Cross-Reactive Neutralizing Humoral Immunity Does Not Protect from HIV Type 1 Disease Progression

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(See the editorial commentary by Doria-Rose, on pages 981–983.)

Broadly reactive neutralizing antibodies are the focus of human immunodeficiency virus (HIV) type 1 vaccine design. However, only little is known about their role in acquired immunodeficiency syndrome (AIDS) pathogenesis and the factors associated with their development. Here we used a multisubtype panel of 23 HIV-1 variants to determine the prevalence of cross-reactive neutralizing activity in serum samples obtained ∼35 months after seroconversion from 82 HIV-1 subtype B–infected participants from the Amsterdam Cohort Studies on HIV Infection and AIDS. Of these patients, 33%, 48%, and 20%, respectively, had strong, moderate, or absent cross-reactive neutralizing activity in serum. Viral RNA load at set point and AIDS-free survival were similar for the 3 patient groups. However, higher cross-reactive neutralizing activity was significantly associated with lower CD4+ T cell counts before and soon after infection. Our findings underscore the importance of vaccine-elicited immunity in protecting from infection. The association between CD4+ T cell counts and neutralizing humoral immunity may provide new clues as to how to achieve this goal.

In individuals infected with human immunodeficiency virus (HIV) type 1, neutralizing antibodies can develop against autologous HIV-1 strains within weeks of infection [1]. In general, antibodies that can neutralize autologous virus variants are strain specific and lack the ability to neutralize heterologous viruses [2]. Some HIV-1–infected individuals, however, mount a potent neutralizing humoral immune response that has the in vitro ability to neutralize HIV isolates from unrelated subjects [3–6]. The exact nature of cross-reactive neutralizing activity in serum is unclear and may be the result of a single high-affinity antibody directed against a highly conserved epitope in the envelope protein. Alternatively, it may reflect the activity of several neutralizing antibodies that in combination give cross-reactive neutralizing activity [7, 8]. Little is known about the protective properties of broadly cross-reactive neutralizing antibodies in vivo. In nonhuman primate studies, passive transfer of broadly neutralizing antibodies completely blocked infection by a chimeric simian-human immunodeficiency virus [9–15], whereas in humans, passive transfer of broadly neutralizing antibodies delayed HIV-1 rebound after cessation of antiretroviral therapy [16].

In our present study, we wished to determine the prevalence of cross-reactive neutralizing humoral immunity in serum samples from participants in the Amsterdam Cohort Studies on HIV infection and AIDS and determine whether the presence of HIV-1–specific cross-reactive neutralizing activity in serum was associated with delayed disease progression. In addition, we
wanted to reveal factors that were associated with the development of such a potent humoral immune response.

In our cohort, 33% of participants had cross-reactive neutralizing serum activity, but no correlation between the presence of potent humoral immunity and disease course could be revealed. The mounting of a potent and cross-reactive neutralizing immune response was significantly associated with a lower CD4+ T cell count at set point but not with viral load at set point. Therefore, although potently neutralizing humoral immunity does not seem to influence disease course, our findings may be relevant for the achievement of optimal vaccine responses.

**MATERIALS AND METHODS**

**Study participants.** The study population consisted of 131 white men who have sex with men who were HIV-1 negative at the time of enrollment in the Amsterdam Cohort Studies on the natural history of HIV-1 infection (between October 1984 and March 1986) and who seroconverted for HIV-1 antibodies between 1984 and 1996 during active follow-up. To obtain the best data on prevalence of cross-reactive neutralizing activity in serum, which generally develops relatively late after seroconversion, we chose serum samples obtained at a mean of 35 months (range, 30–37 months) after seroconversion. Individuals were excluded if they had already reached a CD4+ T cell count of <200 cells/µL blood, had developed AIDS, had begun highly active antiretroviral therapy (HAART), or were unavailable for follow-up at the time of screening, leaving 82 individuals for analysis.

