The effect of sCD4 on the binding and accessibility of HIV-1 gp41 MPER epitopes to human monoclonal antibodies

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Two human monoclonal anti-HIV-1 antibodies, 2F5 and 4E10, were utilized to investigate the accessibility and conservation of gp41 MPER epitopes on five different clades of HIV-1 in the absence and presence of sCD4. The binding of human monoclonal antibodies (mAbs) to HIV-1 was dependent upon the virus clade. Soluble CD4 significantly increased the accessibility of gp41 MPER-binding epitopes on several isolates that previously showed little or no binding with 2F5 and 4E10 mAbs as determined by a modified ELISA-based virus capture assay and surface plasmon resonance. Studies on the relationship between virus binding and neutralization in a TZM-bl pseudovirus assay indicated that in most cases, mAbs that exhibited neutralization also bound the virus. However, neither binding nor the total envelope content per virion was a predictor of neutralization. The hidden or conformational gp41 MPER epitopes unmasked by sCD4 may provide additional targets for vaccine design.

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Introduction

Understanding the generation of immune responses against HIV-1 is critical for the further pursuit of developing an effective HIV-1 vaccine, particularly as they relate to the induction and characterization of virus neutralizing and binding antibodies as well as antibodies that induce cellular cytotoxicity. Even though a strong HIV-1 envelope antibody binding response is induced during the natural course of an infection, these antibodies usually neutralize heterologous HIV-1 isolates only weakly. Despite this, a small number of human monoclonal antibodies (mAbs) that exhibit breadth and potency in terms of neutralization of primary isolates of HIV-1 from several different clades have been identified (Binley et al., 2004; Burton et al., 2005; Zolla-Pazner, 2004). These mAbs bind to conserved regions of the envelope. 2G12 binds to specific oligomannose sequences on gp120, while immunoglobulin G1b12 (b12) binds to the CD4 binding site (Zhou et al., 2007). Three other broadly neutralizing mAbs, 2F5, 4E10, and Z13, all target the membrane proximal external region (MPER) of gp41 (Muster et al., 1993; Zwick et al., 2001).

The use of a high-throughput neutralization screen of antibody-containing culture supernatants from activated memory B cells led to the identification of two human mAbs that exhibit HIV-1 neutralization breadth and potency with specificity to the conserved regions of the V2/V3 loop (Walker et al., 2009). In addition, several human mAbs that recognize V2 and V3 regions of gp120 have been generated and extensively characterized (Cardozo et al., 2007; Gorny et al., 1989; Huang et al., 2005; Nyambi et al., 1998, Pinter et al., 2004; Stanfield et al., 2006). Since the V3 loop is not exposed on envelope trimers, its importance as a strong vaccine target is unclear at the present time (Binley et al., 2004; Burton et al., 2004; Lusso et al., 2005).

HIV-1 entry into a target cell is a well-orchestrated multistep process, initially involving the viral envelope proteins gp120 and gp41. After the initial step of gp120 binding to the CD4 receptor, a conformational change occurs in the gp120 molecule, which exposes the binding sites for the chemokine receptors CCR5 or CXCR4 that serve as the coreceptors (Berger et al., 1999; Doms, 2000). This interaction in turn induces another conformational change that allows the binding of gp41 molecule followed by the fusion of the viral lipid bilayer with the plasma membrane bilayer of the target cell. This fusion results in the entry of the viral RNA into the target cell (Colman and Lawrence, 2003). During the course of these interactions as well as during the budding of the virus, lipid-associated proteins (Wyatt and Sodroski, 1998) and even lipids themselves (Haynes et al., 2005; Sánchez-Martínez et al., 2006) are exposed. Thus, these regions could serve as potential antigenic targets for virus neutralization (Alving et al., 2006) as demonstrated by the production of multispecific murine IgM mAbs that simultaneously bind to lipids and also to gp41 MPER regions and neutralize HIV-1 infection in a PBMC neutralization assay (Matyas et al., 2009b).
The HIV envelope undergoes structural rearrangement during viral entry and various models have been proposed to explain the different conformational states. At least three distinct conformational states, a pre-fusion conformation, a gp41-pre-hairpin intermediate, and a post-fusion trimer of hairpin have been reported (Chan and Kim, 1998; Harrison 2008). There are several studies examining the role of mAbs 2F5 and 4E10 during these conformational states. Using 293T cells transiently expressing HXB2 envelope or HXB2 pseudovirions, de Rosny et al. (2004) demonstrated that mAb 2F5 bound to both native and fusion intermediate forms of gp41 and neutralized virus at a late step in virus entry. In another study using a pseudovirus envelope mutant that consisted of gp120 covalently attached to gp41, Binley et al. (2003) showed that 2F5 and 4E10 neutralized effectively in the post attachment conformation. In contrast, two other studies (Frey et al., 2008; Alam et al., 2009) have proposed a two-step mechanism for the neutralization of HIV-1 by MPER-specific antibodies. In the first step, mAbs 2F5 and 4E10 bind to the lipids on the viral membrane. The second step involves the binding of the mAbs to the receptor-triggered pre-hairpin intermediate conformation. Specific interactions between 4E10 and membrane lipid components and their role in neutralization have also been reported by Xu et al. (2010).

Virus capture assays can be utilized to address the issue of mAbs binding to the native virion surface. The virus capture assay initially developed by Nyambi et al. (2001) primarily concentrated on antigenic epitopes in the gp120 region of the intact virus of different clades and utilized a panel of human monoclonal antibodies directed mainly against this region (Nyambi et al., 2000). The assay was used to map the epitopes exposed on intact native primary HIV-1 and also to study the conformational changes that occurred in the presence of sCD4 (Mbah et al., 2001). Many V3 reactive mAbs do show breadth and potency of neutralization once the site is exposed after binding to sCD4 (Wu et al., 2008). Studies on the exposure of gp41 epitopes on virions in the presence of soluble CD4 are however limited (Crooks et al., 2005; Moore et al., 2006; Nyambi et al., 2000).

