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Information about neutralizing antibody responses in subtype C-infected individuals is limited, even though this viral subtype causes the majority of AIDS cases worldwide. Here we compared the course and magnitude of the autologous neutralizing antibody (NAb) response against viral envelope (Env) glycoproteins present during acute and early infection with subtypes B and C human immunodeficiency virus type 1 (HIV-1). NAb responses were evaluated in 6 subtype B-infected and 11 subtype C-infected subjects over a mean evaluation period of 25 months using a pseudovirus reporter gene assay. All subjects in the C cohort were infected through heterosexual contact, while five of the six subjects in the B cohort were infected via male-to-male contact. The kinetics and magnitude of the NAb responses varied among subjects in the B and C cohorts; however, the median 50% inhibitory concentration (IC50 titer) reached by antibody in the plasma of subtype C-infected subjects, overall, was 3.5-fold higher than in the subtype B-infected subjects (P = 0.06). The higher titers of NABs in the C cohort were associated with viruses having significantly shorter amino acid length (P = 0.002) in the V1 to V4 region of the surface Env glycoprotein, gp120, compared to the B cohort. Despite the potency of the autologous subtype C NAb response, it was not directed against cross-neutralizing epitopes. These data demonstrate that subtype C Env elicits a potent yet restricted NAb response early in infection that frequently reaches IC50 titers in excess of 1:1,000 and suggest that clade-specific differences may exist in Env immunogenicity or susceptibility to neutralization.

Neutralizing antibodies are likely to be an important component of vaccine-induced protective immunity. However, most information concerning antibody-mediated neutralization of human immunodeficiency virus type 1 (HIV-1) has thus far been derived from studies of subtype B HIV-1 infection, which predominates in North and South America, Europe, and Australia (21). The neutralizing antibodies (Nabs) characterized to date by epitope mapping, neutralization breadth, and potency are from subtype B-infected individuals, and of 174 monoclonal antibodies (MAbs) that have been described, only 5 have broad neutralizing activity against diverse primary HIV-1 strains: 2G12, 2F5, 4E10, b12, and 447-52D (14). Nevertheless, a recent study demonstrated that only one of these antibodies, 4E10, possesses significant breadth against non-subtype B viruses (4). This study further demonstrated that HIV-1 group M viruses are polarized based on their neutralization susceptibility to a panel of MAbs. In this study and others, subtype C viruses were characteristically less sensitive to neutralization by the MAbs 2G12 and 2F5, which target a carbohydrate-dependent epitope in gp120 and a linear epitope in gp41, respectively (3, 5; C. Derdeyn, unpublished data).

Because non-subtype B strains of HIV-1 dominate the AIDS pandemic (21), more information is clearly needed about the serology of these infections, especially during the acute/early phase. Recent studies have highlighted potential differences in the biology of transmission between viral subtypes. Viruses belonging to subtypes A and C appear to pass through a genetic bottleneck during or shortly after heterosexual transmission that selects for a virus with compact variable loops (7, 9). This type of selection, however, was not observed in transmission of subtype B viruses, even when transmitted through heterosexual contact (7, 10). Moreover, newly transmitted subtype C viruses were sensitive to neutralization by antibodies in plasma from the chronically infected partner, but newly transmitted subtype B viruses were not (9, 10).

In the presence of humoral and cell-mediated immune responses characteristic of chronic infection, there is evidence that strong positive selection targets distinct regions of gp120, depending on the viral subtype. In HIV-1 sequences from the Los Alamos database, strong positive selection is focused on a region downstream of the third hypervariable domain (V3) of gp120 in subtype C sequences but on V3 itself in subtype B sequences (12). This finding is consistent with the V3 domain’s acting as a principal target for NABs in subtype B infection (13, 5211).
14, 18, 27, 29). Nevertheless, potent selective pressure from autologous NABs during primary infection with subtype B HIV-1 rapidly generates viral variants that escape the initial response (2, 3, 5, 23, 27). In contrast, NAB specificities and escape mechanisms have not been characterized for subtype C infection.

