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Dichotomy in Cross-Clade Reactivity and Neutralization by HIV-1 Sera: Implications for Active and Passive Immunotherapy

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The identification of broadly reactive and cross-clade neutralizing antibodies will facilitate the development of a more universally effective vaccine for human immunodeficiency virus (HIV). Antibodies in sera from individuals infected with Clade B HIV bind native primary viral isolates, and virus binding correlates with neutralization and stable clinical disease. In this study, we quantified cross-clade antibody reactivity and neutralization by Clades B and C sera. Primary viral isolates were captured by serum IgG bound to anti-human IgG and quantitated as p24 released by lysis of captured virus. Neutralization was determined using PHA-stimulated PBMC. Clade B antibodies reacted more frequently with Clade B R5 virus, but positive sera captured quantitatively more X4 virus than R5 and R5X4 virus. Clade B sera reacted less frequently and captured less Clade C virus than Clade B virus. Antibodies in Clade C sera captured Clades B and C isolates with equal frequency and quantity. There was no difference in neutralization of Clade B virus by either group of sera; however, Clade C sera neutralized Clade C virus, whereas Clade B sera were ineffective against Clade C virus. Thus, there are distinct differences in cross-clade reactivity of and neutralization by antibodies induced in response to Clade C infection compared to Clade B infection. Understanding antibody responses to native virions after Clade C infection and cross clade antibody behavior has implications for understanding pathogenesis and vaccine development.

KEY WORDS: human immunodeficiency virus; neutralization; serum antibody; cross-clade

INTRODUCTION

A major goal of human immunodeficiency virus (HIV) vaccine research is to develop an effective vaccine that is protective against all clades of HIV-1. Clade B virus predominates in North America and parts of Europe. Other clades are prevalent elsewhere in the world. Clade C virus constitutes a major fraction of infections in Southern and Eastern Africa, India, Nepal, and China. Clade C is endemic in parts of Africa where infection and transmission rates are very high [Essex, 1993; Oelrichs *et al.*, 2000; Spira *et al.*, 2003]. India and China have the potential to become a major factor in the epidemic in the future, and there are already more than 5 million Clade C infections in India alone [Arora *et al.*, 2004]. While there is minimal interclade diversity within the *pol* and *gag* region of HIV-1, there is considerable inter- and intra-clade variation in the *env* region. To date, a majority of the vaccines being tested are based on, or at least have as one component, the *env* region of HIV, based on Clade B isolates. A specific understanding of limitations of cross-reactive and subtype specific Env responses is required to maximize the development of a broadly effective HIV-1 vaccine.

While both cross-reactive and subtype-specific CTL responses, predominantly to *gag* and *pol* sequences, have been reported [Betts *et al.*, 1997; Cao *et al.*, 1997; Ferrari *et al.*, 1997], the humoral response to vaccines and infections is directed towards Env, where more diversity is seen. Early reports suggested that there is no strict clade dependence of neutralization [Kostrikis *et al.*, 1996; Moore *et al.*, 1996; Nyambi *et al.*, 1996; Weber *et al.*, 1996]. It was shown that while sera from Clades B and E patients pref-

entially reacted with monomeric gp120 from Clades B and E isolates, respectively, this preference was not particularly observed for other clades [Moore *et al.*, 1996]. Also, neutralization of primary isolates in these studies was sporadic, making interpretation difficult. Furthermore, few sera or isolates were derived from Clade C-infected individuals. Recently [Bures *et al.*, 2002], it was shown that Clade C sera neutralized autologous and heterologous Clade C isolates, including geographically diverse isolates. Selected sera displayed cross-clade neutralization activity against Clade B T-cell line adapted isolates (TCLA) and primary isolates. This suggests that antibody responses to Clade C infection may be capable of broad efficacy.

