Longitudinal Antibody Responses in People Who Inject Drugs Infected With Similar Human Immunodeficiency Virus Strains

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Background: Multiple factors influence the human immunodeficiency virus (HIV) antibody response produced during natural infection, leading to responses that can vary in specificity, strength, and breadth.

Methods: People who inject drugs identified as recently infected with HIV (n = 23) were analyzed for clustering of their viral sequences (genetic distance, <2%). Longitudinal antibody responses were identified for neutralizing antibody (Nab) potential, and differences in antibody subclass, specificity, and Fc receptor ligation using pseudovirus entry and multiplexed Fc array assays, respectively. Responses were analyzed for differences between subject groups, defined by similarity in the sequence of the infecting virus.

Results: Viral sequences from infected individuals were grouped into 3 distinct clusters with 7 unclustered individuals. Subjects in cluster 1 generally had lower antibody response magnitudes, except for antibodies targeting the V1/V2 region. Subjects in clusters 2 and 3 typically had higher antibody response magnitudes, with the Fv specificity of cluster 2 favoring gp140 recognition. Nab responses differed significantly between clusters for 3 of 18 pseudoviruses examined (P < .05), but there were no differences in overall Nab breadth (P = .62).

Discussion: These data demonstrate that individuals infected with similar viral strains can generate partially similar antibody responses, but these do not drastically differ from those in individuals infected with relatively unrelated strains.

Keywords: HIV; neutralizing antibody; antibody development; people who inject drugs; cluster linkage.

A successful protective human immunodeficiency virus (HIV) vaccine will most likely need to generate a broad and diverse antibody response that can directly neutralize the virus or direct virions to other cytotoxic mechanisms. An important underlying element of any vaccine construct is the ability to generate a similar immune response in a majority of vaccinees. Previous research examining the development of the natural humoral response to HIV infection has generally focused on individuals infected with naturally diverse viral strains and has found a variable response that is influenced by early events in infection, as well as the ongoing evolution and changes of the viral population [1–3]. Currently, it is not fully understood to what extent individuals initially infected with similar viral strains generate analogous humoral immune responses when exposed for a similar time frame.

The majority of research examining the development of HIV humoral immunity has focused on the neutralizing antibody (Nab) response, and in particular the development of broadly NAb responses from individuals infected with HIV subtype B viruses that the CD4 binding site more potently than non-B subtype viruses, which conversely were superior at targeting the V2 loop in nonhuman primates comparing animals infected with identical inocula suggests that the antibody response is influenced by initial viral genotype. For example, SIVmac239 is poor at eliciting Nab, which SHIVAD8 is able to do [8]. In addition, simian-human immunodeficiency virus–based Nab responses can develop to be specifically targeted to certain areas of the viral envelope, such as the V1/V2 region, similar to the targeting of the CAP256-VRC26 family of NAb [5, 9]. These
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Methods
Study Population
Individuals who enrolled from 1988–1989 in the AIDS Linked to the IntraVenous Experience (ALIVE) study and who were found to be HIV positive on enrollment were screened using a validated multiserological assay algorithm for recent infection (<6 months) [21]. Briefly, patient serum or plasma samples that had a positive viral load (second-generation branched DNA assay; Chiron) were screened for lower antibody titer using the BED HIV titer assay, as well as for low antibody affinity using a modified Bio-Rad HIV enzyme-linked immunosorbent assay [22]. If a sample was found to be below established cutoffs for both values, the patient were determined to be recently infected, and of these patients, those who remained in the study for 3–6 years of follow-up without starting highly active antiretroviral therapy had their initial plasma viral populations sequenced with a validated site-directed next-generation sequencing (NGS) assay for 2 genetic regions (gp41 and pol) [23]. Individuals whose viral populations were successfully sequenced in both regions at baseline and ≥1 subsequent time point were included for subsequent antibody analyses. In addition, the initial diagnosis sample or the closest available sample with adequate volume was sequenced with single-genome amplification (SGA) for either the entire gp160 gene or the C1-V5 region of gp120 if gp160 amplification was not successful. Heparinized plasma or serum samples for 3 time points (0.75–1.5, 2–3, and 5–6 years after diagnosis, or the closest time point available before these windows) were used for subsequent antibody assays. All participants provided informed consent for testing and sample storage for further analysis.