Here, we employed a sensitive and quantitative pseudovirus reporter assay to evaluate the initial autologous NAB response mounted against envelope (Env) glycoproteins present during acute/early infection of 11 subtype C-infected subjects in Zambia and 6 subtype B-infected subjects in the United States (9, 26, 27). Although the course of autologous NAB was similar between the two cohorts, the NAB responses peaked at higher titers in the subtype C-infected subjects. The higher titers were associated with shorter, less glycosylated Envs in early subtype C infection. Thus, unusually potent NAB responses were generated against the virus population present at seroconversion in this cohort of subtype C-infected subjects, providing new information about early subtype C infection and further highlighting potential differences between subtypes B and C. Differences in neutralization susceptibility based on viral subtype could pose further obstacles to the development of practical, widely useful vaccines.

MATERIALS AND METHODS

Study subjects. The Lusaka cohort was established in 1994 to provide voluntary HIV-1 testing and counseling, long-term monitoring, and health care to cohabitating heterosexual couples in the capital city of Zambia. Details of the cohort have been described previously (9, 26). HIV-discordant couples enrolled in studies of transmission are monitored for seroconversion of the negative partner at 3-month intervals, at which time the participants also receive preventative counseling and condoms. The 11 subtype C-infected seroconvertors studied here were derived from this cohort. All but one subject (133 M) were infected by their partner (9, 26; C. Derdeyn, unpublished data). Acutely subtype B-infected subjects were patients at the University of Alabama at Birmingham 1917 Clinic and have been described previously (9, 27). None of the subtype B- or C-infected subjects received antiretroviral therapy during the evaluation period. Blood samples for preparation of plasma or genomic DNA were collected by venipuncture into acid citrate dextrose tubes at the first seropositive or p24 antigen-positive visit (subtype C cohort) or shortly after the onset of symptoms (subtype B cohort). Longitudinal blood samples were collected at routine clinical visits in both cohorts. Steady-state plasma viral loads using the last available plasma sample were determined using the Roche Amplicor HIV-1 Monitor test, version 1.0 or 1.5. Viral loads were determined for all subjects except 153 M. Viral loads using the last available serum samples were collected longitudinally for a mean follow-up time of 129 days of the last seronegative test (C cohort) or within 31 days from the onset of symptoms (B cohort). For each subject, multiple biologically functional env genes were PCR amplified from PBMC DNA or plasma samples collected within 129 days of the last seronegative test (C cohort) or within 31 days from the onset of symptoms (B cohort). Four of the C seroconvertors were viral p24 antigen positive at the last seronegative test or at the time of sampling, indicating recent infection. The env genes from the first HIV-positive time point were cloned into expression plasmids for the production of viral pseudotypes. Plasma and serum samples were collected longitudinally for a mean follow-up time of 25 months and used for neutralization studies against pseudovirus expressing the newly transmitted autologous Envs. The steady-state plasma viral load was determined from the last available plasma sample for all subjects except 153 M.

Sequence analysis. DNA sequencing of env clones was carried out by Lone Star Labs, Inc. (Houston, TX) utilizing an ABI Prism Automated DNA sequencer 377XL and Big Dye Terminator Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) or the UAB CFAR DNA Sequence Analysis Core using an ABI 3100 Genetic Analyzer and dideoxy methodology (Applied Biosystems, Foster City, CA). Nucleotide sequences were edited and assembled using Sequencher, version 3.1, translated using Se-Al version 2.0a11, or DNA Strider, version 1.3, and amino acid alignments were created using Clustal W, version 1.8. The length of each gp120 V1 to V4 (V1-V4) region (based on HXB2-numbered gp120 positions 131 to 418) was determined using Clustal W, version 1.8. The number of glycosylation sites (NXS or NXT where X is any amino acid residue except proline) in each sequence was determined using N-glycosite (28; http://www.hiv.lanl.gov/content/hiv-db/GLYCOSITE/glcyosite.html).

