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Comparison of HIV Detection by Virus Isolation in Lymphocyte Cultures and Molecular Amplification of HIV DNA and RNA by PCR in Offspring of Seropositive Mothers

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Summary: An early and accurate diagnosis of HIV infection is needed in the offspring of seropositive mothers. To this end, we have used two techniques for the direct detection of HIV in 12 newborns tested within 2 weeks after birth and 12 children. HIV isolation was carried out in lymphocyte cocultures and compared with detection of DNA and RNA sequences by molecular amplification using the polymerase chain reaction (PCR). In lymphocyte cocultures, HIV was isolated in 8 of 24 cases (33%), including 3 newborns, 3 symptomatic children, and 2 asymptomatic ones. HIV DNA was detected by PCR in twice as many cases, i.e., in 16/24 cases (66%), including 7/12 newborns, 4/4 symptomatic children, and 5/8 asymptomatic ones, 2 of whom became seronegative, HIV RNA was detected in 10 of 16 cases (60%) with detectable HIV DNA, including all of the cases who had a positive HIV isolation. Only children with clinical or biological signs of HIV infection were positive for HIV RNA. Furthermore, signs of HIV infection appeared within 6 months in three of the four newborns who were positive for HIV RNA at birth. These results indicate that HIV DNA detection by PCR is far more sensitive than HIV isolation in culture for the early diagnosis of HIV infection in offspring of seropositive mothers. HIV RNA detection appears to be a useful prognostic marker since it does correlate with disease progression and may serve as a clue for HIV replication in vivo. **Key Words:** Human immunodeficiency virus—Polymerase chain reaction—DNA—RNA—Culture—Children.

Early detection of human immunodeficiency virus (HIV) infection in offspring of seropositive mothers is crucial for their medical follow-up and treatment. The estimated rate of HIV transmission from mothers to children varies among different studies from 20 to 50% (1–5). More recently, a vertical transmission rate of about 30% was found by two multicenter studies (1,2). These studies are based on clinical observations and give only indirect estimates of the rate of transmission. A more reliable method to diagnose HIV infection as early as possible would be more informative.

The presence of anti-HIV antibodies is not a useful marker of infection because of the passive transmission of maternal antibodies, which may persist

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for several months (3–5). In a multicenter European study, the mean age for the clearance of these antibodies was 10 months but they may persist for up to 15 months. Tests for P24 antigenemia are often negative in newborns and therefore are not helpful for diagnosis. Moreover, it has been reported that some infected children could remain seronegative (1,2). Appearance of clinical or immunological signs of HIV infection is often delayed following a silent period of viral latency as reported in adults. Auger et al. (6) have shown that pediatric AIDS can either have a very short incubation period of less than 1 year or a much longer incubation time with a median of 6 years.

The direct detection of HIV is therefore essential for the diagnosis of infection in children born to seropositive mothers. For this purpose, lymphocytes from children were cultured in order to isolate HIV. The presence of viral DNA was assessed in parallel using the polymerase chain reaction (PCR). A more direct appraisal of HIV replication was also attempted by using PCR to amplify specific HIV RNA sequences in fresh peripheral blood lymphocytes of these children.

METHODS

Patients

Twenty-four neonates and children born to seropositive mothers were studied prospectively for the presence of HIV infection. Twelve of them were tested either at or within 15 days of birth; they will be referred to as newborns hereafter. Ten seropositive children were tested between 2 and 29 months of age. In this group, four children had clinical manifestations of HIV infection that were associated with p24 antigenemia and included encephalopathy, lymphadenopathy, and pneumopathy. They were classified as P2A or P2D according to the Centers for Disease Control classification. Two did not develop symptoms but exhibited hypergammaglobulinemia (P1B). The four other children were indeterminate (P0). Two additional children seropositive at birth became seronegative by 12 and 13 months of age: on that occasion, both were free of any clinical or biological symptoms. One of these children was then lost to follow-up, and the other was still asymptomatic 12 months later (see Table 2).

