

## The Neutralization Breadth of HIV-1 Develops Incrementally over Four Years and Is Associated with CD4<sup>+</sup> T Cell Decline and High Viral Load during Acute Infection<sup>∇</sup>

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**An understanding of how broadly neutralizing activity develops in HIV-1-infected individuals is needed to guide vaccine design and immunization strategies. Here we used a large panel of 44 HIV-1 envelope variants (subtypes A, B, and C) to evaluate the presence of broadly neutralizing antibodies in serum samples obtained 3 years after seroconversion from 40 women enrolled in the CAPRISA 002 acute infection cohort. Seven of 40 participants had serum antibodies that neutralized more than 40% of viruses tested and were considered to have neutralization breadth. Among the samples with breadth, CAP257 serum neutralized 82% (36/44 variants) of the panel, while CAP256 serum neutralized 77% (33/43 variants) of the panel. Analysis of longitudinal samples showed that breadth developed gradually starting from year 2, with the number of viruses neutralized as well as the antibody titer increasing over time. Interestingly, neutralization breadth peaked at 4 years postinfection, with no increase thereafter. The extent of cross-neutralizing activity correlated with CD4<sup>+</sup> T cell decline, viral load, and CD4<sup>+</sup> T cell count at 6 months postinfection but not at later time points, suggesting that early events set the stage for the development of breadth. However, in a multivariate analysis, CD4 decline was the major driver of this association, as viral load was not an independent predictor of breadth. Mapping of the epitopes targeted by cross-neutralizing antibodies revealed that in one individual these antibodies recognized the membrane-proximal external region (MPER), while in two other individuals, cross-neutralizing activity was adsorbed by monomeric gp120 and targeted epitopes that involved the N-linked glycan at position 332 in the C3 region. Serum antibodies from the other four participants targeted quaternary epitopes, at least 2 of which were PG9/16-like and depended on the N160 and/or L165 residue in the V2 region. These data indicate that fewer than 20% of HIV-1 subtype C-infected individuals develop antibodies with cross-neutralizing activity after 3 years of infection and that these antibodies target different regions of the HIV-1 envelope, including as yet uncharacterized epitopes.**

Neutralizing antibodies are thought to be crucial in the protective immune response against many viral infections, yet their role in HIV-1 infection remains controversial. During natural infection, they appear to have little impact on acute viremia, as they arise too late and the virus readily escapes type-specific neutralizing antibodies (35, 41, 42, 55). However, passive transfer of broadly neutralizing monoclonal antibodies (MAbs) has proven to be protective in nonhuman primate models (2, 11, 17, 18, 27, 28, 52), supporting the hypothesis that a vaccine capable of inducing this type of antibodies is likely to be effective. Despite rigorous efforts, designing an immunogen capable of inducing broadly neutralizing antibodies has so far not been feasible. Recently, researchers have turned their attention to understanding the factors associated with the presence of broadly cross-neutralizing antibodies, which develop in a subset of chronically HIV-1-infected individuals. A number

of reports from an assortment of different cohorts have found that the duration of infection, viral load, CD4<sup>+</sup> T cell count, and/or viral diversity is associated with the development of neutralization breadth (10, 37, 44).

The B cell response to HIV-1 infection first appears within 8 days of detectable viremia and initially comprises antigen-antibody complexes (47). This is followed by the detection of circulating anti-gp41 antibodies 5 days later, with anti-gp120 antibodies delayed a further 14 days and targeting primarily the V3 loop. Autologous neutralizing antibodies develop months later (15) and target the variable regions via potent but extremely type-specific neutralizing antibodies (22, 33, 41, 55). Recent data from our laboratory suggest that during the first year of HIV-1 subtype C infection, within a single individual, a limited number of antibody specificities mediate autologous neutralization (34). These arise sequentially and show temporal fluctuations as escape occurs. After years, antibodies with cross-neutralizing potential appear in as many as one-third of chronically infected individuals and target more conserved regions of the HIV-1 envelope (46).

An increasing number of studies have focused on mapping

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the antibody specificities responsible for the cross-neutralizing activity found in selected HIV-1-positive plasmas (3, 16, 25, 44, 45, 54). Using a variety of methodologies, it has been established that some of these neutralizing antibodies recognize epitopes in the context of monomeric gp120, e.g., the CD4 and coreceptor binding sites. In a few cases, the cross-neutralizing activity could be attributed to antibodies recognizing linear epitopes in the membrane-proximal external region (MPER) of gp41 (14, 45). However, many of the antibody specificities responsible for cross-neutralization could not be matched to known epitopes in these studies. More recently, it has become apparent that a quaternary epitope at the tip of the trimeric envelope structure, involving the V2 and V3 loop, is frequently the target of cross-neutralizing antibodies (34, 53, 54). Another cross-reactive specificity, involving the N332 residue in the C3 region at the base of the V3 loop, was also described recently, but the epitope has yet to be defined. Taking these data together, it appears that a limited number of neutralizing antibody specificities are responsible for the broad neutralizing activity in plasma samples (54). Isolation of MAbs from these individuals may delineate important targets on the HIV-1 envelope glycoprotein and thus inform vaccine design. Indeed, naturally occurring broadly neutralizing MAbs, such as b12, 2G12, 4E10, and 2F5 (6), have been crucial reagents for many years, and the more recently isolated MAbs VRC01 and PG9/PG16 (53, 56) are providing new insights into the native envelope structure. However, the mechanism by which these antibodies develop and why this occurs only in some individuals are not clear. Defining when cross-neutralizing antibodies emerge might provide important clues as to their nature and how to elicit them. For this purpose, suitable cohorts of HIV-infected individuals with antibodies able to cross-neutralize heterologous viruses need to be identified and longitudinal samples assessed.

Previously, we reported on the development of early type-specific neutralizing antibodies in the CAPRISA acute infection cohort (15). While autologous neutralizing antibodies were found in all individuals from 3 to 12 months postinfection, little heterologous neutralizing activity was detected in the 14 participants analyzed at 12 months of infection. Here we extended this analysis to 40 participants from the same cohort who had reached at least 3 years postinfection to evaluate the kinetics of the development of cross-neutralizing antibodies. We also analyzed the association between neutralization breadth and clinical factors, such as viral load and CD4<sup>+</sup> T cell count, and explored the epitopes targeted by these cross-reactive antibodies. Our results suggest that cross-neutralizing antibodies targeting different regions on the HIV-1 envelope develop over many years, with low CD4<sup>+</sup> T cell counts and high viral loads in early infection favoring their emergence.

## MATERIALS AND METHODS

**Study subjects.** Plasma and serum samples were obtained from participants in the CAPRISA acute infection cohort (CAPRISA 002), established in 2004 in Durban and Vulindlela, South Africa. Participants in Durban were part of a prospective study of 245 high-risk HIV-negative women who were followed up for subsequent identification of HIV seroconversion (51). A total of 62 women (28 from the HIV-negative cohort and 34 from other seroconversion cohorts) who had a reactive HIV antibody test within 5 months of a previously negative result or detection of HIV-1 RNA by PCR (Roche Amplicor v1.5) in the absence of HIV antibodies were enrolled. Thereafter, participants were monitored and

referred to the antiretroviral (ARV) therapy initiation program when their CD4<sup>+</sup> T cell counts dropped below 350 cells/ $\mu$ l on 2 separate occasions. They were initiated on ARV therapy only once their CD4<sup>+</sup> T cell counts fell below 200 cells/ $\mu$ l or other signs suggestive of WHO stage 4 disease were evident (2004 National Antiretroviral Treatment Guidelines). After the implementation of the 2010 South African Antiretroviral Treatment Guidelines, ARV treatment could be initiated with CD4<sup>+</sup> T cell counts of up to 350 cells/ $\mu$ l in *Mycobacterium tuberculosis*-coinfected individuals. Samples were collected and stored at least every 3 months during chronic infection. This study received ethical approval from the University of Witwatersrand, University of KwaZulu-Natal, and University of Cape Town.

**Envelope clones.** Envelope genes used in this study were either cloned previously in our laboratory (15) or obtained from the NIH AIDS Research and Reference Reagent Program. The ConC plasmid, carrying an envelope gene representing the consensus of all HIV-1 subtype C sequences deposited in the Los Alamos database by 2001, was obtained from Feng Gao (20). Mutations were introduced into envelope clones by use of a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Plasmids encoding HIV-2/HIV-1 MPER chimeras were obtained from George Shaw (15).