Taken together, these results suggest that there may be important differences in the presentation of HIV-1 antigens and epitopes to the immune system resulting in a different outcome in the immune response to Clade C infections. We have also shown that, in addition to clade-specific effects, there is heterogeneity of specific antibody responses to native primary viral isolates as a function of co-receptor usage [Cavacini *et al.*, 2002]. Antibodies reactive with intact primary isolates have not been studied across clades. Therefore, we have analyzed antibody reactivity with and neutralization of primary isolate Clades B and C viruses using sera from individuals infected with Clade B virus and Clade C virus. As described below, more robust antibody responses are observed among Clade C-infected patients; the antibodies were readily cross-reactive with Clades B and C. The reciprocal cross-clade binding of Clade B antibodies to Clade C viruses is less broad and less robust among the Clade B sera tested. These data suggest that studying the humoral immune responses of Clade C patients may lead to more broadly reactive and effective antibodies for therapy and/or vaccine development.

MATERIALS AND METHODS

Clinical Samples and Virus

Subtype B primary isolates, 92HT593, 92US660 from N. Halsey, MACS, HIV-189.6 (89.6) from R. Collman, HIV-1BAL (BAL) from S. Gartner, M. Popovic and R. Gallo, and subtype C primary isolate 93MW960 from Dr. Paolo Miotti and the UNAIDS Network for HIV Isolation and Characterization, and the DAIDS, NIAID were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. Clade B X4 isolate 67970 was obtained from D. Montefiori, Duke University Medical School and Clade C R5 isolate 1157 was from the laboratory of Dr. Wood.

Informed consent was obtained from all participants in this study, and the study was approved by the institutional review boards of the Beth Israel Deaconess Medical Center and Fenway Community Health Center. Sera from Clade B-infected individuals were obtained from

individuals seen either at the New England Deaconess Hospital, Boston MA (now Beth Israel Deaconess Medical Center) or the Fenway Community Health Center, Boston, MA for routine care. Sera were obtained from Clade C-infected individuals attending an HIV clinic at the University Teaching Hospital, in Lusaka, Zambia. While CD4 counts, anti-retroviral history, and probable seroconversion data were available for some of the Clade B-infected individuals, the ability to collect this information for the Clade C-infected individuals was not available at the time of this study. However, all individuals in this study were asymptomatic, without AIDS defining criteria, for a period of time prior to collection of the sera or plasma used in this study. While clade specificity for each individual in this study was not confirmed by sequencing viral isolates for each individual, the clade specificity for these cohorts has been established through other studies. For example, of the hundreds of isolates sequenced from the study site in Zambia, only two were not Clade C with one isolate being Clade A and another a Clade A/C recombinant. Similarly, with the rare exception of an individual being infected outside the United States (and who was not included in this study), sequence analysis of isolates from individuals seen at the study sites in Boston, especially in the time period that the samples were collected, has exclusively been Clade B.

Reactivity With HIV-1 Infected Cells and Virions

Serum IgG binding to primary HIV isolates was measured by ELISA as previously described [Cavacini *et al.*, 1999]. Virus stocks were prepared in PHA-stimulated donor PBMC [Cavacini *et al.*, 1998, 1999]. IgG in serum was captured onto ELISA plates coated with goat anti-human IgG (Fc specific) prior to the addition of virus stock diluted to 100 ng/ml. The concentration of p24 released from bound virus by 1% triton was determined by ELISA. A cut-off value of 200 pg/ml is considered significant and serum from HIV seronegative donors captured less than 100 pg/ml. Low levels of antibody are defined as 200–300 pg/ml, moderate as 300–500 pg/ml, and high titers as >500 pg/ml of p24.

Primary Isolate Neutralization Assays

Primary isolate neutralization was measured using an assay that quantitates the amount of p24 produced by a constant inoculum of virus in PHA stimulated PBMC. In this assay, serum was diluted in growth media (RPMI 1640 with 20%FBS and 5% IL-2), and 50 ml were added to 50 ml of viral stock (200TCID₅₀/ml) and incubated for 1 hr at 37°C. After 1 hr, donor PHA-stimulated PBMC (2 × 10⁵ cells) were added and the plates incubated for 2 hr at 37°C, 5% CO₂. The plates were washed twice, and the medium was replaced with fresh growth medium. Seven days after the infection was initiated, supernatant was

removed and tested for p24 by ELISA. Serum from an HIV-seronegative person was included in each experiment, and the p24 values obtained with those samples were used to determine percent neutralization.