Serology

Sera were tested for HIV antibodies and p24 antigen (Ag) with solid phase enzyme-linked immunosorbent assay (ELISA) (Abbott Laboratories). Results of HIV antibody tests were confirmed by immunoblot assay (Biorad Novapath, Richmond, VA, U.S.A.).

HIV Isolation

Patient's peripheral blood mononuclear cells (PBMCs) were obtained from heparinized blood separated by Ficoll λ-Hypaque gradient. They were then cocultured with normal PBMCs in RPMI 1640 medium, supplemented with 20 U/ml of recombinant interleukin-2, 10% fetal calf serum, and polybrene (2 μg/ml) (7,8). Culture supernatants were tested twice weekly for HIV p24 antigen by ELISA (Abbott).

Polymerase Chain Reaction

PCR was performed on nucleic acid extracted from 2–5 $\times 10^6$ PBMCs. For detection of DNA, cells were treated with proteinase K (100 μg/ml) and 0.5% sodium dodecyl sulfate (SDS) in TEN buffer (10 m*M* Tris at pH 8, 1 m*M* EDTA, 100 m*M* NaCl) at 37°C overnight. DNA was then extracted by phenol–chloroform, precipitated by ethanol, and redissolved in sterile water. RNA was obtained by lysis of cells in 10 m*M* Tris at pH 8, 1.5 m*M* $MgCl_2$, 140 m*M* NaCl, and 0.5% NP40, followed by centrifugation and removal of the cytoplasmic extract.

Two sets of primers were used for PCR. The SK 38 and 39 primers (Cetus Corp., Emoryville, CA, U.S.A.) allowed amplification of a 115 base pair sequence in the *gag* gene of HIV (nucleotides 1089 to 1,204 in the HIV BRU isolate). SK 29 and 30 amplified a 104 base pair, sequence in the long terminal repeat (LTR) (nucleotides 47 to 151). The oligonucleotides SK 19 (nucleotides 1134 to 1174) and SK 31 (nucleotides 92 to 124) were used as internal probe for the gag and LTR sequences, respectively.

PCR was carried out according to the principle of Mullis et al. (9) as follows: 1 μg of genomic DNA was mixed in PCR buffer [50 m*M* Tris at pH 8.3, 6 m*M* $MgCl_2$, 40 m*M* KCl, 1 m*M* dithiotreitol (DTT)] with 100 pmol of each primer, 2.5 units of Taq polymerase (Perkin-Elmer Cetus), and 200 μ*M* of each deoxyribonucleoside triphosphate (dNTP). DNA was subjected to repeated 30 cycles of 25 s at 94°C, 25 s at 55°C, and 1 min at 72°C by using a Perkin-Elmer Cetus thermal cycler.

For RNA detection, synthesis of cDNA from RNA by a reverse transcriptase reaction had to be carried out before PCR amplification with *gag* prim-

ers. Briefly, 10 μl of cytoplasmic extract were incubated in 1× PCR buffer with 200 μ*M* of each dNTP, 100 pmol downstream primer SK 39, 1 unit/ml of RNAsine, and 200 units of MuLV reverse transcriptase (GIBCO) during 15 min at 37°C. After denaturation of reverse transcriptase by heating at 95°C during 5 min, the PCR reaction was carried out as for DNA. To insure specific detection of RNA, samples were treated before reverse transcriptase reaction by 1 unit/ml of RNase-free DNase I (Pharmacia) during 20 min at 37°C. To control the specificity of RNA detection, an amplification reaction was performed on DNase I-treated samples without previous reverse transcription.

