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Neutralization Escape Variants of Human Immunodeficiency Virus Type 1 Are Transmitted from Mother to Infant

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Maternal passive immunity typically plays a critical role in protecting infants from new infections; however, the specific contribution of neutralizing antibodies in limiting mother-to-child transmission of human immunodeficiency virus type 1 is unclear. By examining cloned envelope variants from 12 transmission pairs, we found that vertically transmitted variants were more resistant to neutralization by maternal plasma than were maternal viral variants near the time of transmission. The vertically transmitted envelope variants were poorly neutralized by monoclonal antibodies biz, 2G12, 2F5, and 4E10 individually or in combination. Despite the fact that the infant viruses were among the most neutralization resistant in the mother, they had relatively few glycosylation sites. Moreover, the transmitted variants elicited de novo neutralizing antibodies in the infants, indicating that they were not inherently difficult to neutralize. The neutralization resistance of vertically transmitted viruses is in contrast to the relative neutralization sensitivity of viruses sexually transmitted within discordant couples, suggesting that the antigenic properties of viruses that are favored for transmission may differ depending upon mode of transmission.

Mother-to-child transmission (MTCT) of human immunodeficiency virus type 1 (HIV-1) accounted for more than 1/10 of new infections worldwide in 2004. MTCT occurs in utero, during delivery, and through breastfeeding, at a rate of approximately 30% in the absence of antiretroviral therapy (6, 19). Previous studies have demonstrated that, despite a complex viral population in the mother, only viruses of a restricted subset were typically transmitted to the infant (1, 23, 29, 40, 41, 45, 48). This suggests that some viruses may be favored for transmission in this setting. One obvious source of selective pressure in the setting of MTCT is maternal antibody, which could play a role in limiting transmission of neutralization-sensitive variants. Indeed, studies have shown that nontransmitting mothers had more frequently detected and/or higher-level neutralizing antibody (NtAb) responses than transmitting mothers, suggesting a role for NtAb in reducing MTCT (4, 16, 24, 39, 41). In support of this model, Kliks et al. showed in a small study of six transmission pairs that viral isolates from infants were resistant to neutralization by maternal plasma (21). However, there has not been a detailed study of the neutralization properties of vertically transmitted virus in relation to individual variants within the maternal quasispecies.

Understanding the role of NtAb in MTCT is important for determining whether passive administration of NtAb will be beneficial. One strategy being tested to prevent MTCT is to harness the neutralizing activity of monoclonal antibodies (MAbs) to augment the passive immunization of infants who continue to be exposed to HIV-1 through breastfeeding (27). In these studies, the focus has been on biz, 2G12, 2F5, and 4E10, which were generated from HIV-1 subtype B-infected individuals (5, 7), because a combination of these MAbs, or of just 2G12, 2F5, and 4E10 (also referred to as TriMab), completely protected neonatal rhesus macaques from oral challenge with simian-human immunodeficiency virus 89.6P (13, 14). However, because simian-human immunodeficiency virus 89.6P represents only a single strain of virus, and one that is sensitive to neutralization by these antibodies (2, 10, 17), it is unclear how these findings would translate to vertically transmitted HIV-1 variants, especially those that are not subtype B.

In a small study of envelope sequences from four MTCT pairs (S. M. J. Rainwater, X. Wu, R. Nduati, G. John-Stewart, D. Mbori-Ngacha, and J. Overbaugh, submitted for publication), we found that variants from infants displayed low or undetectable sensitivities to neutralization by maternal plasma, providing some support for the hypothesis that vertically transmitted variants may be resistant to maternal NtAb. However, a caveat to this study was that for two of the four mothers, the maternal viruses examined were also resistant to neutralization by autologous plasma. Moreover, the study focused primarily on envelope clones that encoded only portions of maternal and infant sequences. In the present study, we examined full-length envelope sequences from eight MTCT pairs who were selected from the Nairobi breastfeeding trial (30) based on screening for cases in which maternal viral isolates were sensitive to neutralization by autologous plasma. Because recent studies of heterosexually acquired viruses suggested that they have fewer potential N-linked glycosylation sites (PNGS) and shorter variable loop sequences in envelope (9, 11), we also examined these sequence features and whether they predict neutralization sensitivity.

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MATERIALS AND METHODS

Study subjects. Samples were obtained from participants in the breastfeeding clinical trial in Nairobi, Kenya (30), in which infants born to HIV-1-seropositive mothers were monitored for HIV-1 provirus in blood at birth and at frequent time points thereafter until each infant was 2 years of age (20, 30). Plasma or peripheral blood mononuclear cells (PBMC) were collected and stored in aliquots at −70°C or in liquid nitrogen, respectively. Informed consent and human subject protocols were approved by institutional review boards of the participating institutes.