**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), with Env-pseudotyped viruses (32). Titer was calculated as the reciprocal plasma/serum dilution causing a 50% reduction of relative light units (ID<sub>50</sub>).

**gp120 production and isolation.** The gp120-encoding region of the ConC envelope was inserted into the pPPI4 expression vector (Progenics Pharmaceuticals, Inc., Tarrytown, NY) (4). The D368R and I420R mutations were introduced by site-directed mutagenesis. The resulting constructs were transfected into 293T cells seeded in a HYPERFlask (Corning Inc., Lowell, MA) by using Eugene (Roche Applied Science, Indianapolis, IN). Cell supernatants were 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- $\alpha$ -D-manno-pyranoside (Sigma-Aldrich). Remaining protein contaminants were eliminated through ion-exchange chromatography using FastFlow 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 flow-through, washed in PBS, and concentrated to 5 mg/ml. The purity of the final gp120 preparation was tested by subjecting 10  $\mu$ g of protein to SDS-PAGE. Protein preparations with a purity of >99% were used in subsequent experiments.

**Adsorption of anti-gp120 antibodies from plasma.** Adsorption of plasma anti-gp120 antibodies, using gp120 covalently coupled to tosyl-activated magnetic beads, was done as previously described (16, 25). The depletion of anti-gp120 antibodies was evaluated by enzyme-linked immunosorbent assay (ELISA) as described elsewhere (25).

**Statistical analysis.** Statistical analysis was performed using GraphPad Prism 5.0 and SAS software, version 9.1.3 (SAS Institute Inc., Cary, NC). Friedman's test was performed for pairwise comparisons of the percentages of viruses neutralized for each subtype (A, B, and C). The same data were analyzed using the Kruskal-Wallis rank sum unpaired test. A Wilcoxon matched-pair test was used to compare the medians of viruses neutralized among CAPRISA viruses and Zambian viruses.

The area under the curve (AUC) for viral loads between 6 months and 3 years of infection was calculated using 400 copies/ml as a baseline, which is the limit of detection of the viral load quantification assay used. Spearman rank tests were used to determine the correlation between neutralization breadth and all factors analyzed.

Univariate and multivariate linear regression models were used to study the factors associated with neutralization breadth. First, univariate models were fitted one at a time with each of the predictors, and then potential predictors were analyzed in two multivariate logistic regression models. Model 1 evaluated the significance of each predictor among all the viral load measurements, while model 2 included only the 6-month viral load and CD4<sup>+</sup> T cell decline (difference between preinfection and 6-month CD4<sup>+</sup> T cell counts) as predictors.

A proportional hazard regression model and Kaplan-Meier survival analysis were performed for the time between infection and a CD4<sup>+</sup> T cell count of <200 cells/ $\mu$ l and/or antiretroviral therapy initiation (whichever came first). A log rank *P* value test was used to determine differences between participants who developed cross-neutralizing antibodies and those who did not but had comparable viral loads. Those who did not initiate treatment were censored at their last visit.

## RESULTS

**Broadly cross-neutralizing antibodies develop in a small proportion of HIV-1-infected individuals.** Previous studies have demonstrated that the development of cross-neutralizing antibodies during HIV-1 infection is greatly delayed, appearing in only a subset of individuals (45). To assess the extent of heterologous neutralization in the CAPRISA 002 cohort, serum samples from participants who had documented HIV-1 infection for 3 years were assayed. Of the 62 women enrolled, 9 were referred to and initiated on antiretroviral treatment, 3 were lost to follow-up, and 2 suffered HIV-unrelated deaths before reaching 3 years of infection. Of the remaining 48 individuals, 40 reached 3 years postinfection by June 2010 without requiring antiretroviral treatment and were included in this study. The median CD4<sup>+</sup> T cell count of the study group after 3 years of infection was 442 cells/ $\mu$ l, and the median viral load was 15,700 copies/ml. Seven of the study participants were classified as rapid progressors and five as controllers, with the rest being considered intermediate progressors (Fig. 1).

All serum samples were assayed using the TZM-bl neutralization assay against various standard virus reference panels. These included 6 subtype A (5), 12 subtype B (23), and 12 subtype C (24) envelope-pseudotyped viruses. In addition, 12 pseudoviruses isolated from some of the CAPRISA participants during the acute phase of infection (15), as well as the reference strains ConC and Du151.12 (subtype C), were used. Samples were scored as positive if a virus was neutralized at titers above 1:45. Percent breadth was calculated based on the number of viruses in all panels that each serum sample neutralized (excluding autologous titers).

There was a wide range of activities, with most of the 3-year sera lacking substantial heterologous activity against tier 2 viruses (Fig. 1). However, samples from a few individuals showed extensive cross-neutralization against viruses of different subtypes, while on the other extreme, some sera showed no activity. Based on the percent neutralization, participants were categorized into 3 groups. Samples from 7 individuals able to neutralize more than 40% of the panel were considered to have neutralization breadth (group 1). Those with less than 10% activity ( $n = 19$  [48%]) were considered to have no breadth (group 3), and those with activities between these values ( $n = 14$  [35%]) were considered to have intermediate breadth (group 2). Among group 3 individuals, samples from 3 participants (CAP45, CAP88, and CAP221) did not neutralize any of the heterologous viruses tested, while those from 10 others neutralized only 1 to 3 viruses, often at low titers. Among the individuals with breadth, CAP257 samples neutralized 36/44 (82%) panel members, while CAP256 samples neutralized 33/43 viruses (77%, excluding the autologous virus), the latter with exceptionally high titers against some viruses. Indeed, titers of heterologous neutralization varied greatly, possibly due to differences in the exposure or structural heterogeneity of the epitopes targeted by these antibodies. Alternatively, these polyclonal serum samples contained mixtures of antibodies, with each targeting a different virus. Autologous titers were detected in all cases, as one might expect, given that autologous viruses were isolated at earlier time points from these individuals.

All viruses in the 44-member panel were neutralized by at

least one serum sample. The most resistant viruses to these sera were TRJO4551.58 (subtype B), REJO4541.67 (subtype B), and Q461.e2 (subtype A), while the most sensitive viruses were CAP85.9 (subtype C), ZM109F.PB4 (subtype C), and Q23.17 (subtype A). Interestingly, there was a strong correlation between the ability to neutralize the ConC virus (often at high titers) and the development of neutralization breadth ( $P < 0.0001$ ).

Analyzed all together, sera from CAPRISA participants more frequently neutralized viruses from the subtype C panels (23%) than subtype A (15%) or B (14%) viruses. This comparison was statistically significant using a Friedman test, with a  $P$  value of 0.0003. However, this effect was clearly driven by a subset of the sera that had subtype-specific neutralization, as an unpaired comparison lacked statistical significance (Kruskal-Wallis test;  $P = 0.1213$ ).

Given that many of the subtype C viruses used in this study were from the CAPRISA cohort, we evaluated whether these viruses were better neutralized by serum samples from CAPRISA participants. Thus, we compared the percentage of viruses neutralized and the geometric mean titer obtained with each of the 40 serum samples against the 14 CAPRISA viruses with those for the 7 Zambian viruses from the subtype C tier 2 panel. No statistically significant differences were observed in Wilcoxon matched-pair tests between the percentages of viruses neutralized ( $P = 0.2462$ ) or the geometric mean titers ( $P = 0.2862$ ) for these two groups of viruses (data not shown), suggesting that CAPRISA sera did not preferentially neutralize epidemiologically related viruses obtained from within the same cohort.

