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J Clin Invest. 1996;97(2):323-330. https://doi.org/10.1172/JCI118419.

Research Article

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Dynamics of Viral Replication in Infants with Vertically Acquired Human Immunodeficiency Virus Type 1 Infection

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Abstract

About one-third of vertically HIV-1 infected infants develop AIDS within the first months of life; the remainder show slower disease progression. We investigated the relationship between the pattern of HIV-1 replication early in life and disease outcome in eleven infected infants sequentially studied from birth. Viral load in cells and plasma was measured by highly sensitive competitive PCR-based methods.

Although all infants showed an increase in the indices of viral replication within their first weeks of life, three distinct patterns emerged: (*a*) a rapid increase in plasma viral RNA and cell-associated proviral DNA during the first 4–6 wk, reaching high steady state levels (> 1,000 HIV-1 copies/10^5 PBMC and > 1,000,000 RNA copies/ml plasma) within 2–3 mo of age; (*b*) a similar initial rapid increase in viral load, followed by a 2.5–50-fold decline in viral levels; (*c*) a significantly lower (> 10-fold) viral increase during the first 4–6 wk of age. All infants displaying the first pattern developed early AIDS, while infants with slower clinical progression exhibited the second or third pattern.

These findings demonstrate that the pattern of viral replication and clearance in the first 2–3 mo of life is strictly correlated with, and predictive of disease evolution in vertically infected infants. (*J. Clin. Invest.* 1996. 97:323–330.) Key words: vertical infection • pediatric AIDS • HIV-1 replication • primary infection • competitive PCR

Introduction

Mother-to-child transmission of human immunodeficiency virus type 1 (HIV-1) accounts for most pediatric HIV-1 infections. About one-third of these vertically infected infants will have severe early symptoms of infection, and develop AIDS within the first months of life; in the others, the disease will progress more slowly, and a few will remain clinically asymptomatic for several years (1). To understand the pathogenesis of pediatric AIDS, and define strategies to modify the course of infection, we must know more about the factors that contribute to the different patterns of disease expression.

Although the relatively short interval between birth and disease onset strongly suggests a close relationship between the early phase of HIV-1 infection and the clinical outcome, very little quantitative information is available regarding the pattern of HIV-1 replication soon after birth. By using methods to evidence HIV-1 directly, either by cell culture for virus isolation or polymerase chain reaction (PCR) for HIV-1 DNA sequence detection, we and others (2–6) demonstrated that a consistent proportion of infants who were subsequently recognized as HIV-1 infected do not have detectable levels of virus in their peripheral blood cells at birth. Conversely, > 95% of these infected infants have detectable virus levels when tested at 4–8 weeks of age. Plasma viremia and antigenemia are also undetectable at birth in most infected children, and increase during the first weeks of life (7–9). It was proposed that positive or negative HIV-1 detection at birth might reflect viral transmission in utero or during the intrapartum period, respectively (10), but alternative explanations, such as a latent infection or compartmentalization of virus in lymphoid organs before birth, cannot be excluded. Although the precise timing of vertical transmission cannot be pinpointed, the above findings suggest that HIV-1 replicates and spreads to the peripheral blood cells mainly after birth. Moreover, recent observations indicating that a rapid increase of HIV-1 DNA in cells during the first weeks of life is correlated with a rapid onset of disease (11, 12) support a relationship between the HIV-1 replication pattern early in life and the clinical outcome, and stress the need to elucidate the dynamics of HIV-1 activity soon after birth for further comprehension of pediatric AIDS.

In recent years, several techniques based on nucleic acid amplification have been developed to accurately measure viral burden. Competitive PCR (cPCR)[1] and competitive reverse transcription PCR (cRT-PCR) methodologies have been optimized to quantify the number of HIV-1 DNA and HIV-1 RNA copies, respectively, and have been applied to investigate virus activity in HIV-1 infected adults (13–17).

The present study was aimed at elucidating the dynamics of HIV-1 activity during the first months of life in children with vertically acquired infection, and defining the relationship between the HIV-1 replication pattern and disease outcome. To this end, HIV-1 content in plasma, and HIV-1 provirus content in cells were assayed by cRT-PCR and cPCR, respectively, in serial samples that were obtained early in life from children that showed different patterns of disease progression.

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Received for publication 25 July 1995 and accepted in revised form 23 October 1995.

J. Clin. Invest.

0021-9738/96/01/323/08 $2.00
Volume 97, Number 2, January 1996, 323–330

1. *Abbreviations used in the paper:* cPCR, competitive PCR; cRT-PCR, competitive reverse transcription PCR.

Methods

Patients. This study was performed on 11 HIV-1–infected infants who attended the Pediatric Department of Padova University. These children were all born to HIV-1 seropositive mothers, and were enrolled at birth in a large-cohort study on mother-to-child HIV-1 transmission; they were diagnosed as HIV-1 infected on the basis of positive results in both virus culture and PCR assays, as previously reported (2, 11). Informed consent was obtained for all patients; inclusion in this study was based on the availability of frozen plasma and cell samples collected sequentially from birth up to one year of age. The children were classified as asymptomatic (category N), mildly symptomatic (category A), moderately symptomatic (category B), or severely symptomatic (category C), according to the criteria of the Centers for Disease Control (CDC)(18). Immunological status was defined as class 1 (no immunodepression), 2 (moderate immunodepression), or 3 (severe immunodepression) on the basis of the CD4+ cell count/μl adjusted for age, according to the CDC criteria (18). During the study period, four children started antiretroviral therapy, and 1 was enrolled in the double blind randomized Paediatric European Network for Treatment in AIDS (Penta) trial 1 (19) at the time points reported in Results.

