Vertical Transmission of Human Immunodeficiency Virus Type 1: Seroreactivity by Maternal Antibodies to the Carboxy Region of the gp41 Envelope Glycoprotein

Kenneth E. Ugen, Vasantha Srikantan, James J. Goedert, Robert P. Nelson, Jr., William V. Williams, and David B. Weiner

Maternal antibodies against the envelope glycoprotein of human immunodeficiency virus type 1 (HIV-1) have previously been suggested to be important in influencing the rate of vertical transmission. In this study, serum antibody responses in mothers who did or did not transmit HIV-1 infection to their children were measured against the carboxy region of the transmembrane envelope glycoprotein gp41. Results indicate significantly higher binding reactivity of nontransmitter mothers compared with transmitters to three peptides spanning amino acids 771-810 and 841-856. In addition, high neutralization titers in maternal sera against HIV-1IMB were associated with a nontransmission status. This is the initial report demonstrating a correlation between maternal antibody binding to epitopes within the carboxy region of gp41 envelope glycoprotein and lack of vertical transmission. Immunodetection that identifies antibodies to these regions in gp41 could therefore be considered a strategy to assess the risk of vertical transmission of HIV-1.

In addition to sexual transmission and transmission by intravenous drug use, a major route of contraction of human immunodeficiency virus type 1 (HIV-1) infection is from mother to child, that is, vertical transmission. The routes of vertical transmission include intrauterine as well as peripartum and postpartum transmission [1, 2]. While there is some uncertainty about which route is most important, there is evidence for transmission by all three routes. The number of cases of pediatric AIDS due to vertical transmission has been increasing rapidly and has resulted in AIDS becoming the third leading cause of death in young children.

In the United States, the rate of vertical transmission is ~25% in women not treated with zidovudine. Therefore, a large percentage (75%) of infants of HIV-1-infected mothers escape infection [3]. Previous studies suggest that maternal antibodies to HIV-1 are an important factor in determining transmission status [4-6]. Thus, the vertical transmission system presents a potentially useful model for the study of protective immune responses. In addition, it allows analysis of the potential efficacy of passive immunization strategies, since in effect the HIV-seropositive mother is passively immunizing her unborn child with her immunoglobulins.

Several studies have suggested that maternal antibodies to distinct epitopes of the gp120 V3 region may be associated with a lower vertical transmission rate [4-6]. More recent studies have not confirmed this observation [7-12]. However, most of these studies used peptides derived from the HIV-1IMB envelope glycoprotein sequence, which is divergent from most clinical isolates. We previously demonstrated a correlation between binding of maternal HIV-1-seropositive sera to multiple epitopes from both of the envelope glycoproteins of HIV-1: gp41 and gp120 [12, 13]. In particular, our data, presented in a comprehensive summary of work from the Mothers and Infants Cohort Study, as prepared by Goedert and Dublin [13], suggested the correlation between high reactivity of maternal serum samples to gp41 and a nonvertical transmission status. This study indicated a correlation between broad maternal humoral immune responses to the HIV-1 envelope glycoprotein and lack of vertical transmission. In addition, a correlation between high neutralizing titers in maternal HIV-1-seropositive sera against HIV-1IMB and lack of vertical transmission was demonstrated.

These studies suggested for the first time a possible protective role of maternal immune responses directed against the transmembrane glycoprotein of HIV-1 in terms of prevention of maternal-to-fetal transmission of HIV-1 infection. Our work,
however, was done with peptides derived from HIV-1_MN, a laboratory isolate considerably divergent compared with the clinical isolates responsible for the infection of the majority of persons in North America [14, 15]. Therefore, we decided to perform epitope mapping studies with peptides derived from the gp41 transmembrane glycoprotein of the HIV-1_MN isolate. Of particular interest was the carboxy-terminal region of gp41, since it had previously been found that some neutralizing epitopes were present in this region and that patients mounted immune response to these regions. Specifically, the following regions have been previously demonstrated to be immunoreactive with human HIV-1-seropositive sera: aa 771–802 [16], aa 797–818 [17], and the carboxy-terminal of gp41, aa 846–861 [16, 18–20]. In addition, a region spanning aa 732–746 [18–21] has been suggested to be immunogenic in natural infection by HIV-1 and result in measurable antibody responses in infected persons.