**Viral load at set point and CD4<sup>+</sup> T cell decline strongly correlate with the development of breadth.** Previous studies have found an association between viral load and/or CD4<sup>+</sup> T cell count and the presence of cross-neutralizing antibodies (10, 37, 44). Here we analyzed the correlation between the percentage of viruses neutralized by each individual (as a measurement of neutralization breadth) and the viral load and CD4<sup>+</sup> T cell count at 6, 12, and 36 months postinfection (Fig. 2). In addition, as a measurement of total antigenic exposure, we calculated the area under the curve for the viral loads over time from 6 months to 3 years of infection (viral load AUC). These analyses revealed a significant correlation between the viral load at set point (6 months postinfection) and the percentage of viruses neutralized at 3 years (Fig. 2A). Similarly, a negative correlation was found between the CD4<sup>+</sup> T cell count at 6 months postinfection and the development of breadth (Fig. 2D). However, no correlation was found between neutralization breadth and these two factors at later time points (Fig. 2B, C, E, and F). Furthermore, no significant correlation was observed between neutralization breadth and the viral load AUC (Fig. 2G), suggesting that the antigen load at a particular time postinfection, not total antigenic exposure, predisposes individuals toward the development of breadth. These correlations were confirmed using more robust statistical analyses (Table 1). Thus, while a univariate model analysis showed that viral loads at 6 months and 12 months and the AUC were significantly associated with neutralization breadth, a multivariate analysis revealed that the 6-month viral load was the major driver of this association. For every 1-log increase in viral load

Participant ID	ConC	Subtype C viruses															Subtype B viruses										Subtype A viruses					% of viruses neutralized						
		CAPRISA panel															Subtype C panel <sup>a</sup>										Subtype B panel <sup>b</sup>						Subtype A panel <sup>c</sup>					
CAP257	1401	141	264	142	454	241	336	78	127	99	1068	61	66	158	143	147	59	63	84	137	222	90	99	388	88	55	82	48	320	99	56	615	215	177	373	264	157	82%
CAP256	35496	50	-	345	106	1457	1015	1493	1446	231	755	58	377	721	1363	13479	1307	89	1302	80892	10227	287	13474	520	181	49	-	-	-	56	311	1307	144	2348	658	668	82%	
CAP248	1531	304	841	158	299	841	535	119	-	94	288	46	185	101	190	743	147	183	98	511	601	92	-	210	-	-	-	-	-	117	331	297	-	248	-	668	59%	
CAP177	1094	80	157	719	131	-	-	146	69	56	373	74	-	54	219	49	106	-	162	-	122	-	-	-	386	-	-	-	787	-	60	129	315	-	628	52%		
CAP255	869	-	227	>1245	-	-	-	54	59	725	-	270	-	520	156	334	162	-	-	-	-	-	-	-	112	177	81	78	49	144	55	-	272	-	624	47%		
CAP206	93	105	2368	-	-	-	-	85	>1245	-	-	-	-	173	187	59	75	-	223	161	692	-	-	-	48	48	51	-	108	-	67	81	155	72	169	-	636	42%
CAP8	44896	203	507	314	-	-	-	115	1457	81	-	-	-	58	299	-	48	-	-	-	-	-	-	-	439	61	155	72	169	-	109	-	636	-	311	32%		
CAP274	765	-	94	295	178	-	-	-	-	388	72	-	-	351	-	61	-	-	-	-	-	-	-	-	428	-	-	-	134	-	-	-	-	-	-	32%		
CAP264	-	53	274	423	-	-	-	-	-	-	-	-	-	309	-	50	-	241	88	-	-	-	57	-	-	-	-	-	-	-	-	-	-	-	-	32%		
CAP885	81	104	>1245	76	-	-	-	-	-	-	-	-	-	292	50	-	241	88	-	-	-	57	-	-	-	-	-	-	-	-	-	-	-	-	-	32%		
CAP239	4523	804	753	555	373	-	-	>1245	-	259	-	-	-	80	192	216	49	174	-	201	-	-	-	-	172	67	-	-	-	-	-	-	-	-	-	-	30%	
CAP129	341	901	54	-	-	-	-	166	-	-	-	-	-	172	68	786	-	-	52	243	-	-	-	-	170	-	-	-	-	-	-	-	-	-	-	28%		
CAP37	948	199	78	50	-	-	-	54	801	-	-	-	-	65	383	52	-	132	-	51	-	-	-	-	112	177	81	111	339	-	-	-	-	-	-	27%		
CAP261	289	180	148	104	-	-	-	66	-	-	-	-	-	277	218	388	-	-	-	210	-	-	-	-	288	-	-	-	-	-	-	-	-	-	-	25%		
CAP217	-	-	139	-	-	-	-	634	-	-	-	-	-	110	309	47	138	-	424	-	121	158	-	-	-	-	-	-	-	-	-	-	-	-	-	22%		
CAP257	-	-	75	-	-	-	-	104	-	-	-	-	-	216	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	20%		
CAP211	93	-	82	-	-	-	-	421	-	-	-	-	-	105	-	-	-	54	105	-	-	-	-	-	146	-	-	-	-	-	-	-	-	-	-	18%		
CAP84	100	72	-	48	1004	-	-	-	-	-	-	-	-	76	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	14%		
CAP222	67	150	-	103	-	-	-	-	-	-	-	-	-	67	60	-	-	63	53	-	71	-	-	-	-	-	-	-	-	-	-	-	-	-	-	14%		
CAP188	112	471	72	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	11%			
CAP269	48	-	-	-	-	-	-	77	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	11%			
CAP241	264	85	208	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	9%			
CAP61	88	-	88	-	285	-	-	-	-	289	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7%			
CAP228	-	88	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7%			
CAP262	134	-	-	-	-	-	-	310	-	-	-	-	-	-	-	-	-	46	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7%			
CAP266	48	-	-	-	-	-	-	83	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7%			
CAP40	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7%			
CAP229	-	-	70	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7%			
CAP285	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7%			
CAP268	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7%			
CAP65	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7%			
CAP137	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5%		
CAP137	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5%		
CAP220	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5%		
CAP271	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5%		
CAP225	-	-	48	46	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5%			
CAP237	-	-	77	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5%			
CAP200	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2%			
CAP45	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0%		
CAP88	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0%		
CAP221	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0%		

FIG. 1. Heterologous neutralizing activities in sera from the CAPRISA cohort at 3 years postinfection. The neutralization titer is shown as the reciprocal of the serum dilution required to inhibit 50% of infection for each virus-sample combination. Titers below detection, i.e., those of <1:45, have been omitted. The highest titers are shown in dark red and the lowest in light yellow, following the depicted legend. Autologous neutralization titers are highlighted in gray and were not included in the calculations of percentages of viruses neutralized. Participants were ranked based on cross-neutralizing activity. The pseudoviruses tested were from four panels: CAPRISA subtype C (15), reference subtype C (24), reference subtype B (23), and reference subtype A (5). Viruses are ranked from left to right within each panel based on the number of sera to which they were sensitive. The reference viruses ConC and Du151.12 are depicted separately on the left. Clinical progression is indicated for each participant (\*, slow progressors; †, rapid progressors).



