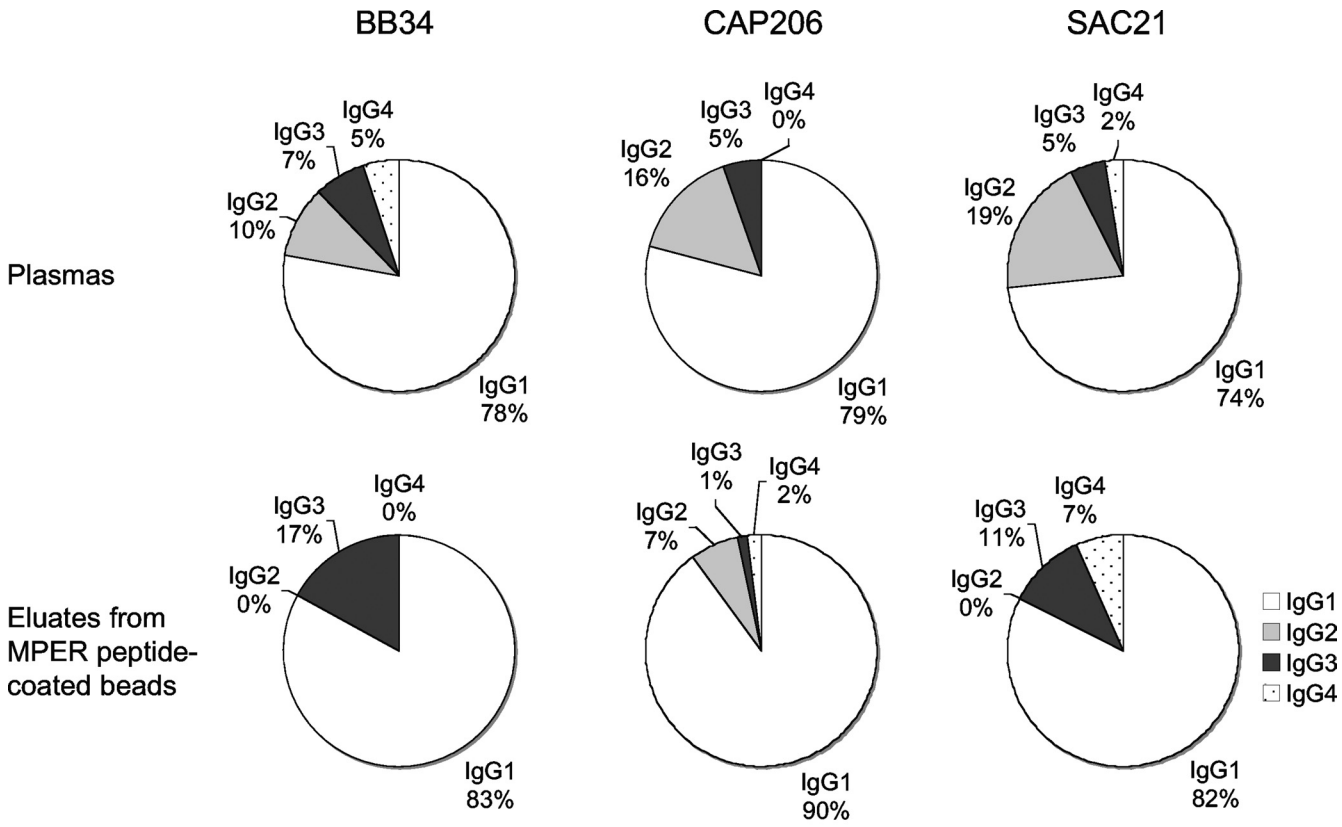
FIG. 2. Antibodies eluted from MPER-coated beads contain cross-neutralizing activity. (A) Neutralization of C1C by eluates from MPER-coated beads of plasmas BB34, CAP206, and SAC21 and MAbs 4E10, Z13e1, and 2F5. conc, concentration. (B) Neutralization of HIV-1 subtype C envelope-pseudotyped viruses COT6.15, ZM197M.PB7, Du156.12, and CAP206.8 and subtype B TRO.11 and JR-FL.

results were found in the neutralization of the viruses COT6.15, Du156.12, JR-FL, and TRO.11. However, for viruses 92Rw0009 and 92UG024, no differences in neutralization were noted between the FTpA and the EpA fractions. This corresponded to our previous observations showing that these viruses were not neutralized via anti-MPER antibodies (Table 2).