We therefore examined binding of sera from mothers who do and do not transmit HIV-1 infection to their child to a series of peptides from the carboxy-terminal region of gp41. These analyses were done by peptide-based ELISAs with peptides derived from the envelope glycoprotein sequence of the MN isolate. In addition, the neutralization activity of these serum samples against HIV-1_MN was assayed by quantitation of syncytia as an end point.

Materials and Methods

**Serum samples.** Ten serum samples from maternal HIV-1 transmitters and 10 from nontransmitters were used in the initial peptide screening analysis. In an expanded analysis, a total of 26 transmitter and 40 nontransmitter serum samples were analyzed against peptides 6 and 7 (described below). These two peptides were selected on the basis of the observation that they demonstrated the most significant ELISA binding differences in the serum samples from maternal transmitters and nontransmitters. All of the samples were from mothers whose vertical transmission status had been confirmed by serologic analysis in the babies after 2 years of age, that is, at a time when accurate diagnosis could be made in these children. Samples from the mothers were collected late in the third trimester of pregnancy. For analysis of neutralization potential, 22 of the transmitter and 28 of the nontransmitter serum samples were used in the cell-free virus assay described below. In addition, none of the mothers were treated with zidovudine during their pregnancy.

**Peptide reagents.** Peptides used in this study were obtained from the AIDS Research and Reference Reagent Program and were derived from the HIV-1 MN envelope glycoprotein sequence. The peptides analyzed in these assays are listed in table 1. Peptides 1 through 7 are from the carboxy-terminal region of gp41, while peptide 8 was derived from the V3 loop of HIV-1_MN.

**ELISA methodology.** Peptide-based ELISA analysis was done by modifications of previously described methods [12, 22]. Briefly, peptides were dissolved in 0.05 M carbonate-bicarbonate buffer, pH 9.6, to a concentration of 10 μg/mL, and 50 μL (500 ng) was added to wells of Immulon II plates and incubated overnight at 4°C. Plates were then rinsed with washing buffer (1× PBS + 0.05% Tween 20) and incubated overnight with blocking buffer (5% nonfat dry milk in PBS + 1% bovine serum albumin [BSA] + 0.05% Tween 20). Serum samples were then diluted in dilution buffer (5% nonfat dry milk in PBS + 0.05% Tween 20) at 1:250 and 1:500 dilutions and incubated in duplicate in peptide-coated plates for 2 h at room temperature (RT), washed, and then incubated for 2 h at RT with a goat anti-human immunoglobulin–horseradish peroxidase conjugate (Sigma, St. Louis) diluted in dilution buffer at the concentration suggested by the manufacturer. After extensive washing, the plates were developed with 3,3′,5,5′-tetramethylbenzidine dihydrochloride substrate (100 μg/mL), the reaction was stopped with 2 N H2SO4, and color development was quantitated at 450 nm. In these assays, a pool of sera from third-trimester HIV-1-seronegative pregnant women was used as the sample control, and BSA-coated wells were used for the negative binding control. The optical density (OD) values given in the figures represent specific binding, that is, nonspecific binding to a control plate had been subtracted, and are given as ΔOD.

**Free virus neutralization assay.** The neutralization assay protocol was essentially that described by Srikantan et al. [23], Montifiori et al. [24], and Nara [25]. Specifically, 50 μL of serial dilutions (1:200, 1:400, and 1:800) of maternal serum samples were mixed with 50 μL of HIV-1_MN cell-free virus titered to 100 TCID50/well in a 96-well microtiter plate. The virus-serum mixture was then incubated at 37°C for 90 min, and at the end of incubation, log-phase human T cell leukemia virus type I–infected MT-2 cells were washed and counted, and the cell suspension was prepared at a concentration of 0.4 × 10⁶/mL. One hundred microliters of this suspension was added to each well in the plate. The plate was then incubated for 1 h at 37°C. The cells were washed twice and suspended in 200 μL of RPMI 1640 medium containing 10% fetal bovine serum. Cell controls (cells + antibody without titered virus) and virus controls (cells plus titered virus + control antibody) were also included in each assay. The plates were incubated at 37°C in a CO2 incubator. After 3 days of incubation, the number of syncytia were counted in each well, and the V0/Vn (mean number of syncytia in wells containing antibody plus virus/mean number of syncytia in virus control wells) and the percentage of neutralization were determined as described [24, 25].

