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SPECIAL REPORT

# Virological, Neurological and Histological Investigations of a Child Born to a Mother with AIDS*

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**HIV-1 was isolated from a child at 6 and 9 months of age, proving the vertical transmission of infection from the mother with AIDS. The p24 antigen test of the plasma at 9 months of age was positive as well. A positive PCR reaction was detected in J34 cells, infected with the supernatant of the peripheral blood lymphocytes of the child. According to phenotypic characterization, the virus proved to be a SI (syncytium inducing) isolate, growing in PBL, MT2, J34 and other T and monocytic cell lines. The isolate was AZT sensitive. Two methods were applied for genotypic characterization: 1. Heteroduplex mobility assay (HMA), 2. Sequence analysis of a part of the env gene. On the basis of both of these methods, this virus belongs to the B subtype of HIV-1, which is prevalent mainly in Europe and in the USA. The neurological status of the child was followed regularly. At autopsy the presence of p24 antigen was detected in glial cells of the frontal cortex, proving the presence of the virus in the brain. A retardation of the development of the central nervous system could be observed as well.** (Pathology Oncology Research Vol 3, No 4, 303–308, 1997)

*Key words:* HIV, mother to child transmission, central nervous system development, delayed myelinization

## *Introduction*

Offsprings of HIV infected mothers become HIV carriers in 20–40 % of cases by infection during pregnancy, delivery or by breast feeding.[7,16] In the first 18 months HIV infection cannot be diagnosed by serological examinations, since one cannot distinguish the antibodies of maternal origin or those produced by the infant. The median age of maternal antibody loss is during the 10th month, and an estimated 75% of infants lose maternal antibody by12 months.[13] Virus culture assay,[8] p24 antigen detection or new methods based on nucleic acid amplification[4] are available to prove infection in the first months of life.

Hungary is still at low risk of HIV infection compared to other countries in Europe. The first case of vertical HIV transmission was proved by virus isolation at 6 months of age in a case of a male infant, born to an AIDS diseased mother. In the present report a summary is given of virus isolation and characterization, neurological investigations, as well as histological examinations – since the child died at 20 months of age.

## *Materials and Methods*

### *Virus isolation*

The T cell coculture method was used for virus isolation.[8] Peripheral blood lymphocytes of a healthy donor were isolated from approximately 10 ml of heparinized blood by Ficoll-Paque solution. The cells were washed and cultured in RPMI medium containing 10 % fetal calf serum, 100 IU/ml penicillin, 100 µg/ml streptomycin and 10 µg/ml Phytohaemagglutinin M (Seromed) for three days. Lymphocytes of the infant were separated from

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heparinized blood as described above, and mixed with the prestimulated donor cells.

The donor-patient cells were cocultured for one month in the presence of 20 U/ml IL-2 (Sigma, Human recombinant IL-2). Aliquots of culture supernatant were collected two times weekly for reverse transcriptase assay (RT),[15] and p24 antigen test. The samples were stored at – 70°C until testing.

*Indirect immunofluorescence assay*

Aliquots from donor and donor–patient cocultured lymphocytes were washed with PBS, dropped on glass slides and fixed with cold acetone.[9] The cells were incubated with known HIV-1 antibody positive serum, then with FITC conjugated anti-human IgG. The results were evaluated at UV light in an Opton microscope.

*Reverse transcriptase assay*

Reverse transcriptase (RT) assay was carried out according to a micromethod developed in our laboratory.[15] Briefly, poly (rA): oligo $(dT)_{12\text{-}18}$ (Sigma) as template primer was immobilized on Whatman DE 81 filter paper squares. $^3H$ TTP and virus-RT buffer mixture were dropped on the squares, incubated for 2 hours, washed, and then dried. Cpm was measured in a scintillation coctail using Packard 1600 CA liquid scintillation analyzer.

*p24 antigen test*

The presence of HIV-1 p24 core antigen in the plasma and in the lymphocyte supernatants was controlled by the use of HIV-1 p24 core profile add kit (Du Pont) and HIV-1 p24 core profile ELISA kit (Du Pont) according to the instructions of the manufacturers.