RESULTS

Prevalence of Virion Specific Antibodies

We have previously reported that the majority of individuals infected with Clade B HIV had either low or no serum IgG antibody reactive with native primary viral isolates [Cavacini *et al.*, 1999]; furthermore, virion-specific antibody in Clade B sera correlated with CD4 counts and with neutralizing antibody activity. To further characterize the virion-specific and neutralizing antibody response, including the antibody response of individuals infected with Clade C HIV, we have analyzed sera from asymptomatic individuals infected with Clades B and C HIV without opportunistic infections, regardless of antiretroviral therapy. Since differential binding of human monoclonal antibodies had a virion dependency upon coreceptor usage [Cavacini *et al.*, 2002], a total of seven isolates, including five Clade B and representing R5, R5X4, and X4 isolates and two Clade C isolates (both R5), were included in the analysis. Of the 32 asymptomatic Clade B-infected individuals, sera from all but one individual had specific antibody (>200 pg/ml) against at least one Clade B isolate of HIV (data not shown). Similarly, sera

from all but 2 of the 32 individuals infected with Clade C HIV had specific antibody reactive with at least one Clade C isolate of HIV (data not shown). Thus, consistent with our previous findings [Cavacini *et al.*, 1999], asymptomatic HIV infection correlates with the presence of virion-specific antibody.

When tested for reactivity with R5X4 primary isolates 92HT593 and 89.6, a large number of sera from both Clade B (Fig. 1A) and Clade C (Fig. 1B) infected individuals were positive for virion-specific antibody. As summarized in Table I, 59% and 41% of sera from Clade B-infected individuals reacted with 92HT593 and 89.6, respectively, while 81% and 59% of Clade C sera were reactive with 92HT593 and 89.6, respectively. These differences did not achieve statistical significance. Similarly, 59% of sera from Clade B and 84% of sera from Clade C-infected individuals reacted with the X4 isolate, 67970 (Fig. 1C,D). Although there were differences in the amount of p24 captured by individual sera, there was no statistically significant difference in the incidence of reactivity. The overwhelming majority of sera from both Clades B and C-infected individuals reacted with Clade B R5 isolates 92US660 and BaL (Fig. 2A,B). In sharp contrast, less than half of the Clade B sera (Fig. 2C) reacted with Clade C R5 isolate (1157 or 93MW960), while more than 80% of the Clade C reacted with Clade C virus (Fig. 2D). This was a statistically significant difference in reactivity against Clade C virus (Table I). It should be noted