Clinical samples and nucleic acid separation. Heparinized peripheral blood samples were centrifuged over a Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient; plasma was recovered from the upper phase, centrifuged at 1000 *g* for 15 min to ensure cell-free specimens, and stored at −80°C until further analysis. Peripheral blood mononuclear cells (PBMC) were recovered from the top of the Ficoll gradient, washed twice with phosphate buffered saline, resuspended in RPMI medium supplemented with 10% fetal calf serum (FCS) and 10% dimethyl sulfoxide, and then cryopreserved until use.

Competitive DNA PCR was performed directly on lysed cells. Briefly, PBMC were washed in PBS, and 2×10^6 were lysed as previously reported (20) in 500 μl of TE buffer (10 mM Tris-HCL [pH 8] and 0.1 mM EDTA) containing 0.001% Triton X-100, 0.0001% sodium dodecyl sulfate, and 600 μg/ml proteinase K.

Plasma virion-associated RNA was obtained by means of an affinity capture method (21) with slight modifications. Briefly, 150 μl of each plasma specimen were incubated for 30 min at 4°C with an anti-gp120 IgG1 mouse monoclonal antibody (Intracel Corporation, Cambridge, MA; 4 μg/100 μl final concentration), and then for an additional 30 min at 4°C with 20 μl of microbeads coated with rat anti–mouse IgG1 (Minimacs; Miltenyi Biotec. Inc., Sunnyvale, CA). Samples were centrifuged at 12,000 rpm for 2 h at 4°C; the pelleted virions were collected and lysed by incubation for 15 min at 37°C in 10 μl of a lysis buffer (10 mM sodium acetate pH 5.5, 0.5 mM $MgSO_4$, 0.01% Triton X-100). To eliminate residual contaminating DNA, 5 U of RNase free DNaseI (Boehringer Mannheim Biochemica, Germany) were added to each sample; DNaseI was then inactivated by incubation at 94°C for 1 min followed by quick cooling on ice.

DNA and RNA competitor templates. Competitive PCR analyses were performed on proviral DNA and viral RNA by using the plasmid pSPLI-II, and its in vitro transcription product as DNA and RNA competitors, respectively. The plasmid pSPLI-II was constructed as detailed elsewhere (22). Briefly, a DNA fragment containing the sequences of four HIV-1 primer-binding sites (highly conserved between different HIV-1 isolates, and corresponding to HIV-1 MN sequence as follows [5′-3′]: 696-723, upstream the first 5′ splice site [primer 1]; 914-889, within the *gag* gene [primer 2II]; 6327-6311, within the *env* gene [primer 3] and 8445-8424, within the second exon of *rev* gene [primer 5][23]) was obtained by recombinant PCR technology, and cloned downstream of the T7 RNA polymerase promoter in the pCRTM II plasmid (TA cloning Kit; Invitrogen Corporation, San Diego, CA). These HIV-1 primer binding sites were chosen to allow the quantification of proviral DNA (using DNA competitor and primer pair 1/2II), and genomic viral RNA, single- and multiple-spliced viral mRNAs (using the RNA competitor and the primer pairs 1/2II, 1/3, and 1/5, respectively), and were arranged in the pSPLI-II in order to produce amplification products which were different in size from the wild-type amplified products, and hence easily distinguishable by gel electrophoresis (22).

To obtain the competitor RNA template, 50 ng of plasmid pSPLI-II were linearized with Kpn1, purified with Microcon microconcentrators (Amicon Inc., Beverly, MA), and transcribed in vitro using a T7 RNA polymerase-based commercial kit (Promega, Madison, WI), in the presence of 1 μl of α-^{32}P dUTP (Amersham, UK; 3000 Ci/mmole; 10 mCi/ml) corresponding to 6×10^6 cpm. After completion of the transcription reaction, template DNA was removed by DNaseI digestion, and competitor RNA was purified by denaturating polyacrylamide gel electrophoresis, eluted from the gel, and resuspended in sterile ribonuclease-free water. An aliquot of the purified competitor RNA preparation was quantified by both spectrophotometric reading at 260 nm, and measurement of bound radioactivity in a counter, as described (24).

Plasmid pBH10 containing the full length HIV-1 genome (25) was obtained from R. C. Gallo (LTBC, NIH, NCI). Plasmid p1-2II, used as template for in vitro transcription of RNA molecules containing the viral sequence amplified by primer pair 1/2II, was obtained by cloning a PCR product derived by amplification with primers 1/2II from pBH10 in plasmid pCRTM II.

Competitive PCR and RT-PCR. To quantify proviral DNA in cells and viral genomic RNA in plasma samples, primer pair 1/2II was used in both cPCR and cRT-PCR reactions. cPCR was performed directly on replicate portions of lysed cells; 15 μl of cell sample (corresponding to 60,000 cells) were amplified along with 2 μl of increasing copies of the plasmid pSPLI-II in 100 μl of PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 0.2 mM of each of the four deoxynucleotide triphosphates [dNTP], 2 mM $MgCl_2$) containing 100 pmol of each of the two primers 1 and 2II, and 2.5 U of Taq polymerase (Perkin Elmer, Norwalk, CT). 45 amplification cycles, each consisting of 50 s at 94°C, 45 s at 62°C, and 50 s at 72°C, were carried out in a Gene Amp PCR System 9600 thermal cycler (Perkin Elmer).