To determine if IgG3-mediated neutralization was a general feature of cross-neutralizing anti-MPER antibodies, we performed similar experiments with the CAP206 plasma. The FTpA fraction of CAP206 was significantly enriched for IgG3 antibodies, similar to BB34 (Fig. 4B). However, the FTpA fraction had little to no neutralizing activity, while the EpA fraction clearly recapitulated the activity of the original IgG pool (Fig. 4D). This suggested that in CAP206, anti-MPER neutralizing antibodies were not IgG3.

MPER epitope mapping. To characterize the epitopes recognized by these anti-MPER antibodies, we tested them against HIV-2/HIV-1 chimeras containing portions of the MPER (3, 10, 11). All three plasmas showed similar patterns of neutralization, mapping to an epitope in the C terminus of the MPER (Table 3). These anti-MPER antibodies were not identical to 4E10, as they failed to neutralize the C6 chimera, which contains the minimal residues for 4E10 neutralization. They were, however, dependent on a tryptophan at position 670 for recognition, as substantial differences in neutralization were observed between the chimeras C4 and C4GW. This is similar to the neutralization pattern seen with MAb Z13e1.

To finely map these novel epitopes, we constructed alanine-scanned mutants from positions 662 to 680 of the MPER in the subtype C virus COT6.15 (Table 4). The alanine at position 662 was changed to a glycine residue. MAb Z13e1 did not effectively neutralize COT6.15, possibly due to a serine substitution in position 671 (19), and therefore we did not use this MAb in the characterization of these mutants. Many of the COT6.15 mutants showed increased sensitivity to neutralization by MAb 4E10 and the three plasmas (Table 4). Similar enhancement has been reported previously using mutants of the JR-2 strain (19, 37), which may be related to distortion of the MPER structure, resulting in increased antigenic exposure. However, we did not observe major changes in the infectivities of the mutant viruses. Neutralization by 4E10 was ablated by previously defined residues with changes at W672, F673, T676, and W680, substantially reducing sensitivity to the MAb (37).



	μg/ml			
	IgG1	IgG2	IgG3	IgG4
BB34 plasma	19,868	2,617	1,822	1,280
BB34 eluate	2.22	b.d	0.45	b.d
CAP206 plasma	33,614	6,580	2,241	b.d
CAP206 eluate	35.21	2.56	0.57	0.78
SAC21 plasma	17,585	4,539	1,221	596
SAC21 eluate	0.63	b.d	0.08	0.05

FIG. 3. Comparison of the IgG subclass profiles between original plasmas and eluates from MPER-coated beads. The pie charts represent the IgG subclasses found in the BB34, CAP206, and SAC21 plasmas and eluates. The table shows the IgG subclass concentrations in plasmas and in eluates. b.d, below detection level.

The three plasma samples effectively neutralized most alanine mutants (Table 4). The mutation W670A affected neutralization by BB34 and to a lesser extent by SAC21, supporting the above findings with the HIV-2 chimeras. However, this mutation did not affect CAP206 neutralization. This is consistent with the observation that CAP206 had the least disparity in titers between the C4 and C4GW chimeras (Table 3). Nonetheless, the decreased sensitivity of C4 to CAP206 may suggest that the residue is more critical for the correct presentation of this epitope in the context of the HIV-2 envelope. The F673A mutation eliminated recognition by SAC21 with no effect on BB34 and CAP206 neutralization. The mutation D674A abrogated neutralization by all three plasmas. As this residue is highly polymorphic among HIV-1 strains, we further mutated D674 to serine or asparagine, the other two common amino acids found at this position. D674N had little effect on neutralization, with only a twofold drop in the ID_{50}, while the D674S mutation affected recognition by all three plasmas. In summary, these plasmas recognized overlapping but distinct epitopes within the C-terminal region of the MPER that did not correspond to the previously defined 4E10 or Z13e1 epitope.

DISCUSSION

In this study, we have clearly demonstrated that anti-MPER antibodies in three broadly cross-neutralizing plasmas were largely responsible for the heterologous neutralization displayed by these samples. For most viruses, the bulk of the neutralizing activity could be attributed to this single antibody specificity. Furthermore, our data suggested that these antibodies were as potent as existing MAbs and defined novel epitopes within the MPER. These data reinforce the potential of the HIV-1 gp41 MPER as a neutralizing-antibody vaccine target.