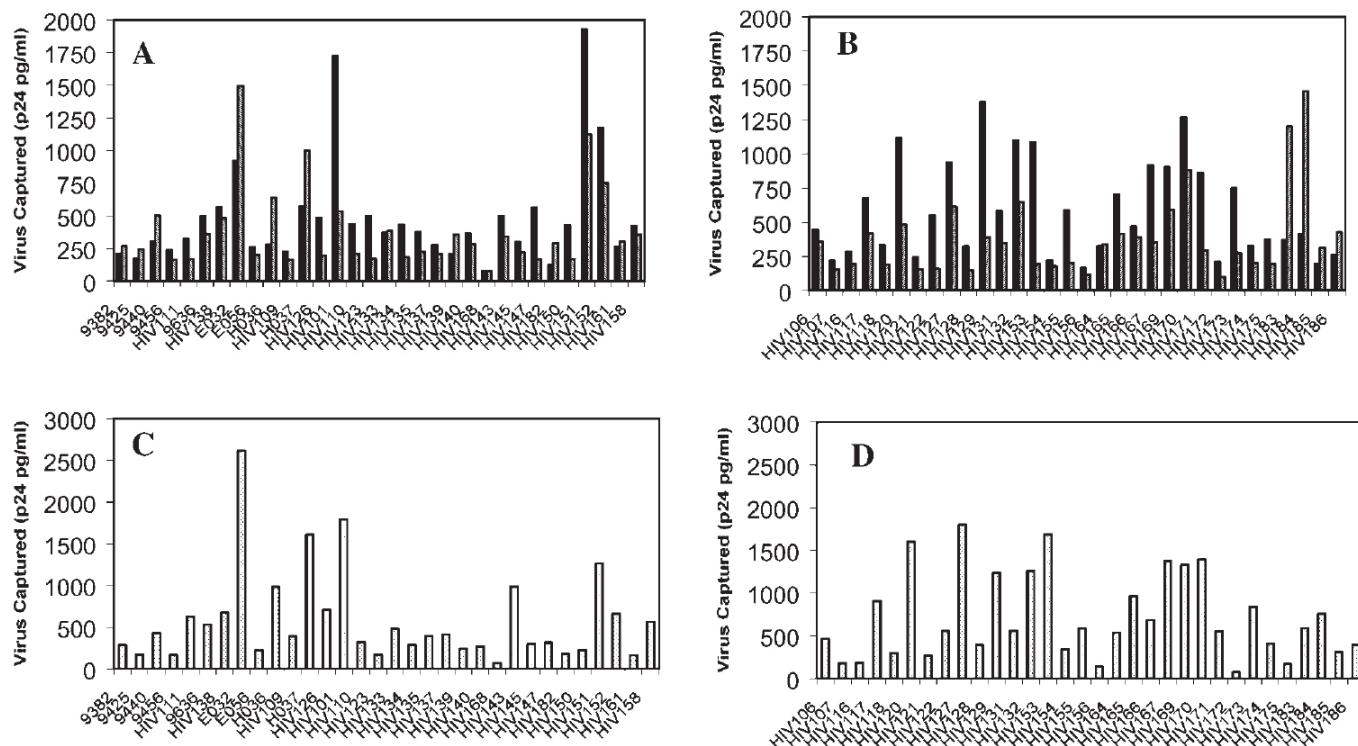


Fig. 1. Serum antibody reactive with R5X4 and X4 primary isolate. IgG in sera from Clade B (A, C) and Clade C (B, D) infected individuals was diluted 1:50 and captured, in triplicate, onto ELISA plates using goat anti-human IgG, Fc-specific antibody. Unbound antibody was removed by washing prior to the addition of primary isolate virions

(100ng/ml p24). In (A) and (B), captured 92HT593 (solid bar) or 89.6 (slashed bar) and, in (C) and (D), captured 67970 virus were lysed using triton and released p24 quantitated by ELISA. Results are expressed as the pg/ml of p24 released and represent the average of a minimum of three experiments. The cut-off for positive sera is 200 pg/ml.

TABLE I. Incidence of Serum IgG Exhibiting Virion Binding to Primary HIV Isolates

	92US660	BaL	93MW960	1157	92HT593	89.6	67970
	Clade B, R5	Clade B, R5	Clade C, R5	Clade C, R5	Clade B, R5X4	Clade B, R5X4	Clade B, X4
Clade B Sera	25/32 (78%) ^a	25/32 (78%)	15/32 (47%)	10/21 (48%)	19/32 (59%)	13/32 (41%)	19/32 (59%)
Clade C Sera	25/32 (78%)	28/32 (88%)	27/32 (84%)	17/21 (81%)	26/32 (81%)	19/32 (59%)	27/32 (84%)
<i>P</i> -value	<0.3 (NS)	<0.3 (NS)	<0.02	<0.02	<0.2 (NS)	<0.4 (NS)	<0.2 (NS)

NS, not statistically significant.

^aValues represent number positive/total tested, with percent positive in brackets.

that fewer sera were analyzed for reactivity with Clade C isolate 1157 due to limited high-titered stocks of this isolate. However, the results obtained with 1157 were similar to that reported for 93MW960. Thus, the HIV antibody response of those infected with Clade C HIV displays broader cross-clade reactivity than the HIV antibody response of those infected with Clade B HIV. This is not a function of co-receptor usage as Clade B sera reacted equally well with R5 Clade B isolates as Clade C sera, yet had lower reactivity with R5 Clade C isolates. Another indication of the difference in the breadth of reactivity is the observation that 34% (11 of 32) of Clade B infected sera reacted with all isolates tested, whereas 56% (18 of 32) of Clade C-infected sera reacted with all isolates (data not shown).