For Kaplan-Meier survival analysis, individuals were censored at their first day of effective antiretroviral therapy or when unavailable for follow-up. When AIDS (Centers for Disease Control and Prevention [CDC] 1993 definition) [17] was used as an end point in Kaplan-Meier survival analysis, 46 individuals had an event, 13 were censored owing to lack of follow-up, and 23 were censored because of initiation of HAART. When the end point was AIDS-related death—defined as death from AIDS-related malignancy, AIDS-opportunistic infection, or an AIDS-related cause not specified by the treating physician—29 individuals had an event, 16 were censored owing to lack of follow-up, and 37 were censored at initiation of HAART. For survival analysis after AIDS diagnosis, 25 individuals had an event, 20 were censored owing to lack of follow-up; and 37 were censored at initiation of HAART.

The Amsterdam Cohort Studies have been conducted in accordance with the ethical principles set out in the Declaration of Helsinki, and written informed consent was obtained from each cohort participant before data collection. The study was approved by the Academic Medical Center institutional medical ethics committee.

**Neutralization assay.** Serum samples from all 82 cohort participants, obtained a mean of 35 months after seroconversion, were tested for cross-reactive neutralizing activity in a pseudo-virus assay involving a single round of viral infection, as developed by Monogram Biosciences [18, 19]. We used 2-tier 2-virus panels (Table 1, which appears only in the electronic version of the Journal) for determining cross-neutralizing activity in serum. The first panel consisted of 20 pseudo-viruses with envelope sequences from HIV-1 subtypes A–D and with 5 viruses per subtype (panel 1). Viruses were obtained recently after transmission or during the chronic phase of infection and were either moderately sensitive or neutralization resistant based on previously determined neutralization sensitivities to serum samples from subtype B–infected individuals and monoclonal antibodies b12, 2G12, and 4E10 [20, 21].

The second panel consisted of 5 pseudo-viruses with envelope sequences from primary isolates of HIV-1 subtypes A, B, C, and CRF01_AE (panel 2) that were resistant (n = 1), moderately resistant (n = 3), or moderately susceptible (n = 1), on the basis of previously determined neutralization sensitivities to serum samples from subtype B–infected individuals and monoclonal antibodies b12, 2G12, and 4E10. This 5-virus panel covered 93% of the variation in neutralization of a larger pseudo-virus panel (n = 15) [21]. Pseudo-typed viral particles were produced by cotransfection of HEK293 cells with an expression vector carrying the HIV-1–derived gp160 gene (eETV) and an HIV-1 genomic vector carrying a luciferase reporter gene (pRTV1.F-lucP.CNDO-DU3). Forty-eight hours after transfection, pseudo-virus stocks were harvested, and small aliquots were tested for infectivity using U87 target cells expressing CD4, CCR5, and CXCR4. Pseudo-virus stocks were tested and normalized for infectivity before testing in the neutralization assay. Diluted pseudo-viruses were incubated for 1 h at 37°C with serial dilutions of the patient serum sample, after which the U87 target cells were added. The ability of patient serum samples to neutralize viral infection was assessed by measuring luciferase activity 72 h after viral inoculation in comparison with a control infection with a virus pseudo-typed with the amphotropic murine leukemia virus (aMLV) envelope.
neutralization titers are expressed as the reciprocal of the plasma dilution that inhibited virus infection by 50% (IC$_{50}$). Neutralization titers were considered positive if they were 3 times greater than the negative aMLV control. The lowest serum dilution used in the assay was 1:40. For calculation of IC$_{50}$ values for viruses that were not inhibited by the 1:40 serum dilution, we assumed that 50% inhibition would have occurred at a 1:20 serum dilution.

**Viral load measurements.** Viral load in plasma was routinely measured at every study visit in the cohort studies by using a quantitative HIV-1 RNA nucleic acid-based sequence amplification (Organon Teknika) with electrochemiluminescently labeled probes [22]. Set-point viral load data were available for all patients. Viral load data were analyzed after log$_{10}$ transformation.