An in-solution virus capture assay was recently developed to examine the accessibility of MPER epitopes on clade B pseudoviruses (Leaman et al., 2010). This assay reduces envelope independent capture of virions. Env-specific antibodies can bind to non-functional gp120 molecules in a virus capture assay and therefore do not necessarily correlate with virus neutralization (Poignard et al., 2003; Moore et al., 2006). Given that p24 is an indirect marker of virus, the use of an infection-based readout would be more desirable (Poignard et al., 2003). IgG anti-Fc-specific antibody was used to coat the wells prior to the addition of the capture antibodies. In our assay, protein G coated plates were used to bind the antibodies through their Fc regions thus allowing the antibody combining sites to be available for virus binding (Bjork and Kronvall, 1984). As shown in Fig. 1A and B, mAbs 4E10 and 2F5 exhibited moderate to high binding to clades A (82–231 pg/ml), B (BAL-PV; 120–340 pg/ml), and D (60–250 pg/ml) pseudoviruses. Clade CRF01_AE pseudoviruses, CM235 and NI bound moderately to 4E10 (55–60 pg/ml) but poorly to 2F5 (0–22 pg/ml) and D (0–25 pg/ml) pseudoviruses. Clade US-1 PV and C (PBL 288) pseudoviruses exhibited extremely low binding to 4E10 (22 pg/ml and 49 pg/ml, respectively) and no binding to 2F5 mAbs (0 pg/ml; Fig. 1B). Another clade C pseudovirus, PBL 286 did not bind 4E10 or 2F5 mAbs.

The 4E10 and 2F5 binding epitopes for all the viral isolates tested are shown in Table 1. Although the 4E10 epitope [NWF(D/N)IT] was intact and variable in the different clades, clades A and B bound 4E10 at high levels compared to CM235. Both the clade C viruses as well as US-1 PV have mutations in positions 1 (asparagine to serine) and 4 (aspartic acid or asparagine to serine) of the 4E10 binding site and exhibited low to no binding to the virus. It appears that the first amino acid in the epitope (asparagine) may be critical for determining the strength of binding to the virus, since both clade D viruses bound 4E10 at high levels, in spite of having serine in position 4 of the epitope.

There was no mutation in the 2F5 binding epitope (ELDKWA) for BAL-PV, US-1 PV, and CM235; yet the 2F5 binding profiles varied greatly. BAL-PV bound 2F5 at high levels while the other two viruses exhibited no binding. Clade C viruses usually do not exhibit 2F5 binding since the 2F5 binding epitope is mutated. The two clade C viruses used in the present study, PBL 286 and PBL 288, are mutated at the first and last amino acid of the 2F5 binding epitope and as predicted did not bind to 2F5. These data indicate that mAbs 4E10 and 2F5 exhibit varying degrees of binding to the viruses even though the 4E10 and 2F5-binding epitopes may be conserved within or between clades.

Variable cross-clade binding (clades A through D; 0–257 pg/ml) was observed with mAb 2G12 (Fig. 1C). Mutations at any of the 5 glycosylation sites on gp120 at positions 295, 332, 339, 386, and 392 that are important for the binding of mAb 2G12 are shown in Table 1.

Table 1

<table>
<thead>
<tr>
<th>Clade</th>
<th>Virus name</th>
<th>Abbreviation</th>
<th>4E10 sequence NWF(D/N)IT</th>
<th>2F5 sequence ELDKWA</th>
<th>2G12 Site mutations</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>93RW_024</td>
<td>93RW</td>
<td>TWFSD5</td>
<td>ALDKWA</td>
<td>S339</td>
</tr>
<tr>
<td></td>
<td>00KE_KNH1144</td>
<td>KHN</td>
<td>NWFD15</td>
<td>ALDKWA</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>BAL</td>
<td>BAL-PV</td>
<td>NWFD1T</td>
<td>ELDKWA</td>
<td>None</td>
</tr>
<tr>
<td>B</td>
<td>US-1 PV</td>
<td>US-1 PV</td>
<td>NWFD1T</td>
<td>ELDKWA</td>
<td>None</td>
</tr>
<tr>
<td>C</td>
<td>PBL 286(496)</td>
<td>PBL 286</td>
<td>NWFD1T</td>
<td>ELDKWA</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>PBL 288(411)</td>
<td>PBL 288</td>
<td>NWFD1T</td>
<td>ELDKWA</td>
<td>None</td>
</tr>
<tr>
<td>D</td>
<td>98UG_57128</td>
<td>57128</td>
<td>NWFD1T</td>
<td>ELDSWA</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>99UG_A07412M</td>
<td>A07412</td>
<td>NWFD1T</td>
<td>ELDKWA</td>
<td>D295 and G 339</td>
</tr>
<tr>
<td>CRF01_AE</td>
<td>CM 235</td>
<td>CM 235</td>
<td>NWFD1T</td>
<td>ELDKWA</td>
<td>E332</td>
</tr>
<tr>
<td></td>
<td>N1149</td>
<td>N1149</td>
<td>NWFD1T</td>
<td>ELDKWA</td>
<td>E332</td>
</tr>
</tbody>
</table>

*N-sites examined 295, 332, 339, 386 and 392. Mutations in amino acids in the 4E10 and 2F5 epitope are underlined.
Clade B pseudovirus BAL-PV exhibited strong binding compared to clade C pseudoviruses (Fig. 1C) even though the 5 glycosylation sites were intact in both cases. A reduction in the binding of the antibody to the virus (with the exception of 93RW) was observed when a mutation was present at any of the glycosylation sites.

Although, USHIV+ plasma pool (Fig. 1D) was derived from clade B infected individuals, it exhibited variable cross-clade binding to clades A through AE viruses (21 pg/ml–476 pg/ml). MAb (Hyb 278-01) specific to tetanus toxoid (negative control) was examined for binding to all 10 of the pseudoviruses used in the study (Fig. 1E). The p24 values obtained with anti-tetanus toxoid for all of the viral isolates tested ranged from 0 to 7.7 pg/ml. Therefore, a p24 value greater than or equal to twice the negative control (15.4 pg/ml) for the test samples was considered positive. The data were analyzed after the p24 value obtained with virus alone (in the absence of antibody) was subtracted from all the test samples.