We previously showed a significant association between the presence of anti-MPER antibodies and neutralization breadth in plasma samples from a cohort of chronically infected blood donors (12). We can now confirm that, at least in some cases, anti-MPER antibodies are primarily responsible for this neu-

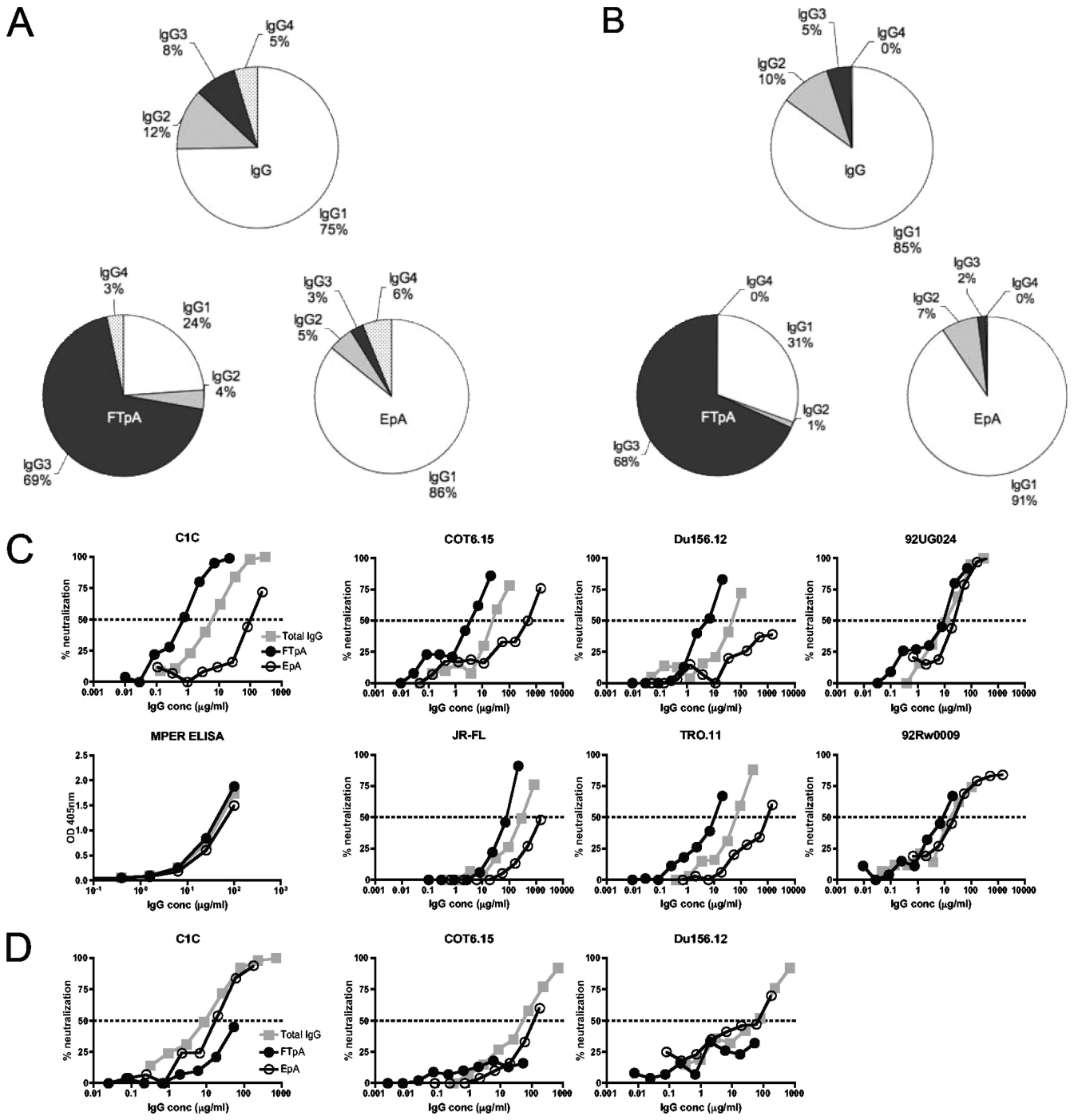


FIG. 4. Neutralizing anti-MPER antibodies are IgG3 in BB34 but not in CAP206. (A and B) IgG subclass profiles of total IgG, FTpA, and EpA of BB34 (A) and CAP206 (B). (C) BB34 fractions were tested for neutralization of C1C and HIV-1 envelope-pseudotyped viruses, as well as binding to the MPER peptide in ELISA. OD, optical density; conc, concentration. (D) CAP206 fractions were tested for neutralization of C1C and HIV-1 envelope-pseudotyped viruses.