Amplification and cloning of HIV-1 *env* genes. HIV-1 *env* genes were amplified by nested PCR (TaqPlus Precision PCR system; Stratagene, La Jolla, CA) from uncultured PBMC DNA (26) or from cDNA obtained by reverse transcription (SuperScript II; Invitrogen, Carlsbad, CA) of viral RNA extracted from plasma. Primers used were primer pair vpr9 and nef34 (round 1) and primer pair vpr11 and nef30 (round 2) as previously described (26). Additional primers used were as follows: for round 1, forward primers vpr1 (5′-GATAGATGGAACAAGCCC CAG-3′) and vpr3 (5′-TCTATGAAACTTATGGGGATAC-3′) and reverse primers nef32 (5′-CATTGGTCTTAAAGGTACCTG-3′) and nef50ab, a mix of equal parts of nef50a (5′-AGAGCTCCCTTGTAAGTCATTGG-3′) and nef50b (5′-AGAGCTGCTTTGTAAGTCATTGG-3′); and for round 2, reverse primer nef52ab, a mix of equal parts of nef52a (5′-GTCATTGGTCTTAGAGGTACT TGTGG-3′) and nef52b (5′-GTCATTGGTCTTAAAGGCACCTGAGG-3′). The round 1 primers used were as follows: for F535, primer pair vpr1 and nef32 and primer pair vpr9 and nef34; for J613, primer pair vpr9 and nef34; for J412, primer pair vpr1 and nef32, primer pair vpr3 and nef32, and primer pair vpr3 and nef34; for G505, primer pair vpr9 and nef32 and primer pair vpr9 and nef34; for L035, primer pair vpr1 and nef34 and primer pair vpr9 and nef34; for K184, primer pair vpr1 and nef34, primer pair vpr9 and nef32, and primer pair vpr9 and nef34; for I206, primer pair vpr1 and nef50ab; and for L274, primer pair vpr1 and nef50ab, primer pair vpr3 and nef50ab, and primer pair vpr9 and nef50ab. The round 2 primers vpr11 and nef30 were used for F535, J613, J412, G505, L035, and K184, and vpr11 and nef52ab (round 2) were used for I206 and L274. The cycling parameters were 1 cycle of 94°C for 4 min, 35 cycles of 94°C for 30 s, 58°C or 60°C for 30 s, and 68°C for 4 min, and 1 cycle of 72°C for 10 min. The PCR product was cloned into pcDNA3.1/V5-His-TOPO vector (Invitrogen). Plasmid DNA was used to transfect 293T cells along with an envelope-deficient HIV-1 subtype A proviral plasmid, Q23Δenv, to generate pseudotyped viral particles (26). The infectivity of the pseudotyped viruses was screened by a single-round infection of TZM-bl cells (AIDS Research and Reference Reagent Program, National Institutes of Health) (46). Only a fraction of these envelopes were capable of mediating viral infections (26), and 78 of these functional envelope clones, each from an independent PCR, were sequenced and used for further studies.

Phylogenetic tree analysis. A total of 78 envelope sequences were obtained from the study subjects, and 150 reference sequences from group M HIV-1 data were downloaded from the Los Alamos National Laboratory HIV sequence database (http://www.hiv.lanl.gov). Codon-aligned nucleotide sequences spanning the V1-to-V5 region of envelope were analyzed by a neighbor-joining tree based on distance using the general time-reversible model implemented in PAUP* 4.0b10 (D. L. Swofford, Sinauer Associates, Inc., Sunderland, MA). After gap stripping was performed, 705 positions remained. Two subtype K reference sequences were used as an outgroup. The reliability of branching orders was assessed by bootstrap analysis with 1,000 replicates. Maximum-likelihood trees were constructed using nucleotide sequences spanning the V1-to-V5 region of envelope. After codon-optimized alignment and gap stripping were performed, Modeltest (33) was used to identify the optimal evolutionary model for each transmission pair. PAUP* 4.0b10 was then used to construct the likelihood tree. Two unrelated sequences from the same clade (clade A or D, as appropriate) were included as an outgroup. For F535 and L035, who were infected with D/A recombinants, unrelated sequences of both clades A and D were used as an outgroup.

Neutralization assay. Neutralization was assessed using envelope-pseudotyped viruses to infect TZM-bl cells as described previously (Rainwater et al., submitted, and reference 28). Briefly, the titer of each pseudotyped stock was determined in a single-round infection of TZM-bl cells by directly counting β-galactosidase-positive "blue" foci at 48 h postinfection. About 500 infectious units of pseudotyped viruses were incubated with twofold serial dilutions of antibody or heat-inactivated plasma, or with growth medium alone, in a total volume of 50 μl at 37°C for 1 h. TZM-bl cell suspension in 100 μl of growth medium containing 30 μg/ml diethylaminoethyl-dextran was added to the virus-antibody conjugates. In 48 h, infection levels were determined using Galacto-Light Plus (Applied Biosystems, Foster City, CA), a quantitative assay measuring β-galactosidase activity present in the cell lysate. Infections were performed in triplicate within each experiment, and the results shown are averages from two or more independent experiments using viral stocks generated by at least two separate transfections. Differences between β-galactosidase activity in the presence of antibody or plasma and growth medium alone were calculated as the percentage of neutralization. No neutralization activity above 50% was observed when plasmas from HIV-seronegative individuals were used. The 50% inhibitory concentration (IC_{50}) was calculated from a dose-response curve using the logarithmic function of Microsoft Excel and is expressed as the reciprocal dilution of plasma or the concentration of MAb required to inhibit infection by 50%. The highest amounts of antibody tested were 1:25 for plasma and 50 μg/ml for MAbs. In cases in which the IC_{50} for plasma was <25, the midpoint value between 0 and 25, 12.5, was assigned for statistical analysis. The antibody biz (7) was kindly provided by Dennis Burton (The Scripps Research Institute, La Jolla, CA). The antibodies 2G12 (5, 44), 2F5 (5, 34), and 4E10 (5, 43) were kindly provided by Hermann Katinger (Polymun Scientific, Vienna, Austria).

Statistical analysis. To compare the V1-to-V5 length and number of PNGS between infant and mother sequences, we used generalized estimating equations (GEE) with a Gaussian link and exchangeable correlation structure, after assessing whether the distribution of V1-to-V5 lengths and number of PNGS in the envelope followed the Gaussian distribution. On the basis of previous findings that different subtypes differed in envelope length and number of PNGS (9), we also controlled for different subtypes in these analyses to assess whether any differences seen were independent of subtype. To compare neutralization sensitivities between infant and maternal envelope variants, due to the fact that neutralization IC_{50}s did not follow a Gaussian distribution, we dichotomized the neutralization sensitivity of each envelope variant, using the detection limit of the neutralization assay, an IC_{50} of 25, as the cutoff. We then used GEE with a logit link and exchangeable correlation structure to analyze the data. In addition, to retain the information about the magnitude of neutralization sensitivity, the median IC_{50} was calculated for each infant and mother subject, and the difference between infant and mother medians was tested using a two-sided Wilcoxon signed-rank test. Correlation of neutralization IC_{50}s to the V1-to-V5 lengths or number of PNGS was examined with Spearman's correlation test. Correlation between the viral genetic distance and difference in neutralization IC_{50}s was examined by Mantel testing using the Spearman's correlation. Significance was reported when $P \leq 0.05$, and a trend was reported when $0.05 < P \leq 0.1$. All statistical analyses were done with Intercooled Stata version 8.0 (Stata Corporation, College Station, TX) except for the Mantel test, for which analysis was done with XLSTAT (Addinsoft, New York, NY) for Microsoft Excel.