To quantify RNA, replicate portions of RNA samples (2 μl corresponding to 30 μl of plasma) were reverse-transcribed along with 2 μl of increasing-copy numbers of competitor RNA molecules in 20 μl final volume of reverse transcription buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 0.2 mM of each dNTP, 5 mM of $MgCl_2$) containing 50 U of Moloney murine leukemia virus reverse transcriptase (Perkin Elmer), 20 U of RNase Inhibitor (Perkin Elmer), and 100 pmol of antisense primer 2II. RNA samples were preheated for 5 min at 65°C, and then incubated with the reaction mixture for 60 min at 42°C. The reaction was stopped by incubation at 90°C for 5 min, and the samples were cooled on ice. 80 μl of PCR reaction mixture (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 0.2 mM of each dNTP, 2 mM $MgCl_2$) containing 2.5 U Taq polymerase and 100 pmol of the sense primer 1 were added to each tube, and 45 amplification cycles were carried out using the PCR profile described above. One portion of each sample was analyzed without reverse transcription and in the absence of competitor molecule to ensure that DNA contamination was avoided.

To increase the sensitivity of the assay, the sense oligonucleotide primer 1 was radiolabeled with [γ^{33}P]ATP using 1 U of T4 polynucleotide kinase (United States Biochemical, Cleveland, OH) in 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine for 45 min at 37°C, and then inactivated at 90°C for 3 min; 100 pmol of radiolabeled primer were used in each amplification reaction.

A 30-μl aliquot of each 100 μl PCR reaction mixture was run on a polyacrylamide gel and exposed to an x-ray film for 2–4 h at −80°C. The sizes of the competitor and wild-type amplified products were 240 and 218 bp, respectively. The peak areas of the amplified bands were measured by densitometric scanning (Enhancer laser densitometer ULTRASCAN XL, LKB, Pharmacia, Uppsala, Sweden). The logarithm of the ratio between the optical density values of competitor and wild-type amplified products (on the *y*-axis) was plotted against the logarithm of the competitor copy number (on the *x*-axis) and a linear regression curve was extrapolated.

P24 antigen assay. HIV-1 p24 acid-dissociated (immune complex-

dissociated [IC]) antigen was determined using the Coulter Assay (Coulter Immunology, Hialeah, FL) as previously described (8).

Results

Sensitivity of cPCR and selected conditions for clinical sample analyses. The sensitivity of cPCR in quantifying DNA was first assessed in reconstruction experiments using the plasmid pBH10, which contains a full-length wild-type DNA sequence. Replicate samples containing defined amounts of pBH10 plasmid were coamplified with increasing copy number of competitor plasmid pSPLI-II. As shown in Fig. 1 *A*, the amplified products of the target (218 bp) and competitor plasmid (240 bp) were easily visualized by gel electrophoresis starting with 1,000 copies of template. At a lower target input, such as 100 or 10 copies, competitor and target PCR products were not efficiently visualized in the ethidium bromide stained gel; however, the use of the radioactive-labeled sense primer in the amplification reaction and subsequent autoradiography increased the sensitivity of cPCR up to enable the detection of 10 copies (Fig. 1 *B*).

To assess sensitivity in quantifying RNA, 1,000, 100, or 10 target RNA molecules, obtained by in vitro transcription from plasmid p1-2II, were retro-transcribed and amplified along with an increasing copy number of the competitor RNA. As shown in Fig. 1 *C*, 1000 molecules of target were reliably visualized on the ethidium bromide-stained gel, and positive signals could be detected using as few as 10 molecules of target after autoradiography (Fig. 1 *D*). Calculations, performed as detailed in Methods, gave the expected number of target copies, with a coefficient of variation of 10% in all competitive PCR reactions (not shown).

Due to the limited quantity of blood available from newborns, the choice of the competitor copy number range to be tested for competitive amplification was critical. Based on previous evidence that most infected children at birth have an undetectable HIV-1 infection either by DNA-PCR, virus culture or p24IC antigen assay, we used a set of four reactions containing replicate portions of cell lysate (equivalent to 60,000 PBMC) and 0, 10, 50, and 250 competitor DNA molecules to quantify HIV-1 DNA in the first perinatal specimen; samples obtained thereafter were tested against 0, 50, 250, and 1,250 competitor DNA molecules. To quantify RNA in plasma, a set of 4 reactions containing replicate portions of RNA samples (equivalent to 30 μl of plasma) was tested against 0, 10, 100, and 1,000 RNA competitor molecules in the perinatal samples, and against 0, 1,000, 10,000, and 100,000 RNA competitor molecules in later samples. To ensure a higher assay sensitivity and avoid variability due to different sample treatment, all amplifications were performed using the radio-labeled sense primer, and quantitative determinations were achieved by densitometric scanning of x-ray films. Data obtained by cPCR and cRT-PCR were then normalized to 1×10^5 PBMC, and to 1 ml of plasma, respectively. A representative quantification experiment for a DNA and RNA sample is shown in Fig. 2 (*A* and *B*, respectively) and detailed in the legend.

Molecular data from HIV-1 infected newborns. Three of the 11 children developed severe symptoms of disease as listed in category C of the CDC classification within 6 mo of age. Another child, that was classified clinically in category B, had severe immunodepression by 6 mo old (i.e., CD4 cell count below 750/μl). These four children were defined as rapid progressors according the definition of the IIIrd Consensus Workshop on Pediatric AIDS (manuscript in preparation). The remaining seven children have a mean follow-up of 4.2 yr (range 3.1–6 yr); none has developed severe immunodepression, but two progressed to category C of the CDC classification at 3 and 4 yr old, respectively.