Despite a difference in the incidence of reactivity between Clades B and C sera, analysis of the raw individual data (not shown) indicated that there is little difference

in the amount of virus captured by sera for most isolates. Using the criteria of capture of more than 500 pg/ml as indication of high titer virion specific antibody, similar results were obtained with sera from both Clades B and C for isolates 92US660 (13% for Clade B, 9% for Clade C), 89.6 (22% for Clade B, 19% for Clade C), BaL (22% for Clade B, 31% for Clade C), and 93MW960 (19% for Clade B, 16% for Clade C). For the R5X4 isolate 92HT593, more individuals infected with Clade C HIV (47%) had high primary isolate-specific antibody than individuals infected with Clade B HIV (25%). Similarly, 59% of individuals infected with Clade C had high primary isolate-specific antibody reactive with the X4 isolate 67970, whereas fewer Clade B sera (38%) had high specific antibody reactive with this isolate. Ten and 13 of the 32 individuals infected with Clade C or Clade B HIV, respectively, did not mount a high titer virion specific antibody response against any isolate.

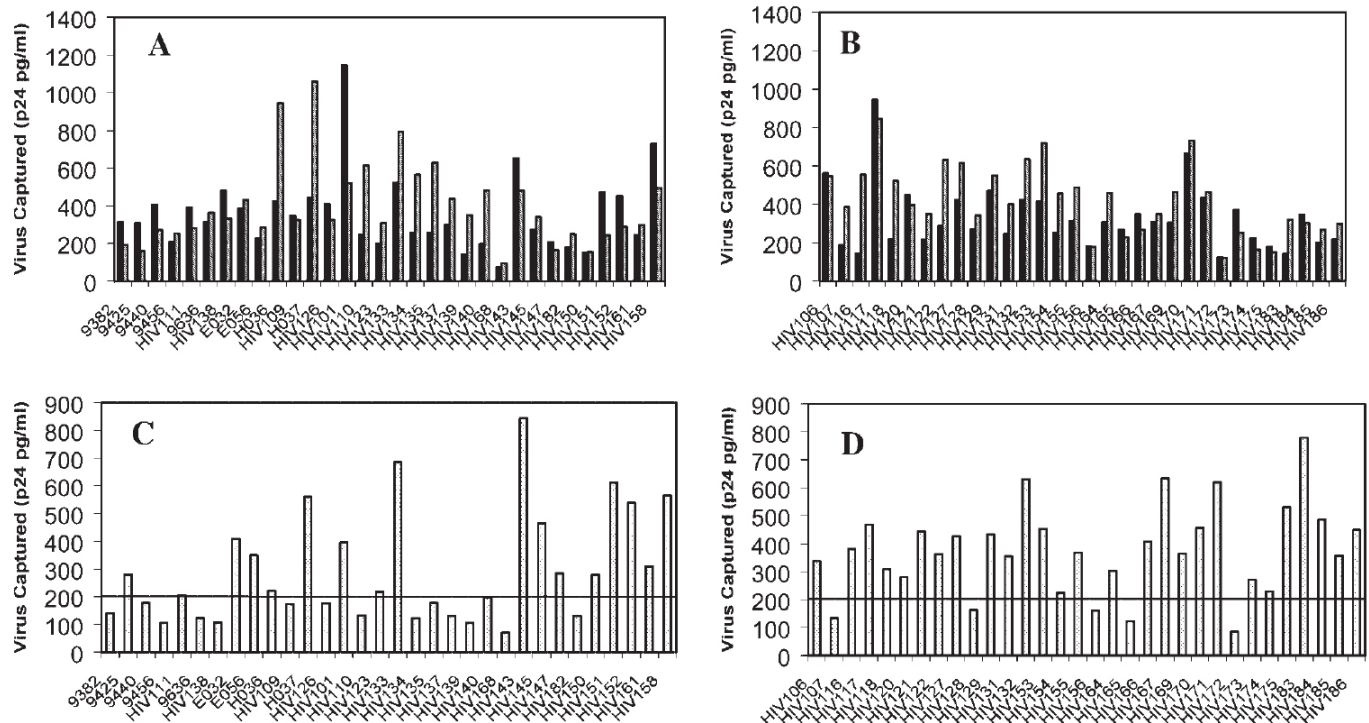


Fig. 2. Serum antibody reactive with R5 primary isolate virions. IgG in sera from Clade B (**A**, **C**) and Clade C (**B**, **D**) infected individuals was diluted 1:50 and captured, in triplicate, onto ELISA plates using goat anti-human IgG, Fc-specific antibody. Unbound antibody was removed by washing prior to the addition of primary isolate virions (100 ng/ml p24). In (A) and (B), captured clade B virus 92US660 (solid bar) or BaL

(slashed bar) and, in (C) and (D) captured Clade C 93MW960 virus were lysed using triton, and released p24 was quantitated by ELISA. Results are expressed as quantity of p24 released in pg/ml and represent the average of a minimum of three experiments. The cut-off for positive sera is 200 pg/ml.

Neutralization of Primary Isolates by Sera