*Infection of different cell lines with RT positive PBL supernatant of FN.*

Aliquots of supernatant were incubated with MT4, H9 and HIV-1 tat c DNA transfected J34 cells,[3] for 1 hour at 37 °C. The cells were then cultured and regularly controlled for syncytia forming and for the presence of HIV. The infection procedure was repeated using the RT positive J34 cell culture supernatant, and MT2, H9, MT4, CEM and U937 cells.

*AZT sensitivity test*

AZT (3'-azido-3'-deoxythymidine) is an antiviral agent that inhibits the infectivity of HIV in vitro.[12] The AZT sensitivity of the isolated virus was tested in 96 well plates, on MT2 cells ($5x10^4$cells/well), cultured in the presence of 1, 5 and 10 μM AZT, and different dilutions of cell free virus supernatant (total volume was 200 μl/well). The different concentrations for AZT and virus were controlled in triplicates. Appearance of syncytia and/or RT activity of the supernatant was controlled one week later.

*PCR amplification*

DNA from control J34 cells and J34 cells infected with cocultivated PBL supernatant of the patient were isolated by standard phenol extraction method.

A nested PCR was used to amplify viral genomic regions for heteroduplex mobility assay (HMA) and sequencing. For HMA, ED3 and ED14[6] as outer primers which amplify an approximately 2 kb fragment, spanning from the first exon of rev and the complete surface (SU) protein (gp 120) coding sequence of env. Two sets of second round primers were used : 1. ED5 and ED12, to amplify the 1.2 kb V1 through V5 coding domains of the SU protein; 2. ES7 and ES8, to amplify the 0.7 kb V3 through V5 region of gp 120.

The PCR mixture (50 μl for the first round and 50-100 μl for the second round) contained 10 mM TRIS-HCl (pH 8.3), 50 mM KCl, 1.25 mM $MgCl_2$ for ED3/ED14 and ED5/ED12 or 1.8 mM $MgCl_2$ for ES7/ES8, 200 μM of each dNTP, 10 pmol primer and 1U Taq polymerase.

The first round PCR mixture also included 1μg of target DNA. Second round reactions (50–100 μl volume) were initiated with 2 μl of the first round reactions as template.

Subtype references were amplified using second round primers and 10 ng of plasmid as template. Reference plamids containing HIV-1 gp120 clones of subtypes A (RW20, IC144, SF170), B (BR20, TH14, SF162), C (MA959, ZM18, IN868, BR25), D (UG21, UG38, UG46), E (TH22, TH06, CAR7), F (BZ162, BZ163), G (RU131, LBV21-7, VI525) and H (CA13, VI557) were provided by the NIH AIDS Research and Reference Reagent Program.

The thermocycling conditions were 3 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min followed by 32 cycles of 94°C for 15 sec, 55°C for 45 sec, 72°C for 1 min, with a final extension at 72°C for 5 min.

For sequencing, amplifications were performed on the same starting material with the previously described primers ED5/ED12 for outer primers and V3L1 (5'-Biotin-GTACAATGTACACATGGAATTAGGCCA at positions 6956-6982)[1] and ES8 (5'-caggaaacagc-tatgaccCACTTCTCCAATTGTCCCTCA at positions 7667-7647-lower case letters are complementary to the reverse M13 primer) for the inner primers, which allow direct sequencing of the PCR product.

The reaction mixture for second round PCR contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 μM

each dNTP, 10 pmol primer and 1U Taq polymerase. Cycling conditions were: 94°C for 5 min., followed by 30 cycles of 94°C for 1 min. 55°C for 1.5 min. and 72°C for 7 min. The PCR amplification products were detected by electrophoresis on a 1% agarose gel and by ethidium bromide staining.