**Immunologic assays.** CD4$^+$ T cell counts in peripheral blood were first measured at the first visit after entry in the Amsterdam Cohort Studies, and were routinely measured at every subsequent study visit using flow cytometry. Set-point CD4$^+$ T cell and CD8$^+$ T cell percentages from 62 patients were available from a previous study [23]. CD4$^+$ T cell counts data were available for all patients. Data on CD4$^+$ T cell and CD8$^+$ T cell percentages before and 1 and 5 years after seroconversion (normally distributed) was tested with the Kruskal-Wallis test. Spearman’s rank correlation coefficient was used to assess the association between geometric mean titers obtained with the 2 viral panels for each patient serum sample. Analyses were performed with GraphPad Prism software, version 4 (GraphPad Software).

**RESULTS**

Prevalence of cross-reactive neutralizing activity in serum in the natural course of HIV-1 infection. We first screened serum samples from participants in the Amsterdam Cohort Studies on HIV infection and AIDS for the presence of cross-reactive neutralizing activity. Because neutralizing serum activity is mounted relatively late after seroconversion [24], we chose to test serum samples obtained ∼35 months (range, 30–37 months) after seroconversion, which also allowed sufficient follow-up time to perform survival analysis from the time of screening onward. Cohort participants who at this time point had already progressed to disease or begun HAART were excluded from the study. The remaining group of 82 participants had a median AIDS-free follow-up time of 8.31 years (95% confidence interval, 5.95–10.5 years) after seroconversion, left truncation for the time point of screening. HIV-1–specific cross-reactive neutralizing activity in the serum samples of these patients was measured in a cell-based infectivity assay using a panel of 20 pseudo-viruses carrying a luciferase reporter gene and the envelope proteins from tier 2 HIV-1 subtypes A, B, C, and D (panel 1) and a panel of 6 pseudo-viruses with envelope proteins from JRCSF and tier 2 HIV-1 subtypes A, B, C, and CRF_01 AE (panel 2) [21] (Table 1 and Figures 1 and 2, which appear only in the electronic version of the Journal).

Cross-neutralizing activity of patient serum samples on these 2 virus panels (Figures 1 and 2) were strongly correlated (Spearman $r = 0.91$) (Figure 3, which appears only in the electronic version of the Journal). Therefore, data from the 2 panels were combined for further analysis, excluding the data on CRF_01 AE, because we had only 1 variant of this subtype (panel 3) (Figure 4). The analysis of the combined data sets showed strong correlations between cross-reactive neutralizing activity in serum and geometric mean titer (Spearman $r = 0.89$; data not shown) and the number of viruses neutralized (Spearman $r = 0.79$; data not shown). Strong cross-reactive neutralizing activity in serum, defined as the ability to neutralize HIV-1 variants at an IC$_{50}$ titer of $\geq 100$ to $\geq 1$ virus from $\geq 3$ subtypes, was observed in 27 patients (33%), similar to the prevalence in other cohorts [25, 26]. Serum samples from 39 patients (48%) neutralized HIV-1 at an IC$_{50}$ titer of $\geq 100$ to $\geq 1$ virus from 1 or 2 subtypes (moderate cross-reactive neutralizing activity), whereas serum samples from 16 patients (20%) completely lacked cross-reactive neutralizing activity (Figure 4).
Figure 4. Breadth and titer of human immunodeficiency virus (HIV) type 1–specific neutralizing activity in serum. Values represent plasma dilution that inhibits virus infection by 50% (IC\textsubscript{50}), given as the reciprocal serum dilution; serum samples were obtained ∼3 years after seroconversion. Patient identification (ID) numbers for the 82 HIV-1–infected individuals are provided in the left column; patients are ranked based on the breadth and titer of the neutralizing activity in serum. Virus panel 3 included 23 viruses from subtypes A, B, C, and D, with controls on the far right (JRCSF, NL4–3, and amphotropic murine leukemia virus [aMLV]). IC\textsubscript{50} titers are color coded as follows: white, IC\textsubscript{50} $\leq 1:40$; green, IC\textsubscript{50} $>1:40$ but $\leq 1:100$; orange, IC\textsubscript{50} $>1:100$ but $\leq 1:1000$; and red, IC\textsubscript{50} $>1:1000$.

