Quantitation of human immunodeficiency virus type 1 during pregnancy: Relationship of viral titer to mother-to-child transmission and stability of viral load

(vertical transmission/perinatal infection)

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ABSTRACT To develop strategies to prevent mother-to-child transmission of human immunodeficiency virus type 1 (HIV-1), it is important to define the factors determining it. We examined the relationship between maternal HIV-1 titer and the occurrence of mother-to-child transmission. In addition, we quantitated HIV-1 longitudinally in mothers during pregnancy, at delivery, and up to 1 year postpartum. To examine transmission, we prospectively studied 19 mother-child pairs; in 5 pairs, HIV-1 transmission occurred. We used endpoint dilution culture of peripheral blood mononuclear cells to determine maternal viral titer and found that although 4 of 6 (67%) women with viral titers of ≥ 125 HIV-1 infectious units per 10^6 cells transmitted HIV-1 to their infants, only 1 of 13 (7.6%) women with lower viral titers transmitted ($P = 0.01$). Twelve of the 19 mothers had HIV-1 loads determined serially 3–8 times over periods ranging from 18 to 65 weeks. Viral titers varied greatly between the 12 women, but the viral load in each woman remained stable over time. In this cohort, HIV-1 viral load remained stable during pregnancy and the greater the maternal viral burden, the more likely that transmission occurred. These two related findings suggest that determination of HIV-1 titers early in pregnancy may predict which women are at high risk of transmitting to their infants and may be used to counsel HIV-1-infected women of childbearing age. These data identify maternal viral titer as a major determinant of mother-to-child HIV-1 transmission and thereby provide the scientific rationale for therapeutic strategies designed to interrupt transmission by lowering viral load.

Human immunodeficiency virus type 1 (HIV-1) transmission from mother to child is now a major route of infection worldwide (1, 31). To develop strategies to prevent mother-to-child transmission, it is important to define the factors determining it (2). The rate of transmission averages 25% (1), but why some mothers transmit to their infants and the majority do not is not well understood. Studies to date suggest there are multiple determinants including maternal immune response, properties of the mother's virus, and obstetric factors (1–8). Women are more likely to transmit during primary HIV-1 infection and advanced disease (2–6), both associated with high viral loads (9–11). Results of a National Institutes of Health AIDS Clinical Trial Group (NIH ACTG) trial of zidovudine to prevent mother-to-child transmission demonstrated efficacy of antiviral therapy in reducing transmission (12), consistent with the concept that transmission is related to maternal viral burden. Systematic determination of maternal HIV-1 load and its relationship to mother-to-child

transmission, however, has not been reported. We therefore quantitated maternal HIV-1 load and prospectively examined its role in mother-to-child transmission.

Mother-to-child transmission can occur *in utero*, at birth, or postnatally through breast-feeding (1–4, 7, 13–16). Independent of HIV-1 infection, pregnancy is an immunodeficient state characterized by decreased numbers of CD4⁺ T lymphocytes and greater severity of viral infection (17). In addition, there are reports of a more rapid rate of decline of CD4⁺ lymphocyte counts in HIV-1-infected pregnant women than in other HIV-1-infected populations and accelerated HIV-1-related disease during or after pregnancy (18–20). Because an increase in HIV-1 load has been correlated with advanced disease and CD4⁺ cell decline (9–11), it was suspected that HIV-1 titers might increase with pregnancy. For these reasons, this study included longitudinal quantitation of HIV-1 during pregnancy and delivery as well as postpartum.

MATERIALS AND METHODS

Subjects. Nineteen HIV-1-infected women participated in a prospective study of mother-to-child transmission at Stony Brook University Hospital in Stony Brook, NY, and St. Joseph's Medical Center in Paterson, NJ, enrolling during pregnancy or at delivery. The institutional review boards at each hospital and the New York State Department of Health approved the investigation and each woman provided informed consent for herself and her child. Four mother-child pairs participated in NIH ACTG protocol 076 (12), a randomized, controlled trial of zidovudine administered to mothers during pregnancy and labor and to their infants during the first 6 wk of life to prevent maternal-child HIV transmission (protocol cochairs, Rhoda Sperling and Edward Connor). T-cell subset analyses were determined locally by flow cytometry.