**Statistical analysis.** Calculations of statistical significance (Student’s t test) as well as the measurements of test specificity and sensitivity were made by standard methods [26]. The results of these analyses are shown in table 2.

### Table 1. Amino acid sequences of peptides used in study (single-letter code).

<table>
<thead>
<tr>
<th>Peptide no.</th>
<th>First amino acid no.</th>
<th>Last amino acid no.</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>721</td>
<td>740</td>
<td>RPVPVRGDPPEGIEEGGE</td>
</tr>
<tr>
<td>2</td>
<td>731</td>
<td>750</td>
<td>PEGIEEGGERDRTGRGLV</td>
</tr>
<tr>
<td>3</td>
<td>741</td>
<td>760</td>
<td>RDRDTRGRLVHIGFLAIWVD</td>
</tr>
<tr>
<td>4</td>
<td>771</td>
<td>790</td>
<td>HIRDLLIAARIVELLLGRGW</td>
</tr>
<tr>
<td>5</td>
<td>781</td>
<td>800</td>
<td>IVELLGGRGWELKLYWNNLL</td>
</tr>
<tr>
<td>6</td>
<td>791</td>
<td>810</td>
<td>EKLKYWNNLLOYWSQELKSS</td>
</tr>
<tr>
<td>7</td>
<td>841</td>
<td>856</td>
<td>LIHPTIRQGRELALL</td>
</tr>
<tr>
<td>8</td>
<td>301</td>
<td>320</td>
<td>CTRPNYNKRKRIIHIGPRAF</td>
</tr>
</tbody>
</table>
Table 2. Test specificity and sensitivity for vertical nontransmission status based on ELISA peptide binding and neutralization titers.

<table>
<thead>
<tr>
<th>Peptide binding</th>
<th>Specificity, %</th>
<th>Sensitivity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>73</td>
<td>78</td>
</tr>
<tr>
<td>7</td>
<td>85</td>
<td>55</td>
</tr>
<tr>
<td>6 or 7</td>
<td>65</td>
<td>82.5</td>
</tr>
<tr>
<td>6 and 7</td>
<td>96</td>
<td>50</td>
</tr>
<tr>
<td>Neutralization (≥50% inhibition)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:200</td>
<td>86.4</td>
<td>89.3</td>
</tr>
<tr>
<td>1:400</td>
<td>100</td>
<td>71.4</td>
</tr>
<tr>
<td>1:800</td>
<td>100</td>
<td>57.1</td>
</tr>
</tbody>
</table>

NOTE. Specificity = 100 × (no. of transmitter sera with negative binding or <50% inhibition)/(total no. of transmitter sera); sensitivity = 100 × (no. of nontransmitter sera with positive binding or ≥50% inhibition)/(total no. of nontransmitter sera). n = 26 and 40 (peptide reactivity), and 22 and 28 (neutralization) for transmitters and nontransmitters, respectively.