*Heteroduplex formation*

Heteroduplex molecules were formed by thermal denaturation and reannealing of 10 µl of the patient's DNA and 10 µl of known subtype reference DNA from the second round PCR products. Melting and annealing conditions were 94°C for 2 min followed by rapid cooling on ice. The reaction was performed in 2.2 µl of heteroduplex annealing buffer (100 mM NaCl, 10 mM Tris (pH 7.8), 2mM EDTA) in a final volume of 22.2 µl.

The electrophoretic mobilities of all generated homoduplex and heteroduplex DNA fragments were determined in 5% (30:0.8 acrylamide:bisacrylamide) polyacrylamide gel. 1.2 mm thick gel was poured using 19×12 cm plates and run in a vertical gel apparatus with 1 TBE (88 mM Tris-borate, 89 mM boric acid, 2 mM EDTA) for 1.5 h at 150 V. The heteroduplex formation was detected after being stained with ethidium bromide. The electrophoretic mobility of the heteroduplexes was inversely proportional to the sequence divergence of two annealed strands.

*DNA sequencing*

Fourty microliters of the nested PCR product were purified using streptavidin coated magnetic beads (Dynal AB, Norway) according to the protocol of the manufacturer. Biotinylated strand was suspended in 13 µl of distilled water. Sequencing reactions were performed according to the instructions of Auto Read kit (Pharmacia Biotech, Sweden) concerning single stranded DNA sequencing with fluorescent primer. Reaction products were separated and analyzed by ALF DNA sequencer and by ALF program version 2.5 (Pharmacia Biotech, Sweden).

*Histological investigations*

Formaldehyde-fixed material was embedded in paraffin from the frontal and parietal as well as occipital cortex and subcortical white matter. Samples of the basal ganglia and brain stem in different levels and 4 segments of the spinal cord were investigated. Stainings: haematoxylin-eozin, cresyl fast violet, Woelcke method for myelin, PAS, Prussian blue, Gomori-Grocott silver staining. Immunohistochemistry was performed with HIV p24 and HIV gp120 antibody (Dako).

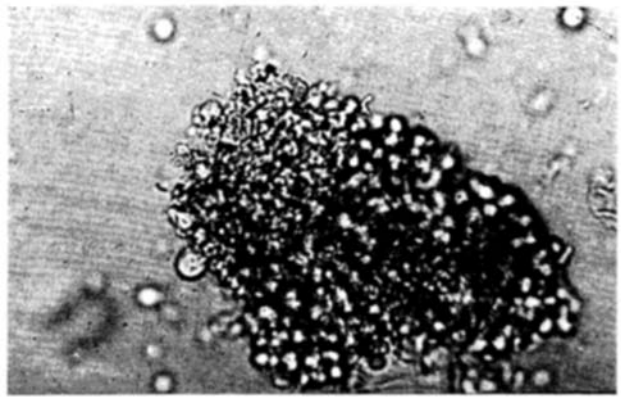

***Figure 1.*** *Syncytium in the cluster of lymphocytes from the infant and donor at 7th day of culturing.*

***Results***

Lymphocytes were isolated twice (at the age of 6 and 9 months) from the heparinized blood of the child. Syncytia appeared both times between the 7–10 days of culturing *(Figure 1)*. At the same time positive cells were detected by immunofluorescence as well.

RT assay results of the first blood specimen showed a peak on the 11$^{th}$ day of culturing. In correlation with the RT results, the p24 antigen test was positive in the PBL culture supernatant on the 7$^{th}$ and 10$^{th}$ day of culturing.

The plasma of the second blood sample (at 9 months of age) proved to be positive by p24 assay as well. On the basis of these results we proved the HIV infection of the infant in a time period when serological methods do not give correct result.