Viral Linkage
NGS-derived consensus sequences for pol and gp41 from each individual at their initial diagnosis sample were combined into a single consensus sequence for each region and concatenated. The concatenated viral sequences were aligned with HIV subtype B reference sequences and examined for linkage. Sequences that were genetically linked (<2% total genetic distance between the whole group) were classified into clusters or determined to be unclustered (HIV-TRACE) [24]. Clusters were phylogenetically confirmed using SGA-derived sequences from either the initial diagnosis sample or the closest time point available.

Antibody Binding, Immunoglobulin Class, and Fc Receptor Screen
A subset of plasma and serum samples from all 16 of the subjects that were clustered and 7 of the 9 unclustered subjects were analyzed for antigen binding and Fc interactions, as reported elsewhere [25, 26]. Briefly, antibodies specific for 40 antigens derived from the HIV envelope and other structural and accessory genes were probed with 15 Fc receptor and other detection reagents to profile the antibody response [27]. Samples were analyzed at a dilution of 1:5000 for FcgRs, anti-human (aHu) IgG, and aHu IgG1 detection reagents, whereas the dilution used for analysis with aHu IgA, IgD, IgM, IgG2–4, and C1q detection reagents was 1:1000. Detection with aHu IgG was also performed using 1:1000 and 1:25 000 dilutions. The median fluorescence intensity was reported for each measurement.

NAb Responses
Neutralization was measured using single-round-of-infection HIV-1 Env pseudoviruses and TZM-bl target cells, as described...
Neutralization curves were fit by nonlinear regression using a 5-parameter hill slope equation. The 50% and 80% inhibitory dilutions (ID50 and ID80) were reported as the reciprocal plasma dilutions required to inhibit infection by 50% and 80% respectively. Plasma from all available time points for clustered and unclustered individuals were screened for neutralization activity using a standard 20 pseudovirus panel, as reported elsewhere [27]. Results from 2 of the pseudoviruses (231965.c1 and 242-14) were removed from analysis because they were sensitive to residual heparin in the plasma, rendering the data unreliable. Samples that demonstrated >30% neutralization breadth were analyzed for their monoclonal NAb fingerprint, and these were compared for correlations between samples as reported elsewhere with a modification for using the 18 virus panel [28].

**Statistical Analyses**

Fold changes in mean values per each group and t tests were used to compare antigen binding, immunoglobulin class, and Fc receptor binding measurement. Excel software was used to calculate fold change and perform t tests. Fc array heat map and Volcano plots were generated in R using the “gplots” package. Longitudinal NAb potency for individual pseudoviruses tested and overall breadth (median inhibitory dilution, >40:1 dilution) for each individual were analyzed using a linear mixed-effects model for differences between the clusters overall, as well as the for each individual were analyzed using a linear mixed-effects model for differences between the clusters overall, as well as the clusters versus the unclustered group.

Sequence data for the study are available through GenBank (accession nos. MN412134–MN412403). The full neutralizing antibody and binding antibody data sets are available on request (aredd2@jhmi.edu).

**RESULTS**

Of the individuals identified as being recently infected on enrollment into the original ALIVE cohort (n = 80), 23 had their viruses successfully sequenced in both pol and gp41 genetic regions at multiple time points (Figure 1). These individuals were all African American, mostly men (19 of 23), and they were grouped according to the genetic similarity of their NGS-derived viral sequences at their initial study time point (Figure 1). These clusters were confirmed by means of SGA of the viral envelope (Supplementary Figure 1). The demographics and HIV disease status for the individuals in the clustered groups (clusters 1, 2, and 3; n = 5, 5, and 6 respectively) did not differ significantly from the unclustered individuals (n = 7) (Table 1). It should be noted that the baseline viral loads for these patients were determined using a second-generation branched DNA assay, which has been shown to report viral loads at 2-fold lower levels than reverse-transcription polymerase chain reaction–based assays [30].

Using a high-throughput antibody binding assay that examines epitope targets (n = 40), as well as antibody Fc characteristics (n = 15), “snapshots” of the humoral response for each subject were captured. Visualizing individual feature measurements relative to one another at the earliest time point tested (mean, 1.06 years after diagnosis; interquartile range, 1.02–1.14 years) for each subject, and sorting the features by Fc and Fv specificity revealed that some antibody attributes were shared by subjects belonging to the same viral cluster (Figure 2). It was also apparent from this analysis that at a biophysical level, there were large differences in both the level of responses and the specificity of those responses between subjects, regardless of cluster. These differences may be related to different environmental factors or the distinct genetic profiles of each individual that participated in this study, among others. As anticipated, subjects typically had a wide range of responses, both to the envelope glycoprotein as well as to the other accessory, regulatory, and structural proteins of the HIV proteome; these responses were typically observed across time points.