Neutralization assay. Plasma samples were assayed for NAB activity against a range of pseudotypes Envs using a single-round pseudotype reporter assay described previously (9, 27). Briefly, JC53BL-13 cells were plated and cultured overnight. A total of 2,000 infectious units of each pseudotyped virus were combined with fivefold dilutions of heat-inactivated test plasma or serum and incubated for 1 h at 37°C. Noninfected heat-inactivated human plasma was added as necessary to maintain a constant overall concentration. The virus–Ab mixture was then added to JC53BL-13 cells, and after 2 days, the cells were lysed, and the luciferase activity of each well was measured using a luciferase assay reagent (Promega, Madison, WI) and an ABI Tropix (Applied Biosystems, Foster, CA) or Synergy HT luminometer (Bio-Tek, Winooski, VT). Background luminescence was determined in uninfected wells and subtracted from all experimental wells. Cell viability and toxicity were monitored by basal levels of luciferase expression and by visual inspection. Relative infectivity (percentage of control) was calculated by dividing the number of luciferase units at each plasma dilution by the values in wells containing no test plasma. The dilution of test plasma or serum that inhibited 50% of virus infectivity (IC50 titer) was determined using a linear regression-least squares fit method (27).

RESULTS

Autologous NAB responses reach high titers in early subtype C infection. Development of the initial autologous NAB response was evaluated in 11 subjects infected through heterosexual contact in Zambia, 5 subjects infected through male-to-male contact in the United States, and 1 subject (FASH) presumably infected through heterosexual contact in the United States (Table 1). For each subject, multiple biologically functional env clones were PCR amplified from PBMC DNA or plasma samples collected within 129 days of the last seronegative test (C cohort) or within 31 days from the onset of symptoms (B cohort). Analysis of the early env sequences revealed a mean of 6 subtype C-infected subjects from this cohort of subtype C-infected subjects, providing new information about early subtype C infection and further highlighting potential differences between subtypes B and C. Differences in neutralization susceptibility based on viral subtype could pose further obstacles to the development of practical, widely useful vaccines.
pseudotyped with Env proteins from one to five representative env clones (9, 27). Figure 1A shows that the NAb response varied in both course and magnitude for the 11 subtype C-infected subjects. Neutralizing activity was low or undetectable at the time of seroconversion in all C-infected subjects but increased over the next several months. Thus, a potent autologous NAb response could be detected as early as 3 months after the last seronegative test. Peak IC50 titers against the newly transmitted Envs occurred between 12 to 27 months after the last seronegative test and ranged from 1:284 to 1:28,571, with all but two subjects exceeding titers of 1:1,000 (Table 1). The median IC50 titer for the C cohort was 1:2,363.

For subjects infected with subtype B virus, a detectable NAb response was present as early as 1 to 2 months after the onset of symptoms (Fig. 1B). Peak IC50 titers in these subjects occurred between 7 and 22 months later and did not exceed 1:3,000 (Table 1). In contrast to the C cohort, peak IC50 titers for five of the six B subjects were less than 1:1,000, and the median IC50 titer for the B cohort was 1:682. Thus, the median peak IC50 titer was 3.5-fold higher for the C cohort compared to the B cohort (Fig. 2A), and this difference reached the borderline of significance using a Mann-Whitney test ($P = 0.06$). The timing of the peak NAb response, as well as the set point plasma viral load, did not differ significantly for the two cohorts (Fig. 2B and C).

Newly transmitted subtype C Envs are shorter and less glycosylated than subtype B Envs. Although transmission of subtype C viruses has been associated with shorter V1-V4 length, less glycosylation, and neutralization sensitivity to plasma from the chronically infected partner, this has not been observed in transmission of subtype B viruses (9, 10). We therefore compared the median length of the V1-V4 region and number of N-linked glycosylation (N-gly) sites in Envs from subjects in the B and C cohorts studied here. Because there were different numbers of Envs analyzed for each subject, a single median value was used for each subject (the V1-V4 length and number of N-gly sites was heterogeneous in two subjects). The median V1-V4 length was greater for the subtype B Envs (290 versus 278 amino acids) (Fig. 2D), and this difference reached significance using a Mann-Whitney test ($P = 0.002$). The median number of N-gly sites was also greater in the subtype B Envs (22 versus 20 sites), but this difference only reached borderline statistical significance ($P = 0.08$). Note that all of the subtype B Env sequences contained an N-gly site at position 130 (adjacent to the first Cys of V1), while Envs from only four subtype C subjects conserved this site (data not shown).