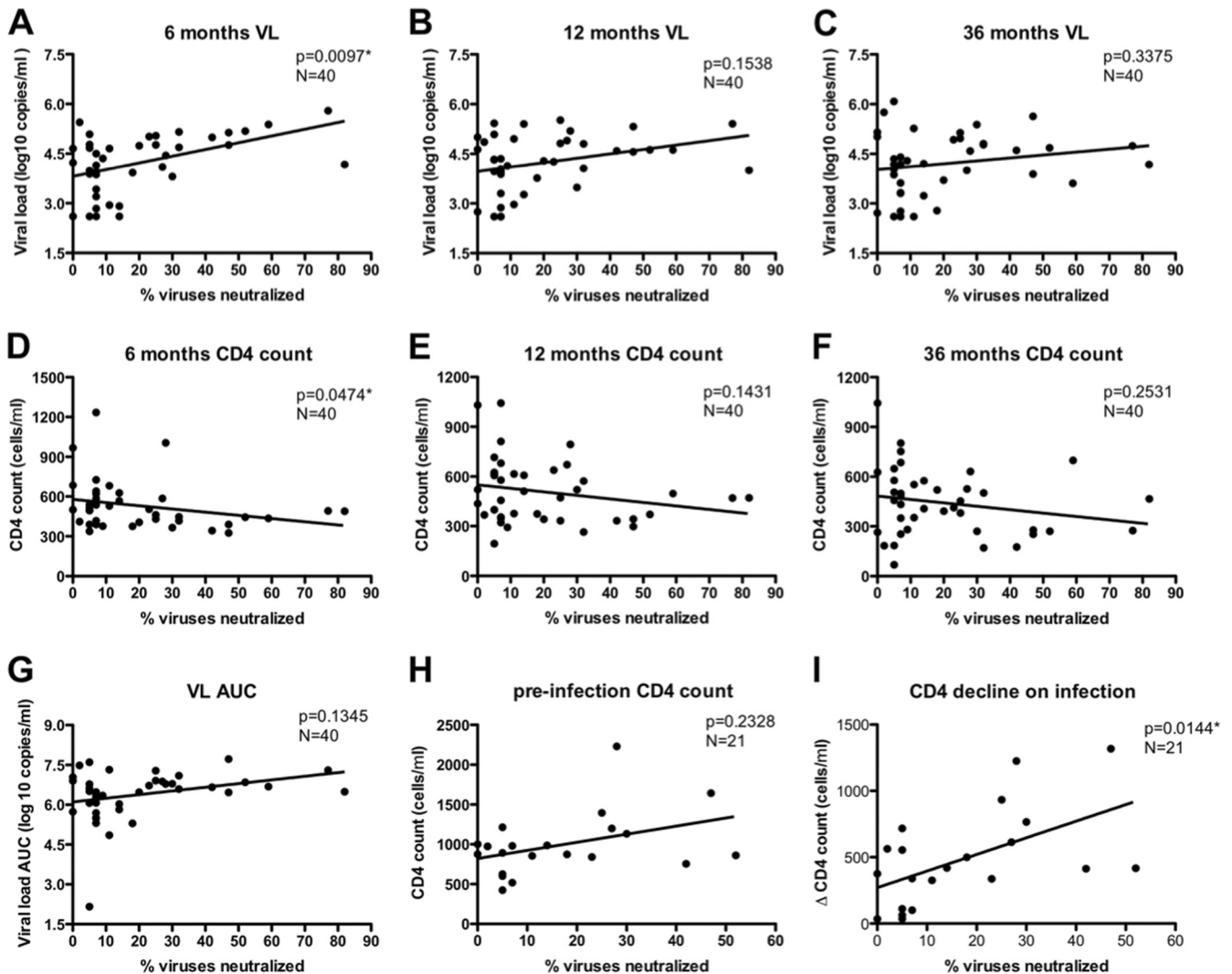


FIG. 2. Factors correlated with the development of cross-neutralizing antibodies. The percentage of viruses neutralized by each serum was correlated with the 6-month (set point), 12-month, and 36-month (contemporaneous) viral loads (VL) (A, B, and C) and  $CD4^+$  T cell counts (D, E, and F). Neutralization breadth was also correlated using the viral load AUC from 6 to 36 months postinfection (G), the preinfection  $CD4^+$  T cell count (H), and the decline in  $CD4^+$  T cell count between preinfection and 6 months postinfection (I). Each correlation was analyzed using a Spearman nonparametric test. The number of pairs ( $N$ ) and  $P$  value for each correlation are shown. Statistically significant  $P$  values are marked with asterisks.

at 6 months, the percentage of cross-neutralization increased by 12.4% ( $P = 0.002$ ).

Interestingly, the correlation between  $CD4^+$  T cell count prior to infection and neutralization breadth showed a positive trend, although it was not statistically significant (Fig. 2H). Of note, preinfection  $CD4^+$  T cell counts were available for only 21 participants in the seronegative cohort, which included 3 of the 7 individuals with the broadest cross-neutralizing activities. These data are in stark contrast to the negative slope of the  $CD4^+$  T cell count at 6 months postinfection (Fig. 2D). Furthermore, the decline in  $CD4^+$  T cell number upon HIV-1 infection, measured as the difference between  $CD4^+$  T cell counts preinfection and at 6 months postinfection, strongly correlated with the development of heterologous neutralization ( $r = 0.52$ ;  $P = 0.0144$ ) (Fig. 2I). This was confirmed in a univariate model analysis (Table 1). Given the existing corre-

lation between viral load and  $CD4^+$  T cell decline, both variables were fitted into a second multivariate model, which revealed a significant association between the development of breadth and  $CD4^+$  T cell decline ( $P = 0.0474$ ) but not viral load (Table 1).

As shown above, the development of breadth was associated with factors such as high viral load and steep  $CD4^+$  T cell decline in early infection, which are well known markers of disease progression (12, 13, 29). Of the 40 participants tested for heterologous neutralization, 11 were initiated on treatment during the course of this study, including 5 of the 7 women in group 1. To investigate the potential relationship between the development of neutralization breadth and disease progression, a Kaplan-Meier survival analysis was performed from the time of infection until the first  $CD4^+$  T cell count below 200 cells/ $\mu$ l or therapy initiation as a marker of clinical AIDS.

TABLE 1. Factors correlated with neutralization breadth

Variable	Univariate model		Multivariate model 1		Multivariate model 2	
	Estimate (95% CI)	P value <sup>a</sup>	Estimate (95% CI)	P value <sup>a</sup>	Estimate (95% CI)	P value <sup>a</sup>
Viral load						
6 mo	0.11 (0.077, 0.143)	<b>0.0020</b>	0.124 (0.071, 0.178)	<b>0.0259</b>	0.032 (-0.008, 0.073)	0.4308
12 mo	0.077 (0.040, 0.114)	<b>0.0437</b>	-0.005 (-0.065, 0.055)	0.9366		
36 mo	0.044 (0.008, 0.080)	0.2219	-0.045 (-0.096, 0.006)	0.3836		
Viral load AUC	0.080 (0.043, 0.117)	<b>0.0389</b>	0.026 (-0.045, 0.098)	0.7145		
Preinfection CD4 count <sup>b</sup>	0.016 (0.008, 0.024)	0.0710				
CD4 decline <sup>b</sup>	0.024 (0.015, 0.032)	<b>0.0094</b>			0.021 (0.030, 0.011)	<b>0.0474</b>
CD4 count						
6 mo	-0.029 (-0.046, -0.012)	0.0998				
12 mo	-0.024 (-0.041, -0.007)	0.1564				
36 mo	-0.021 (-0.037, -0.005)	0.1923				

<sup>a</sup> Statistically significant P values are shown in bold.

<sup>b</sup> Data are available for only 21 of the 40 study participants.

Because of the link between viral load and disease progression, we excluded participants with low viral loads from this analysis and included only the 22 individuals for whom the viral load AUC exceeded 5 million copies per ml. These were divided into two groups: 7 participants with cross-neutralizing antibodies (BCN) and 15 participants without breadth but with similar viremia (no BCN) at 3 years postinfection (Fig. 3). There were no significant differences between the median viral loads and CD4<sup>+</sup> T cell counts of both groups at all time points. A Cox proportional hazard model showed that those who developed breadth had a hazard ratio of 1.04 (95% confidence interval [95% CI], 0.32 to 3.34) and a log rank test P value of 0.9508. These results indicated that the development of cross-neutralizing antibodies did not preclude or promote disease progression.

**Evolution of cross-neutralizing antibodies in individuals with the greatest breadth.** Early serum samples from the 7 individuals with the greatest neutralization breadth (group 1)