To examine how the humoral responses of individuals differed between subjects infected with similar strains and those infected by disparate strains, the Fc array data were compared between subjects in viral clusters. Subjects in cluster 1 typically exhibited lower-intensity gp140-specific responses. In some cases, IgM antibodies that were specific for gp120 were elevated in cluster 1, particularly in comparison with cluster 2 (Figure 3A). Although the magnitude of these differences tended to decrease over time, they were still present at the later time points tested (data not shown). Similar to those in cluster 2, cluster 3 subjects had higher responses against many of the Env antigens, although there was a subset of responses to 1 specific sequence of the V1/V2 region of the viral envelope, as defined by a V1V2 epitope probe (gp70 from strain 62357) that was higher in cluster 1 than in cluster 3 (Figure 3B). These differences also held over time, with subjects in cluster 1 increasing in V1/V2 response magnitude longitudinally (data not shown). Antibodies with high C1q ligating capacity often appeared elevated among subjects in cluster 3 when compared with cluster 2, suggesting an elevated antibody-associated complement response in this cluster. Consistent with distinctions in the genetic sequence of the infecting virus, antibody profiles from nonclustered subjects did not seem to strongly support their inclusion in clusters 1, 2, or 3 (data not shown).

NAb responses were measured using an 18-psuedovirus panel, and the longitudinal ID50 values were examined for differences between viral groups as compared with the unclustered control group (Figure 4). NAb responses increased over time, culminating in a relatively broad response by 5–6 years after infection; however, the potency of the NAb response to specific pseudovirus strains differed dramatically between subjects. In addition, some NAb responses waned later in disease compared with the second time point tested.
Figure 1. Maximum likelihood phylogenetic tree of concatenated consensus sequences derived from next-generation sequencing of pol and gp41. Clusters of sequences with <2% genetic distance are indicated by color (cluster 1 [red], cluster 2 [green], and cluster 3 [purple]), along with a group of unclustered individuals (blue). Subtype B reference sequences are shown in black. The scale of the phylogenetic tree is 0.007.
The longitudinal breadth of the NAb response did not differ between the 4 groups ($P = .62$) (Figure 5A). However, significant differences were observed between the 4 groups for 3 individual pseudoviruses: KER2008.12 ($P = .02$), X26191.2.48 ($P = .02$), and X0013095.2.11 ($P = .002$) (Figure 5B–5D). For these 3 pseudoviruses, the NAb responses for each group were compared alone with the unclustered group. For X0013095.2.11, each of the 3 clustered groups differed significantly from the unclustered group ($P < .02$), but only group 3 differed significantly from the unclustered group for KER2008.12 ($P = .04$). No individual clustered group was significantly different from the unclustered group for X26191.2.48.

A serum neutralization fingerprinting analysis with the 18-pseudovirus panel was performed for all sample time points that demonstrated >30% breadth (Supplementary Figure 2). Despite the observed viral sequence similarities within each cluster, the neutralization fingerprints of individuals within a cluster were varied and were not found to be more similar than between clusters, suggesting a diversity of antibody-specificities in the different clusters (Supplementary Figure 3).

**DISCUSSION**

Genetic analysis of viral strains in individuals who entered the ALIVE cohort study after being recently infected with HIV identified 3 groups of individuals who were infected with highly similar viruses, suggesting they may have been part of transmission clusters. In several other subjects, the sequence similarities of the pol and gp41 regions of their infecting strains were not similar enough to each other or the 3 groups to be classified as members of a cluster. It should be noted that genetic analysis of multiple HIV isolates reveal that the highest rate of mutation is in the gp120 region, with reduced mutation rates in gp41 and
pol [31]. In addition, the NGS protocol used here does not allow for determining whether the 2 regions examined are collinear on the same viral isolate. Therefore, gp160 or partial gp120 SGA-derived sequences were analyzed as well, and they found to agree with the cluster groupings.

Interestingly, the SGA and NGS data showed that the subjects were infected with relatively uniform viral inocula, in contrast to previous findings in acutely infected people who inject drugs [32]. This may be because these patients were infected early in the epidemic in Baltimore, when there were limited numbers of viral strains circulating in the community. Alternatively, these patients were identified early in infection but not during the acute stage, and the viral populations that we examined may therefore represent the strain that grew out after initial infection with multiple transmitted strains. It should also be noted that the multiasay algorithm used in our study to identify recently infected individuals may have biased our findings by excluding those who rapidly developed a potent anti-HIV antibody response [33]. This bias, together with the lower viral loads seen with the branched DNA assay, may have contributed to the lower viral loads observed in this cohort, because it has been shown that individuals with low viral loads develop mature antibody responses at slower rates [33]. However, the initial viral loads did not differ significantly between groups.