Results

Maternal Serum Binding Reactivity to gp41 Peptides

Figure 1 shows the mean ± SE binding (measured as OD at 450 nm spectrophotometrically) of sera from vertical transmitters and nontransmitters to the eight different peptides listed in table 1. In particular, there were significant differences between binding of maternal transmitters and nontransmitters to peptides 4 (aa 771–790, figure 1D), 6 (aa 791–810, figure 1F), and 7 (aa 841–856, figure 1G) at both of the dilutions (1:250 and 1:500) shown. Peptide 5 (aa 781–800, figure 1E) demonstrated a significant difference in binding between the transmitters and nontransmitters only at the 1:250 serum dilution. These differences were significant at P < .05 using Student’s t test. Peptides 1 (aa 721–740, figure 1A), 2 (aa 731–750, figure 1B), and 3 (aa 741–760, figure 1C) failed to demonstrate significant binding reactivity with serum samples from either transmitters or nontransmitters (P > .05). To eliminate the possibility that the low binding noted with serum samples from transmitters to peptides 4, 6, and 7 was not due to a low overall level of immunoglobulins in these samples, binding analysis was done with the serum samples from both groups against an immunodominant HIV-1MN V3 loop peptide (peptide 8, aa 301–320, figure 1H). Similar levels of binding to this peptide were noted for both groups (i.e., no significant differences between transmitters and nontransmitters at the 95% confidence level using Student’s t test).

Overall, these results indicate a significant difference between the ability of sera from nontransmitters to bind to sequences in the carboxy-terminal region of the transmembrane glycoprotein gp41. Specifically, three of these peptides showed significantly higher mean binding of serum antibodies, in both dilutions tested, from nontransmitters compared with serum antibodies from transmitters.

In addition, we performed an expanded analysis of binding of maternal antisera to peptides 6 and 7 (figure 2). The total number of nontransmitter and transmitter samples analyzed was 40 and 26, respectively. These sample numbers include those shown in figure 1.

Figure 1. Binding of maternal serum immunoglobulins from HIV-1 vertical transmitters and nontransmitters to HIV-1MN peptides from carboxy-terminal region of gp41 transmembrane envelope glycoprotein. Sera from transmitters and nontransmitters were diluted, and binding to peptides was measured by ELISA. gp41 peptides analyzed were as follows: A, peptide 1; B, peptide 2; C, peptide 3; D, peptide 4; E, peptide 5; F, peptide 6; G, peptide 7; H, peptide 8 (see table 1 for peptide sequences). Mean ± SE binding (measured as OD at 450 nm spectrophotometrically) is shown for 10 transmitters and 10 nontransmitters.
The results indicate and confirm a statistically significant difference in OD values between nontransmitters and transmitters at \( P < .05 \) as measured by Student's \( t \) test for both peptides at a serum dilution of 1:250. In addition, at a 1:500 dilution, statistical significance was attained for peptide 6.

**Free Virus Neutralization Assay**

Figure 3 shows the mean \( V'/V_0 \) values for sera from transmitters versus nontransmitters when incubated with the HIV-1MN cell-free virus stock and MT-2 cells. These results indicate that, on average, sera from nontransmitters were able to more significantly neutralize infection of MT-2 cells by cell-free HIV-1MN at all dilutions tested than were sera from transmitters. In fact, at a serum dilution of 1:800, transmitter sera failed to neutralize at all, while nontransmitter sera still neutralized infection by at least 50%. Through analysis of individual \( V'/V_0 \) values from transmitters and nontransmitters, the following observations are made: The percentage of transmitter sera showing >50% neutralization at 1:200, 1:400, and 1:800 was 13.6%, 0%, and 0%, respectively, whereas the percentage of nontransmitter sera having >50% neutralization at 1:200, 1:400, and 1:800 was 89.3%, 71.4%, and 57.1%, respectively.

**Statistical Analyses of Sensitivity and Specificity of Binding Reactivity and Neutralization**

*ELISA peptide-binding analysis.* The specific OD values at 450 nm for the normal human serum samples for all of the peptides analyzed was \( \sim 0.05 \). For the purposes of these analyses, any individual serum sample whose OD at 450 nm for a particular peptide was 3 SD above the mean value for normal human serum was considered to exhibit positive binding to that peptide. Conversely, any OD value at 450 nm below this number was considered negative binding for that peptide. In making these calculations, it was assumed that a positive value is associated with nontransmission status while a negative value is associated with transmission status. The sensitivity (ability to detect true cases of nontransmission) and specificity (ability to detect true cases of transmission) for peptides 6 and 7 are given in table 2. Peptides 6 and 7 have test sensitivities of 78% and 55%, respectively, whereas test specificities for these peptides were 73% and 85%, respectively. In addition, if binding to either peptide 6 or 7 was considered positive for the individual sample, the test specificity and sensitivity were 65% and 82.5%, respectively. If binding to both peptides 6 and 7 was considered necessary for each sample, the test specificity and sensitivity were 96% and 50%, respectively. These results indicate that binding of serum antibodies from HIV-1-infected mothers to peptide 6 has the greatest ability to detect cases of nontransmission, whereas binding to peptide 7 has the greatest ability to detect cases of transmission. Likewise, when a positive binding outcome is defined as reactivity to peptides 6 or 7 as well as to peptides 6 and 7, the test specificity and sensitiv-
ity are increased and decreased, respectively. When a positive outcome is defined as reactivity to peptide 6 or 7, the test specificity and sensitivity decrease and increase, respectively.