The molecular findings in the sequential samples collected from these infants during their first year of life are reported in Fig. 3, together with the CD4 cell counts. In seven cases, a first specimen was available within 48 h of birth, and in four cases

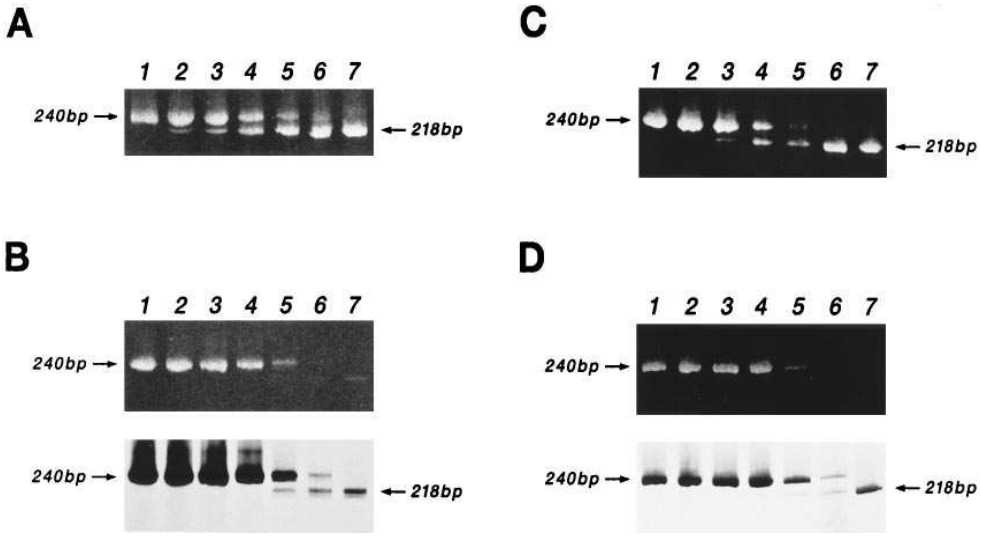


Figure 1. Sensitivity of cPCR and cRT-PCR. (*A*) Ethidium bromide-stained gel of PCR products derived from the competitive template pSPLI-II (*upper band*, 240 bp), and the plasmid pBH10 containing a wild-type target sequence (*lower band*, 218 bp). Lane *1*, 10,000 copies of pSPLI-II; lanes *2–7*, coamplification of pBH10 wild-type sequence (1,000 copies) with a decreasing copy number of pSPLI-II (10,000, 5,000, 1,000, 500, 100, 0). (*B*) Ethidium bromide-stained gel (*upper panel*), and autoradiography (*lower panel*) of PCR products from the pSPLI-II. Lane *1*, 1000 copies of pSPLI-II; lanes *2–7*, coamplification of pBH10 (10 copies) with a decreasing copy number of pSPLI-II (1,000, 500, 100, 50, 10, 0). (*C*) Ethidium bromide-stained gel of RT-PCR products derived from the competitive RNA template (*upper band*, 240 bp) and the RNA product from plasmid p1-2II, containing wild-type target sequence (*lower band*, 218 bp). Lane *1*, 100,000 copies of HIV-1 competitor; lanes *2–7*, coamplification of wild-type sequence (1,000 copies) with a decreasing copy number of HIV-1 competitor (100,000, 10,000, 1,000, 100, 10, 0). (*D*) Ethidium bromide-stained gel (*upper panel*) and autoradiography (*lower panel*) of RT-PCR products. Lane *1*, 100,000 copies of HIV-1 competitor; lanes *2–7*, coamplification of wild-type sequence (10 copies) with a decreasing copy number of HIV-1 competitor (100,000, 10,000, 1,000, 100, 10, 0).