These subjects’ participation in the ALIVE cohort study allowed for longitudinal analysis of the natural development of their anti-HIV humoral response before initiation of highly active antiretroviral therapy. To clarify the relationship between the infecting strain of a virus and the humoral responses it induces, tools to characterize the antibody repertoire were implemented and provided insights into the mechanisms of these differences. The Fc array data revealed distinct patterns between subject clusters for the binding antibody responses, such as stronger V1/V2 binding and weaker gp140 binding in cluster 1 subjects. Responses among subjects in clusters 2 and 3 typically had similar magnitude responses toward gp140s, whereas cluster 2 seemed to have lower-magnitude gp120-specific responses. Not only did Fv specificities diverge, but the Fc characteristics of antibodies also differed between the subject clusters, with complement binding and IgM antibodies being some of the most noticeable points of distinction between subject groups.
In most cases, these differences persisted throughout later time points. Interestingly, the binding responses of individuals with unclustered viral sequences did not present with the same patterns as the members of the cluster with which they were most closely genetically related. The neutralization patterns on a heterologous virus panel showed higher similarity within the clusters than the unclustered viruses in only 1 instance (X0013095.2.11). Neutralization patterns are derived from the set of epitopes targeted by NAb, a subset of those targeted by binding antibodies; these data suggest that the infecting viruses in this cohort did not trigger highly similar antibodies in different individuals.

Figure 4. Heat map of longitudinal serum and plasma median inhibitory dilution (ID50) measurements of neutralizing antibody potential for all patient samples tested, grouped by cluster and time since diagnosis. The potency of neutralization for each pseudovirus tested (labeled above) is color coded, showing no neutralization (red), and high (green), yellow, white for each pseudovirus tested (labeled above) is color coded, showing no neutralization (red), and high (green), yellow, white.
These data support the concept that antibody characteristics and repertoire are mildly affected by the infecting strain, an observation also made as part of an analysis of the Swiss HIV cohort study, in which it was estimated that up to 19% of the IgG binding response may be due to antibody imprinting of the infecting strain [19]. There are some critical differences, however, between these 2 studies. First, the Swiss study was significantly larger, providing it with statistical power that was not available in the current analysis. Second, several unclustered patients in our study were infected with viral sequences similar to those in some of the clustered patients, although they were greater than the 2% cutoff used. This could have diluted the effect seen between the unclustered subjects and the clustered groups.

The ALIVE cohort had several advantages compared with the Swiss cohort, particularly when we tried to investigate the effects of antibody imprinting from the infecting strain. In the ALIVE study, subjects were followed up longitudinally, whereas

Figure 5. Longitudinal plots of the median inhibitory dilution (ID50) neutralizing antibody (Nab) potential for all samples tested. A, Overall breadth did not differ between groups (cluster 1 [red], cluster 2 [green], cluster 3 [purple], and unclustered individuals [blue]). B–D, The Nab responses to 3 pseudo typed viral strains differed between all clusters and the unclustered group (overall P values shown; linear mixed effects). In addition, individual clusters that differed significantly from the unclustered group are indicated.
the Swiss analysis was cross-sectional. More importantly, the patients whose findings we analyzed are people who inject drugs and were most likely infected intravenously, whereas the Swiss cohort comprised patients infected through a variety of modes of transmission, which may affect the types of humoral responses seen in these patients [19]. One limitation of both studies is that humoral response attributes are affected by host genetics, among other factors, which were not controlled for or investigated in our study but are expected to affect observations independent of infection strain.

The results presented here further support the findings in the Swiss cohort that a portion of the NAb response may be attributable to antibody imprinting by the infecting strain [19]. The NAb responses in the clustered groups differed significantly from those in the unclustered in 3 of the 18 pseudoviruses tested, and they also trended toward differences for 3 other viruses ($P < .10$; data not shown). The Swiss cohort study found that the NAb responses to 50% of the pseudoviruses tested (7 of 14) differed significantly in putative transmission clusters [19]. The associations between NAb response, binding antibody profiles, and viral genetic similarity presented here provide evidence that the infecting viral strain may have a small but significant effect on the resulting anti-HIV NAb response.

Supplementary Data
Supplementary materials are available at The Journal of Infectious Diseases online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes
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