Nucleotide sequence accession numbers. Nucleotide sequences are available under GenBank accession numbers DQ208424 to DQ208501.

RESULTS

To specifically examine cases in which the mother harbored NtAb-sensitive variants, we first screened viruses isolated by primary cell culture from 16 transmitting mothers for sensitivity to neutralization by autologous plasma. Eight transmitting mothers whose viruses displayed some sensitivity to autologous contemporaneous plasma taken near the time of transmission were selected for study. These subjects represented infections with a range of CD4 counts and viral loads (Table 1). Infants born to these mothers were HIV-1 negative at birth but became HIV-1 positive at 6 weeks after birth. Thus, transmission presumably occurred either during delivery or through early breastfeeding.

Full-length envelope genes were amplified from uncultured patient PBMC DNA (or plasma RNA in one case in which DNA amplification was unsuccessful). Samples collected near the estimated transmission time (for infants, 6 weeks after birth; for mothers, within 1 week of delivery) were used (Table 1). A total of 78 functional envelope clones, each from an independent PCR, were obtained and sequenced. A neighbor-joining tree was constructed by alignment of nucleotide sequences spanning the V1-to-V5 region of envelope (Fig. 1). The resulting tree revealed clear epidemiological linkage between each

TABLE 1. Summary of subjects

Identification no.	Subject	Subtype(s)	Time of infant's first HIV-positive result[a]	Envelope cloning time[a]	Maternal plasma time[a]	Maternal CD4 (cells/μl)	Maternal viral load (copies/ml)	Estimated mode of transmission	No. of clones	% Maximum distance[b]
G505	Mother	A		W0	W0	Not done	29,210	Delivery or breastfeeding	7	3.8
	Infant	A	W6	W6					3	0.5
L274	Mother	A		W0	W0	548	383,833	Delivery or breastfeeding	7	7.0
	Infant	A	W6	W6					3	0.2
I206	Mother	A		W0	W0	262	131,745	Delivery or breastfeeding	6	3.0
	Infant	A	W6	W6					3	0.6
J613	Mother	A		W1	W1	104	438,175	Delivery or breastfeeding	3	7.5
	Infant	A	W6	W6					2	0.2
F535	Mother	D/A		W0	W0	690	337,338	Delivery or breastfeeding	7	9.5
	Infant	D/A	W6	W6					3	0.6
L035	Mother	D/A		W0	W0	250	28,913	Delivery or breastfeeding	10	5.7
	Infant	D/A	W6	W6					5	3.0
K184	Mother	C/D		W0	W0	568	77,670	Delivery or breastfeeding	6	4.8
	Infant	C/D	W6	W6					3	0.2
J412	Mother	C		W0	W0	293	71,795	Delivery or breastfeeding	7	9.0
	Infant	C	W6	W6					3	0.5
B201[c]	Mother	A		P32	P32	521	701,650	In utero	2	4.0
	Infant	A	W0	W0					2	0.8
I369	Mother	A		M12	M9	302	Not done	Breastfeeding	1	11.4
	Infant	A	M15	M15					1	0.4
B539	Mother	A		M6	M6	260	403,300	Breastfeeding	3	9.3
	Infant	A	M9	M9					3	1.6
S208	Mother	A		W6	W14	219	Not done	Breastfeeding	5	3.6
	Infant	A	M6	M6					1	0.8

[a] W0, delivery; W, week after delivery; P32: 32 weeks of pregnancy; M, month after delivery.
[b] Maximum distance among nucleotide sequences spanning the V1 to V5 of envelope within each subject.
[c] For each mother-infant pair whose identification number is in boldface characters, maximum distances were among nucleotide sequences of V1, V2, and V3 of envelope within each subject. Data for those mother-infant pairs have been submitted for publication by Rainwater et al.

mother and her infant, with no evidence of cross-subject contamination. Phylogenetic and Bootscan analyses with subtype reference sequences indicated that G505, J613, L274, and I206 were infected with subtype A, J412 with subtype C, L035 and F535 with D/A recombinants, of which the V1-V2 loops of envelope were subtype D and the V4 loops were subtype A, and K184 with a C/D recombinant, of which C1 of envelope was subtype C and the V1 to V5 of envelope was subtype D (data not shown).

In all but one case, L035, sequences from the infant formed a subcluster within the framework of sequences from the paired mother, supporting the notion that a single variant was transmitted. The maximum nucleotide distance in the V1-to-V5 region of envelope ranged from 3.0% to 9.5% for each mother and 0.2% to 0.6% for each infant, excluding L035 (Table 1). L035 infant sequences formed two subclusters, suggesting that two distinct variants, with a 3.0% distance in envelope V1-to-V5 nucleotide sequences, were transmitted. To verify this, envelope clones were obtained from the infant plasma RNA, and both variants were found, suggesting that the results with PBMC DNA were unlikely to be the result of contamination with maternal sequences.

Sensitivity of envelope variants to neutralization by maternal plasma. The cloned envelopes were used to generate pseudotyped viral particles, of which infectivity was examined by infecting TZM-bl cells (46). Each of the 78 envelope variants capable of infecting TZM-bl cells was examined for sensitivity to neutralization by maternal plasma. Typically, the mothers harbored a mix of NtAb-sensitive and -resistant envelope variants, and variants from the infants were resistant to neutralization by maternal plasma. This is perhaps best illustrated by transmission pair G505 (Fig. 2A), for whom four of seven variants from the mother were neutralized (>50%) by autologous plasma but none of the three variants from the infant were neutralized by the same maternal plasma.