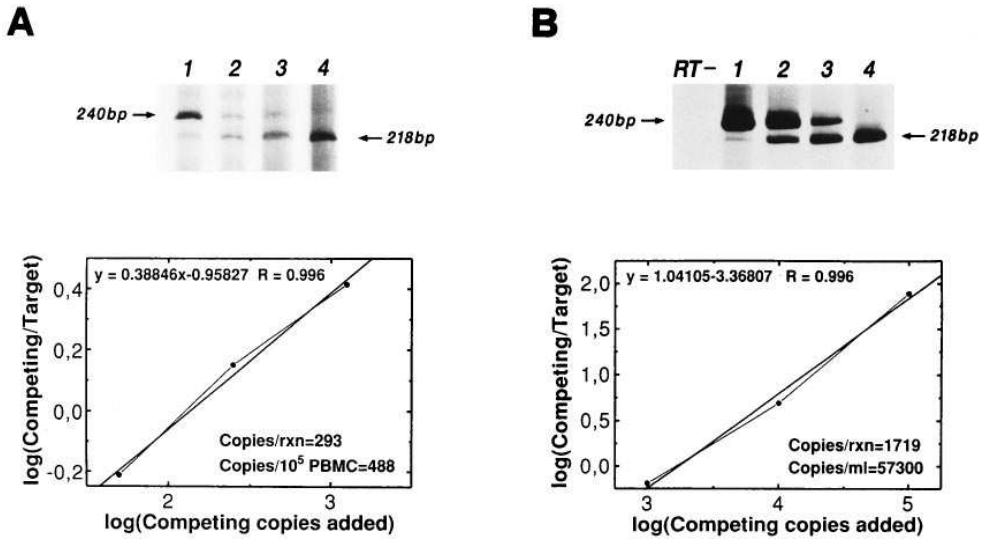


Figure 2. Representative quantification of HIV-1 DNA and HIV-1 RNA molecules. (*A*) DNA corresponding to 60,000 PBMC of child No. 8 (30 d old) was amplified with 1,250, 250, 50, 0 (lanes 1–4) molecules of the pSPLI-II competitor. (*B*) RNA equivalent of 30 μl of plasma from child No. 8 at 30 d old was amplified with 100,000, 10,000, 1,000, 0 (lanes 1–4) competitor RNA molecules obtained from pSPLI-II competitor. Lane *RT-* PCR amplification without reverse transcription (the absence of signal indicates the absence of contaminating DNA). Linear regression analysis to obtain competition equivalent points is reported at the bottom of each autoradiogram. The peak areas of the amplified bands were measured by densitometric scanning. The logarithm of the ratio between the optical density values of competitor and wild type amplified products (*y*-axis) was plotted against the logarithm of competitor copy number (*x*-axis), and a linear regression curve was extrapolated. The concentration of each target was estimated according to the equation of the fitting line, considering the value of 0 (log 1) on the *y*-axis as the point at which the number of competitor molecules was equal to the number of target molecules. The correlation coefficient and the equation of the fitting line are also indicated.

within 5–15 d old. Only one infant who was tested at birth (No. 3, Fig. 3 *A*), and all the infants tested between 5 to 15 d old were found to be positive for HIV-1 sequences by cPCR analysis, in agreement with previous results obtained by virus culture and/or DNA-PCR (2, 8). The results of cRT-PCR analysis of plasma paralleled those obtained in cells, and all children with undetectable levels of HIV-1 DNA in their first specimen also had undetectable levels of HIV-1 RNA. It is noteworthy that, regardless of positive or negative HIV-1 detection at or near birth, all the children showed an increase in viral burden during the first month of life, although with different slopes. Both viral DNA and RNA parameters increased quickly in the four rapid progressor infants and, at 4–6 wk old, values of 410–1,050 HIV-1 DNA copies/10^5 PBMC, and 219,640–500,000 HIV-1 RNA molecules/ml were recorded (Fig. 3 *A*). In three cases, an initial high level of plasma viral RNA ranging from 419,087 to 500,000 RNA molecules/ml could be detected between days 40 and 69, while in infant No. 4 a high level of plasma viral RNA (3,330,000 molecules/ml) was recorded on day 104; interestingly, p24IC was also undetectable in this child up to this age. The increase in viral burden in cases No. 1 and 4 was paralleled by a significant drop in the CD4 cell count, while counts in the other two cases were invariably less than 2,000 CD4+ cells/μl since birth. After the first rapid increase in plasma and cellular viral content, the quantity of both HIV-1 DNA in cells and HIV-1 RNA in plasma showed a further 2–5-fold increase in the following one to two months; a steady state level was subsequently observed in all the infants, during which the viral parameters fluctuated within a narrow range of 1,000–3,000 HIV-1 DNA/10^5 PBMC and 1,000,000 to 4,000,000 HIV-1 RNA molecules/ml (Fig. 3 *A*). Three infants started early treatment with AZT, but only in one case (No. 2) a transient decline in plasma viremia paralleled by a transient CD4 cell increase was observed. As none of the mothers underwent AZT treatment during pregnancy or at delivery, possible drug-resistant variants could have selectively emerged in these infants only after the start of their own therapy. As AZT escape mutants could emerge in a period as early as 25 d on treatment, and plasma viral load could rebound to pre-therapy levels as early as 27 d (26), a transient drug efficacy could be missed, at least in case No. 1, given the time interval between the start of antiretroviral therapy and the collection of the subsequent samples.

Different HIV-1 replication patterns could be identified among the slow progressors. In four infants, the slopes and rates of viral increase were similar to those recorded in the rapid progressors (Fig. 3 *B*); in particular, in two cases (Nos. 5 and 6) a high level of plasma viral RNA (500,000 and 166,000 copies/ml, respectively) was observed at 4 wk old, while in two others (Nos. 7 and 8) the increase in plasma viral RNA occurred later on and was detected in samples collected on days 103 and 140 (1,485,260 and 1,660,000 RNA molecules/ml, respectively). In all four cases, an increase in p24IC concentration paralleled the one detected for viral RNA. The CD4 cell count decline paralleled the slope of the viral increase; the minimum CD4 cell level coincided with the highest detected level of viral RNA in two cases, and was detected in the subsequent sample in two others. On the whole, a mean CD4 cell reduction of 57% (range 48 to 69%) compared with the baseline values at birth was observed. At the time of the highest detected level of plasma viral RNA, all but one of these infants had a high level of HIV-1 DNA in their cells (from 314 to 1,230 HIV-1 DNA copies/10^5 PBMC). In case No. 5, who showed a low copy number of HIV-1 DNA (12 /10^5 PBMC) at 30 d old in concomitance with the highest detected plasma HIV-1 RNA level, an upsurge in the HIV-1 DNA copy number (327/10^5 PBMC) was detected in the subsequent cellular sample on day 77, most likely a consequence of the high level of circulating virus. All four cases showed a decline in both p24IC antigen and plasma viral RNA in the absence of antiretroviral therapy, although with different slopes and rates of viral decrease. In three cases (Nos. 5, 6, and 7) HIV-1 RNA levels underwent a