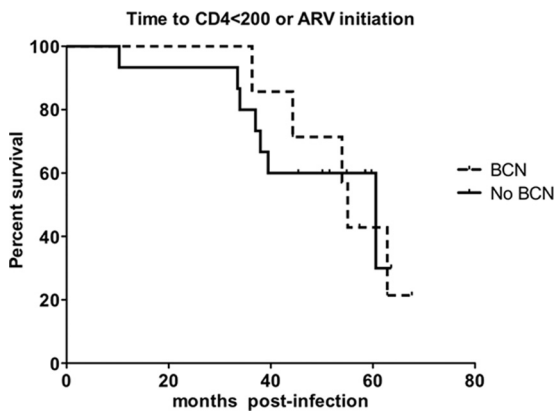
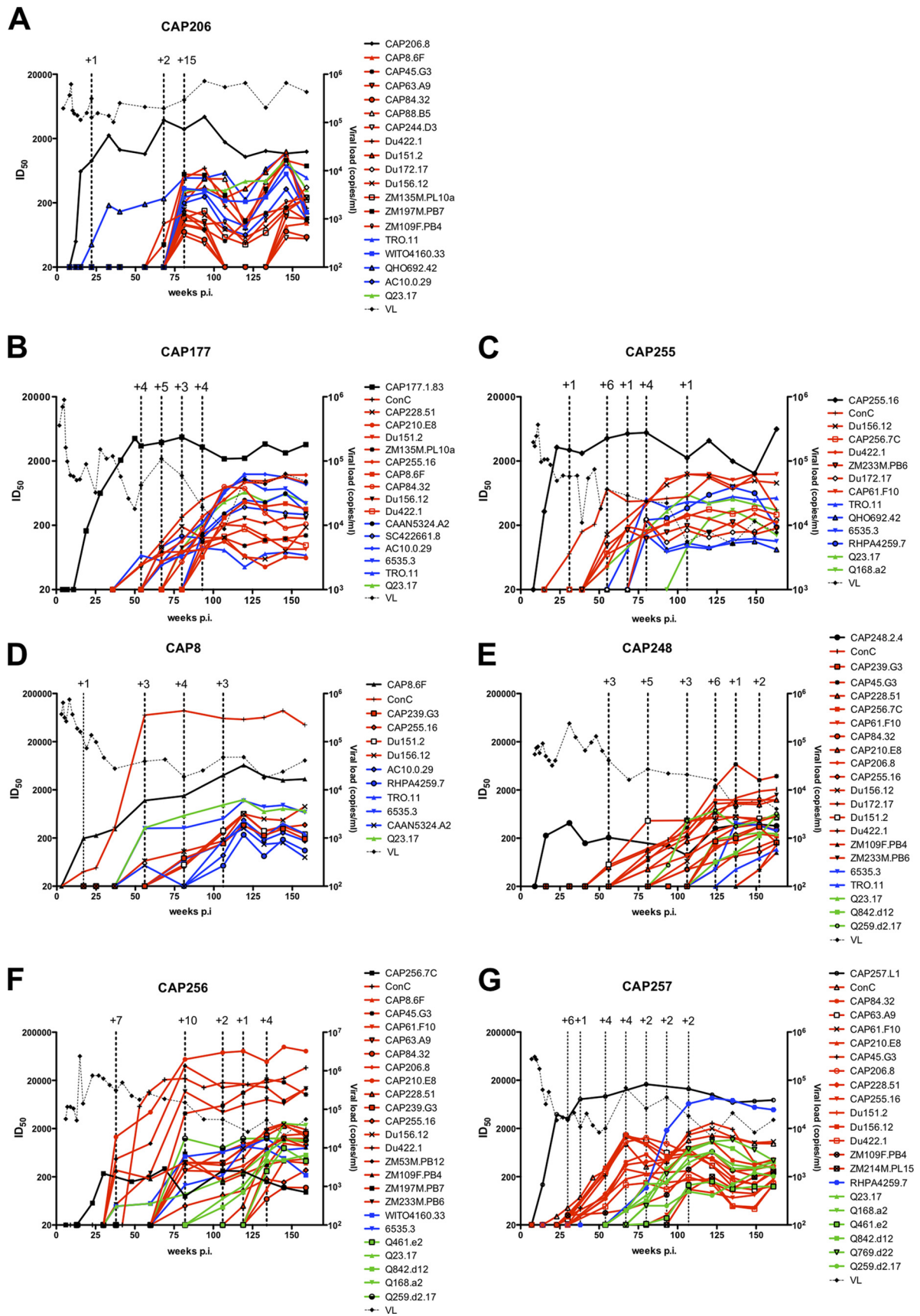


FIG. 3. Kaplan-Meier analysis of time from seroconversion until ARV initiation for CAPRISA participants with and without neutralization breadth. Study participants with similar viral loads were segregated based on their cross-neutralizing activity at 3 years postinfection, into BCN (group 1) (Fig. 1) and no BCN (groups 2 and 3) groups. A Cox proportional hazard analysis was used to compare the two groups based on time of infection until the first CD4<sup>+</sup> T cell count below 200 cells/ $\mu$ l or therapy initiation.

were assayed against all the viruses neutralized by the 3-year samples with titers above 1:100. On average, sera from 13 time points (range, 10 to 19 time points) over the first 3 years of infection were included. The longitudinal neutralization profiles against both autologous and sensitive heterologous viruses for each of the 7 participants are shown in Fig. 4. As described previously (15), antibodies to the infecting virus appeared within months of infection and titers remained high over 3 years. This suggested that despite multiple cycles of escape, sufficient neutralizing determinants remained on the infecting virus to stimulate new autologous specificities. Some of these new specificities acquired the ability to neutralize heterologous viruses of different subtypes (Fig. 4 [viruses from different subtypes are color coded]).

Analysis of the individual neutralization patterns showed that in one case (CAP206), the neutralizing activities against the majority of viruses occurred simultaneously (at 81 weeks postinfection), suggesting that a single dominant antibody specificity with cross-neutralizing activity emerged at this time point. Interestingly, this response waned at 107 weeks but reappeared later, targeting the same viruses (Fig. 4A). In participants CAP177 and CAP255, heterologous activity appeared mostly during the second year of infection (52 to 90 weeks postinfection), with titers reaching a plateau thereafter (Fig. 4B and C). This might indicate affinity maturation of existing antibodies resulting in increased potency and breadth. The same can be said for the remaining four individuals, in whom the neutralizing activity increased gradually over an extended period (Fig. 4D, E, F, and G). Alternatively, the cross-neutralizing activity in these four participants might involve the appearance of new specificities which target different epitopes on different viruses. Some of these new antibodies appeared to be subtype specific, as the early sera from participants CAP248, CAP255, CAP256, and CAP257 preferentially neutralized subtype C viruses, with cross-subtype neutralization appearing at later stages (Fig. 4).

**Heterologous neutralization increases over time, peaking at 4 years of infection.** The above data from the 7 individuals in group 1 suggested that neutralization breadth continued to increase over 3 years of infection. In order to more fully explore the dynamics of the development of breadth, we tested



sera from all 40 individuals at 1, 2, and 3 years postinfection. In addition, samples were collected from 27 of the 40 participants at 4 years of infection (7 participants, including CAP8 and CAP255, had initiated antiretroviral therapy, and 6 had not yet reached 4 years of infection). By 5 years of infection, samples from 12 participants were available (3 participants, including CAP256 and CAP257, started treatment before 5 years of infection, and 12, including CAP177, had not yet reached this time point). Individual CAP206 was initiated on treatment shortly after 5 years of infection, while subject CAP248 remained therapy naive over the course of the study. For this analysis, we used a smaller panel of 12 viruses, which included 4 viruses of each subtype (A, B, and C). We found a good correlation between the percentages of viruses neutralized using the reduced panel and the full panel of 44 viruses with sera from 3 years postinfection ( $R^2 = 0.9012$ ;  $P < 0.0001$ ).

The heterologous neutralizing activity increased gradually over time for the first 4 years (Fig. 5A), consistent with earlier reports that neutralization breadth was associated with duration of infection (44). The titers at which viruses were neutralized also increased over this period. An analysis of the 27 individuals at 4 years indicated that in addition to those previously shown to have neutralization breadth (i.e., CAP177, CAP256, and CAP257), 2 other individuals (CAP37 and CAP267) showed a breadth of >40% of viruses neutralized at this time point (Fig. 5B). CAP37 also showed more than 40% neutralization at 3 years of infection, reflecting minor differences in the 2 viral panels used for Table 1 and Fig. 5. Most of the other individuals showed slight increases in breadth between 3 and 4 years, but all remained below 40%. At 5 years of infection, no additional samples were found to have developed neutralization breadth. Only CAP206 and CAP248, previously shown to have breadth, had neutralization percentages above 40% at this time (Fig. 5C). Indeed, there appeared to be no increase in heterologous neutralization between 4 and 5 years of infection in this small subset, even among those with breadth, suggesting that no new further maturation or new specificities were generated after 4 years of infection.

**Mapping the antibody specificities in broadly neutralizing plasmas.** We previously reported the presence of anti-MPER antibodies at high titers ( $ID_{50}$ s above 1:1,000) in participant CAP206 (15). Removal of these anti-MPER antibodies using MPER peptide-coated beads resulted in a significant loss of neutralizing activity against most heterologous viruses. Plasmas from CAP248, CAP255, and CAP257 had undetectable neutralization titers against the HIV-2/HIV-1 MPER chimeric virus, while plasmas from CAP8, CAP177, and CAP256 had low anti-MPER neutralization titers, of 1:73, 1:280, and 1:274, respectively. The removal of these low-titer anti-MPER antibodies did not affect heterologous neutralization (data not shown).