The neutralization sensitivities of envelope variants were compared between infants and mothers, including the eight cases described here as well as the four cases ($n = 18$ clones) from this cohort described previously (Rainwater et al., submitted). In 10 transmission pairs, the median IC_{50} of variants

FIG. 1. Neighbor-joining tree based on distance, showing the epidemiologic linkage between envelope sequences from mother (◆) and infant (Δ) subjects within each transmission pair. A total of 78 nucleotide sequences spanning the V1-to-V5 region of envelope, with 150 reference sequences from group M HIV-1, were codon aligned and subjected to phylogenetic tree construction using the general time-reversible model. Two subtype K references were used as an outgroup. Horizontal branch lengths are drawn to scale. Subtypes and bootstrap values are indicated to the left of each node. The location of each mother-infant transmission pair is indicated.

from the infant was lower than that from the mother; in the other two pairs, I369 and B539, which were cases from the previous study (Rainwater et al., submitted), the IC_{50}s for variants from both infants and mothers were low or undetectable, indicating resistance to neutralization by maternal plasma (Fig. 2B). To formally test whether variants differ between infants and mothers, we compared the 32 aggregate variants from infants to the 64 aggregate variants from mothers (Fig. 2C). Because the IC_{50} of 25 was the detection limit, we transformed the IC_{50}s into binomial data grouped as either <25 or ≥ 25. We then used GEE with a logit link and exchangeable correlation structure to analyze the data. The analysis showed a significant difference in IC_{50}s of variants between infants and mothers ($P = 0.02$), with an odds ratio of 0.43 (95% confidence interval, 0.21 to 0.87), indicating that compared to maternal variants, variants from infants were less likely to have an $IC_{50} \geq 25$. Because this analysis ignored data relating to the magnitude of IC_{50}s, we used Wilcoxon signed-rank testing to compare the median IC_{50} of each infant to the median IC_{50} of the paired mother (Fig. 2D). The result of this comparison indicated that the median IC_{50}s of variants from infants were significantly lower than those from mothers ($P = 0.02$). This association was also observed when the two transmission pairs with more limited number of clones (I369 and B201) were excluded from the analysis (data not shown). This suggests that variants from the infant are more resistant to maternal NtAbs than the overall population of viruses in the mother.

Lengths of envelope variable loops and number of PNGS. For eight transmission pairs, the mean V1-to-V5 length of envelope sequences from the infant was smaller than that from the mother; for the other four pairs, the value from the infant was either equal to or greater than that from the mother (Fig. 3A). The V1-to-V5 envelope sequences from nine infants had a smaller mean number of PNGS than those from their mothers; in the other three pairs, envelope sequences from the infant had mean numbers of PNGS equal to or greater than those from the mother (Fig. 3B). To formally test whether the V1-to-V5 lengths and numbers of PNGS in this region of envelope were different between sequences from infants and mothers, we used GEE with a Gaussian link and exchangeable correlation structure for analysis (Fig. 3C and D). Results from these analyses indicated that the V1-to-V5 lengths of envelope did not differ between infants and mothers ($P = 0.377$). However, the numbers of PNGS in this region significantly differed between the two groups ($P = 0.004$; slope $= -1.07$; 95% confidence interval, -1.83 to -0.35), despite the fact that the V1-to-V5 length and the number of PNGS were correlated (data not shown). The difference in number of PNGS was independent of viral subtype. Restriction of these analyses to V1 to V2 or V1 to V4 of envelope also indicated no significant difference in length but significantly fewer PNGS in sequences from infants compared to those from their mothers (data not shown). Similar results were also obtained when the cases in which there may not have been representative sampling of sequences due to small number of clones (I369 and B201) were excluded (data not shown).

To specifically map where the absence of PNGS in infant sequences occurred, we recorded the positions of PNGS in envelope sequences spanning V1 to V5 from mother-infant pairs according to the HXB2 sequence (GenBank accession number K03455). The positions where PNGS were absent in sequences from infant but present in maternal sequences are summarized in Fig. 3E. The absence of PNGS at positions in V1 and V2, at 339 and 363 in C3, at 392 in V4, and at 465 in V5 was common to at least two infants. The absence of these PNGS was mostly caused by deletions in V1 and by mutations in other regions.