### Comparing virus binding and neutralization

The relationship between the binding of the mAbs 4E10 and 2F5 to the virus, and their ability to neutralize the virus in a TZM-bl pseudovirus assay is shown in Table 2. The data for the virus binding are derived from Fig. 1. The binding and the neutralization experiments were conducted independently. The TZM-bl pseudovirus assay neutralization data is presented as IC_{50} (pg/ml). The highest concentration of mAbs tested for neutralization was 25 μg/ml. As shown in Table 2, the different clades of HIV-1 tested, showed varying degrees of binding and neutralization to 2F5 and 4E10. Based on the binding and neutralization by 4E10 mAbs (Table 2), viruses can be grouped into three categories. (i) viruses (KNH, BAL-PV, A07412, CM235, and NI) that bind to and are neutralized by 4E10; (ii) viruses (93RW, US-1 PV) that bind but are not neutralized by 4E10; (iii) virus (PBL 286) that does not bind but is neutralized by 4E10. Based on the binding and neutralization by 2F5 mAb, the different clades of HIV-1 tested could be grouped into four categories. (i) viruses (93RW, KNH, BAL-PV, A07412, and CM235) that bind to and are neutralized by 2F5; (ii) virus (57128) that binds to but is not neutralized by 2F5 mAb; (iii) viruses (PBL 286, USHIV+ plasma pool, panel D) and control antibody Hyb 278-01 against tetanus toxoid (panel E) were examined. The amount of virus bound to each of the mAbs was determined by measuring the amount of viral p24 bound (pg/ml) as described in the Materials and Methods. The p24 value obtained with virus alone (in the absence of antibody) was subtracted from all the test samples. The p24 values for the negative control Hyb278-01 (anti-tetanus toxoid antibody) for all viral isolates tested ranged from 0 to 7.7 pg/ml (background value). Therefore a p24 value ≥ 15.4 pg/ml, which was twice the background value was considered positive. The average of duplicate wells of 3–5 experiments ± S.D. is shown.

### Table 2

Comparison of 2F5 and 4E10 binding to HIV-1 and TZM-bl pseudovirus neutralization assay.

<table>
<thead>
<tr>
<th>Clade</th>
<th>Virus</th>
<th>Virus capture</th>
<th>TZM-bl Neut IC_{50}, pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4E10</td>
<td>2F5</td>
</tr>
<tr>
<td>A</td>
<td>93RW</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>KNH</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>BAL-PV</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>US-1 PV</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>C</td>
<td>PBL 288</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>PBL 288</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>57128</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>A07412</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CM 235</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>NI</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>B</td>
<td>93RW</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>KNH</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>BAL-PV</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>US-1 PV</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>C</td>
<td>PBL 288</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>PBL 288</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>57128</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>A07412</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CM 235</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>NI</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

* p24 concentrations: –, no binding (< 15.4 pg/ml); +, weak binding (16–75 pg/ml); ++, moderate binding (76–150 pg/ml); ++++, strong binding (>221 pg/ml). See Material and Methods for more details.

Fig. 1. Binding patterns of various human monoclonal antibodies to different HIV-1 clades. The binding of HIV-1 pseudoviruses (100 ng/ml) to 4E10 (panel A), 2F5 (panel B), 2G12 (panel C), USHIV+ plasma pool (panel D) and control antibody Hyb 278-01 against tetanus toxoid (panel E) were examined. The amount of virus bound to each of the mAbs was determined by measuring the amount of viral p24 bound (pg/ml) as described in the Materials and Methods. The p24 value obtained with virus alone (in the absence of antibody) was subtracted from all the test samples. The p24 values for the negative control Hyb278-01 (anti-tetanus toxoid antibody) for all viral isolates tested ranged from 0 to 7.7 pg/ml (background value). Therefore a p24 value ≥ 15.4 pg/ml, which was twice the background value was considered positive. The average of duplicate wells of 3–5 experiments ± S.D. is shown.
(iv) virus (US-1 PV) which does not bind but is neutralized by 2F5 mAb.

Quantitative measurements of envelope and p24 from pseudoviruses

Fig. 2A, B, C, and D shows the results from SDS-PAGE analysis of viral proteins stained with fluorescent dyes. Concentrated virus preparations were lysed and the viral proteins were analyzed on a gradient gel under reducing conditions. The figure shows images for Pro-Q® Emerald 300 (panels A and B) and SYPRO® Ruby (panels C and D) staining for quantification of glycoprotein and total protein, respectively. Panels A (gel stained with Pro-Q® Emerald 300) and C (gel stained with SYPRO® Ruby) contain the following: Molecular weight markers (MW range from 180 kDa to 14 kDa); lane 1: 93RW (clade A); lane 2: KNH (clade A); lane 3: BAL-PV (clade B); lane 4: PBL 288 (clade C); lane 5: US-1 PV (clade B). Panels B (gel stained with Pro-Q® Emerald 300) and D (gel stained with SYPRO® Ruby) contain the following: Molecular weight markers (MW); lane 1: PBL 286 (clade C); lane 2: 57128 (clade D); lane 3: A07142 (clade D); lane 4: CM235 (clade CRF01_AE); lane 5: NI (clade CRF01_AE). Lanes 6 through 10 in panels A, B, C, and D contain the standards, gp140 and p24. Lane 6: (90 ng gp140; 1200 ng p24); lane 7: (45 ng gp140; 600 ng p24); lane 8: (22.5 ng gp140; 300 ng p24); lane 9: (11.25 ng gp140; 150 ng p24); lane 10: (5.6 ng gp140; 75 ng p24). Arrows denote the position of gp140 and p24 on the gel. The position of gp120 and gp160 are depicted by (*) and (**), respectively.

Table 3
Semi-quantitative envelope/p24 analysis.