Interestingly, 1 patient had an average log-transformed neutralizing titer of 2.9 with panel 2, for which he ranked in the top 3 of recently identified elite neutralizers (average log-transformed HIV-1 neutralizing titer, $>2.5$) [21].

Association between cross-reactive neutralizing activity in serum and clinical course of HIV-1 infection. Next, we investigated the potential relationship between cross-reactive HIV-1–specific neutralizing activity in serum and the rate of HIV-1 disease progression. Kaplan-Meier and Cox proportional hazard analysis were performed for the period after cross-reactive neutralizing activity in serum was measured, using clinical AIDS (1993 CDC definition [17]) and AIDS-related death...
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Figure 5. Kaplan-Meier survival analysis for time from seroconversion until occurrence of AIDS (Centers for Disease Control and Prevention [CDC] 1993 definition [17]) (A) or AIDS-related death (B) and for time from AIDS diagnosis to AIDS-related death (C) for individuals with strong (red lines) \((n = 27)\), moderate (dashed yellow lines) \((n = 39)\), or absent (green lines) \((n = 16)\) cross-reactive neutralizing activity. \(P\) values (log-rank test) are denoted. Median survival times for groups of individuals with strong, moderate, or absent cross-reactive neutralizing activity at \(\sim 35\) months after seroconversion were 7.5 ± 2.2, 8.5 ± 3, and 10.5 ± 4 years, respectively, for AIDS-free survival; 9.9 ± 2.5, >7.9, and >8.5 years for time from seroconversion to AIDS-related death; and 2.3 ± 0.5, 2.4 ± 0.2, and 2 ± 0.4 years for time from AIDS diagnosis to death.

The presence of cross-reactive neutralizing activity in serum was not associated with delayed progression to AIDS according to the 1993 CDC definition (\(P = .29\), by log-rank test; median AIDS-free survival times [from screening onward] for individuals with strong, moderate, or absent cross-reactive neutralizing activity in serum 35 months after seroconversion, 7.5 ± 2.2, 8.5 ± 3, and 10.5 ± 4 years, respectively) (Figure 5A). The time from screening to AIDS-related death was also similar for the groups with strong, moderate, or absent cross-reactive neutralizing activity in serum 35 months after seroconversion (\(P = .69\), by log-rank test; median survival times, 9.9 ± 2.5, >7.9, and >8.5 years, respectively) (Figure 5B). Finally, survival time after AIDS diagnosis was also the same for the 3 patient groups (\(P = .5\), by log-rank test; median survival time, 2.3 ± 0.5, 2.4 ± 0.2, and 2 ± 0.4 years, respectively) (Figure 5C).

Factors associated with the presence of cross-reactive neutralizing activity in serum. We subsequently investigated the potential relationship between the breadth of the HIV-1–specific neutralizing activity in serum and the viral RNA load in plasma at set point and the CD4+ T cell counts at set point. In our cohort, cross-reactive neutralizing activity in serum at 35 months after seroconversion was not associated with the level of plasma viremia at set point, which was defined as the average viral load between months 18 and 24 after seroconversion (Figure 6A).