Sera from HIV Clades B and C-infected individuals were tested for the ability to neutralize three Clade B (92HT593 {R5X4}, 92US660 {R5}, and 67970 {X4}) isolates, and one Clade C (93MW960 {R5}) isolate. Values are expressed as the dilution of sera resulting in 90% neutralization (IC₉₀). The higher the IC₉₀ value, the more the sera can be diluted indicating more potent neutralizing activity. As shown in Table II, regardless of co-receptor use, there were no significant differences in neutralization between Clades B and C sera for the Clade B isolates. In contrast, there was a significant difference in the neutralization of Clade C. Sera from Clade B infected individuals did not neutralize Clade C virus. In contrast, 40% of Clade C sera neutralized Clade C virus with a range of IC₉₀ titers of 0–117 ($P < 0.04$).

When neutralization titers and virion specific antibody are correlated, additional cross-clade differences are apparent. For the Clade B R5X4 isolate 92HT593, sera with virion-specific antibody had neutralization activity (IC₉₀ > 10) as shown in Figure 3A. However for Clade B sera, the correlation was not tight. While neutralizing, a higher virion-specific antibody level did not necessarily indicate a greater neutralization activity. Similar results were obtained for Clade B sera and the Clade B R5 isolate 92US660 (Fig. 3B). Moreover, despite high HIV specific antibody, only a quarter of the Clade C sera with virion-specific antibody neutralized 92US660 (Fig. 3B). Also, only half of Clade B or C sera with virion-specific antibody neutralized the X4 isolate 67970 (Fig. 3C) and none of the Clade B sera neutralized Clade C R5 virus 93MW960 (Fig. 3D), despite more than 40% of the sera having virion specific antibody. In addition, only half of the virion specific antibody positive Clade C sera neutralized Clade C virus. Despite a lack of direct correlation between the concentration of virion specific antibodies and neutralization titers, with the exception of two sera with the Clade B R5 isolate, 92US660, all neutralizing sera had virion-specific antibodies. Thus, while the presence of virus-specific antibody did not predict neutralization activity, specific antibody was essential for neutralization activity to be present in the sera. This implies that some virus-specific antibody is not directed at neutralizing epitopes.