mean reduction of 89% (range 80 to 98) over a mean time period of 49 d (range 46 to 55) and fell to 50,000 molecules/ml (children Nos. 5 and 6) or less (child No. 7); this decline was paralleled by a rapid decrease in p24IC antigen, which fell to undetectable levels. In case No. 8 plasma viral RNA declined over a period of 35 d by a factor of only 2.5 (from 1,660,000 molecules/ml at day 140 to 650,000 molecules/ml at day 175), but further decreased in the absence of antiretroviral therapy to a level of 300,000 molecules/ml at day 243. Although p24IC antigen remained at high levels (200 pg/ml) a reduction in its value (from 880 pg/ml at day 140 to 320 pg/ml at day 175, and then to 241 pg/ml at day 243) could be documented in parallel with the plasma HIV-1 RNA decrease. In all four cases, the viral decline was paralleled by a rise in the CD4 cell number, which underwent a mean increase of 40% (range, 34 to 60%) compared with minimum values over a mean time period of 54 d (range, 35 to 74 d) (Fig. 3 *B*).

Three other infants showed a slower increase in plasma viral RNA, which reached consistently lower levels at 4–6 wk old (28,000 RNA copies/ml [No. 9] 35,000 RNA copies/ml [No. 10] and 57,000 RNA copies/ml [No. 11]); only in two cases was there a concomitant transient increase in p24IC (Fig. 3 *C*). At this time point, all three infants had less than 50 HIV-1 DNA copies/10^5 PBMC. After the initial rise in plasma viral RNA, only one child (No. 9) showed a decrease in the CD4 cell count, and the RNA levels remained fairly stable (in No. 11), or increased steadily but very gradually (No. 9 and 10) over the first year of life. The proviral HIV-1 DNA copy number also increased very gradually over the study period.

A statistical comparison of the HIV-1 RNA levels among the three groups of children displaying the different patterns of HIV-1 infection (i.e., patterns A, B, and C, shown in Fig. 3, *A*, *B*, and *C*, respectively) was carried out applying the Student *t* test to the mean values of HIV-1 RNA calculated from the entire period of study, as well as from different time intervals. For each individual, a mean value was derived by transforming the RNA values to natural logarithms and calculating the ratio between the area under the curve described by the transformed series (considered as a function of time) and the length of the corresponding time interval of observation. When the entire period of study was considered, the mean RNA value of pattern A, displayed by rapid progressors, significantly differed from those of patterns B ($P = 0.0087$) and C ($P = 0.0046$), displayed by slow progressors, while no significant difference was found between the mean RNA values of patterns B and C ($P = 0.331$). Similar findings were obtained when analysis was performed from 4 mo old up to the end of study period (A vs. B, $P = 0.0087$; A vs. C, $P = 0.0081$; B vs. C, $P = 0.4036$). However, when analysis was performed in the first 4 months of life, although the differences between the RNA values of pattern B and C were not statistically significant, the mean values of HIV-1 RNA of pattern A were significantly different from those of pattern C ($P = 0.005$), but not from those of pattern B ($P = 0.05$), thus supporting the two different profiles of viral increase early in life in the slow progressor infants.