To examine the relationship of maternal viral load and vertical transmission, we studied 19 mother-child pairs who met the following criteria: (i) the mothers had HIV-1 titers determined at the time of delivery or during gestation and at delivery and (ii) the children born to these mothers have been followed ≥ 6 mo and are HIV-1 uninfected as determined by both viral culture and PCR or had HIV-1 infection documented by positive culture. A 6-mo virologic follow-up period was chosen because recent studies demonstrated detection of HIV-1 by using culture alone or culture and PCR in $>90\%$ of infected babies by 1–3 mo and in 100% of infected babies by 6 mo (15, 21, 22). We performed serial cultures for

HIV-1 on blood from each child starting at birth and including the sample obtained at the latest follow-up visit. We performed PCR on the last follow-up sample to confirm culture results. Twelve of the 19 mothers included in the transmission study had HIV-1 titers determined at least three times during pregnancy, at delivery, and postpartum and were also studied to determine the effect of pregnancy on viral load. We confirmed the HIV-seropositive status of one woman with repeatedly negative cultures by performing an ELISA and Western blot.

HIV-1 Quantitation and Culture. We quantitated HIV-1 in mothers by using endpoint dilution culture of peripheral blood mononuclear cells (PBMCs), employing the procedure of the NIH ACTG (23, 24). Subjects' PBMCs were serially diluted 1:5 (range, 1×10^6 to 3.2×10^2 cells per ml) in duplicate and then cocultivated with 1×10^6 stimulated normal donor PBMCs per ml. After 2 wk, culture supernatants were tested for production of HIV-1 p24 antigen by ELISA (Coulter or DuPont). The smallest number of PBMCs required to produce a positive culture was taken as the endpoint titer, which was expressed as 1, 5, 125, 625, and 3125 infectious units per 10^6 PBMCs (IU/ 10^6 PBMCs). Because the inherent variation of this assay is $1.2 \log_{10}$, a change in titer of $<1 \log$ is considered virologically insignificant (23). We also used cocultivation to detect HIV-1 in infants. To recover virus from infants <1 mo old, we cocultivated $1.5\text{--}3.0 \times 10^6$ infant PBMCs with an equal number of donor PBMCs. For older children, quantitative cultures were performed as described above. To increase HIV-1 detection in children, we maintained cultures for 30–35 days and used the method of Burgard *et al.* (13) to concentrate supernatants and thereby increase the sensitivity of the p24 assay.

PCR. We used nested PCR to detect HIV-1 in uncultivated PBMCs from infants and children, amplifying a 114-bp fragment from a highly conserved region of the HIV-1 *gag* gene. Procedures were primarily those outlined by Simmonds *et al.* (25). Briefly, total cellular DNA was extracted from $0.5\text{--}2.0 \times 10^6$ PBMCs by using the Elu-Quik DNA purification kit (Schleicher & Schuell), with extraction of 10^6 PBMCs typically yielding 6–10 μg of DNA. We first amplified HIV-1 DNA from 1 μg of template DNA by performing 35 cycles (25) of PCR with outer primers GAG1 (sense: nt 582–599 = GCA ACC CTC TAT TGT GTG) and GAG2 (antisense: nt 1238–1302 = CCA ACA AGG TTT CTG TCA TC). Primers were synthesized by the Wadsworth Center Molecular Genetics Core Laboratory and their sequences match that of the reference sequence HIV-1_{HXB2R}. The amplified products were then subjected to another 25 cycles of PCR by using the inner primer pair SK38 and SK39 (nt 1090–1117 and 1177–1204; Perkin-Elmer). In addition, a pair of GenAmp primer- β -globin primers (Perkin-Elmer), which initiated amplification of a 268-bp fragment of human DNA, were used in a parallel reaction to monitor for the presence of DNA and amplification conditions. Approximately 20% of the final amplified products were electrophoresed on a 2% agarose gel and detected by ethidium bromide staining. We routinely detected one copy of HIV-1 control DNA mixed with 1 μg of cellular DNA. Samples were considered positive or negative only if the following criteria were met: negative controls were negative, positive controls were positive, globin amplification was positive, and results of duplicate PCR assays of each sample were concordant. HIV-1 cultivation and PCR yielded concordant results.

Statistical Analysis. We compared the magnitude of maternal viral titers, CD4⁺ counts, and CD8⁺ counts to the occurrence of mother-to-child transmission by using the Mann-Whitney test. Fisher's exact test was used in the analysis of contingency tables for examination of the 5 mothers with high HIV-1 titers and low CD4⁺ counts. Slopes of viral titers over time were calculated by converting the

titers to ranks and using the STATISTIX program (Analytical Software, Tallahassee, FL).