<table>
<thead>
<tr>
<th>Virus</th>
<th>gp 160 (ng)</th>
<th>gp 120 (ng)</th>
<th>gp 120 (fmol)</th>
<th>p24 (ng)</th>
<th>p24 (fmol)</th>
<th>p24 /gp120 fmol/fmol</th>
<th>Trimers per virion (1400 Gag/viron)</th>
</tr>
</thead>
<tbody>
<tr>
<td>93RW</td>
<td>173</td>
<td>139</td>
<td>1.16</td>
<td>536</td>
<td>22.33</td>
<td>19.3</td>
<td>24.9</td>
</tr>
<tr>
<td>KNH</td>
<td>126</td>
<td>77</td>
<td>0.64</td>
<td>216</td>
<td>9.0</td>
<td>14.1</td>
<td>34</td>
</tr>
<tr>
<td>US-1 PV</td>
<td>33</td>
<td>42</td>
<td>0.35</td>
<td>126</td>
<td>5.0</td>
<td>8.0</td>
<td>106.7</td>
</tr>
<tr>
<td>BAL-PV</td>
<td>31</td>
<td>14</td>
<td>0.12</td>
<td>12.9</td>
<td>0.54</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>PBL 288</td>
<td>105</td>
<td>60</td>
<td>0.50</td>
<td>221</td>
<td>9.21</td>
<td>18.4</td>
<td>26.1</td>
</tr>
<tr>
<td>PBL 286</td>
<td>1593</td>
<td>306</td>
<td>2.55</td>
<td>684</td>
<td>28.5</td>
<td>11.2</td>
<td>42.9</td>
</tr>
<tr>
<td>57128</td>
<td>943</td>
<td>146</td>
<td>1.22</td>
<td>349</td>
<td>14.54</td>
<td>11.9</td>
<td>40.3</td>
</tr>
<tr>
<td>A07142</td>
<td>996</td>
<td>149</td>
<td>1.24</td>
<td>318</td>
<td>13.25</td>
<td>10.7</td>
<td>44.9</td>
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<tr>
<td>CVX335</td>
<td>1388</td>
<td>196</td>
<td>1.63</td>
<td>441</td>
<td>18.4</td>
<td>11.3</td>
<td>42.5</td>
</tr>
<tr>
<td>NI</td>
<td>608</td>
<td>88</td>
<td>0.73</td>
<td>247</td>
<td>10.29</td>
<td>14.1</td>
<td>34.0</td>
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PBL 288, and NI) that neither bind to nor are neutralized by 2F5; (iv) virus (US-1 PV) which does not bind but is neutralized by 2F5 mAb.

Although we could detect the p24 band for US-1, it was not in the linear portion of the standard curve and was therefore omitted from the calculation. The number of trimers per virion (Table 3) were calculated based on an estimate of approximately 1400 gag molecules per virion and an average Gag:Env ratio of approximately 60:1 (mol/mol), corresponding to about 8 envelope trimers per virus particle (Louder et al., 2005). The number of trimers/virion were fairly consistent among all the pseudoviruses tested with the exception of BAL-PV which had approximately 107 trimers/virion compared to an average of 36 trimers/virion. Western blotting (Fig. 3) confirmed the results of the fluorescent SDS-PAGE analysis (Fig. 2). The gels were probed with USHIV+ sera (Fig. 3A and B). The HIV protein specificity of the bands was confirmed by probing a separate blot with normal human sera (Fig. 3C). A major band observed at approximately 65 kDa in the
fluorescent gels (Fig. 2) was not an HIV protein as it was not detected in the Western blot and might represent serum albumin from the cell culture supernatants.

**Identification of different forms of envelope on pseudoviruses by Native Blue-PAGE gels**

The presence of envelope monomers and trimers on all the pseudoviruses used in this study were examined on a 4–16% Bis-Tris NuPAGE gel followed by Western blotting (Fig. 4). To extract envelope proteins from the pseudoviruses, 1% digitonin was used. The data from 5 of the 10 pseudoviruses examined (93RW, KNH, US-1 PV, BAL-PV, and PBL 288) are shown in Fig. 4. BN-PAGE blots gave weaker bands than those observed with SDS-PAGE Western blots (Fig. 3). The blots in Fig. 4 were probed with either anti-gp120 mAb cocktail (Fig. 4A) or with anti-gp41 cocktail (Fig. 4B). The blots also contained 5 ng to 40 ng of CHO-expressed gp140. Two bands were detected with the anti-gp120 mAbs (Fig. 4A, lanes S1–S4). These corresponded to gp140 trimers and gp140 monomers (indicated by arrows). Two additional bands (indicated by arrows) corresponding to gp41 trimers and gp41 monomers were detected with anti-gp41 mAbs (Fig. 4B, lanes S1–S4). All 5 pseudoviruses showed a band around 440 kDa, which is likely to be gp120/gp41 trimers (Figs. 4A and B). The second band around 160 kDa appears to be gp120/gp41 monomers as it was detected by both mAb cocktails. In Fig. 4B, two faint bands could be distinguished, which could correspond to trimeric and monomeric gp41.

**Effect of soluble CD4 on the binding of mAb 2F5 and 4E10 to HIV-1**

The accessibility of gp41 epitopes on intact virions to 2F5 and 4E10 was examined in the virus capture assay in the absence and presence of sCD4. As shown in Fig. 5, the binding of 2F5 and 4E10 in the absence (closed bars) or presence of 15 μg of soluble CD4 (open bars) to clades A, B, C, D, and CRF01_AE viruses was examined. The presence of sCD4 resulted in a significant increase ($p ≤ 0.05$) in the binding of 2F5 to clade A (93RW and KNH; panel A) and clade CRF01_AE (CM235 and NI; panel E) viruses. No significant increases were observed in binding to clade B (US-1 PV and BAL-PV; panel B), clade C (PBL 288, PBL 286; panel C), and clade D (57128, A07412; panel D) viruses, although there was a trend towards significant binding of 2F5 to PBL 288 in the presence of sCD4. In the presence of 15 μg of sCD4 (Fig. 5), a significant increase ($p ≤ 0.04$) in the binding of 4E10 to clade A (93RW; panel F) and clade C (PBL 286; panel H) viruses was observed. The binding of another clade A (KNH; panel F), clade B (US-1 PV and BAL-PV; panel G), clade C (PBL 288; panel H), clade D (57128 and A07412; panel I), and clade AE (CM235 and NI; panel J) viruses to 4E10 was not significantly affected by the addition of sCD4. The above results indicate that binding of sCD4

**Fig. 3.** Western blot analysis of viral proteins. Proteins from the pseudoviruses separated by SDS-PAGE were blotted onto PVDF membranes and probed with USHIV+ sera (panels A and B) or with normal human sera (panel C). The molecular weight markers (MW range from 180 kDa to 6 kDa) are shown to the left of each of the panels. Panel A contains the viral proteins from the following pseudoviruses: 93RW, KNH (clades A); US-1 PV, BAL-PV (clade B); PBL 288 (clade C). Panels B and C contains the viral proteins from the following pseudoviruses: PBL 286 (clade C); 57128, A07412 (clade D); CM235, NI (clade CRF01_AE). The standards (S1–S5) are depicted in each of the panels. S1: 400 ng gp140 and 1200 ng p24; S2: 200 ng gp140 and 600 ng p24; S3 100 ng gp140 and 300 ng p24; S4: 50 ng gp140 and 150 ng p24; S5: 25 ng gp140 and 75 ng p24.