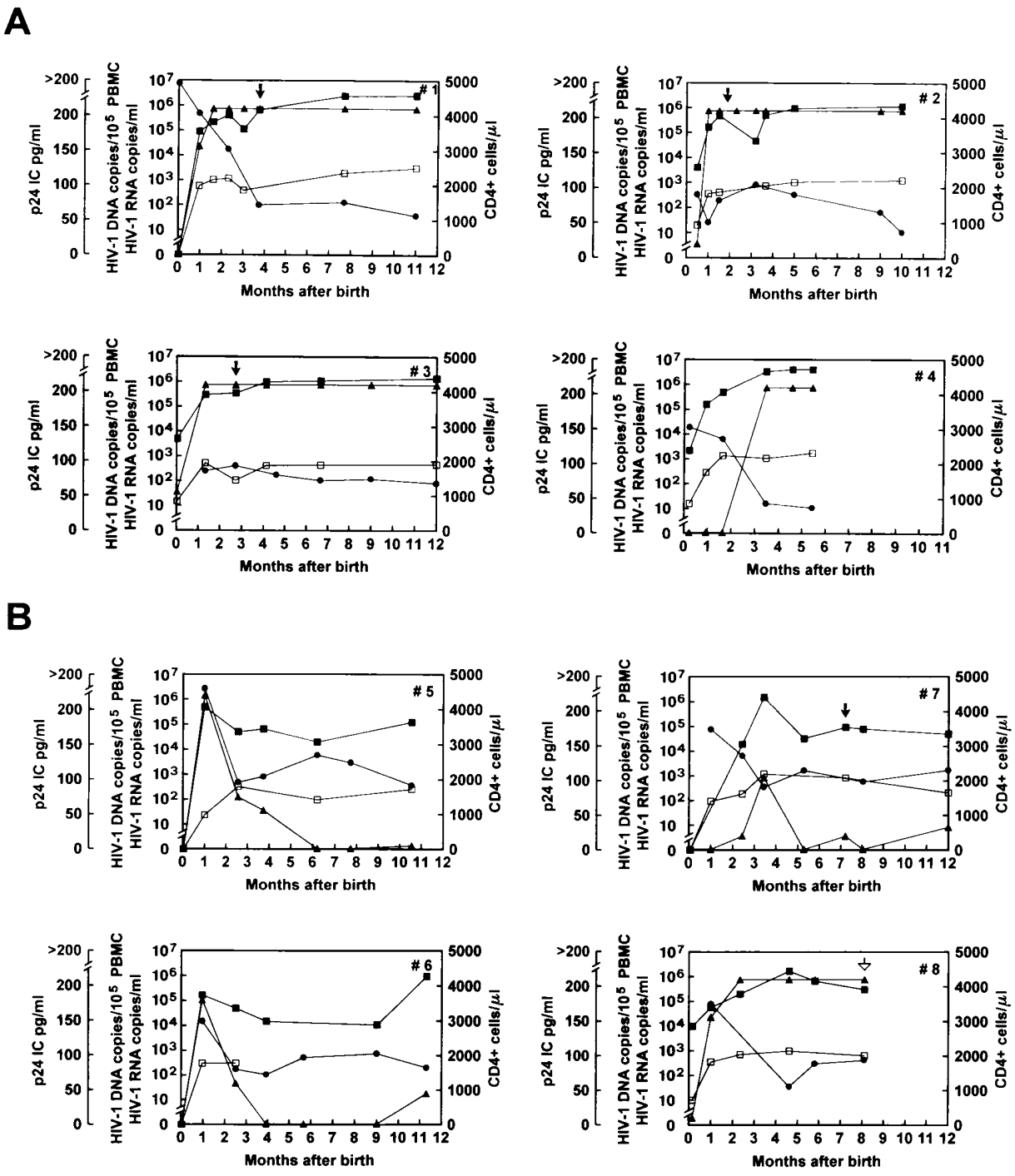
Discussion

We evaluated the dynamics of HIV-1 replication by assessing of the number of HIV-1 DNA copies in cells and HIV-1 RNA molecules in plasma in 11 infants who were sequentially studied since birth. All of the children showed an increase in the indices of viral replication activity within their first weeks of life, regardless of their positive and negative HIV-1 detection at birth.

Besides an individual variability in the different viral activity markers, three distinct patterns of HIV-1 replication could be recognized early in life. In the first pattern (Fig. 3 *A*), both plasma viral RNA and cell-associated proviral DNA levels rose rapidly during the first month of life, continued to increase gradually in the second and third months of life, and then remained steadily at high levels, ranging from 1,000 to 3,000 HIV-1 DNA copies/10^5 PBMC, and 1,000,000 to 4,000,000 HIV-1 RNA molecules/ml. The CD4 cell number fell dramatically in two cases (Nos. 1 and 4) in concordance with the rapid increase in viral load, and thereafter gradually declined over time. In the second pattern (Fig. 3 *B*), an initial rapid increase in the indices of HIV-1 replication activity was accompanied by a decrease in the CD4 cell number during the first weeks of life. After this early phase, comparable to that observed in the first pattern, virus levels in the blood declined by a factor ranging from 2.5 to 50, and then fluctuated at levels 10–100-fold lower than those recorded in the first pattern. A partial restoration of the CD4 cell number in blood was observed in parallel with the decline in viral load. In the third pattern (Fig. 3 *C*), viral replication activity in the first weeks of life was lower than that seen in the first two patterns. After the initial viral increase during the first 4–6 wk of age, plasma viral RNA reached levels significantly lower (10–100-fold) than those observed in the first pattern, and then remained fairly stable or increased very gradually over first year of life. All rapid progressor infants showed the first pattern of HIV-1 replication, while the slow progressors exhibited the second or third pattern.

These data support and extend previous observations addressing HIV-1 replication activity in newborns and their clinical outcome. A rapid increase in viral load, as assessed by the HIV-1 DNA copy number, was found to be strictly associated with the early onset of symptoms; conversely, infants with slower disease progression showed a very gradual increase in the proviral DNA content (12). By studying a larger number of samples obtained early in life, and using a sensitive cRT-PCR technique to determine the HIV-1 RNA content in plasma, we were also able to identify transient increases in plasma viremia, and delineate a more complex pattern of HIV-1 replication in the slow progressors. Our observation of a rapid increase in viral titers during the first weeks of life followed by a decline in blood viral load in four slow progressors agrees with the findings of Luzuriaga et al. in children studied monthly since birth by means of virus culture from PBMC and plasma (7). On the whole, the rate of virus increase, and the plasma viremia peaks observed early in life in our infants may be consistent with the hypothesis that infected newborns experience a primary infection (27–29), thus suggesting that HIV-1 transmission mainly occurs late in pregnancy or during the intrapartum period.

The factors that determine the different patterns of HIV-1 replication early in life remain an open question. That the viral load did not decrease after the earlier viremia peak in the rapid progressors strongly suggests that these infants were not able to control viral replication, as usually occurs in adults after the primary infection (30–31). The presence of autochthonous antibodies binding to several HIV-1 gag and envelope



epitopes, could be documented in 3 out of 4 rapid progressors, and 4 out of 5 slow progressors early in life (8; and unpublished results); therefore, in agreement with other studies (32, 33), such antibodies did not appear to play a critical role in determining the pattern of infection. As viral decline after primary infection in adults has mainly been associated with the emergence of HIV-1 specific cytotoxic T lymphocytes (CTL) (34–36), the viral decline observed in slow progressors might indicate that these infants were able to mount a cellular immune response, and partially control HIV-1 replication. Nonetheless, in agreement with a recent observation (37), it is noteworthy that the viral clearance in these infants was consistently lower than that observed in adults after primary infection, in whom a viral decline by a factor of 100 to 1000 was described (15, 16). This different pattern might reflect a different efficiency of the immune system in controlling infection, and could explain why most infants, including those in the slow progressor group, have a shorter clinical latent phase (2–4 yr) than usually observed in adults (8–10 yr) (1). The finding that HIV-1 specific CTL are only seldom detected in infected newborns (38, 39) is in line with the above suggestion. The selective loss of naive CD8+ cells, recently demonstrated in HIV-1 infected infants (40), may also contribute to the defects in cell-mediated immunity.