Interestingly, strong cross-reactive neutralizing activity in serum was significantly associated with a low median CD4+ T cell count at set point (\(P = .011\)) (Figure 6B). To analyze whether the association between more potent humoral neutralizing activity in serum and CD4+ T cell counts may have potential significance for vaccine efficacy, we next analyzed whether this same association could be observed between pre-seroconversion CD4+ T cell numbers and the titer of the neutralizing humoral immune response after HIV-1 infection. For this purpose, we compared the mean percentages of CD4+ and CD8+ T cells before seroconversion (\(\geq 6\) months before seroconversion) and 1 and 5 years after seroconversion within groups of individuals with strong, intermediate, or absent cross-reactive neutralizing activity in serum. Individuals with strong neutralizing activity had lower percentages of CD4+ T cells (\(P = .011\)) (Figure 6C) and higher percentages of CD8+ T cells (\(P = .0082\)) (Figure 6D) before seroconversion than HIV-infected individuals who lacked cross-reactive neutralizing activity in serum. This trend was still observed 1 year after seroconversion but was absent at year 5 of infection (Figure 6C and 6D).

DISCUSSION

Previous studies have shown that autologous strain-specific neutralizing activity does not contribute significantly to the control of HIV-1 infection [27–29]. In the current study we showed that even cross-reactive neutralizing activity in serum is not associated with prolonged time to AIDS or death. This observation is in line with the finding that administration of broadly neutralizing antibody b12 before viral challenge could protect animals from infection, but administration after in-
occlusion had no effect on the control of established HIV-1 infection in vivo [30]. Moreover, it confirms recent findings in a cohort of Kenyan women in which cross-reactive neutralizing activity was not associated with time to AIDS or initiation of antiviral therapy [31].

Cross-reactive neutralizing activity is known to accumulate with time of infection [26]. For this reason, we chose to screen for serum neutralizing activity at ~35 months after seroconversion, when an adequate cross-reactive humoral immune response could have been developed, and excluded cohort participants who at that time point had already developed AIDS, begun HAART, or reached a CD4+ count of <200 cells/μL of blood. As a consequence, individuals with very rapid disease progression were excluded from analysis, and our study design therefore only allows for the conclusion that cross-reactive neutralizing activity has no long-term protective effect on HIV-1 disease progression.

The prevalence of strong cross-reactive neutralizing activity in serum in our study population was 33%, similar to observations in recent studies [21, 25, 26]. Simek et al [21] tested the neutralizing activity in serum samples from ~1800 individuals on different pseudo-virus panels and reported that screening for a panel of only 5 selected viruses (panel 2 in our study) provided similar information on the presence of cross-reactive neutralizing activity as screening for a large pseudo-virus panel. Indeed, the results obtained with serum samples from patients in our study for either panel 1 (20 viruses from subtypes A–D) or panel 2 were highly concordant in geometric mean titer (Spearman $r = .91$). This not only confirms the suitability of our large pseudo-virus panel for characterization of HIV-1 neutralizing activity in patient serum samples, but it also allows for a direct comparison of our data with previous studies.

Interestingly, Simek et al [21] identified 15 so-called elite neutralizers who had an average log-transformed titer of $\geq 2.5$ on panel 2 (including JRCSF). In our cohort, we identified 1 elite neutralizer who reached a log-transformed titer of 2.9 on this same virus panel. Compared with the elite neutralizers in the study by Simek et al [21], our patient ranked third. Because the prevalence of elite neutralizers is considered to be only 1%, the biomaterial from this Amsterdam Cohort participant is definitely interesting for the identification of potentially novel cross-reactive neutralizing antibodies.

It has been reported that the prevalence of cross-reactive neutralizing activity in serum from elite controllers was much lower than that for long-term nonprogressors or slow pro-
gressors [32]. A certain level of antigen is apparently required to drive the humoral immune response. Previous studies have indeed demonstrated a correlation between the breadth of neutralizing activity in serum and viral load at set point or at time of testing for neutralizing activity [26, 31, 33]. In our present study, we did not observe a correlation between the presence of cross-reactive neutralizing activity in serum at ∼35 months after seroconversion and the viral load at set point or at the time of screening for HIV-1-specific humoral immunity. We currently have no explanation for this apparent discrepancy. However, of the 10 patients with the lowest viral load at set point, 6 lacked cross-reactive neutralizing activity in serum, indicating that a certain level of antigen is indeed required to stimulate neutralizing humoral immunity [31, 32]. However, absent cross-reactive neutralizing activity in patients with higher viral loads in plasma indicates that additional factors may be critical for the development of a cross-reactive neutralizing antibody response.