**Fig. 4.** BN-PAGE Western blots of pseudovirus envelope species. Proteins from the pseudoviruses separated by BN-PAGE were blotted onto PVDF membranes and probed with anti-gp120 cocktail (panel A) or with anti-gp41 cocktail (panel B). The molecular weight markers (MW range from 669 kDa to 66 kDa) are shown to the left of each of the panels. Panels A and B contain the viral proteins from the following pseudoviruses: 93RW, KNH (clades A); US-1 PV, BAL-PV (clade B); PBL 288 (clade C). The standards (S1–S4) are depicted in each of the panels. S1: 40 ng gp140; S2: 20 ng gp140; S3: 10 ng gp140; and S4: 5 ng gp140. Monomers and trimers are indicated by arrows.
increases the accessibility of the 2F5 and 4E10 epitopes in certain viral isolates.

Binding of 2F5 and 4E10 to AT-2 inactivated virus as determined by surface plasmon resonance

We further verified the effect of sCD4 on the binding of 2F5 and 4E10 to HIV-1 by surface plasmon resonance using AT-2 inactivated virus. In order to establish that AT-2 inactivation of the virus did not impact the binding of mAb 2F5, AT-2 clade D virus A07412 was immobilized on a C1 Biacore chip (2800 RU). The binding of different concentrations of 2F5 (0–1000 nM) to the virions was analyzed and is represented as a sensogram (Fig. 6A). There was a dose-dependent increase in the binding of 2F5. Alternatively, 2F5 and 4E10 or USHIV+ sera or normal human sera were immobilized on a CM5 Biacore chip and the binding (RU) to AT-2 clade D virus A07412 was analyzed. A representative of two separate experiments is shown in Fig. 6B. A 5- to 6-fold higher binding of mAbs 2F5 and 4E10 as well as USHIV+ sera to AT-2 clade D virus A07412 was obtained compared to pooled normal human sera. Fig. 6A and B demonstrates that AT-2 inactivation did not destroy the 2F5 or the 4E10 binding epitopes on the virions.

Effect of soluble CD4 on the binding of mAbs 2F5 and 4E10 to HIV-1 as determined by surface plasmon resonance

Only two of the pseudoviruses (clade A KH; clade D A07412) used in Fig. 5 were available as AT-2 inactivated viruses and were utilized in the Biacore studies. Protein G was immobilized on a CMS Biacore chip followed by the addition of 25 μg/ml of 2F5, 4E10, or control antibody Hyb 278-01. AT-2 clade A (KH) and clade D (A07412) viruses were incubated with sCD4 (0, 1, 5, and 10 μg/ml) before addition to the CM5 chip containing the bound mAbs. As shown in Fig. 7, the addition of 10 μg/ml of sCD4 significantly increased (p = 0.04) the binding of 2F5 to KH virions. Clade D A07412 virus did not show a similar significant increase in binding to 2F5 or 4E10 mAbs in the presence of sCD4. The results with clades A and D viruses were similar to those obtained with sCD4 in the virus capture assay (Fig. 5). These data indicate that accessibility of 2F5 and 4E10 epitopes depends upon the virus clade.

Discussion

The envelope protein of HIV-1 has always been considered as a potential vaccine target for the generation of neutralizing antibodies. In particular, the envelope protein gp41 has been the focus of attention as three broadly neutralizing mAbs that bind to the MPER region of gp41 have been described. mAbs 2F5 and 4E10 not only demonstrate specificity to the MPER region of gp41 but also specifically bind to various lipids (Haynes et al., 2005; Matyas et al., 2009a, 2009b; Sánchez-Martínez et al., 2006). Binding of the antibodies to lipids could be part of the neutralizing mechanism (Sánchez-Martínez et al., 2006; Alving et al., 2006). It has been demonstrated that murine IgM mAbs as well as several human IgG mAbs that exhibited binding to pure phospholipids neutralized HIV-1 infection in a PBMC assay (Brown et al., 2007; Moody et al., 2010). Similarly, murine multispecific IgM mAbs that simultaneously bind to lipids and gp41 MPER regions also neutralized HIV-1 infection in a PBMC assay (Matyas et al., 2009a, 2009b).
Although, the exact mechanism(s) of neutralization by 2F5 and 4E10 are not fully understood, they appear to interfere with the fusion of the virus with the target cell and not with the initial binding step. Since the binding, fusion, and entry of HIV-1 is a highly complex and intricate process involving protein–lipid interactions, there are several studies that have reported that the mAbs 2F5 and 4E10 bind to both the native and fusion intermediates of gp41 (Chan and Kim, 1998; Harrison 2008; de Rosny et al., 2004; Binley et al., 2003) and thus are available for binding throughout the viral entry process, while other studies have reported that the neutralization epitopes on the virus may be visible only for very short periods of time (Frey et al., 2008; Alam et al., 2009). In order to design better immunogens as potential vaccine candidates, it is important to map the epitopes visible on intact virions and also to examine if epitopes that are critical for virus binding or fusion can be made accessible.

Previously, a virus capture assay developed by Nyambi et al. (2000) was utilized to map the epitopes exposed on intact virions using human anti-gp41 cluster I and II antibodies in addition to antibodies directed against the V2, V3 loop, and CD4 binding domain. In their study, the six mAbs tested to the cluster II region did not bind or bound poorly to the isolates examined. In the present study, the virus-binding assay was modified to determine the binding of human mAbs that recognize HIV-1 gp41 epitopes. Protein G coated ELISA plates were used to bind the antibody before capture of the virus. Using this assay, we analyzed the binding of three human anti-envelope mAbs to pseudoviruses from clades A, B, C, D, and CRF01_AE (2 pseudoviruses from each of the clades). We chose to concentrate only on pseudoviruses as this eliminates the potential complexities, which could be introduced by primary and T cell adapted viruses.