Analysis of Amplified DNA

Ten microliters of amplified products were subjected to electropheresis on a 3% Nusieve/1% agarose gel in Tris–borate–EDTA buffer containing ethidium bromide and then analyzed under ultraviolet light. After gel denaturation in 0.5 *M* NaOH and 1.5 *M* NaCl for 20 min, DNA was transferred by Southern blot to a nylon membrane (Amersham) in 20× SSPE buffer (0.18 *M* NaCl, 10 m*M* sodium phosphate, 1 m*M* EDTA, pH 7.4). Hybridization with a specific phosphorus-32-labeled oligonucleotide probe was carried out at 42°C overnight in a 3× SSPE, 5× Denhardt solution, 30% formamide, 0.5% SDS mixture. The filters were then washed twice in 2× SSPE, 0.5% SDS for 15 min at 42°C, and autoradiographed at −80°C with intensifying screens.

The specificity of HIV detection by PCR in this study was insured by the use of internal negative controls in each series of tests. Contamination, a constant concern when using PCR, was avoided by strict care in sample preparation in an isolated room and checked by carrying out the PCR reaction without nucleic acid for each run.

RESULTS

In this study, three tests were used to diagnose HIV infection: HIV isolation from PBMC cocultures, and direct detection of both HIV DNA and RNA by PCR. The results of the three assays were negative in eight case: five newborns (Table 1) and three seropositive children younger than 1 year (Table 2). By contrast, all three seropositive children older than 1 year were positive by all three methods.

HIV was isolated from PBMC cultures in 8 of the 24 cases studied (33%), including 3/12 newborns and 5/12 children. The two children who became seronegative were negative by the culture assay.

Twice as many cases [16/24 (66%)] harbored HIV DNA sequences in their PBMCs using PCR, including 7 of the 12 newborns. HIV DNA was found in all children with clinical or biological signs of HIV infection, including four symptomatic children with p24 antigenemia and two others asymptomatic with hypergammaglobulinemia (Fig. 1).

HIV proviral DNA was also detected in an asymptomatic seropositive child and in the two children who had lost antibodies. These three cases and two newborns were positive for HIV DNA only with the *gag* primer pair. HIV DNA was always detectable whenever HIV was isolated by culture.

HIV RNA sequences were amplified using *gag* primers in cells from 10 newborns and children (Fig.

TABLE 1. *Results of HIV isolation and HIV DNA/RNA detection by PCR amplification in newborns*

Case number	HIV serology		HIV isolation	PCR DNA		PCR RNA	Follow-up (months)	Outcome
	Ab	Ag		gag	LTR			
4	+	−	+	+	+	+	10	P2A
8	+	−	+	+	+	+	6	P2E
22	+	−	+	+	+	+	2	P1B
2	+	−	−	+	+	+	10	P0
11	+	−	−	+	+	−	4	P0
19	+	−	−	+	−	−	3	P0
21	+	−	−	+	−	−	12	P0
24	+	−	−	−	−	n.d.	9	P0
3	+	−	−	−	−	−	10	P0
20	+	−	−	−	−	−	3	P0
23	+	−	−	−	−	−	12	Seronegative
13	+	−	−	−	−	−	15	Seronegative

n.d., not done.

TABLE 2. *Correlation of results of HIV isolation and DNA/RNA detection by PCR amplification with the clinical stage and outcome in children of seropositive mothers*

Case number	Clinical stage	HIV serology		HIV isolation	PCR DNA		PCR RNA	Follow-up (months)	Outcome
		Ab	Ag		gag	LTR			
Babies younger than 12 months									
12	P2D	+	+	−	+	+	+	8	P2D
17	P2D	+	+	+	+	+	+	8	P2D
10	P2A	+	+	+	+	+	+	8	P2F
16	P0	+	−	−	+	−	−	Lost to follow-up	
15	P0	+	−	−	−	−	−	4	Seronegative
1	P0	+	−	−	−	−	−	12	Seronegative
18	P0	+	−	−	−	−	−	2	P0
Children older than 12 months									
6	P1B	+	−	+	+	+	+	5	P2D
7	P1B	+	−	+	+	+	+	1	P2A
14	P2A	+	+	+	+	+	+	9	P2F
9[a]	Asymp	−	−	−	+	−	−	Lost	
5[a]	Asymp	−	−	−	+	−	−	12	Asymptomatic

[a] Both children became seronegative.