In order to characterize the virus, we tried to infect different cell lines with cell free supernatant of the PBL cultures. In the first experiment only J34 cells could be infected. On the 4$^{th}$ day following infection syncytia were recog-

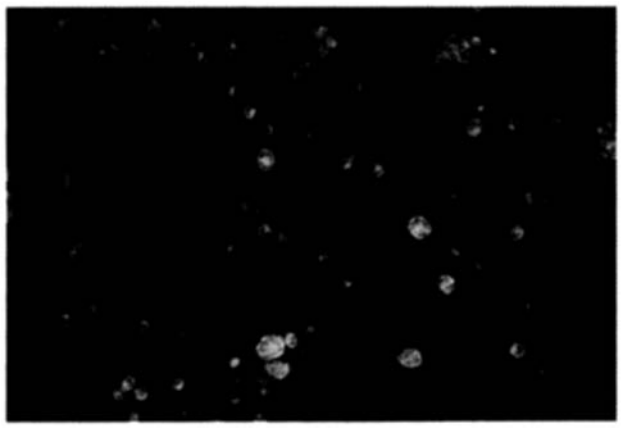

***Figure 2.*** *Immunofluorescent picture of J34 cell infected with the supernatant of lymphocyte coculture from the infant and donor. 4th day of culturing.*

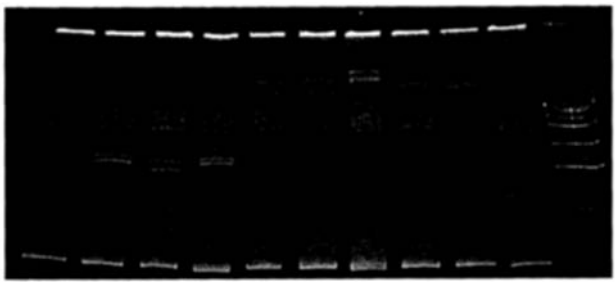

*Figure 3. Heteroduplex mobility assay of the the infant HIV-1 sample. PCR amplified HIV-1 env gene fragments spanning the V3-V5 region were mixed and reannealed with PCR fragments derived from HIV-1 subtype references. Lanes 1-9, heteroduplex analysis of patient's sample reannealed with A-SF170 (lane1); B-BR20 (lane 2); B-TH14 (lane 3); B-SF162 (lane 4); C-ZM18 (lane 5); D-UG38 (lane 6); E-CAR7 (lane 7); F-BZ163 (lane 8); G-RU131 (lane 9); TH14+SF162 (lane 10); 1kb molecular weight marker (lane 11). He, position of heteroduplexes; ss, position of single-stranded DNA; Ho, positions of homoduplexes.*

nized by microscopical investigations and RT results also showed strong positivity *(Figure 2, and data not shown).*

The infection experiment was repeated using the high RT active J34 cell supernatant.

Syncytia appeared in J34, MT2 and MT4 cells. Positive cells were also seen in U937 cells by immunofluorescence. In correlation with the immunofluorescence assay RT activity could be detected in the injected J34, MT2, MT4 and U937 cells.

On the basis of these results, the virus isolated belongs to the SI (syncytium inducing) phenotype of the virus.

The isolate proved to be AZT sensitive, as syncytia were not detected in the presence of 1 μM AZT. HIV-1 env gene fragments spanning the V1-V5 (1.2 kb), as well as the V3-V5 (0.7kb) regions were PCR amplified from the patient's proviruses. The nested DNA protocol yielded correct size amplification products as determined by agarose gel electrophoresis.

Aliquots from each PCR reaction were mixed with the amplified product from 9 previously sequenced reference HIV-1 isolates from subtypes A through F. The resultant DNA heteroduplexes were analyzed on the basis of their electrophoretic mobility in neutral polyacrylamide gels, and the genetic relationships between viral sequence and reference HIV-1 sequences were compared. A representative experiment is shown in *Figure 3.* The FN strain is identified as subtype B by the more rapid relative migration of heteroduplexes with subtype B isolates compared to other subtypes. In order to complete the analysis started by HMA analysis, approximately 110 base-pairs (the V3-V5 region of env gene) were directly sequenced from the PCR products.The nucleotide sequence of the patient's (accession number: AJ001618) was compared with databank sequences and the highest homology was observed with the type B strains in accordance with the HMA analysis *(Figure 4).*