RESULTS

Patient Population. The 19 HIV-1-infected mothers participating in this study represented a broad spectrum of clinical and immunologic states (Table 1). Twelve had CD4⁺ cell counts of >200 cells per mm^3 , all of whom were asymptomatic. Seven women had CD4⁺ cell counts of <200 cells per mm^3 and 3 of these 7 had a history of severe opportunistic infections. Nine women were treated with zidovudine during pregnancy; in most cases, the therapy was intermittent. Eight women were prescribed the drug by their physicians and 1 was randomized to receive it as part of NIH ACTG protocol 076 (Table 1) (12). Three additional subjects were enrolled in NIH ACTG protocol 076 but were randomized to receive placebo. None of the mothers breast-fed.

Viral Load and Transmission. To examine the relationship between maternal viral load and mother-to-child transmission, we determined HIV-1 titers in the 19 women by endpoint dilution culture and prospectively followed their infants to determine whether they became HIV-1 infected (Table 1). Five of the 19 women transmitted HIV-1 to their infants. Four of 6 (67%) women with high viral titers, designated as ≥ 125 HIV-1 IU/ 10^6 PBMCs, transmitted to their infants, although only 1 of 13 (7.6%) women with lower titers transmitted (Fig. 1; Table 1). The relationship of the magnitude of viral titer to occurrence of mother-to-child transmission in this cohort was statistically significant ($P = 0.01$) as analyzed by using the Mann-Whitney test.

CD4⁺ T-lymphocyte counts in this cohort were in general inversely correlated with HIV-1 viral load (Table 1; Fig. 2), as observed in other patient populations (11). Because previous studies of determinants of mother-to-child transmission had observed a correlation between HIV-1 transmission and low maternal CD4⁺ T-lymphocyte count (1, 6), we examined that relationship in our patients. We documented a correlation between low CD4⁺ cell count and transmission in this cohort as well (Table 1) but found that CD4⁺ count was not as strong a predictor of transmission as viral titer ($P = 0.04$ by using the Mann-Whitney test). It is noteworthy that there were 2 women, mothers 14 and 19, who had CD4⁺ cell counts of <200 cells per mm^3 but relatively low viral titers (15 and 5 IU/ 10^6 PBMCs, respectively); these women did not transmit HIV-1 to their infants. Women with the lowest CD8⁺ cell counts were most likely to transmit HIV-1 but this association was not statistically significant. In addition, there was no clear relationship between antiviral therapy and transmission in this population.

To begin to identify a group of women who are at greatest risk of transmitting HIV-1 to their children, we stratified this cohort according to both viral load and CD4⁺ cell count. Of the 5 women who had both high HIV-1 titers (≥ 125 IU/ 10^6 PBMCs) and low CD4⁺ cell counts (<200 cells per mm^3), 4 (80%) transmitted HIV-1 to their infants as compared to only 1 (7%) of the remaining 14 women ($P = 0.0061$ by using Fisher's exact test). This stratification was highly effective in identifying HIV-1-infected women who are at very high risk for mother-to-child transmission.

Serial Viral Titers. To examine the effect of pregnancy on HIV-1 titer and disease progression, we performed a longitudinal study of 12 of the 19 women described above; these women had HIV-1 titers determined between 3 and 8 times during pregnancy, at delivery, and up to 1 yr postpartum (Fig. 2). The average duration of follow-up was 39 wk, with a range of 18–65 wk; the mean number of serial samples was 4.6. One woman, mother 16, had PBMCs cultured for HIV-1 on four separate occasions, spanning a 1-yr period from 15 wk of gestation to 27 wk postpartum; on each occasion, the viral