DISCUSSION

We have shown previously that the majority of individuals infected with Clade B HIV had either low or no serum IgG antibody reactive with native primary isolate virions [Cavacini *et al.*, 1999]. In this report, we studied virus-specific antibodies from asymptomatic individuals infected with either Clade B or C HIV, and cross-clade virion binding and neutralization by serum antibodies from these two populations. Consistent with our previous report, native virion-specific antibody was necessary for viral neutralization, albeit it did not predict neutralization activity. That is, sera with robust neutralization activity had viral specific antibody, while only some sera with virus-specific antibody had neutralizing antibody. It is not surprising that among the multiple assays and individuals studied, there are rare individuals with low titers of virion specific antibody but with high levels of neutralizing antibody to an individual isolate. This can be explained by heterogeneity in both the polyclonal antibody response and virus (autologous virus vs. test virus in assay).

It has been suggested that the failure of most neutralizing human monoclonal antibodies to react substantially with native primary viral isolates is the result of non-functional spikes on the virions suggesting that the viral capture assay is a limited assay for the study of antibody/viral interactions resulting in viral neutralization [Poignard *et al.*, 2003]. However, it can be argued that the utility of the viral capture assay is a function of the parameters of the assay [Cavacini and Posner, 2004]. For example, differences in the incorporation of specific cellular host components in the viral membrane as a function of the cell type used to produce progeny virus has major implications for infectivity, gp120/41 structure, and viral neutralization. More importantly, the use of a neutralizing antibody to capture virus has been shown to result in reduced viral output, which is to be expected, and which may influence interpretation of the results. Failure of neutralizing antibodies to bind substantially to primary isolate virions may reflect virion camouflage. Non-V3 neutralizing epitopes are not exposed on the primary HIV isolates until the virus is in proximity of a target cell or in the post-CD4 binding state. Further, the immune system is confounded by the immunogenicity of

TABLE II. Neutralization of Primary Isolates by HIV Sera

	Average IC ₉₀ ^a			
	92US660	92HT593	67970	301960
	Clade B, R5	Clade B, R5X4	Clade B, X4	Clade C, R5
Clade B Sera	88 (2–235)	79 (2–257)	27 (0–106)	0.3 (0–2)
Clade C Sera	44 (0–331)	78 (0–207)	23 (0–88)	27 (0–117)
<i>P</i> -value ^b	<0.2	<0.5	<0.4	<0.04

^aValues represent the reciprocal dilution of the average IC₉₀ value for each group with the range in parentheses.

^bSignificance of difference between Clades B and C sera for each isolate was determined by Student's *t*-test.