Studies of subtype C Envs in our laboratory demonstrated an inverse correlation between the length of the V1-V4 region and neutralization susceptibility (Rong et al., unpublished data). We therefore evaluated whether the median V1-V4 length and number of N-gly sites in all 17 subjects was correlated with the peak IC50 titer using a nonparametric Spearman rank correlation test. Figure 3A shows that V1-V4 length was inversely correlated with peak IC50 titer ($r = -0.59$, $P = 0.002$).
FIG. 1. Course and magnitude of autologous neutralizing antibody responses against Envs in subtype C- and B-infected subjects. The IC_{50} plasma dilution against virus pseudotyped with autologous Envs derived during acute/early infection is plotted vertically on a log 10 scale. The collection time (in months) of each plasma sample tested is shown on the horizontal axes. Envs were cloned at the first time point only. The Env pseudotypes tested for each subject are indicated in each panel, and NAb responses against each Env are represented by colored lines with symbols. M or F indicates a male or female subject, respectively; PB or PL indicates that the Env was cloned from PBMC DNA or plasma, respectively. (A) Autologous NAb responses in 11 subtype C-infected seroconvertors are shown against individual Env pseudotypes. The initial samples were collected when the subjects were first seropositive, within 4 months (129 days) of the last seronegative test (time zero). Subjects 201 M and 242 F were viral p24 antigen positive at the first time point analyzed; subjects 185 F and 205 F were viral p24 antigen positive at time zero. (B) Autologous NAb responses in six subtype B-infected acutely infected subjects against individual Env pseudotypes. The initial samples were collected when the subjects were first seropositive, within 4 months (129 days) of the last seronegative test (time zero).
inversely correlated with the peak IC_{50} (r = -0.62, P = 0.008). Since increases in V1-V4 length are frequently associated with insertion or duplication of sequence containing N-gly sites, we also evaluated whether V1-V4 length was correlated with the number of N-gly sites. Figure 3B shows that these two parameters were highly correlated (r = 0.68; P = 0.0003). Our previous studies have shown that, despite a correlation between length and number of N-gly sites in V1-V4, the latter is not significantly correlated with neutralization susceptibility (Rong et al., unpublished data). In the 17 subjects studied here, a significant correlation between number of N-gly sites, which fell within a narrow window of 18 to 22 sites, and IC_{50} titer was not observed (Fig. 3C).

Subtype C plasma samples lack cross-neutralizing activity. A previous study reported that subtype C isolates from South African subjects were sensitive to cross-neutralization by heterologous serum samples (6). We evaluated whether plasma samples from a subset of the Zambian seroconvertors studied here could neutralize heterologous subtype C Envs by using a checkerboard approach (Table 2). For each subject, the plasma sample containing peak autologous NAb activity was paired with an autologous Env and a representative Env from the other subjects. For subtype C, only two of the six plasma samples tested contained antibodies that neutralized one or more heterologous Envs with an IC_{50} titer above background (1:20). In contrast, low levels of cross-neutralizing activity were detected in plasma samples from all six subtype B-infected subjects (Table 3). Antibodies in plasma from subject INME were able to cross-neutralize all five heterologous subtype B Envs tested. Paradoxically, Env INME 6-1 was not subject to cross-neutralization by any of the other plasmas tested. Inspection of the V3 domain sequence for INME 6-1 revealed that the last residue of the well-conserved GPGR motif had been replaced with a G, and all of the other subtype B Envs contained the more common R at this position (data not shown). The remaining subtype B Envs were neutralized by at least four of the six heterologous plasmas. Thus, cross-neutralizing activity was lacking in most of the subtype C
plasmas tested but was common among plasma samples from subtype B-infected subjects.