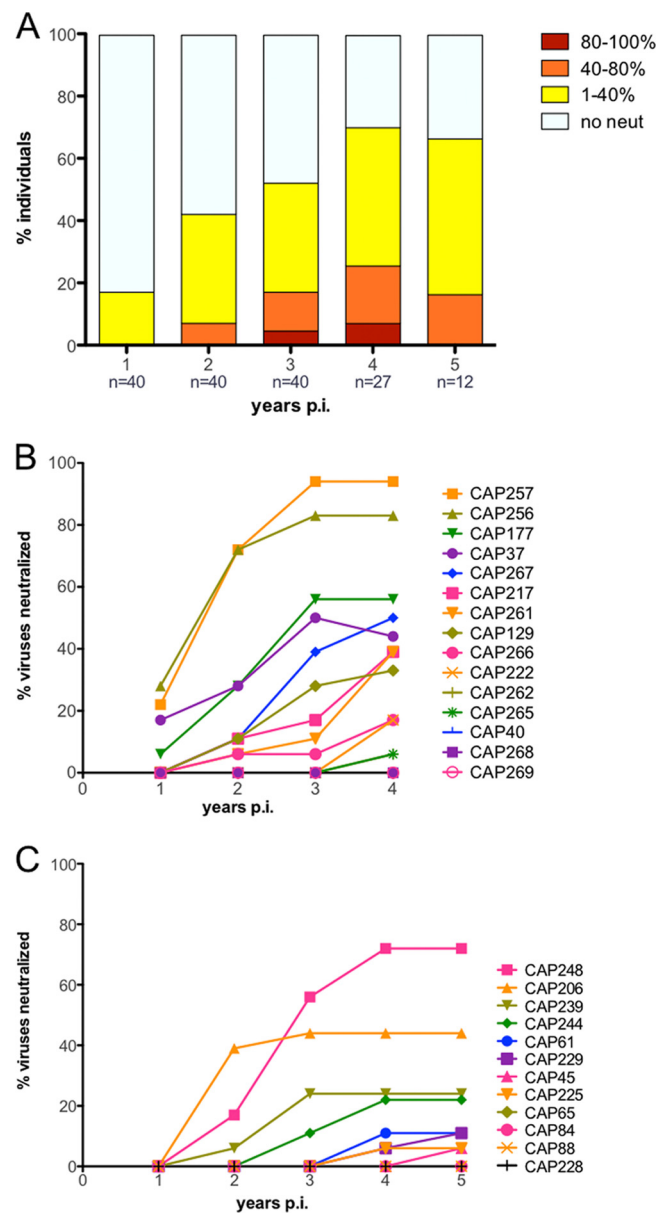


FIG. 5. Development of cross-neutralizing antibodies over 5 years of infection. The sera obtained at 1, 2, 3, 4, and 5 years of infection were tested for neutralization against a panel of 12 viruses of subtypes A, B, and C (4 of each subtype). The percentage of viruses neutralized was calculated for each sample. (A) Percentages of individuals capable of neutralizing more than 80%, 40 to 80%, 1 to 40%, and none of the 12 viruses at 5 different time points. (B and C) Percentages of viruses neutralized over time for the 15 and 12 participants that reached 4 and 5 years postinfection, respectively.

FIG. 4. Kinetics of development of heterologous neutralization in individuals with breadth. Sequential serum samples from 0 to 3 years of infection (10 to 19 samples) from all 7 participants with breadth were tested against all the viruses previously shown to be sensitive to the 3-year plasma samples. The  $ID_{50}$  titers versus weeks postinfection (p.i.) are represented for each virus, with the autologous viruses shown in solid black, subtype C viruses in red, subtype B viruses in blue, and subtype A viruses in green. The time points when detectable heterologous activity emerged are indicated using dashed vertical lines, with the number of additional viruses neutralized displayed above. The viral loads over time are shown as dashed black curves.



Participant	% Binding depleted	
	ConC gp120	ConC gp145
CAP8	>97%	>98%
CAP177	>97%	>97%
CAP248	>99%	>99%
CAP255	>99%	>99%
CAP256	>99%	>99%
CAP257	>99%	>99%

% Neutralization depleted by ConC gp120			
ConC	Q23.17	TRO.11	CAP45.G3
14%	44%	27%	(-)
>85%	>80%	>69	(-)
46%	23%	n.d.	36%
80%	>70%	>68%	(-)
0%	10%	(-)	2%
31%	43%	n.d.	0%

% Neutralization depleted by ConC gp145	
ConC	
20%	
>85%	
42%	
79%	
0%	
39%	

n.d.: Not done  
 (-): Virus not sensitive to neutralization by the plasma

FIG. 6. Adsorption of neutralizing antibodies using recombinant envelope proteins. Plasmas obtained at 3 years postinfection were adsorbed using recombinant ConC monomeric gp120- or trimeric gp145-coated beads. Blank beads were used as a negative control. (A) Depleted plasmas were tested for binding to ConC gp120 or ConC gp145 by ELISA. The percentage of antibody depleted was calculated using the following equation:  $[1 - (\text{midpoint titer of plasma treated with gp120-coated beads} / \text{midpoint titer of plasma treated with blank beads})] \times 100$ . (B) Plasmas adsorbed with ConC gp120 were tested for neutralizing activity against various envelope-pseudotyped viruses. The percent depletion was calculated as follows:  $[1 - (\text{ID}_{50} \text{ of plasma treated with gp120-coated beads} / \text{ID}_{50} \text{ of plasma treated with blank beads})] \times 100$ . Data represent the means for two separate neutralization experiments. (C) Plasmas adsorbed with ConC gp145 were tested for neutralizing activity against ConC and analyzed as described for gp120.

To further explore the specificities of the cross-neutralizing antibodies in the other 6 individuals, anti-gp120 antibodies in the 3-year postinfection plasmas were removed using magnetic beads coated with recombinant ConC gp120. More than 97% of the gp120-binding antibodies were depleted in an ELISA (Fig. 6A). For CAP177 and CAP255, neutralizing activities against at least three viruses were significantly decreased by the removal of anti-gp120 antibodies (Fig. 6B). Since no effect was seen with the samples from the other four individuals, we purified ConC trimers by gel exclusion chromatography and immediately used these to coat magnetic beads for adsorption experiments. However, despite the removal of most gp145-

binding antibodies (Fig. 6A), this protein also failed to adsorb significant amounts of the neutralizing activity in plasmas from CAP8, CAP248, CAP256, and CAP257 (Fig. 6C), suggesting that neutralizing antibodies in these four plasmas recognized epitopes only apparent on the quaternary structure of the envelope glycoprotein.

Further experiments were performed to determine the epitope(s) within gp120 that was recognized by the CAP177 and CAP255 neutralizing antibodies. We found that gp120 mutated in the CD4 (D368R) or coreceptor (I420R) binding site effectively removed the heterologous activity (Fig. 7A), suggesting that the epitopes recognized by these plasmas do