tralizing activity. The levels of breadth displayed by these three HIV-1 subtype C plasma samples varied, with BB34 being the broadest and CAP206 and SAC21 neutralizing about half the viruses tested. Of those viruses neutralized by BB34 and CAP206, approximately 70% were neutralized via anti-MPER antibodies, and in the majority of cases, these antibodies mediated almost all the activity. The anti-MPER antibodies in SAC21 neutralized fewer viruses, and often they only partially contributed to the overall neutralization, probably due to smaller amounts of specific IgG in the sample. For all three plasmas, there were examples where the adsorption of anti-MPER antibodies did not remove all the neutralizing activity

TABLE 3. Mapping of anti-MPER neutralizing antibodies

Chimera	MPER sequence[a]	Neutralization[b]			Plasma ID_{50}		
		2F5	4E10	Z13e1	BB34	CAP206	SAC21
7312A	NMYEL$_{660}$QKLNSWDVFG$_{670}$NWFDLASWVK$_{680}$YIQYGVYIV	−	−	−	<20	21	<20
C1	NMYEL$_{660}$*LALDKWASLW*$_{670}$*NWFDITKWLW*$_{680}$*YIK*YGVYIV	++	++	++	5,560	3,903	3,871
C1C	NMYEL$_{660}$*LALD***S***W***KN***LW*$_{670}$*NWFDITKWLW*$_{680}$*YIK*YGVYIV	−	++	++	3,945	2,867	2,733
C1C F/L	NMYEL$_{660}$*LALD***S***W***KN***LW*$_{670}$*NW***L***DITKWLW*$_{680}$*YIK*YGVYIV	−	−	+	1,779	2,449	1,802
C3	NMYEL$_{660}$*LALDKWASLW*$_{670}$NWFDLASWVK$_{680}$YIQYGVYIV	++	−	−	<20	<20	<20
C7(2F5)	NMYEL$_{660}$Q***A***L***DK***W***A***VFG$_{670}$NWFDLASWVK$_{680}$YIQYGVYIV	++	−	−	<20	<20	<20
C6(4E10)	NMYEL$_{660}$QKLNSWDVFG$_{670}$NWFD***IT***SW***I***K$_{680}$YIQYGVYIV	−	++	−	<20	<20	<20
C4	NMYEL$_{660}$QKLNSWDVFG$_{670}$*NWFDITKWLW*$_{680}$*YIK*YGVYIV	−	++	+/−	<20	723	189
C4GW	NMYEL$_{660}$QKLNSWDVFW$_{670}$*NWFDITKWLW*$_{680}$*YIK*YGVYIV	−	++	++	7,482	3,067	2,987
C8	NMYEL$_{660}$QKLNSWD*SLW*$_{670}$*NWFDITKWLW*$_{680}$*YIK*YGVYIV	−	++	+	3,351	2,538	1,199

[a] Grafted amino acids are indicated in italics, with the 7312A residues in lightface. Further mutations on the chimeras are in boldface.
[b] Neutralization by MAbs 2F5, 4E10, and Z13e1 are qualitatively indicated relative to the titers obtained with the C1 chimera. −, no neutralization; ++, neutralization similar to that of C1; +, neutralization within 3-fold of that of C1; +/−, neutralization within 10-fold of that of C1.

or in some cases had no effect. The latter suggests that other specificities distinct from the adsorbed anti-MPER antibodies were also present in these plasmas. The residual neutralization of C1C by depleted CAP206 and SAC21 plasmas suggested that in some cases they may also be MPER antibodies that failed to bind the linear peptide. This is in line with the observations by others that more than one specificity may be involved in the neutralization breadth displayed by plasmas from some HIV-1-infected individuals (3, 7, 16, 28).