2). PCR DNA results were negative after DNase I treatment of cytoplasmic extracts, which eliminated the possibility of DNA contamination of the sample and insured the specificity of RNA detection. HIV RNA was detected in all eight cases in whom HIV was isolated by culture. All of the cases positive for HIV RNA in PBMCs were also positive for HIV DNA.

In the group of seropositive children, HIV RNA was detected only in those six who had clinical or biological signs of infection (P1 and P2 stage). Among newborns, clinical and biological signs of HIV infection occurred in three cases within the 6 month follow-up period. One developed encephalopathy and died at 6 months of age, the second exhibited multiple lymph node enlargement with hypergammaglobulinemia, and the other had hypergammaglobulinemia. These babies were positive for HIV DNA, HIV RNA, and HIV isolation at birth. By contrast, two babies (case numbers 23 and 13) who were negative by the three tests at birth were seronegative after 12 months of age.

Among the 12 children, 3 were negative by PCR and 2 of them were seronegative at 12 months of age (case numbers 1 and 15). Both remained clinically and biologically healthy. The serology of the other children remained identical after an average follow-up of 10 months. Remarkably, all six children positive for HIV RNA deteriorated rapidly: two progressed from stage P1B to P2A and P2D, two P2A cases turned P2F, while the two remaining cases had already developed AIDS.

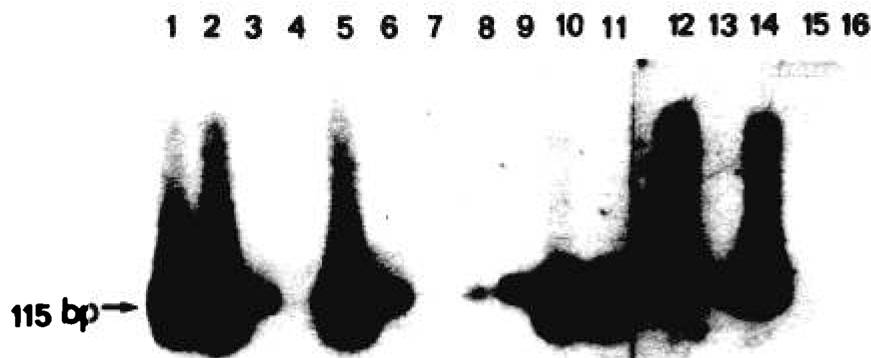

FIG. 1. HIV DNA detection in children using gag primers: Southern blot analysis of PCR products. Lanes 1, 2, 3, 5, 6, and 13: seropositive children numbers 12, 17, 16, 10, 21, and 19, respectively. Lanes 4 and 7: PCR negative children numbers 15 and 18, respectively. Lanes 8 and 9: seronegative case numbers 5 and 9, respectively. Lanes 10 to 12: seropositive children older than 12 months. Lane 14: positive control. Lane 15: uninfected control cells. Lane 16: negative control without DNA.

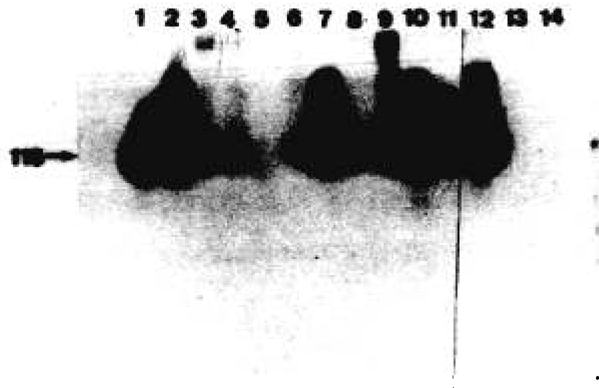

FIG. 2. HIV RNA detection by PCR in newborns and children of seropositive mothers: Southern blot analysis of PCR products. Lanes 1 to 4: newborn numbers 4, 8, 22, and 2, respectively. Lane 5: case number 11 negative for RNA PCR. Lanes 6 to 11: seropositive children numbers 12, 17, 10, 6, 7, and 14, respectively. Lane 12: positive control. Lane 13: uninfected control cells. Lane 14: negative control without RNA.