In our studies, mAb 2G12 bound to at least one of the viral isolates from each of the 5 clades tested. The binding of mAb 2G12 is dependent upon 5 glycosylation sites on gp120 at positions 295, 332, 339, 386, and 392. Mutations at any of the glycosylation sites resulted in a reduction in binding of the antibody to the virus with the exception of 93RW. Our data suggest that the glycosylation sites as well as the accessibility of the mannose residues on the virion influence the binding characteristics. Similarly, HIV-1 clade B positive plasma pool USHIV+ bound all of the 10 isolates tested. Although the plasma pool was from persons infected with clade B virus, it also recognized clades A, B, C, D, and CRF01_AE viruses. The plasma pool therefore contained polyclonal antibodies that cross-reacted with envelope proteins from other clades.

93RW and BAL-PV were captured at a higher level by all Abs tested, whereas US-1 PV and PBL 288 were captured at significantly lower levels. In order to determine if these differences in virus capture were related to the envelope concentrations, the amount of envelope on the pseudoviruses were quantified. 93RW and BAL-PV had 139 ng and 14 ng of gp120, while US-1 PV and PBL 288 had 42 ng and 60 ng of gp120, respectively. These data demonstrated that there was no correlation between the envelope content and virus binding. In the pseudovirus preparations tested, the number of envelope spikes per virus ranged from 25 to 107. The trimers per virion also were not predictive of high or low binders in the virus capture assay.

To account for alternative forms of the envelope to explain the different amounts of virus captured, we examined the pseudovirus envelope proteins on native blue gels followed by Western blotting. Probing with an anti-gp120 mAb cocktail revealed the presence of two bands in the lanes loaded with commercially obtained gp140. These bands are likely to be trimers of gp140 and gp140 monomers. When the...
isolates tested. Five of the 6 viral isolates that 2F5 bound were also sCD4. Therefore, 4E10 bound and neutralized 8 out of the 10 viral neutralized by 4E10 but was bound by 4E10 only in the presence of 4E10 in 7 out of the 10 viral isolates tested. Two of the isolates were and/or viral assembly.

Of the 4E10 binding epitope. The amino acid sequence could contribute strong binding and a lack of neutralization could be due to the mutation that are incorporated into the viral envelope. Another possibility for the addition of sCD4 thereby suggesting that there was no further increase that there were no changes in the cluster I and II regions of gp41 after the binding in 7 out of the 8 intact virions tested. Their study also reported and V3 regions of gp120 resulting in a 2- to 22-fold increase in antibody mAbs and the neutralization pro

It is interesting to note that although 4E10 bound very strongly and 2F5 exhibited moderate binding to 93RW in the virus capture assay, 4E10 did not neutralize the virus in a TZM-bl pseudovirus assay when tested at 25 μg/ml, however, 2F5 did neutralize the virus. The lack of correlation between binding of the different mAbs and neutralization may be the due to several reasons. If binding to non-functional trimers (Crooks et al., 2005; Poignard et al., 2003) was the sole reason for this discrepancy, then both 4E10 and 2F5 should have exhibited similar neutralization characteristics, which was clearly not the case. It is well established that the lipid binding profiles for 2F5 and 4E10 are different (Haynes et al., 2005; Matyas et al., 2009a). Therefore, the binding of the mAbs and the neutralization profiles could be influenced by the lipids that are incorporated into the viral envelope. Another possibility for the strong binding and a lack of neutralization could be due to the mutation of the 4E10 binding epitope. The amino acid sequence could contribute to variations observed between binding and neutralization. Even though the antibodies bind the virus they may not be able to inhibit viral entry and/or viral assembly.

There was an association between binding and neutralization by 4E10 in 7 out of the 10 viral isolates tested. Two of the isolates were bound but were not neutralized by 4E10. Another isolate (PBL 286) was neutralized by 4E10 but was bound by 4E10 only in the presence of sCD4. Therefore, 4E10 bound and neutralized 8 out of the 10 viral isolates tested. Five of the 6 viral isolates that 2F5 bound were also neutralized by 2F5, while 4E10 bound and neutralized 4 of these same viral isolates. The above results indicate that binding per se does not determine the neutralization ability of the mAb.

Mbah et al. (2001) have demonstrated that sCD4 binding causes conformational changes leading to the exposure of epitopes in the V2 and V3 regions of gp120 resulting in a 2- to 22-fold increase in antibody binding in 7 out of the 8 intact virions tested. Their study also reported that there were no changes in the cluster I and II regions of gp41 after the addition of sCD4 thereby suggesting that there was no further increase in the exposure of gp41 epitopes. We found similar results (data not shown) as Mbah et al. (2001), in terms of a lack of increase in 2F5 binding to 92UG in the presence of sCD4. However, our study showed a significant increase in both 2F5 and 4E10 binding in the presence of sCD4 with 93RW, a different clade A isolate. In addition, a significant increase in 2F5 binding in the presence of sCD4 was observed with clade A (KNH) and clade AE (CM 235 and NI) viruses.

Addition of sCD4 to clade C PBL 286 virus resulted in binding to 4E10, which was previously absent suggesting that although the 4E10 epitope was present, it was inaccessible and not exposed on the surface of the virion. This may be due to the fact that the 4E10 antibody-binding site on gp41 is close to the viral lipid bilayer and is normally not easily accessible. In clade C viruses, since the 2F5-binding site is mutated, the mAb did not bind to the virus and the binding characteristics also did not significantly change with the addition of sCD4. Similarly sCD4 did not significantly enhance the binding of clades B and D viruses to either 2F5 or 4E10. It is possible that sCD4 not only induces conformational changes in the gp120 region of the envelope but also in the gp41 region. There might be subtle differences in the conformational changes between the different HIV-1 clades that could not only provide greater accessibility of the gp41 epitopes for antibody binding in some clades and not in others but also influence the neutralization profiles. It has been reported that pre-incubation of the virus with sCD4 substantially increased the sensitivity of subtype B viruses to neutralization by the V3 mAbs (Wu et al., 2008).