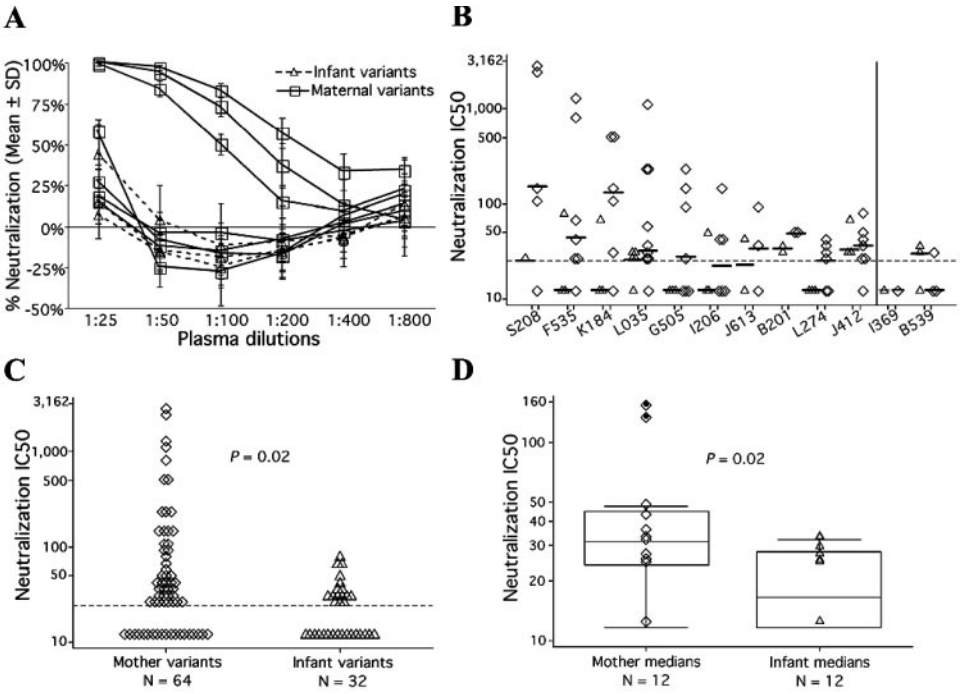


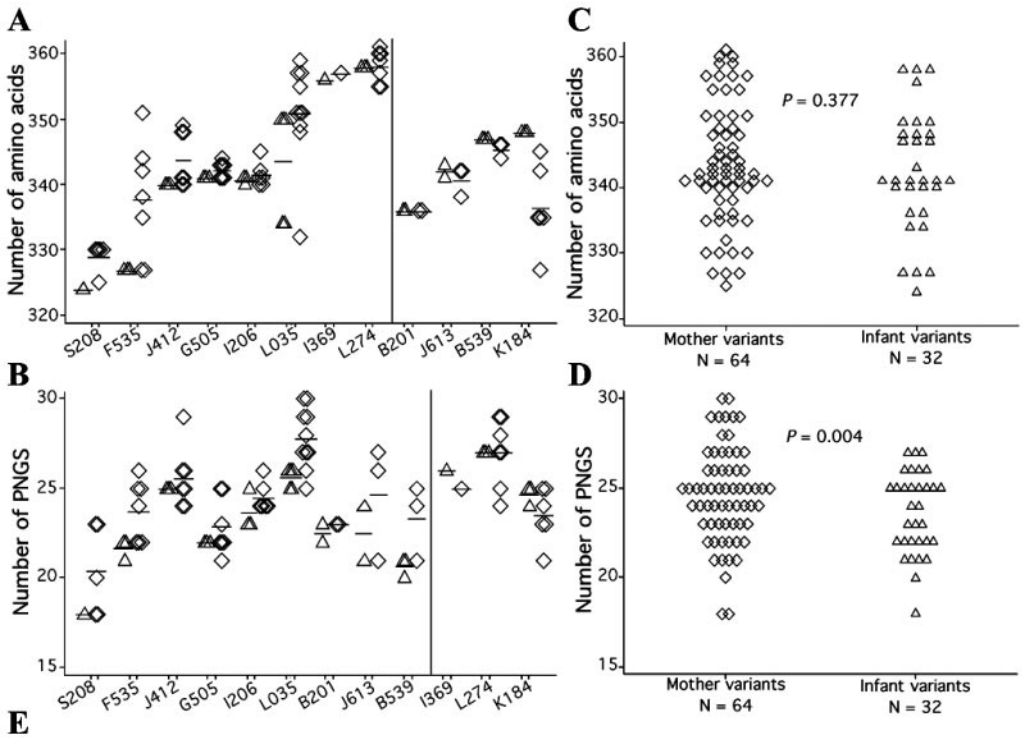
FIG. 2. Neutralization sensitivity of envelope variants for maternal plasma. (A) Percent neutralization versus plasma dilution of viruses pseudotyped with envelope variants from transmission pair G505. (B) Comparison of neutralization IC_{50}s between variants from mothers (◇) and those from infants (Δ) within each transmission pair. The horizontal bars indicate the median IC_{50} for each subject. The vertical line divides the transmission pairs into two groups: to the left are pairs with lower median IC_{50}s of variants from infants; to the right are pairs with median IC_{50}s of variants from infants equal to or higher than the median IC_{50}s of variants from each paired mother. The dashed horizontal line indicates the lowest dilution tested. (C) Comparison of IC_{50}s between variants from mothers (◇) and from infants (Δ) performed by GEE using a logit link and exchangeable correlation structure. The *P* value of the comparison is shown. (D) Box plot of median IC_{50} of variants from mothers and infants. The *P* value is for the comparison of the median IC_{50} of variants from each infant to the median IC_{50} of variants from the paired mother (two-sided Wilcoxon signed-rank test).

Because both escape from maternal NtAb and envelope sequence features such as number of PNGS contribute to the selection of vertically transmitted variants, we further examined the relationship between NtAb sensitivity and sequence features such as length and number of PNGS of envelope. We found that shorter V1-to-V5 length correlated with greater neutralization sensitivity to autologous plasma (or maternal plasma for infant variants) ($P = 0.006$) (Fig. 4A) but that the number of PNGS did not predict neutralization sensitivity ($P = 0.34$) (Fig. 4B). Restriction of the length and PNGS numbers within V1 to V2 or V1 to V4 of envelope also indicated that shorter variable loops correlated with greater NtAb sensitivity and that the number of PNGS did not predict NtAb sensitivity (data not shown).

Because an increase in sequence diversity of envelope is driven in part by NtAbs, we also examined the relationship between genetic distance and NtAb sensitivity of envelope variants from six mother-infant transmission pairs in which the mother subject harbored at least one NtAb-sensitive variant with an IC_{50} above 100 (Fig. 5). Of the six mother subjects analyzed, we found that for two subjects, L035 and K184, the NtAb-sensitive variants clustered or tended to cluster together (Mantel test of genetic distance and difference in NtAb sensitivity: for L035, $r = 0.48$, $P = 0.006$; for K184, $r = 0.43$, $P = 0.1$), whereas in the other four subjects, F535, G505, I206, and S208, the NtAb-sensitive variants scattered in different branches in the phylogenetic tree (Mantel test: for F535, $r = -0.18$, $P = 0.5$; for G505, $r = 0.16$, $P = 0.5$; for I206, $r = -0.09$, $P = 0.8$; for S208, $r = 0.3$, $P = 0.3$). Similar observations were reported by Frost et al., who also found both patterns existed in chronically infected individuals and described one case for each pattern (15). These findings indicate that NtAb-resistant variants could arise from a single founder strain within an individual or from different founder strains by multiple mechanisms.