Neutralization titer analysis. For the neutralization assay, test specificity and sensitivity were determined on the basis of the number of samples having >50% neutralization (i.e., inhibiting >50% of the number of syncytia quantitated in the virus control wells). At serum dilutions of 1:200, 1:400, and 1:800, the test specificity was 89.3%, 71.4%, and 57.1%, respectively. In contrast, test sensitivity at these serum dilutions was 86.4%, 100%, and 100%, respectively. In summary, at all serum dilutions tested, the neutralization assay was able to detect to a high degree (>85%) true cases of vertical transmission. In contrast, only at a dilution of 1:200 was the neutralization assay able to detect a nontransmission status to the same degree.

Discussion

Since the largest-growing populations of AIDS patients are women of childbearing age and children, the role of maternal-to-fetal transmission of HIV-1 infection is becoming more important. Of particular interest has been the potential role of maternal humoral immune responses to the envelope glycoprotein of HIV-1 in modulating the incidence of vertical transmission of this retrovirus. It has been suggested that passive or active vaccination (or both) may be useful in reducing the incidence of vertical transmission of this virus, as has been shown for hepatitis B [27]. Much of this speculation is based on the ability of hyperimmune anti-HIV-1 serum or monoclonal antibodies against the V3 loop to protect chimpanzees from infection [28, 29]. In addition, it has been shown that immunodecient (SCID) mice reconstituted with human peripheral blood mononuclear cells and challenged with HIV can also be protected by anti-HIV-1 monoclonal antibodies [30]. Since a large proportion of babies born to HIV-1-seropositive mothers remain uninfected, regardless of established transplacental exposure to virus, the vertical transmission system provides an excellent model for the study of potential protective immune responses to this retrovirus.

Previously, we had shown that there was a quantitative difference between transmitters and nontransmitters in terms of the number of envelope glycoprotein peptides (gp120 and gp41) to which sera from these groups bound, with nontransmitters binding significantly more peptides [12, 13, 31, 32]. In particular, we were able to demonstrate in that investigation that overall maternal serologic responses to regions in gp41 were correlated with protection of the babies from infection by HIV-1. This is a potentially important finding in light of reports suggesting that antibody reactivity to conserved epitopes of gp160 is narrower in patients with late-stage AIDS than in asymptomatic patients [20]. In particular, these investigators examined binding of HIV-1 patient serum samples to a peptide corresponding to aa 846–860 in gp41 in relationship to CD4 cell counts. They showed that significantly fewer patients with <200 CD4 cells/mm³ had antibody reactivity to this region than did asymptomatic patients with >400 CD4 cells/mm³. They suggest that the failure to generate a significant immune response to this region may result in disease progression.

Based on these findings, we decided to perform a mapping analysis of specific regions of the gp41 transmembrane glycoprotein, which had been demonstrated by other investigators to show reactivity with serum samples from HIV-1–infected persons [16, 18–20]. One such region selected was at the carboxy-terminal of gp41 as described by Warren et al. [20]. In addition, our original study was done with envelope glycoprotein peptides based on the HIV-1MN sequence. However, in the study reported here, peptides were based on the HIV-1MN sequence, a laboratory isolate that has been suggested to be more reflective of the clinical isolates that circulate in nature [14, 15]. Most of these sequences are located in the carboxy-terminal region of gp41. Specifically, in this report the analysis covered an 86-aa segment of gp41 that comprised a total of seven peptides of 15 or 20 aa in length. This analysis indicated that there was a significant difference in binding of serum samples between transmitters and nontransmitters to four of the gp41 peptides examined. It is of interest that binding of maternal antibodies to a peptide similar to the one studied by Warren et al. [20] (peptide 7: aa 841–856) was inversely correlated with infection status in the infant. The overall significance of binding of HIV-1–infected patient sera to this epitope needs to be further analyzed.