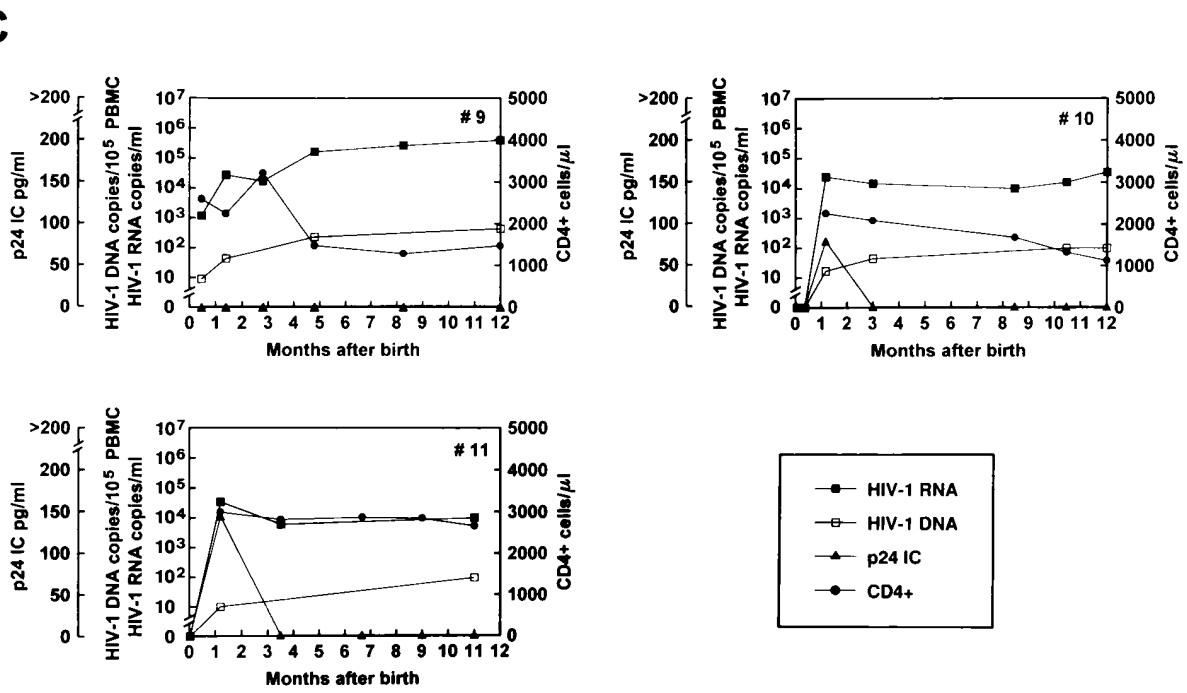


Figure 3. Molecular parameters of HIV-1 activity in children born to HIV-1 infected mothers, monitored during their first months of life. (*A*) Rapid progressors infants; children Nos. 1, 2, and 3 developed severe symptoms of disease as listed in category C of the CDC classification at 4, 4, and 2 mo, respectively. Child No. 4 was clinically in category B, but had severe immunodepression at 6 mo old. (*B* and *C*) Slow progressor infants; children have a mean follow-up of 4.2 yr (range 3.1–6 yr). None developed severe immunodepression; children Nos. 5 and 7 progressed to category C at 3 and 4 yr, respectively. Copy numbers of HIV-1 DNA (□) and HIV-1 RNA (■) are shown in log scale. p24IC levels (▲) and CD4+ cell number (●) are also shown. Closed arrow indicates start of AZT treatment; open arrow indicates enrollment in the PENTA 1 trial.

The emergence of viral variants with increased growth capacity (i.e., rapid/high variants), either with or without syncytium-inducing capability in the MT-2 indicator cell line, has been consistently correlated with an increasing viral burden, and consistently detected in adults (41–43) and in infants (11, 44) who showed a rapid clinical progression. In our study, all of the rapid progressors, but only one slow progressor (No. 8), had a viral isolate with a rapid/high phenotype at 4–8 wk old (11, and unpublished results); the remaining infants had viral isolates with a slow/low (Nos. 5, 10, and 11) or an intermediate slow/high phenotype (11, and unpublished results). Interestingly, child No. 8 showed the highest viral increase and the slowest viral decline among the slow progressor infants. While these findings strongly suggest that the replicative capacity of infecting strains contributes to determining the pattern of infection, it is conceivable that the wide variability in the dynamics of HIV-1 increase and clearance may reflect differences in both the rate of viral replication and the maturation status of the immune system in newborns. Based on the above considerations, it appears likely that a rapid spread of virus before the child is capable of mounting an immune response would determine the pattern of HIV-1 replication described in the rapid progressor infants, and that early AIDS is a clinical manifestation of the primary infection.

Finally, the results presented in this work clearly indicate that the pattern of viral DNA and RNA accumulation and clearance in infected infants is strictly correlated with the evolution of disease. This observation stresses the notion that the rate of viral replication is a major determinant of AIDS pathogenesis, and a faithful predictor of the clinical outcome.

Acknowledgments

We thank Dr. Sandro Tognazzo for Statistical analysis, and Patricia Segato for help in preparing the manuscript.

This work was supported by Istituto Superiore di Sanita' Progetto AIDS No. 9202-05, No. 9302-04.

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