Development of NtAb in infants. To examine whether variants that escaped NtAb in the mother were capable of eliciting NtAb in the infant, we examined autologous response in six infected infants from whom the follow-up plasmas were available. The transmitted variants induced variable NtAb response, with peak IC_{50}s ranging from 91 to a strikingly high titer of 21,650 (Fig. 6). The de novo production of NtAbs was evident at about 6 months after the infants became infected. The observed IC_{50}s for infants BF535 and BJ613 at early time points (weeks 6 and 14) were likely to represent passive transfer of maternal antibodies, because the corresponding maternal plasmas at week 6 and 14 also contained detectable NtAbs against the transmitted variants (data not shown), and the IC_{50}s for infant BF535 and BJ613 plasmas later (at month 6 and week 14, respectively) dropped to undetectable levels before rising again.

Sensitivity of the transmitted variants to neutralization by MAbs. Ten full-length envelope variants from nine infants (one from eight infants and two distinct sequences from the



E

ID		Number of clones	N-terminal part of V1		C-terminal part of V2		C-terminal part of C2				N-terminal part of V3	N-terminal part of C3				N-terminal part of V4	Central V5
			135/136	140-146	184	186/188	241	276	289	295	301	332	339	356	363	392	465
B539	Infant	3						M			M					M	
	Mother	3						2(67%)			2(67%)					2(67%)	
S208	Infant	1	D	D		M									M		
	Mother	5	3(60%)	2(40%)		1(20%)									4(80%)		
G505	Infant	3			M		M					M					M
	Mother	7			2(29%)		6(86%)					2(29%)					5(71%)
L274	Infant	3		D				M					M		M		
	Mother	7		6(86%)				2(27%)					1(14%)		2(29%)		
I206	Infant	3			M											M	
	Mother	6			3(50%)											2(33%)	
J613	Infant	2		M						M			M	M			D
	Mother	3		3(100%)						2(67%)			3(100%)	3(100%)			2(67%)
F535	Infant	3	D	D		D							M		M		
	Mother	7	5(71%)	4(57%)		3(43%)							5(71%)		2(29%)		
L035	Infant	5	D	D													
	Mother	10	3(30%)	5(50%)													
J412	Infant	3	D			M										D	
	Mother	7	3(43%)			4(57%)										4(57%)	

FIG. 3. Comparison of amino acid V1-to-V5 sequences between maternal (◇) and infant (Δ) variants. (A) Plot of the length of sequences from each transmission pair. (B) Plot of the number of PNGS of sequences from each transmission pair. In panels A and B, the horizontal bars indicate the mean value for each subject. The vertical line divides the transmission pairs into two groups: to the left are pairs with lower mean values from infant; to the right are pairs with equal or higher mean values from infants compared to the mean value from each paired mother. (C) Comparison of length of the aggregate sequences from mothers and infants. (D) Comparison of number of PNGS of the aggregate sequences from mothers and infants. In panels C and D, *P* values are from analysis using a GEE model with a Gaussian link and exchangeable correlation structure. (E) Positions within the V1-to-V5 region of envelope where a PNGS was absent in variants from infant but present in variants from the paired mother. The number and proportion of maternal envelope variants containing the PNGS at the indicated positions are shown. The absence of PNGS caused by deletions or mutations is indicated by D or M, respectively. The PNGS positions were assigned according to the HXB2 amino acid sequence.

ninth infant, who received two distinct variants from the paired mother) were tested for neutralization sensitivity by MAbs. Three cases from the previous study (Rainwater et al., submitted) were excluded because the available envelope clones were chimeras encoding a heterologous transmembrane sequence which included the epitopes targeted by two of the MAbs. All 10 envelope variants were resistant to 2G12 (Table 2), consistent with the lack of PNGS at positions 295, 332, 339, and 392, which are critical for 2G12 recognition (37, 38, 44). The MAb biz, which recognizes a complex epitope in the CD4-binding domain of envelope surface unit gp120 (36), neutralized 3 of the 10 variants tested, with IC_{50}s of 0.8, 9.6, and 41.7 μg/ml (Table 2). The 2F5 and 4E10, which target the DKW and WFXI motifs, respectively, in the envelope transmembrane unit gp41 (3, 32, 34, 43, 49), neutralized nine and seven variants, respectively, with various potencies. Variants with changes within the antibody epitope (e.g., BF535.A1 and BJ412.S3) were resistant to neutralization by that MAb. In addition, variants BJ613.E1 and BK184.D2 were resistant to neutralization by 4E10, even though they contained sequences in the 4E10 epitope NWFDIT identical to those of variants that displayed sensitivity to neutralization by 4E10, suggesting that regions outside of NWFDIT also influence

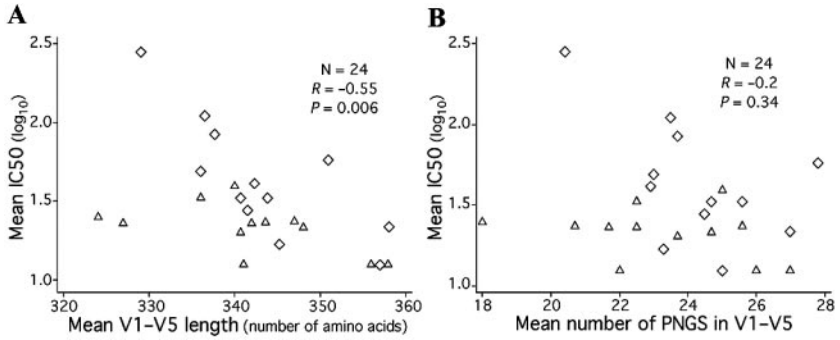


FIG. 4. Correlation of neutralization sensitivity with V1-to-V5 length (A) and PNGS (B) in this region. The mean values of envelope variants from each mother (◇) and infant (Δ) were used. The coefficient *R* and *P* values were generated using Spearman's correlation.