We recently demonstrated that in serum samples of subtype B–infected patients, the neutralizing activity was stronger against the subtype B viruses in our panel than against the subtype A, C, and D viruses in our panel [34]. We could confirm this observation in our present study, because neutralization of subtype B variants was seen significantly more often than neutralization of viruses from other subtypes (P < .001, by χ² test). Indeed, in serum samples from 42 of 82 patients, neutralizing activity against >50% of the subtype B viruses in the panel was observed, whereas neutralization of >50% of subtype A, C, or D viruses was seen in serum samples of only 27, 23, and 19 individuals, respectively.

Interestingly, we observed a correlation between cross-reactive neutralizing activity and a lower CD4+ T cell count at set point and a lower CD4+ T cell percentage before HIV-1 infection. In another study, this correlation was not seen [26], but in that study the within-subject average of CD4+ T cells from different time points was compared with the breadth of neutralizing activity and can therefore not be compared with the CD4+ T cell count at set point or before HIV-1 infection.

Our data are in line with a study in a lymphocytic choriomeningitis virus mouse model, in which either partial CD4+ T cell depletion before infection or exclusion of dominant CD4+ T cell epitopes from the vaccine enhanced the generation of neutralizing antibody responses, owing to reduced polyclonal B cell activation [35, 36]. In analogy, decreased CD4+ help may prevent polyclonal B cell activation and hypergammaglobulinemia in HIV infection [37, 38], favoring the production of neutralizing antibodies. Although the differences in percentages or numbers of CD4+ T cells between groups may be small, they could reflect a critical threshold for proper B cell help.

The absent association between cross-reactive neutralizing immunity and the clinical course of HIV-1 infection is suggestive of rapid viral escape from humoral immune pressure [1, 2, 19, 39], despite the fact that cross-reactive neutralizing antibodies are considered to be directed against conserved epitopes. We have indeed observed that HIV-1 can rapidly escape from autologous humoral immunity with cross-reactive neutralizing activity (authors’ unpublished data). Apparently, these escape mutations do not come at a fitness cost to the virus [40], as has been described for certain escape mutations in conserved epitopes for cytotoxic T lymphocytes [41–44]. In agreement, we previously reported that the replication rates of viruses that were resistant to broadly neutralizing antibodies b12, 2G12, 2F5, and/or 4E10 were similar to the replication kinetics of the co-existing neutralization sensitive viruses from the same patient [40].

In conclusion, cross-reactive neutralizing activity in serum does not seem to have an effect on the clinical course of HIV-1 infection. Possibly, and as observed for other viral infections, cytotoxic T lymphocytes rather than neutralizing antibodies may contribute to the control of already established infections, whereas neutralizing antibodies may be essential for protection from infection [14, 15]. Our data suggest that a broadly neutralizing humoral immune response may be best achieved in the face of reduced CD4+ T cell numbers. Although arguably this may be unrealistic to achieve deliberately as part of a vaccination regimen, it could provide clues for achieving a more efficacious antibody vaccine. Apart from that, the relatively large proportion of individuals with cross-reactive neutralizing humoral immunity elicited by the native HIV-1 envelope may already predict a satisfying response rate once a vaccine becomes available.

Acknowledgments

We thank Jannie van der Helm and Ronald Geskus for statistical support and Angélique van ’t Wout, Neeltje Kootstra, and René van Lier for critical reading of the manuscript.

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