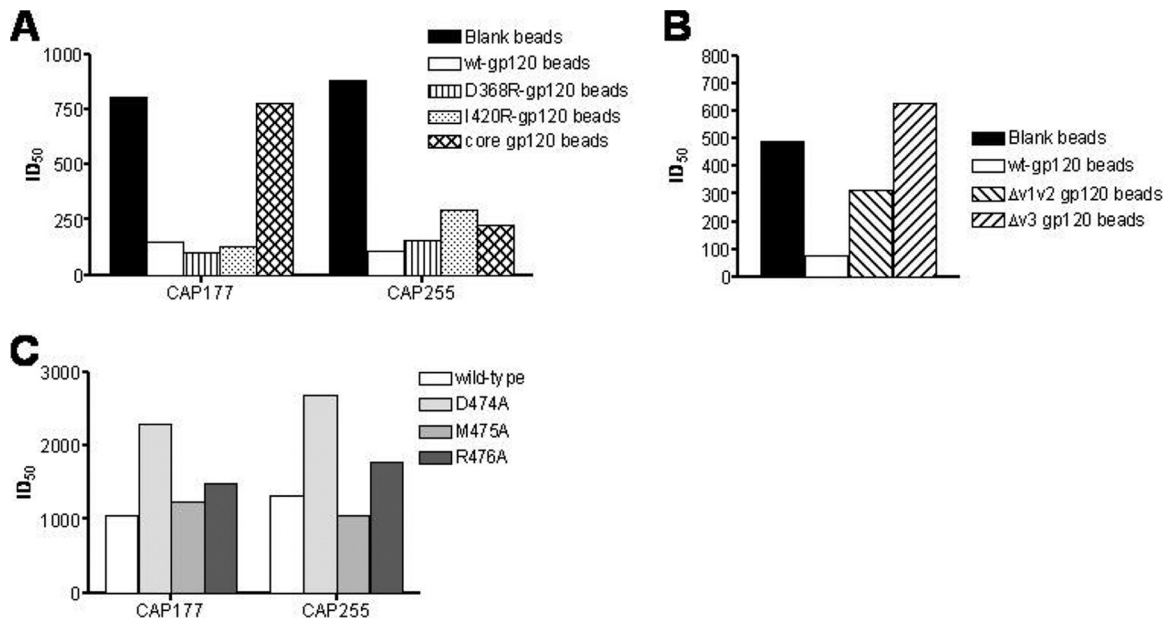


FIG. 7. Epitope mapping of CAP177 and CAP255 anti-gp120 neutralizing antibodies. (A) The 3-year plasmas of these two participants were adsorbed using gp120 mutated in the CD4 binding site (D368R) or coreceptor binding site (I420R) and gp120 with the V1V2 and V3 loops deleted (core gp120). Wild-type gp120-coated beads and blank beads were used as positive and negative controls, respectively. Depleted plasmas were tested for neutralization of ConC. (B) CAP177 plasma was adsorbed with V1V2- or V3-deleted gp120 prior to testing of ConC neutralization. (C) Both plasmas were tested for neutralization against wild-type ConC and three mutants in the core DMR epitope. Data represent the means for two separate neutralization experiments.

TABLE 2. Effects of single point mutations on neutralization sensitivity and summary of antibody specificities

Plasma or control MAb	Fold effect of mutation <sup>a</sup>							Antibody specificity conferring breadth
	ConC N160A	CAP45 N160	ConC I165A	CAP45 I165A	ConC N332A	TRO.11 N332A	Q23.17 N332A	
CAP206	1.0	1.0	0.1	1.0	0.9	1.8	0.7	MPER (14)
CAP177	0.9	NS	0.2	NS	<b>3</b>	<b>4</b>	<b>&gt;11</b>	gp120, N332
CAP255	1.0	NS	0.1	NS	0.2	<b>&gt;3</b>	<b>&gt;4</b>	gp120 core, N332
CAP8	<b>13</b>	NS	0.9	NS	0.5	1.5	0.7	Quaternary, PG9/16-like
CAP248	1.4	0.9	0.2	1.0	0.4	1.1	2.1	Quaternary, unknown
CAP256	1.1	<b>15</b>	<b>5</b>	<b>5</b>	0.4	1.1	0.9	Quaternary, PG9/16-like (34)
CAP257	0.1	1.0	0.5	1.0	0.3	0.6	0.5	Quaternary, unknown
PG16	<b>&gt;1,000</b>	<b>&gt;1,000</b>	2.4	2	0.3	NS	1	
2G12	NS	NS	NS	NS	NS	<b>9</b>	NS	

<sup>a</sup> Calculated as wild-type ID<sub>50</sub>/mutant ID<sub>50</sub> for the plasmas or as mutant IC<sub>50</sub>/wild-type IC<sub>50</sub> for the MABs. Changes in titer of >3-fold are shown in bold. NS, wild-type virus not sensitive to plasma or MAb being tested.

not overlap these regions or at least are not affected by these two mutations. However, use of a core gp120 in which the V1V2 and V3 loops had been deleted affected the epitope recognized by CAP177 but not that recognized by CAP255 (Fig. 7A and B), suggesting that elements present in the variable loops were important for CAP177 neutralization. To determine if these two samples targeted an epitope in the gp120 core defined by the HJ16 MAb (7, 38), which also overlaps the CD4 binding site, plasmas were tested for neutralization of the DMR ConC mutants (D434A, M435A, or R436A). None of these mutants showed reduced sensitivity to the neutralizing antibodies in these 2 samples; in fact, the D434A mutant was more sensitive to neutralization than the wild-type virus (Fig. 7C).

A recent study reported a number of sites commonly targeted by cross-neutralizing antibodies found in the plasmas of HIV-1-infected individuals with neutralization breadth. These included the N160 and L165 amino acids in the V2 region and the N332 glycan at the base of the V3 loop (54). To assess whether these types of antibodies were present in the cross-neutralizing plasmas from the CAPRISA cohort, we tested the 3-year samples against single-point mutants made in either the ConC or CAP45 background (Table 2). The introduction of the N160A mutation into ConC reduced CAP8 neutralization 13-fold, with no decrease observed for any of the other samples. However, the same mutation in the CAP45 virus reduced neutralization by CAP256 plasma 15-fold. In addition, CAP256 neutralization was sensitive to the L165A change in both ConC and CAP45, with a 5-fold drop in titers. This site overlaps the PG9/16 epitope (53). These observations, together with the lack of binding to recombinant envelope proteins, suggested the presence of PG9/16-like antibodies in these two plasmas. Indeed, this proposition was further demonstrated in a recent published detailed study on this participant (34). CAP177 titers were reduced 3-fold with the introduction of an N332A mutation in ConC, but this mutation did not affect neutralization by any of the other 6 plasmas (Table 2). To confirm this, the same mutation was also introduced into two other viruses. N332A mutants of TRO.11 and Q23.17 were also resistant to neutralization by CAP177 and, in addition, to CAP255 plasma. CAP206, CAP248, and CAP257 plasmas showed no resistance to any of the gp120 mutants tested. However, as mentioned above, CAP206 cross-neutralizing antibodies recognized predominantly the

MPER and the antibodies in subjects CAP248 and CAP257 were largely dependent on epitopes that were apparent only on the envelope trimer. A summary of the antibody specificities in these 7 plasma samples is shown in Table 2.

### DISCUSSION

In this study, we established that after 3 years of infection, the frequency of individuals with neutralization breadth in the CAPRISA cohort was 17.5% (7/40 participants). In some individuals, cross-neutralizing antibodies appeared to target subtype-specific determinants, while in others these antibodies were aimed at more universal epitopes. Heterologous neutralizing antibodies first appeared in some individuals as early as 1 year postinfection but peaked at 4 years, with no increases thereafter. The number of viruses neutralized was associated with the viral load and CD4<sup>+</sup> T cell count at set point (6 months postinfection) as well as with the drop in CD4<sup>+</sup> T cell count between preinfection and 6 months, suggesting that early events in HIV infection set the stage for the development of breadth.

Broadly cross-neutralizing antibodies were produced in a small proportion of individuals within the CAPRISA cohort after 3 years of follow-up. The frequency and extent of neutralization breadth found in this study were slightly lower than those described in other studies, which reported up to 30% of samples with this activity (9, 10, 16, 37, 44). However, many of these other studies were cross-sectional studies that made use of samples collected from chronic infections where the exact duration of infection was not always available. Because of the nature of the CAPRISA cohort, which is a prospective study of HIV-negative individuals with documented seroconversion, the timing and clinical features of infection are well known. Furthermore, virus panels and sensitivities of various neutralization assays vary considerably between studies, possibly contributing to this difference. The availability of longitudinal viral load data allowed us to analyze associations over time with the development of breadth. Interestingly, only the set point viral load at 6 months postinfection, not the overall antigenic stimulation (as measured by viral load AUC), correlated with the development of breadth. This agrees with a report by Piantadosi and colleagues in which a multivariate analysis found the set point, but not the contemporaneous viral load, to be asso-

ciated with neutralization breadth (37). As they described, the association of contemporaneous viral load and breadth observed by them and others (37, 44) may have been driven by the correlation of viral load within individuals at different stages of infection. However, a high viral load is not an overall predictor of neutralization breadth, as some highly viremic individuals did not develop cross-neutralizing antibodies, even after 5 years of infection. This has also been noticed in previous studies (10), suggesting that other factors in addition to high viremia determine the development of breadth.