4E10 neutralization or that exposure of the 4E10 epitope may depend on structural context (3). TriMab, which contained both anti-gp41 antibodies and 2G12, exhibited breadth and potency of neutralization comparable to that seen with the anti-gp41 antibodies; neutralization was not detected for three variants, BJ613.E1, BF535.A1, and BJ412.S3.

DISCUSSION

It is generally agreed that a potent and broad NtAb response by effective vaccines is needed to confer protection against HIV-1 infections. Thus, to aid in the design of vaccines, we characterized variants that are vertically transmitted, in a setting where antibodies are present, and examined the role of NtAb in selecting these variants during transmission. We examined the sensitivity of a total of 96 envelope variants from 12 MTCT pairs, each from an independent PCR amplification, to neutralization by maternal plasma. We compared 32 variants from the infants to 64 variants from the mothers and found that near the time of transmission, variants from infants were more resistant to neutralization by maternal plasma than was the overall virus population from their mothers.

The maternal viral variants displayed a wide range of neutralization sensitivities, even within the quasispecies from a single subject. The neutralization-sensitive variants may reflect the fact that these viruses are expressed from cells that have not undergone rapid turnover, such as "quiescently" infected resting T cells and/or monocytes/macrophages. Alternatively, the contemporaneous presence of both neutralization escape and sensitive variants may suggest the emergence of escape variants before the clearance of sensitive strains. Indeed, this dynamic has previously been observed during chronic HIV-1 infection (35, 42, 47). The presence of variants that were sensitive to neutralization was not surprising given that we selected cases in which there was evidence for neutralization of the maternal primary isolate by her autologous plasma.

The selection of NtAb-resistant variants during MTCT is in apparent contrast to the results seen with five cases of heterosexual transmission in discordant couples in which the viruses that were transmitted were among the more neutralization-sensitive variants in the index case (11). One explanation for the difference is that sexual transmission occurs in the absence of preexisting NtAb in the exposed person; thus, the fitness of a variant for transmission may be determined by other properties of the virus. In contrast, in the setting of MTCT, infants passively acquire maternal antibodies that usually persist for approximately 10 months after birth (12, 18). In this regard, MTCT represents a model of transmission that occurs in the face of NtAb pressure. In this setting, it may be more difficult for vaccines that induce NtAb with suboptimal breadth or

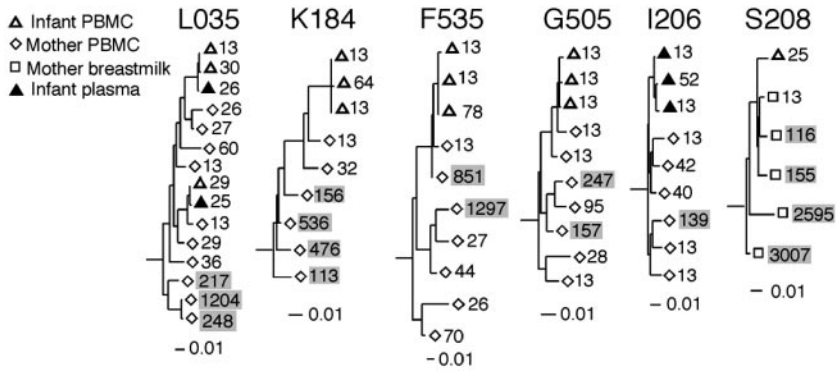


FIG. 5. Maximum likelihood analysis of nucleotide sequences spanning the V1-to-V5 region of envelope from six mother-infant transmission pairs. Horizontal branch lengths are drawn to scale. Each symbol represents an individual variant sequence. The panel at the top left corner is the key to the type of samples from which the envelope sequences were derived. The neutralization sensitivity of each variant to maternal plasma is indicated by the IC_{50} value (rounded to integers) next to the corresponding symbol of the variant. IC_{50}s over 100 are highlighted.

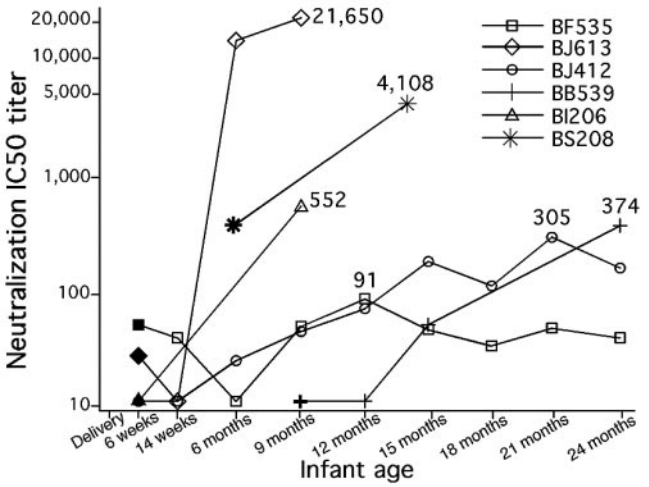


FIG. 6. Neutralization IC_{50}s of six envelope variants vertically transmitted by autologous infant longitudinal plasmas. Plasma collection times are indicated on the horizontal axes. Neutralization IC_{50}s are plotted on the vertical axes. The peak IC_{50} values are indicated. Symbols in boldface indicate the time points when each infant first tested positive for HIV.

potency to block the transmitted strains, as the bar for neutralization may be high.

The transmitted variants were capable of eliciting NtAb response in the infants. In fact, in two cases, NtAbs responses were high (IC_{50}s of 4,108 and 21,650). These infant NtAbs were unlikely to be derived from maternal antibodies because in the limited maternal follow-up plasmas tested, the NtAb titers of maternal plasmas were much lower than those of the infant plasmas from the same time points (data not shown). These results suggest that the transmitted envelopes are able to elicit NtAb response in the newly infected hosts.