**DISCUSSION**

In this study, we compared the course and magnitude of the autologous NAb response against Envs present during acute/early infection in 11 subjects infected with subtype C HIV-1 and 6 subjects infected with subtype B HIV-1. Overall, the course of the autologous NAb response was similar between the two cohorts but varied between individual subjects. A notable finding was the 3.5-fold-greater magnitude of the response in the C cohort compared to the B cohort. This observation could imply that the subtype C Envs are more susceptible to neutralization by autologous antibody in early infection than the subtype B Envs or that they are more immunogenic, eliciting more potent or higher titer NAbs. An important caveat to this finding is the small number of subtype B-infected subjects studied, and evaluation of additional subtype B-infected subjects will be necessary to validate and extend this finding. The subtype B titers described here are consistent with those reported by Richman et al., although it is important to note that a direct comparison of timing and magnitude between the two studies may not be valid due to the different assay formats (23). A biological basis for the higher peak NAb titers in the C cohort is not directly apparent, but the V1-V4 lengths of the subtype C Envs were significantly shorter than the subtype B Envs. The inverse correlation between V1-V4 length and peak IC50 shown here, coupled with the preferential transmission of neutralization-sensitive subtype C HIV-1, suggests that the newly transmitted subtype C Envs could be inherently more sensitive to autologous neutralization than newly transmitted subtype B Envs. The B and C cohorts studied here also differ by route of infection. Five of the six subjects in the B cohort were infected through male-to-male sexual contact, while all subjects in the C cohort were infected through heterosexual contact. Thus, the local cytokine environment (1, 15), the availability of resting versus activated

![FIG. 3](image-url)

**TABLE 2. Intrasubtype cross-neutralizing activity for subtype C Env**

<table>
<thead>
<tr>
<th>Env of subtype C subject</th>
<th>IC50 titer for Env-subtype C plasma combination by subject at the indicated time pointa</th>
</tr>
</thead>
<tbody>
<tr>
<td>133 M 109 F 106 F 55 F 53 M 135 M</td>
<td>Month</td>
</tr>
<tr>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>3.008</td>
<td>&lt;20</td>
</tr>
<tr>
<td>2,801</td>
<td>&lt;20</td>
</tr>
<tr>
<td>2,882</td>
<td>&lt;20</td>
</tr>
<tr>
<td>2,941</td>
<td>&lt;20</td>
</tr>
</tbody>
</table>

a Values in boldface represent the autologous plasma-Env combinations. <20, below background levels.

**TABLE 3. Intrasubtype cross-neutralizing activity for subtype B Env**

<table>
<thead>
<tr>
<th>Env of subtype B subject</th>
<th>IC50 titer for Env-subtype B plasma combination by subject at the indicated time pointa</th>
</tr>
</thead>
<tbody>
<tr>
<td>BORI FASH HOB R INME SUMA WEAU</td>
<td>Month</td>
</tr>
<tr>
<td>18</td>
<td>13</td>
</tr>
<tr>
<td>474</td>
<td>25</td>
</tr>
<tr>
<td>1,695</td>
<td>25</td>
</tr>
<tr>
<td>1,042</td>
<td>93</td>
</tr>
<tr>
<td>877</td>
<td>&lt;20</td>
</tr>
<tr>
<td>495</td>
<td>26</td>
</tr>
<tr>
<td>662</td>
<td>26</td>
</tr>
</tbody>
</table>

a Values in boldface represent the autologous plasma-Env combinations. <20, below background levels.
CD4 T cells (16, 24), or the presence of other susceptible target cells (20, 22, 25) in the genital versus rectal mucosa of the new host could influence the properties of the newly transmitted Envs. Another distinction between the B and C cohorts that could augment differences in the NAb response is that the C subjects were identified through routine visits in a voluntary testing and counseling program, whereas the B subjects were identified based on presentation with symptoms. The Envs from the B cohort were also derived exclusively from plasma virus, while the Envs were derived from the PBMC compartment \((n = 6)\), plasma \((n = 2)\), or both \((n = 3)\) in the C cohort. We did not, however, observe any compartment-specific differences in the neutralization susceptibility of Envs derived from plasma versus PBMC of the same subject (Fig. 1A, subjects 205, 201, and 185). Different NAb titers between viral subtypes could result from (i) differences in the B cell repertoire of the host populations, (ii) differential exposure of common epitopes, and (iii) antibodies that target subtype-specific epitopes. It will therefore be important to determine the specificities of NAbs from subtype C-infected subjects during the acute/early phase of infection.