A low CD4<sup>+</sup> T cell count at set point was also found to correlate with the development of cross-neutralizing antibodies. Moreover, the drop in CD4<sup>+</sup> T cell count from preinfection to 6 months postinfection was a better predictor of the extent of heterologous neutralization than the set point viral load, despite the limited number of individuals in this analysis. This association was noted in another study in which the CD4<sup>+</sup> T cell count at set point was also analyzed (10). It is intriguing that a drop in CD4<sup>+</sup> T cell count was associated with the production of cross-neutralizing antibodies given the importance of T cell help for B cell function. However, with the lymphocytic choriomeningitis virus (LCMV) model, it has been reported that depletion of CD4<sup>+</sup> T cells improves virus neutralizing antibody production by reducing the CD4<sup>+</sup> T cell-dependent virus-nonspecific polyclonal hyperglobulinemia (21, 40). HIV and hepatitis C virus infections, like LCMV infection, induce similar dysfunctions (8, 19, 43), suggesting that depletion of CD4<sup>+</sup> T cells in these infections might improve the neutralizing antibody response by reducing B cell polyclonal activation. This is an interesting observation and raises further questions such as whether low CD4<sup>+</sup> T cell counts are associated with a quicker upsurge and/or stronger autologous neutralizing responses, whether hyperglobulinemia is lower in participants who develop cross-neutralizing antibodies, and whether experimental depletion of CD4<sup>+</sup> T cells in nonhuman primates may enhance the production of neutralizing antibodies.

Our results suggest that neutralization breadth is acquired at 2 to 3 years postinfection. This agrees with a recent study done with subtype B-infected individuals that showed that breadth developed, on average, 2.5 years following infection (30). The reason for the delay in the development of neutralization breadth is not understood. However, the association of breadth with high viral loads suggests that antigen-driven selection is crucial in the development of these antibodies. This could drive affinity maturation, as suggested in a study by Toran and coworkers (48), who showed that all anti-gp120 neutralizing antibodies in a long-term nonprogressor were clonally related, with considerable somatic hypermutation. It has been proposed that HIV-1 has evolved to avoid germ line-like antibody recognition, possibly delaying the appearance of cross-neutralizing antibodies (57). Furthermore, immunogenetic analysis of existing broadly neutralizing MAbs suggests that they have undergone multiple rounds of affinity maturation to achieve cross-neutralizing activity (36, 58). This is in contrast to other virus infections, such as severe acute respiratory syndrome (SARS) coronavirus and henipavirus infections, where cross-neutralizing antibodies are induced early but carry very few somatic mutations (39).

Our observation that no further increase in neutralization

breadth occurred after 4 years is particularly intriguing and rather unexpected, although this was also recently reported for the subtype B cohorts mentioned above (30). We hypothesized that cross-neutralizing capacity would increase steadily over time even after 4 years of infection. If neutralization breadth occurs stochastically, with relentless waves of escape and the development of new autologous neutralizing antibodies in the presence of high antigenic loads resulting in the appearance of antibodies that recognize a generally conserved epitope, then this should happen at any stage of disease. Alternatively, dysregulation of the immune system over time (31) may result in a reduced ability to mount new antibody responses at later stages of infection. This is suggested by preliminary data from our laboratory (data not shown) and by reports from others that suggest that the capacity to mount new autologous responses is impaired after 2 to 3 years of infection, independent of disease progression (49). However, this needs to be explored further by measuring *de novo* autologous responses at various times postinfection in individuals with a range of viral loads.

Interestingly, 5 of the 7 individuals identified here as having cross-neutralizing activity were initiated on antiretroviral treatment because they had low CD4<sup>+</sup> T cell counts and/or other signs of clinical AIDS. Only CAP177 and CAP248 were still ARV naive after 4 and 5 years of infection, respectively. This observation is consistent with the report by Euler et al. that cross-reactive antibodies do not protect against disease progression (10), probably because the virus readily escapes even cross-neutralizing antibodies. Indeed, factors associated with the development of neutralizing antibodies, such as high viral load and low CD4<sup>+</sup> T cell count, are strongly correlated with disease progression (12, 13, 29). These factors herald a decline of immune function which, in the absence of successful antiretroviral therapy, results in progression to AIDS. It is possible that the individuals with high viral loads and low CD4<sup>+</sup> T cell counts who failed to mount a cross-neutralizing response within this time frame "lost their opportunity" with the collapse of the immune system. On the other hand, participants with low viral loads and high CD4<sup>+</sup> T cell counts did not develop breadth, probably due to a lack of sufficient antigenic stimulation and/or the overwhelming nonspecific hyperglobulinemia. It would be interesting to explore whether these individuals develop breadth at later stages of infection, either as waves of autologous neutralization continue to develop against a diversifying virus or through a sudden collapse in control and drop in CD4<sup>+</sup> T cell count that result in the development of cross-neutralizing antibodies.

A summary of what is currently known about the antibody specificities mediating breadth at 3 years postinfection in the 7 individuals in group 1 is shown in Table 2. Only one participant had neutralizing antibodies against the MPER of gp41. These plasma antibodies recognized an epitope centered on D674 and were shown to be distinct from other antibodies with MPER specificities, such as 4E10 and Z13e1 (14). Confirmation of this comes from the recent isolation of a neutralizing MAb (CAP206-CH12), using circulating blood memory B cells from CAP206, which showed similar epitope recognition (L. Morris et al., unpublished data). Two participants had antibodies that recognized epitopes in gp120 that involved the glycan at position 332. However, these two specificities are likely to be different from one another given that the CAP255

epitope is present on the core gp120, while the CAP177 antibody depends on variable loops. Walker and colleagues also found N332-dependent neutralizing antibodies in 5 of the 19 plasmas that they analyzed. Only one of these plasmas was shown to have 2G12-like activity, despite their epitope convergence on the N332 glycan (54). Antibodies targeting trimer-specific epitopes were the most common among the 7 individuals with neutralization breadth. Plasmas from two individuals, CAP248 and CAP257, could not be mapped using available methods. However, in two cases, CAP8 and CAP256, dependence on the N160 glycan suggested the presence of PG9/16-like antibodies. Although N160- and L165-dependent neutralization has been described recently as two distinct specificities (54), both affected neutralization by CAP256 plasma. The fine mapping of the CAP256 quaternary specificity indicated that it depended on various residues in the V2 loop which overlapped the PG9/16 epitope (34).

Three of the four individuals whose antibodies recognized quaternary neutralizing epitopes, i.e., CAP248, CAP256, and CAP257, neutralized subtype C viruses preferentially, and this discrimination was more evident at early time points. However, CAP8 antibodies preferentially neutralized subtype B viruses. In contrast, the anti-MPER antibodies in CAP206 and the anti-gp120 antibodies conferring breadth in CAP177 and CAP255 neutralized viruses from different subtypes equally. Preferential intrasubtype neutralization was reported previously by van Gils and colleagues (50). In their study of subtype B-infected individuals, participants with intermediate breadth showed more subtype B-restricted responses, which suggested that subtype specificity was related to low neutralization titers. In contrast, CAP256 and CAP257, the two participants with the greatest breadth, had high titers against subtype C viruses despite their subtype specificity. Therefore, we concluded that subtype-specific neutralization is not a general feature of intermediate breadth but instead depends on the epitope recognized by the cross-neutralizing antibodies. Furthermore, our data suggest that antibodies targeting quaternary neutralizing epitopes are more likely to be subtype specific than those with other targets.

The V1V2 and C3 regions have previously been shown to be common targets for autologous neutralizing antibodies in subtype C infection (26, 33, 35, 42). It is interesting that most heterologous activity found in this study overlapped with these two regions. CAP8 and CAP256 plasmas contained cross-neutralizing antibodies that recognized epitopes within the V2 loop, while CAP177 and CAP255 appeared to recognize an epitope in C3. This suggests that the V1V2 and C3 regions are particularly immunodominant regions of the envelope and are the focus of the neutralizing antibody response from early times on, with these type-specific antibodies sometimes developing into antibodies with cross-neutralizing specificities. Ongoing studies in our laboratory aim to determine the relationship between the evolution of autologous neutralizing antibodies and the development of broadly neutralizing activity. Interestingly, the N332 glycan targeted by the heterologous neutralizing antibodies in CAP177 was not present in the transmitted founder virus isolated from this participant (1). This glycan appeared later, at 6 months postinfection, as an escape mutation driven by the primary autologous neutralizing antibody response that targeted the alpha-2 helix of C3 (35). This

suggests that, at least in this case, later variants rather than the infecting virus elicited antibodies with cross-neutralizing activity. Elucidating the path between these two responses may guide the design of immunization strategies that mimic this process, focusing the response on the conserved motifs in the envelope glycoprotein.

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