Another recent report has suggested that low levels of CD4 binding site (located in gp120) antibodies correlate with an increased risk of HIV-1 vertical transmission [33]. Therefore, antibodies generated against more conserved regions, such as the CD4 binding site on gp120, as well as the relatively conserved carboxy-terminal regions of gp41 analyzed in this study may be useful as passive immunotherapies to decrease the incidence of HIV-1 vertical transmission. In particular, the amino acid sequence of the four gp41 MN peptides, whose binding to maternal HIV-1–positive sera was shown to be correlated with vertical transmission status, had an average homology of 90% among 39 different HIV-1 isolates (based on the HIV Sequence Database from Los Alamos National Laboratory, Los Alamos, NM).

Our findings are the initial documentation that there are site-specific serologic binding differences between transmitters and nontransmitters within the transmembrane glycoprotein gp41. The results also confirmed our earlier published studies that indicated that high HIV-1 neutralizing activity of maternal serum antibodies against laboratory isolates, such as MN, can correlate with nontransmission status. Much recent work in this area has suggested that maternal antibodies can neutralize autologous virus isolates (autologous neutralization), which may be of particular relevance if neutralization-resistant mutants arise under selective immune pressure and result in vertical transmission of these isolates. In relation to neutralization studies, our analysis suggests that other epitopes outside of the gp120 V3 loop and perhaps residing in gp41 may explain the
correlation between high anti–HIV-1MN neutralization potential and lack of vertical transmission. This statement is based on the finding that binding reactivity to the HIV-1MN V3 loop peptide is not significantly different between transmitter and nontransmitter sera. Additional studies examining the role of maternal immune responses to other regions of the gp41 glycoprotein, including the 2F5 epitope [34] as well as an epitope within the immunodominant region [35] recently described by our group, are being pursued. Results of these analyses as well as those already garnered on the carboxy region of gp41, as presented here, should identify distinct regions that may have value for assessing the likelihood of vertical transmission.

The specific values in table 2 on sensitivity and specificity suggest that analysis of a larger group of maternal serum samples, particularly to peptides 6 and 7, is warranted. In addition, in this particular study, the test specificity and sensitivity value of neutralization titers of maternal sera against HIV-1MN are quite high, again suggesting that further analysis may indicate the contribution of regions within the carboxy terminal of gp41 in the neutralization activity observed.

It is interesting to speculate as to the mechanism regarding the correlation between the diversity of immune responses and nontransmission status. The differences were apparent even in the cases in which CD4 levels are uninformative. It is well established that the majority of immunoglobulin responses are T cell–dependent. In this case, the serologic determination of binding diversity may represent a simple method for determination of the overall T cell health of the mothers and their general ability to control viral infection. In this sense, a higher anti–HIV-1 cytotoxic T lymphocyte response might be expected to accompany these serologic findings. Therefore, further analysis of the potential role of immune responses against these regions of gp41 in lowering virus load and therefore transmission of infection is warranted.

In conclusion, this study indicates that maternal immune responses to regions within the transmembrane envelope glycoprotein gp41 may have a role in predicting vertical transmission of HIV-1. The role of the gp41 regions in preventing or modulating HIV-1 vertical transmission is currently unknown; however, data presented here indicate that further study on the question is warranted. Even though zidovudine administration to pregnant HIV-1–infected mothers has recently been shown to decrease the vertical transmission rate of HIV-1 [36], it is likely that immunologic modulations such as active and passive immunotherapies will have utility in further decreasing the incidence of pediatric AIDS caused by this route of infection.

Acknowledgments

The technical assistance of C. Noble, S. Nyland, and J. Lamont is acknowledged.

References