Surface plasmon resonance was used to confirm the sCD4 results obtained with the modified virus capture assay. Binding of AT-2 inactivated viral isolates to both 2F5 and 4E10 in the presence and absence of sCD4 was analyzed using the Biacore. Due to the unavailability of the isolate-specific AT-2 inactivated virus, all of the isolates used in the virus capture assay could not be tested using the Biacore. Chemical inactivation of the virus with AT-2 did not destroy or alter the 2F5 or 4E10 binding epitopes as demonstrated by Biacore analysis. A dose-dependent increase in binding of 2F5 to clades D virus, A07412, was observed. Addition of sCD4 did not significantly enhance or decrease the binding of both 2F5 and 4E10 to clade D virus A07412. These results were similar to the results obtained with the modified virus capture assay. A significant increase in the binding of 2F5 but not 4E10 to clade A isolate KNH in the presence of sCD4 was observed. Our studies demonstrate that sCD4 binding causes a significant increase in the accessibility of gp41 epitopes on the intact virus of certain isolates, leading to an increase in binding of 2F5 and 4E10 to the virus. The virus capture assay and the Biacore studies demonstrate that sCD4 facilitates the accessibility of gp41 epitopes on intact virions of certain isolates. However, it is not clear whether the increase in binding in the presence of sCD4 is because of binding to functional trimers or to sCD4 induced non-functional trimers as reported by Haim et al. (2009). It has been proposed that 2F5 and 4E10 epitopes are only exposed for antibody binding within the gp41 pre-hairpin intermediate (Frey et al., 2008; Alam et al., 2009). However, our data demonstrate that CD4 engagement is not required for MPER epitope binding since both 2F5 and 4E10 mAbs recognize viral structures before and after CD4-induced envelope activation.

It is still not clear what determinants on the envelope should be the target of neutralizing antibodies. It might be important to target several regions of the envelope for the induction of neutralizing antibodies in order to prevent entry, replication, and budding of the virus. Thus it is important to identify the protein and lipid epitopes on the surface of intact virions that are cross-clade binding. Some of these epitopes may be conformational and exposed only after interaction with the CD4 receptor. These are some important aspects to consider while designing a HIV-1 vaccine that would generate cross-clade neutralizing antibodies.

Materials and methods

Pseudoviruses and antibodies

Pseudoviruses were prepared by transfecting 5 × 10^6 293 T cells with 8 μg of env expression plasmid and 24 μg of an env-deficient HIV-1 backbone vector (pSG3ΔEnv) using FuGene transfection reagent (Roche). Culture supernatants were harvested on day 3, stored at −80 °C overnight, and then transferred to a liquid nitrogen freezer. The p24 concentration in each stock was determined by using a commercially available p24 ELISA kit (ABL Inc., Kensington, MD). Dr. Jeff Lifson (AIDS Vaccine Program/NCI, Frederick, MD) provided the AT-2 inactivated HIV.

HIV-1 clade B positive plasma pool (abbreviated as USHIV+), collected under an approved IRB protocol was generated as previously described (Brown et al., 2005). Murine monoclonal IgG antibody (mAb Hyb 278-01) specific to tetanus toxoid was purchased from Abcam Inc. Human anti-HIV-1 monoclonal antibodies 2F5 and 4E10 specific to gp41 and lipids and 2G12 specific to manose residues on gp120 were purchased from Polymun Scientific GmbH.

Virus capture assay for IgG monoclonal antibodies in the presence and absence of soluble CD4 (sCD4)

The virus capture assay was modified from Nyambí et al. (1998). Protein G coated 96-well microtiter plates (Thermo-Fisher) were
washed and 100 μl of a 10-μg/ml solution of human anti-HIV-1 monoclonal antibody (2F5, 4E10, 2G12) or control antibody (antitetanoxoid mAb Hyb 278-01) in sodium carbonate buffer pH 9.6 was added to duplicate wells. The plates were sealed and incubated overnight at 4 °C. The next day, plates were washed once with PBS before the addition of 250 μl of blocking buffer (0.5% casein–0.5% BSA in PBS) for 1 h at 37 °C. Plates were washed once with RPMI-1640 media. Culture supernatant containing HIV-1 (100 ng of p24/ml) was diluted in RPMI-1640 media and 100 μl was added to each well. The plates were incubated for 1 h at 37 °C. Plates were washed 5 times with RPMI-1640 media before the addition of 200 μl of 1:5 dilution of lysis buffer in RPMI-1640 media (p24 kit, ABL Inc.). Samples were stored at −80 °C until assayed for p24 by ELISA according to the manufacturer’s instructions.

The binding of the antibodies to the virus was determined by the amount of viral p24 present in each case. Data are expressed as pg/ml ± SD. Each experiment was repeated a minimum of 3 times and all data points were done in duplicate. In each experiment, virus only and mAb only were also included. USHIV+ (1:100 dilution) and Hyb 278-01, an irrelevant monoclonal antibody, served as the positive and negative controls, respectively for the antibodies. The p24 values for the negative control Hyb278-01 (anti-tetanoxoid antibody) for all viral isolates tested ranged from 0 to 7.7 pg/ml (background value). Therefore, a p24 value of ≥15.4 pg/ml, which was twice the background value was considered positive.

Soluble CD4 (15 μg/well, Progenics Pharmaceuticals) was either pre-incubated for 1 h at 37 °C with 100 μl of supernatant of different HIV-1 clades (100 ng/ml of p24) and then added in duplicate to the protein G coated plates containing mAbs 2F5 and 4E10 or sCD4 and HIV-1 were simultaneously added to the plates. The remainder of the procedure for the determination of p24 was carried out as described above. The data obtained from the pre-incubation and the concurrent addition of sCD4 and HIV-1 were indistinguishable, thus concurrent addition was chosen as the standard method.