Table 1. Virologic and clinical characteristics of mother-child pairs

Mother-child pair	Characteristics of mothers during pregnancy and delivery						Characteristics of children	
	Age, yr/ HIV risk	CDC class*	CD4 ⁺ count, cells per mm ³	CD8 ⁺ count, cells per mm ³	Anti-HIV therapy	HIV-1 titer, [†] IU/10 ⁶ PBMCs	Clinical status*	Follow-up, mo
Transmission-positive families								
1	28/hetero	C3	160	730	—	625	P2A,B	16
2	28/hetero	A3	146	334	AZT	225	P2A,E	9
3	36/hetero	C3	43	122	AZT	125	P2A	6
4	21/hetero	A2	470	680	076 placebo	25	P2A,B	6
5	34/IDU	B3	30	NA	—	125	P2A,B	12
Transmission-negative families								
6	29/hetero	A2	380	513	—	5	Well	15
7	23/hetero	A2	467	759	AZT	125	Well	17
8	27/hetero	A1-A2	420	784	AZT	25	Well	16
9	25/IDU	A2	400	1154	AZT	17	Well	10
10	29/hetero	A2	267	748	—	25	Well	6
11	36/IDU	A1	650	720	076 placebo	25	Well	15
12	34/transfusion	A3	154	514	AZT	125	Well	6
13	29/IDU	A1	537	531	AZT	75	Well	7
14	35/hetero	A3	160	1370	AZT	15	Well	6
15	33/IDU	A2	285	635	076 AZT	1	Well	10
16	36/IDU	A1-A2	571	951	—	No growth	Well	6
17	33/hetero	A2	380	720	076 placebo	25	Sickle cell disease	6
18	28/hetero	A1	520	NA	—	1	Well	6
19	29/IDU	C3	76	450	—	5	Well	14

IDU, injecting drug use; hetero, heterosexual transmission; AZT, zidovudine; 076, NIH ACTG protocol 076; NA, not available.

*HIV-1 infection status of adults was described using the 1993 Revised Centers for Disease Control (CDC) classification system (26), which classifies individuals according to both clinical status and CD4⁺ count into nine exclusive categories. Clinical category: A, symptomatic infection; C, severe AIDS-defining disease; B, symptomatic conditions not included in A or C. CD4⁺ cell category: 1, counts ≥ 500 per mm³; 2, counts between 200 and 499 per mm³; 3, counts < 200 per mm³. Pediatric HIV-1 infection was described according to the CDC classification system for HIV in children (27): P2A, symptomatic infection with nonspecific findings; P2B, progressive neurologic disease; P2E, other diseases including thrombocytopenia.

[†]The HIV-1 titer for 7 women (mothers 4, 5, 6, 10, 17, 18, and 19) was determined at delivery; for the other 12 mothers, the HIV-1 titer is a mean of the values determined at ≥ 2 time points during pregnancy and at delivery.

culture was negative. The other 11 women had detectable virus at each time point (Fig. 2). Viral titers in the 11 culture-positive women varied from a low of 1 to a high of 625 IU/10⁶ PBMCs. The viral load in each woman, however, remained stable during pregnancy, at delivery, and postpartum (Fig. 2). Four women showed no change in viral titer at all (mothers 3, 11, 12, and 16) and the others in general showed a change of only 1 dilution in magnitude (Fig. 2). Furthermore, in each of the 12 women, the first viral titer was the same as the last or differed by only one 1:5 dilution, a level of change encompassed by the inherent variation of the assay (23). The minor

fluctuations in viral titer in PBMCs in this small number of patients did not bear a clear relationship to antiviral therapy.

Statistical analysis of the variation in viral titer by determination of the slope over time showed that 7 of 12 women had titers with a slope of 0, the median slope was 0, and the mean slope was 0.006 (slopes for each woman are indicated in Fig. 2). This analysis underscores the stability of viral load with passage of time. Clinical status in these women remained stable during the period of observation as well. None of the women who were asymptomatic when they entered the study developed symptoms during pregnancy or postpartum and none of the women with a history of severe opportunistic infection (Centers for Disease Control clinical category C) (26) died.

DISCUSSION

This systematic study identified maternal HIV-1 titer as a major determinant of mother-to-child transmission. These data help to elucidate the pathogenesis of HIV-1 mother-to-child transmission and are directly relevant to its prevention. A clinical trial recently showed that antiviral therapy with zidovudine was effective in reducing the mother-to-child HIV-1 transmission rate (12) and trials of other chemotherapeutic and immunoprophylactic interventions are under development worldwide (28). No consensus has been reached, however, regarding treatment recommendations to prevent transmission of HIV-1 during pregnancy and delivery (12). The identification of high maternal viral load as a major determinant of mother-to-child transmission provides the scientific rationale for implementing strategies to interrupt transmission by reducing viral titer (28).

FIG. 1. Relationship of maternal HIV-1 titer to mother-to-child transmission. The maternal titer is the titer obtained at delivery (7 mothers) or the mean titer obtained during pregnancy and at delivery (12 mothers), as indicated in Table 1.