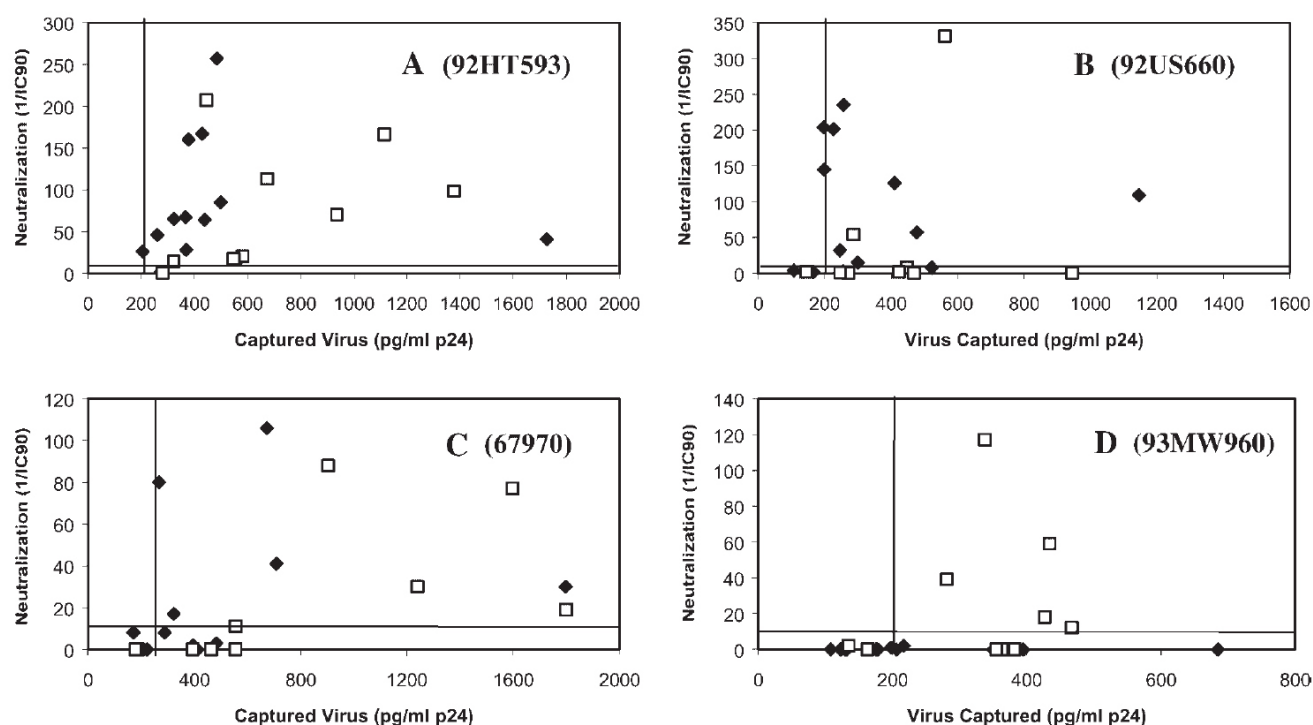


Fig. 3. Relationship of virion-specific antibody with viral neutralization. For each serum, virion-specific antibody, as pg/ml of p24, is plotted on the x-axis as a function of the reciprocal dilution of the IC_{90} value for neutralization. Isolate 92HT593 is shown in (A), 92US660 in (B), 67970 in (C), and 93MW960 in (D). Closed symbols represent Clade B sera and open symbols represent Clade C sera. The vertical line represents the cut-off for positive virion specific antibody (200 pg/ml) and the horizontal line represents the cut-off for neutralization activity (10).

the exposed variable loops while neutralizing epitopes are not generally available to immune cells or serum antibody. For those sera with both high virion binding antibody and neutralization, it can be hypothesized that V3 loop antibody, which readily reacts with virions, is a major contributor to virion binding and neutralization for these sera. Further analysis of the epitopes recognized by the antibodies that capture virus, and the contribution of those antibodies to *in vivo* viral inactivation or neutralization are underway. Finally, the contribution of synergy between non-neutralizing serum antibodies, which bind primary HIV isolates and neutralizing antibodies in *in vitro* viral neutralization remains to be determined, although synergy in this setting has been demonstrated for human monoclonal antibodies [Cavacini *et al.*, 2002].

Antibodies raised in response to HIV-1 vaccines in humans and animals react with HIV but rarely neutralize primary isolates [Mascola *et al.*, 1996; Beddows *et al.*, 1999; Bures *et al.*, 2000]. Early reports suggested that there is no strict clade dependence of neutralization [Kostrikis *et al.*, 1996; Moore *et al.*, 1996; Nyambi *et al.*, 1996; Weber *et al.*, 1996]. In this study, serum antibodies in Clade C-infected individuals reacted with Clade C HIV as well as cross-reacted with Clade B HIV. However, virion-reactive antibodies in individuals infected with Clade B HIV were not as robustly cross-reactive with Clade C HIV. Consistent with this result, sera from Clade C-infected individuals have been shown to broadly neutralize autologous

and heterologous Clade C isolates and Clade B isolates [Bures *et al.*, 2002]. Of interest, it has been reported that a disproportionate number of African women, as compared to African men and European men and women, had high-titer, broadly cross-reactive neutralizing antibodies [Beirnaert *et al.*, 2000; Donners *et al.*, 2002].

In conclusion, there is a significant difference in the quality of the humoral response to Clade C virus compared to Clade B virus. Antibodies from individuals infected with Clade C HIV-1 have broader cross-reactivity in both primary virion binding and neutralizing activity than antibodies from individuals infected with Clade B HIV. Further study of the humoral response of Clade C infected individuals, in terms of antibody reactivity and presentation of viral epitopes to the immune system, may lead to more broadly reactive and effective antibodies for therapy and/or vaccine development.

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