Transmembrane-directed antibodies 2F5 and 4E10 displayed greater breadth of neutralization against the transmitted variants than did antibodies directed to the envelope surface unit (biz and 2G12), which is consistent with previous observations that 2F5 and 4E10 generally show greater breadth of neutralization than biz and 2G12 (3, 25). None of the viruses were neutralized by 2G12, and biz was also mostly ineffective, with a few exceptions. Most importantly, TriMab (mix of 4E10, 2F5, and 2G12), which is being tested for efficacy in blocking MTCT, failed to neutralize 3 of 10 variants and neutralized several others with limited potency. On the basis of these observations, we conclude that the MAbs tested in this study may have limited benefit in protecting against most vertically transmitted non-subtype B viruses. It is unclear whether the vertically transmitted variants are more resistant to these MAbs and other NtAbs than viruses transmitted by other routes or viruses present during chronic infection. The fact that infant viruses could be neutralized by the infants' antibodies suggests that vertically transmitted viruses are capable of being neutralized by antibodies of the proper specificity. The lack of potency of the MAbs tested here may in part reflect the fact that the viruses under study are subtype A, C, and D variants, whereas the MAbs were derived from subtype B-infected persons. Thus, future passive strategies may benefit by combining the most potent of these with additional MAbs from subjects infected with diverse strains.

We also found that compared to variants in the index case, vertically transmitted variants contained significantly fewer PNGS in envelope, specifically in regions near the stem of the V1V2 loop, at positions 339 and 363 in C3, 392 in V4, and 465 in V5. This was somewhat surprising given that neutralization resistance is typically associated with increased PNGS in viruses that evolve during chronic infection (8, 47). Yet in the case of the transmitted viruses examined here, an inverse correlation appears to exist, suggesting that there may be other advantageous properties for less-glycosylated viruses during transmission. As predicted on the basis of the predicted structure of CD4-bound HIV-1 gp120 (22), glycans in V1V2 loop may play a role in occlusion of the CD4 binding site, and glycans at positions 276 in C2, 363 in C3, and 465 in V5 are located proximally to residues directly involved in CD4 binding (22, 31). Removal of these glycans could potentially expose the CD4 binding site, thereby increasing viral infectivity.

There was no difference in the lengths of envelope variable loops between mother and infant viruses. Yet greater sensitiv-

TABLE 2. Neutralization sensitivity of the transmitted envelope variants to MAbs and comparison of the antibody epitopes to sequences of the transmitted variants

Envelope	Subtype(s)	Neutralization IC_{50} titer (μg/ml)					Epitope[b]																
							2G12					2F5						4E10					
		Biz	2G12	2F5	4E10	TriMab[a]	**295N**	**332N**	339N	386N	**392N**	E	L	**D**	**K**	**W**	A	N	**W**	**F**	D	**I**	T
BS208.B1	A	0.8	>50	5.9	26.2	12.1	−	+	+	+	+	A	·	·	·	·	·	·	·	·	G	·	S
BG505.B1	A	>50	>50	24.7	38.6	38.2	+	−	+	+	+	A	·	·	·	·	·	·	·	·	·	·	S
BL274.A3	A	9.6	>50	10.7	19.2	12.9	+	+	−	+	+	S	·	·	·	·	·	·	·	·	·	·	S
BI206.P.A1	A	>50	>50	8.6	20	20.5	+	+	+	+	−	A	·	·	·	·	·	T	·	·	·	·	S
BJ613.E1	A	>50	>50	37.5	>50	>50	−	Shift	−	+	+	A	·	·	·	·	T	·	·	·	·	·	S
BF535.A1	D/A	>50	>50	>50	21.4	>50	−	Shift	−	+	+	Q	·	·	**Q**	·	·	·	·	·	S	·	·
BL035.A1	D/A	>50	>50	7.5	13.4	9.7	+	+	+	+	−	A	·	·	·	·	·	·	·	·	·	·	·
BL035.C1	D/A	>50	>50	0.3	1.9	1.5	+	+	+	+	−	·	·	·	·	·	·	·	·	·	S	·	·
BK184.D2	C/D	41.7	>50	14.3	>50	27.2	−	+	+	+	+	Q	·	·	·	·	·	·	·	·	S	·	·
BJ412.S3	C	>50	>50	41.5	>50	>50	+	+	+	+	−	A	·	·	·	·	·	·	·	·	N	·	S

[a] TriMab, mix of equal parts of 2G12, 2F5, and 4E10.
[b] Boldface characters are used to represent the minimum residues required for antibody neutralization; Symbols: −, absent; +, present; shift, absent at position 332 but present at position 334; raised dot, conserved amino acids; underline, creation of a PNGS.

ity to neutralization by maternal plasma was found to correlate with shorter variable loops in the envelope sequence, suggesting that length variation may be one mechanism for altering neutralization sensitivity of these viruses. However, features of envelope other than neutralization sensitivity, such as glycosylation and affinity for CD4 or coreceptor, may also be important characteristics of viruses that are transmitted.

In summary, we identified two mechanisms in the selection of vertically transmitted variants: escape from maternal NtAb and reduced glycosylation in envelope. The latter feature, represented by fewer PNGS in envelope, was also characteristic of variants transmitted heterosexually (9, 11), indicating that this may be a common property of transmitted variants independent of transmission mode. However, escape from NtAb was observed in vertically transmitted variants but the opposite—namely, neutralization sensitivity—was observed in some cases of sexually transmitted variants (11) although not in others (15). It remains to be determined whether there are common characteristics of all transmitted variants, independent of viral subtype and mode of transmission, that may provide insights into the selective pressures that occur during HIV-1 transmission.

For vaccines that aim to block HIV-1 spread by all modes of transmission, the variability in sensitivity to NtAb among variants of different subtypes or selected by different modes of transmission may need to be considered. The panel of vertically transmitted viruses characterized here may represent a useful screen for defining the ability of vaccine immunogens to elicit NtAbs against strains transmitted in regions of high HIV-1 prevalence. These viruses may also constitute a useful panel for assessing the potency of antibody cocktails under consideration as tools for passive therapy to prevent MTCT of HIV-1.

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