Testing of the antibodies eluted from the MPER peptide allowed us to conclusively show that these antibodies mediated cross-neutralization. The potency of the eluted antibodies recapitulated the activity in the original plasma samples, although the IC_{50} and ID_{50} values did not always correlate. This may be due to other non-MPER neutralizing antibodies present in these samples, as described above, or perhaps loss of activity during the elution process. Eluates are likely to contain mixtures of MPER-specific antibodies that may differ in binding affinity, as well as neutralization capacity, and thus represent considerably more of a technical challenge than testing purified MAbs. Even if the elution data are more qualitative than quantitative, they nevertheless show that the potencies of these antibodies are in the range of the current MAbs. Interestingly, the CAP206 eluate efficiently neutralized the autologous virus, despite the fact that no significant reduction in the ID_{50} was observed after depletion of anti-MPER antibodies from the plasma sample (Table 2). It is possible that other autologous neutralizing-antibody specificities overshadowed the activities of the anti-MPER antibodies in this plasma sample.

The neutralizing anti-MPER antibodies in plasma BB34 were found to be mainly IgG3. It is interesting that the original

TABLE 4. Relative neutralization of pseudotyped COT6.15 envelope MPER mutants[a]

COT6.15	4E10		BB34		CAP206		SAC21	
	IC_{50}	Ratio[b]	ID_{50}	Ratio[c]	ID_{50}	Ratio[c]	ID_{50}	Ratio[c]
Wild type	0.9	1.0	1,392	1.0	1,256	1.0	317	1.0
A662G	0.12	0.1	4,899	0.3	2,443	0.5	978	0.3
L663A	0.02	0.0	8,714	0.2	7,971	0.2	5,660	0.1
D664A	0.77	0.9	1,149	1.2	844	1.5	238	1.3
S665A	0.14	0.2	5,495	0.3	1,562	0.8	1,787	0.2
W666A	0.51	0.6	5,554	0.3	4,294	0.3	446	0.7
K667A	0.05	0.1	3,261	0.4	1,694	0.7	1,734	0.2
N668A	1.3	1.4	831	1.7	425	3.0	208	1.5
L669A	0.05	0.1	3,847	0.4	3,138	0.4	1,195	0.3
W670A	0.11	0.1	**132**	**10.5**	1,054	1.2	**105**	**3.0**
S671A	0.04	0.0	3,102	0.4	1,614	0.8	928	0.3
W672A	**>25**	**>25**	2,959	0.5	2,244	0.6	468	0.7
F673A	**>25**	**>25**	779	1.8	498	2.5	**<50**	**>6.3**
D674A	1.4	1.6	**<50**	**>25**	**<50**	**>25**	**<50**	**>6.3**
D674S	2.49	2.8	**<50**	**>25**	**90**	**14.0**	**<50**	**>6.3**
D674N	0.33	0.4	663	2.1	643	2.0	149	2.1
I675A	0.04	0.0	4,069	0.3	2,065	0.6	718	0.4
T676A	**21.77**	**24.2**	2,380	0.6	895	1.4	524	0.6
K677A	0.05	0.1	4,671	0.3	2,151	0.6	1154	0.3
W678A	0.05	0.1	3,842	0.4	1,885	0.7	1,007	0.3
L679A	0.09	0.1	2,085	0.7	1,448	0.9	225	1.4
W680A	**10.89**	**12.1**	731	1.9	904	1.4	142	2.2

[a] Cases with more than a 3-fold drop in the ID_{50} or IC_{50} are in boldface.
[b] (Mutant IC_{50})/(wild-type IC_{50}) ratio.
[c] (Wild-type ID_{50})/(mutant ID_{50}) ratio.

hybridoma-derived broadly neutralizing anti-MPER MAbs 4E10 and 2F5 were of the IgG3 subclass (14) and the neutralizing fraction of a polyclonal human HIV immune globulin was also reported to be IgG3 (29). IgG3s have a highly flexible hinge region that has been proposed to facilitate access to the MPER and that is thought to be partly buried in the viral membrane and enclosed by the gp120 protomers. However, for both MAbs, a change to IgG1 did not affect the neutralization capacity, suggesting that IgG3s are not essential for MPER-mediated neutralization (14, 15). Indeed, for CAP206, the IgG3-enriched fraction had less activity, and in this case, neutralization was due to either IgG1 or IgG2. While there was an enrichment of IgG3 in SAC21 eluates, the low potency of these antibodies precluded them from being tested further. Both BB34 and SAC21 were from blood donors with an unknown duration of infection, while CAP206 has been followed prospectively for 3 years since seroconversion. Although IgG3 has been reported to appear early in infection, we will continue to monitor the anti-MPER response in CAP206 to see if the IgG subclass profile, antibody specificities, or neutralization titers change over time.