DISCUSSION

HIV isolation by coculture of PBMCs is instrumental in the detection of HIV infection in the offspring of seropositive mothers, as previously reported (10,11). Furthermore, HIV isolation at birth seems to be associated with poor prognosis (12). Conversely, in previous reports (11) as well as in ours, some infected children may remain negative for HIV by coculture. In this study, for example, one child at stage P2D was initially found to be negative by culture. HIV isolation by culture is therefore insufficient for early diagnosis of an HIV vertical transmission. Genomic amplification using PCR is, in principle, the most sensitive method for detecting HIV in infected individuals (13). This sensitivity indeed enabled Loche and Mach to disclose HIV infection in some subjects several months before seroconversion (14).

In the present study, genomic amplification yielded positive results in all children who had clinical, immunological, and/or virological signs of HIV infection. Moreover, HIV DNA sequences were detected by PCR in five neonates and children without any evidence of HIV infection except serology. Since the specificity of the PCR assay was tested in each run on negative and positive controls, these results are unlikely to be false positive. Similar results were found by Laure et al. (15) using the same technique.

A discrepancy between the HIV DNA results obtained with the two different sets of primers was observed in five cases. This could be explained either by mutations in the primer sites or by a greater efficiency of annealing with the gag primers than with LTR primers. Since these five cases had no other evidence of HIV infection, one likely possibility is that they had too few HIV copies in their PBMCs for efficient amplification when using LTR primers.

These results indicate that PCR amplification allows one to distinguish, among children born from seropositive mothers, those who are indeed infected by HIV from those who are not, regardless of their serological status. The detection of HIV proviral DNA sequences in seronegative children is unlikely to correspond to a false-positive reaction, as discussed above. It may be explained by a silent HIV infection that can persist for a long time before seroconversion, as recently described in adults by Imagawa (16). However, several years may be necessary to verify this hypothesis. In our study, 67% (7/12) of newborns were found to be infected by HIV using PCR. A similar high rate (64%) was also found by Paterlini (17). Further studies using PCR on more cases are needed to define better the exact rate of HIV transmission from infected mothers to children.

Genomic amplification of HIV RNA sequences allows for direct assessment of HIV replication in infected individuals. The presence of HIV RNA in lymphocytes indicates that a replicative cycle of HIV, with production of virions, does take place. In our study, HIV RNA sequences were detected in 10 cases of the 16 who were positive for HIV DNA. This included all of the symptomatic children but also those with only biological signs of HIV infection such as hypergammaglobulinemia. These results confirm that HIV RNA is detectable whenever active HIV replication responsible for immunological disorders takes place. The rapid deterioration observed in children with HIV RNA sequences at birth confirms the predictive value associated with the detection of HIV RNA. HIV RNA sequences were detected in four newborns younger than 15 days, three of whom later developed AIDS or signs of HIV infection. Hart et al. (18) studied HIV RNA by PCR in infected adults and found that 65% of the sample positive for HIV DNA were also positive for HIV RNA. Their results, like ours, would indicate that HIV replication fluctuates in infected individuals. Longitudinal studies with more cases are needed to determine if the lack of HIV RNA sequences is associated with the latency period of

HIV infection. In conclusion in this preliminary study, direct detection of HIV genomic sequence by PCR is an important tool for the early diagnosis of HIV infection in the offspring of infected mothers. This technique appears to be more sensitive and faster than HIV isolation in culture, since only 50% of the cases with HIV proviral sequences have a positive culture.

The detection of HIV RNA provides a clue for the recognition of the replicative stage of HIV infection, and may therefore serve as a useful prognostic marker in adults as well as in newborns and children. HIV RNA detection will also help in monitoring antiviral therapy.

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