Analysis of 2F5 and 4E10 binding to AT-2 inactivated virus in the presence or absence of sCD4 by surface plasmon resonance (SPR)

Measurement of AT-2 inactivated HIV-1 (Rossio et al., 1998; Rutebemberwa et al., 2007) binding to 2F5 was performed with a Biacore 2000. AT-2 inactivated clade D virus, A07412, was coupled to a flow cell of a C1 sensor chip (Biacore) via primary amines according to the manufacturer’s specifications. HIV-1 A07412 was injected over the activated surface at a concentration of 12.3 nM. Unbound free amine groups were then quenched with ethanolamine. This immobilization yielded approximately 2800 response units (RU) of virus. All experiments were performed at 25 °C with 20 mM Tris–154 mM NaCl, pH 7.4. Association of 2F5 (0–1000 nM) was measured for 180 s and dissociation was measured for 90 s. Non-specific binding was subtracted. After each injection of 2F5, the chip was regenerated with 50 mM NaOH followed by blocking and capture of AT-2 inactivated D virus, A07412. The data (RU) from a representative experiment of two separate experiments is presented as a bar graph.

Additional experiments were conducted utilizing a Biacore T100. In these experiments, protein G was immobilized on a CM5 chip (~4000 RU). Control mAb Hyb 278-01 specific to tetanoxoid, mAb 2F5, or mAb 4E10 was then captured on the immobilized protein G chip. The non-specific virus binding sites were then blocked with 10% normal goat serum in PBS (120 s, 50 μl/min), AT-2 inactivated clades D (A07412, 42.4 μg/ml) and A (KHN, 71 μg/ml) viruses were pre-incubated with sCD4 (0, 1, 5, 10 μg/ml) at 37 °C for 1 h before injecting over the CM5 chip containing 2F5, 4E10, or Hyb 278-01 (flow rate of 50 μl/min with a 60 s association). After each injection of virus/sCD4, the chip was regenerated with 50 mM NaOH for 45 s at a flow rate of 50 μl/ml. Non-specific virus/sCD4 binding to Hyb 278-01 was subtracted from each reading. The data (RU) of the average of three separate experiments is presented as a bar graph.

TZM-bl assay for TCID50 and neutralization (IC50)

The 50% tissue culture infectious dose (TCID50) was determined by generating eight serial 4-fold dilutions of pseudovirus in triplicate in 96-well culture plates in a total volume of 50 μl of growth medium (DMEM), TZM-bl cells (1 × 104) were suspended in 50 μl of growth medium containing 60 μg/ml DEAE-dextran and added to each of the wells. After a 48 h incubation at 37 °C in a humidified 5% CO2–95% air environment, the plates were analyzed using Brite-Light Luciferase kit (Perkin-Elmer Life Sciences). 100 μl of the reconstituted substrate was added to each well and the relative luminescence units (RLU) were measured by a Victor 2 luminometer (Perkin-Elmer Life Sciences). Wells producing RLU greater than 2.5 times background (signal of cells without virus) were scored as positive (Li et al., 2005).

Pseudovirus neutralization assay

Virus (25 μl) was incubated with dilutions of test mAbs (25 μl) in duplicate for 1 h at 37 °C in a total volume of 50 μl growth medium in 96-well flat-bottom culture plates (Corning-Costar). A 50 μl aliquot containing 1 × 104 TZM-bl cells and 60 μg/ml DEAE-dextran was added to each well. Each plate included wells with cells and virus (virus control) or cells alone (background control). After 48 h, cells were lysed, luminescence was measured as above and the 50% inhibitory dose (ID50) was defined as the dilution that resulted in a 50% reduction in RLU compared to virus control wells after subtraction of background RLU (Mascola et al., 2005). Titers were calculated as previously described (Brown et al., 2008).

Quantitative measurements of envelope and Gag-p24 from pseudoviruses

293 T cell supernatants containing pseudoviruses for each of the 5 clades were collected and the p24 concentration determined as mentioned earlier. The clarified cell culture supernatants containing the pseudoviruses were concentrated by centrifugation at 150,000 rpm for 1 h. Proteins from each of the lysed virus preparations were resolved by SDS-PAGE on 4–20% gels (Invitrogen, Carlsbad, CA) under reducing conditions and stained with fluorescein dyes (Loudet et al. 2005). Known amounts of Escherichia coli expressed clade B-LAIIIIB-HXB2 p24 (provided by Dr. Venagilia Rao, Catholic University of America) and CHO expressed HIV-1gp140 (ABL Inc.) were run on the same gels along with the test virus preparations to estimate the amount of p24 and gp120 content of the viruses. Gels were stained first with the fluorescent dye Pro-Q® Emerald 300 and visualized on a Kodak GEL Logic 200 Imaging System followed by SYPRO® Ruby (Molecular Probes/Invitrogen) to detect and quantify glycoproteins and total protein, respectively. The fluorescence intensity of the bands for p24 and gp120 were analyzed using ImageJ software (NIH) and the amount (ng) of p24 and gp120 were calculated from the standard curves. A representative gel of three separate experiments is shown.

In separate experiments, proteins from each of the lysed virus preparations were resolved by SDS-PAGE on 4–20% gels under reducing conditions and transferred onto PVDF membranes. The PVDF membranes were probed with USHIV+ sera or normal human sera followed by rabbit anti-human IgG-labeled with HRP (Thermo Scientific/Fierce, Rockford, IL). The blots were developed with TMB membrane peroxidase substrate system (KPL, Gaithersburg, MD).

Blue Native PAGE

The envelope proteins derived from the various pseudoviruses were analyzed under native conditions using blue native gels followed by Western blotting according to the manufacturer’s instructions with
modifications from the procedure of Moore et al. (2006). Samples were analyzed on 4–16% Bis-Tris NuPAGE gels (Invitrogen). The blots were probed using an anti-gp120 cocktail or anti-gp41 cocktail, which consisted of 1 μg/ml each of the mAbs 2G12, b12, and 447–52d, or 20 μg/ml each of the mAbs 2F5 and 4E10, respectively. Rabbit anti-human-HRP conjugate (1:2,500, Thermo Scientific/Pierce) was used as the secondary antibody. The blots were developed with TMB membrane peroxidase substrate system (KPL).

Statistical analysis of experimental data

The data was analyzed for significance using paired t-tests. A p value ≤0.05 was considered significant and is depicted by an * in the figures.

Conflict of interest statement

The views and opinions expressed in this article are those of the authors and do not reflect the official policy of the Department of the Army, the Department of Defense, or the U.S. Government.

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