FIG. 2. Serial maternal HIV-1 viral titers and CD4⁺ counts. Data are expressed as HIV-1 IU/10⁶ PBMCs (●) and number of CD4⁺ cells per mm³ (○). Data, including slope of viral titer over time, are presented here for the 11 HIV-1 culture-positive women who had viral loads determined ≥ 3 times. Mother 16 had negative viral cultures on four occasions and her viral titers were not graphically represented. Because the four cultures were all negative, the slope of viral titer over time for mother 16 was judged to be 0.

Identification of high maternal viral titer as one of the determinants of mother-to-child transmission provides a quantitative virologic foundation for studies linking increased risk of transmission to maternal primary infection, advanced HIV-1 disease, and surrogate markers such as HIV-1 p24 antigenemia and low CD4⁺ count (1–6); these factors are associated with high viral burden (9–11) but earlier studies did not measure viral titer. The present study documented a correlation between low CD4⁺ cell count and transmission but found that CD4⁺ count was not as strong a predictor of transmission as viral load. Other immunologic and viral factors

have also been shown to play a role (1–3, 8). Mothers with phenotypically virulent HIV-1 strains *in vitro* were found to have an increased risk of transmission as compared to those with viruses that replicate more slowly (8). Biologic phenotype of the mother's isolate may be an independent factor influencing transmission, but because virulent phenotype has been correlated with severity of HIV-1-related disease, it is likely that virulent phenotypes occur in women with high viral loads.

Although the data presented here identify maternal viral load as a key determinant in mother-to-child transmission, they also underscore the concept that multiple factors con-

tribute to such transmission. One mother in this cohort, mother 4, had a viral titer in the middle range (25 IU/10⁶ PBMCs) yet transmitted HIV-1 to her infant *in utero* as judged by a positive viral culture in the baby on the day of birth. Mothers 7 and 12, by contrast, had high viral loads (125 IU/10⁶ PBMCs) but did not transmit. These findings suggest that factors other than maternal HIV-1 titer such as maternal viral phenotype, viral tropism for placental macrophages, immune response, and obstetric factors resulting in exposure of the infant to maternal blood also play a role in determining whether and when an infant becomes infected (1–3, 6–8, 14).

The findings that maternal HIV-1 titer is a determinant of mother-to-child transmission and that viral titer remains stable during pregnancy may provide the scientific underpinnings for counseling of HIV-1-infected women of child-bearing age. These two related results suggest that determination of HIV-1 titers early in pregnancy may predict which women are most likely to transmit to their infants. Although larger studies will be necessary to quantify the precise risk of HIV-1 transmission associated with a particular viral titer, stage of disease, and antiviral therapy, it is noteworthy that by stratifying women according to both CD4⁺ count and viral titer, we were able to identify a subset of infected women in this cohort who had an extremely high risk (80%) of transmitting HIV-1 to their infants. This high transmission rate contrasts sharply with the overall rate of 26% in this cohort, which closely resembles the average mother-to-child transmission rate in the United States (1). The data presented here suggest that, for women with high viral loads, the HIV-1 mother-to-child transmission rate is much greater than average, and for those with very low viral titers it is lower.

We also addressed the question of whether pregnancy is associated with an acceleration of HIV-1 infection and found that viral load was stable during an average follow-up period of 39 wk. It is particularly interesting that one woman, mother 16, had four consistently negative viral cultures performed during a 1-yr period. If pregnancy had the effect of increasing HIV-1 viral load, it would be predicted to increase it to the level of detection by viral culture. Although normal gestation is considered an immunosuppressive state and early reports raised the possibility that pregnancy might lead to acceleration of HIV-1-related disease (18–20), prospective studies encompassing asymptomatic women did not show an adverse effect of pregnancy on clinical status (20, 29, 30). Our findings provide a virologic basis for these data.

Two reports describing the procedure for endpoint dilution culture examined stability of viral load in PBMCs by studying several nonpregnant patients serially for 4–20 wk (9, 24). These studies reported the same small degree of variation in viral load observed in our cohort. One of these early studies also examined the effect of a 4-wk period of zidovudine treatment on HIV-1 titer (9). Although viral titers in plasma decreased significantly after therapy, the HIV-1 titer determined by endpoint dilution culture of PBMCs remained the same in 5 of 7 patients and decreased by only 1 log in the other 2. These data are consistent with our finding that initiation of antiviral treatment did not result in a significant change in HIV-1 titer in PBMCs during the period of observation. Particularly because both HIV-1 infection in women and heterosexual transmission are increasing (1, 31), more extensive studies of the effect of pregnancy on HIV-1 infection as well as strategies to interrupt mother-to-child transmission are warranted.

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