Interestingly, intrasubtype cross-neutralizing activity was much more prevalent in the B cohort than in the C cohort. This argues that much of the neutralizing activity in the plasma samples from B subjects could be attributed to shared epitopes, such as those in V3 or other epitopes that are absent in the subtype C Envs (such as 2F5 or 2G12). The observation that Env INME 6-1 lacks the conserved GPGR motif at the tip of V3 and is not cross-neutralized by any of the other five plasmas tested argues that the V3 crown is a common target for heterologous neutralization in this cohort of B-infected subjects. Thus, while the epitopes targeted in subtype B Envs appear to have low immunogenicity, inducing generally lower autologous NAb titers, they are more broadly recognized across the different isolates. In contrast, the neutralization epitopes targeted in the C Envs appear to be highly immunogenic, inducing a more potent response that is nonetheless restricted to the autologous Env in most cases. These findings raise the possibility that the NAb response induced by subtype B and C viruses during early infection is fundamentally different in specificity. Furthermore, the higher-titer NAb responses in early subtype C infection could drive more rapid and extensive Env evolution, in a manner similar to that described in the context of subtype B infection (11). It will, however, be important to determine if the newly transmitted subtype C Envs are immunogenic when used in an immunization setting.

The observations reported here follow several recent studies that have highlighted differences between viral subtypes with regard to transmission and neutralization susceptibility. First, in the setting of heterosexual transmission of subtype C viruses in Zambia, we reported that viruses present in newly infected partners had shorter amino acid length in the gp120 V1-V4 region and fewer N-gly sites in this region than the quasispecies in the matched chronically infected partners (9). Only five seroconvertors from the previous report were included in the present study, indicating that newly transmitted Envs from an additional six seroconvertors seem to follow this trend. Furthermore, the viruses pseudotyped with Envs from newly subtype C-infected partners were significantly more sensitive to neutralization by plasma from the chronically infected partner than viruses pseudotyped with chronically infected Envs (9). In contrast, Frost et al. reported no consistent differences in V1-V4 or neutralization susceptibility for sexual transmissions involving subtype B virus (10). However, in the Frost et al. study, half of the index cases were recently infected, and most of the source partners exhibited lower sequence divergence and length variation in the V1-V4 region than we observed in the subtype C chronically infected partners (9). In the four subtype B transmissions that did involve chronically infected source partners, increased neutralization susceptibility was found in two recipients, and shorter Envs were found in three recipients (10). Thus, the inherent differences between these two cohorts notwithstanding, these studies provide evidence that transmission could differ based on route of infection and/or viral subtype. Chohan et al. recently compared the amino acid length and extent of glycosylation of the V1-V2 domain of gp120 in subjects recently infected with subtype A HIV-1 through heterosexual contact in Kenya (7). Although sequences from the index case were not available for comparison, the newly transmitted subtype A sequences were found to be significantly shorter and less glycosylated than subtype A sequences in the Los Alamos database. In contrast, newly transmitted subtype B sequences were not different from subtype B sequences in the database with respect to V1-V2 length and glycosylation, even in subjects infected through heterosexual contact. Moreover, the early A sequences were significantly shorter and less glycosylated than the early B sequences (7). Comparison of a larger number of sequences in the database also demonstrates that the hypervariable domains are shorter in early infection in subtypes A and C, but not subtype B (Bette Korber, personal communication). Taken together, these observations suggest that lineage-specific differences in transmission exist between viral subtypes that could influence neutralization susceptibility and/or immunogenicity in early infection, as reported here, and are therefore important from a vaccine perspective. Chohan et al. also demonstrated that the number of N-linked glycosylation sites in the V1-V2 domain during early subtype A infection was directly correlated with steady-state plasma viral load, a marker of disease progression (7, 19). In contrast, we did not observe a significant correlation between V1-V4 length and set point plasma viral load, but the number of subjects studied here could have been too small to rigorously support this type of analysis (data not shown).

The results reported here suggest that when subtype C HIV-1 is transmitted heterosexually, the responsible virus (i.e., the virus that was transmitted or that begins to replicate in the new host) frequently has a shorter and less heavily glycosylated Env protein. These differences appear to increase the immunogenicity of the Envs and could also affect the susceptibility of the transmitted Envs to autologous antibody-mediated neutralization in the newly infected partner. The data presented here also suggest that subtype C Envs are highly susceptible to neutralization in early infection (prior to viral escape) but that the NAb response is highly focused on strain-specific epitopes. Even so, reproducing the potent NAb response against a panel of newly transmitted subtype C Envs in the absence of escape (i.e., immunization) could be of potential value. Examination of these NAb responses in more detail is therefore clearly needed to validate whether there are biological differences that could have relevance from a vaccine perspective.
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