The binding of all three anti-MPER plasma antibodies depended on the residue at position 674 in the MPER, which has been shown to be the most critical for Z13e1 recognition (21). The immunogenicity of this residue may be related to its location in the hinge region of the MPER (21, 31, 33). However, the high level of polymorphism at this position is considered to be one of the main reasons why the Z13e1 MAb neutralizes a narrower set of viruses than the 4E10 MAb. In contrast to MAb 2F5, which seldom neutralizes subtype C viruses due to a subtype-associated polymorphism at position 665 (4, 9), the residue at position 674 is not associated with a particular subtype. This is consistent with our finding that subtype B and C viruses were equally neutralized by MPER antibodies present in all three plasmas. In addition to this common residue, BB34 and SAC21 also depended on W670, which is not implicated in either 4E10 or Z13e1 recognition. SAC21 showed some overlap with the 4E10 MAb, since it was affected by the F673A mutation. However, the identities of the precise residues required by these antibodies indicated that they are distinct from 4E10 and Z13e1. Furthermore, analysis of the MPER sequences of the viruses neutralized by these plasmas suggested that the residue at position 674 affects their sensitivity, with the majority of viruses harboring a serine showing resistance (see Fig. S1 in the supplemental material). However, not all viruses with an aspartic or asparagine residue at position 674 and, even more, with the same MPER sequence were neutralized equally, suggesting that features outside this region may modulate the presentation of this epitope, as suggested by previous studies (4, 10).

The presence of anti-MPER antibodies in broadly cross-neutralizing subtype B plasmas has been reported recently by others. Li and colleagues found that neutralization of the JR-FL virus by plasma no. 20 was outcompeted by a peptide covering the 4E10 epitope, although the extent of the contribution of this specificity to breadth was not determined (16). Sather and coworkers found 4E10-like activity in plasma VC10008 (28); however, this sample did not neutralize some 4E10-sensitive viruses, suggesting differences in their specificities. Neither of these studies investigated the precise epitopes recognized by these potentially novel antibodies, so it is not possible to determine if they differ from the ones identified here. A third study described an individual who developed antibodies that recognized a region overlapping the 2F5 epitope (30). Anti-MPER affinity-purified antibodies from this individual, SC44, displayed broad neutralizing activity. Similar to our study, which identified three samples from among 156 chronically infected individuals, the 2F5-like antibody found by Shen and colleagues was 1 of 311 plasmas analyzed (30).

The scarcity of these samples supports the notion that broadly neutralizing anti-MPER antibodies are seldom developed by HIV-1-infected individuals. Haynes et al. proposed that such antibodies are autoreactive and therefore eliminated through B-cell tolerance mechanisms (13). While CAP206 did not have detectable levels of autoreactive antibodies, BB34 was positive for anti-double-stranded DNA antibodies and rheumatoid factor (12). Another explanation for the paucity of such antibodies may be the short exposure time of this epitope during the formation of the fusion intermediate (8). Consistent with this, MAbs 2F5, 4E10, and Z13e1, as well as plasma BB34, neutralize JR-FL after CD4 and CCR5 attachment, when this occluded epitope is exposed (2, 3). Furthermore, BB34 neutralization was potentiated by coexpression of FcγRI on JC53bl-13 cells, also a feature of 2F5 and 4E10, possibly by providing a kinetic advantage through prepositioning of these antibodies close to the MPER (22). However, it remains unclear how these antibodies are induced in the context of natural infection despite the exposure constraints of this epitope. Perhaps these antibodies are elicited by more open conformations of the envelope glycoprotein that expose the MPER. Analysis of the autologous viruses that induce such responses may help to answer these questions.

It is noteworthy that the three cross-neutralizing antibodies identified here, while sharing some common residues, had distinct fine specificities. This suggests that the MPER can be recognized in a variety of conformations by the human immune system. It is therefore critical to isolate MAbs that define these novel epitopes within the MPER in order to facilitate a better understanding of the immunogenic structure of this region of gp41 and to identify new targets for HIV vaccine design.

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