*Clinical and neuorological examination*

In the first three months the patient seemed to be healthy, without neurological abnormalities. At 10 weeks of age lymphadenopathy and hepatosplenomegaly appeared. The CD4 cell count was 324/μl, showing a decrease in the following months. At 23 weeks of age 50 CD4 cells/μl were measured. In the 4th months of age a rigidity in the lower extremities was noticed. At 10 months

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                                                    109          99          89
FN0429                                              GGACAAATTAAATGTTCATXXXATATTACA
                                                    | | | | | | | | | |  | | | | | | | | : : : | | | | | | | |
TH014   GGAAAAGCAATGTATGCCCCTCCCATCAGAGGACAAATTAGATGTTCATCAAATATTACA
        1250          1260          1270          1280          1290          1300

              79          69          59          49          39          29
FN0429  GGGCTGATATTAACXXXXXATGGTGGTGTTAGCTCGAACAACACCACCGAGACCTTCAGA
        | | |  | | | | | | | : : : : : | | | | | | | |  | | |  | | | | | |  | | | | | | | | | | | | | | | | | | |
TH014   GGGTTGCTATTAACAAGAGATGGTGGTAATAGTGAGAACAAGACCACCGAGACCTTCAGA
        1310          1320          1330          1340          1350          1360

              19          9
FN0429  CCTGGAGGAGGAGATATGAGGGA
        | | | | | | | | | | | | | | | | | | | | | | | | |
TH014   CCTGGAGGAGGAGATATGAGGGACAATTGGAGAAGTGAATTATATAAATATAAAGTAGTG
        1370          1380          1390          1400          1410          1420

        85.0% identity in 113 bp overlap
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*Figure 4. Alignment of nucleotide sequences of HIV-1 env genes. The sequence derived from (FN0429) is compared with reference sequence of subtype B (TH014).*

*Figure 5. Underdeveloped myelin structure in the medulla oblongata (Woelcke stain, x40).*

the muscular rigidity involved the upper extremities as well, although it was more pronounced in the lower extremities. The tendon reflexes were hyperactive. A delayed mental development was noted: his mental activity was at the level of an approximately 5 months old infant. At the age of 16 months the symptoms were similar: in addition, patella and Achilles clonus and a positive Babinski reflex (on both sides) was recorded. Mental development was still delayed. At 20 months the increased muscle tone was significant: flexion in the upper, extension in the lower extremities. The muscles of the neck and back were hypotonic. Clonus in the patella and Achilles tendon reflexes and a positive Babinski reflex occurred bilaterally. The patient was listening to his surroundings but did not grasp for toys, he could not play or speak. The EEG showed a desorganized cortical activity. The CT showed signs of a severe brain atrophy with the dilatation of extra- and intracranial CSF spaces. The child died due to respiratory and cardiac failure. The clinical diagnosis were AIDS and pneumonia.

*Pathological findings*

The pathological and histological findings supported the clinical diagnosis, the pneumonia affected both lungs, trophozotes and cysts of Pneumocystis carinii could be detected in the cysudate. Invasive candidiasis damaged the oesophagus. Histology of the brain is described below.

At autopsy the brain weighed 850 g. It was not swollen. The vessel walls seemed normal, the leptomeninges were transparent. The striatal area on the right side was brightened, and the white matter of the occipital lobes was soft. Microscopically a mild inflammation was found in the molecular layer of the cerebellar cortex: fungal elements were surrounded by a few macrophages and lymphocytes. No other region was affected by the inflammatory process. In the hemispheres, brain stem and spinal cord there were signs of delayed development: a layer of primitive nerve cell elements was revealed subependymally and delayed myelinization *(Figure 5)* was observed. Widespread reduction in the cortical volume was found. In the parietal and occipital lobes large cortical regions of spongionecrotic alteration were revealed. The nerve cells of the brain stem nuclei showed chronic alterations: these were altered in shape and size.

Only the motoneurons of the spinal cord seemed to be formally intact. Mineralization of the vessel walls occurred in the basal ganglia: furthemore, pigment granules were seen in both the parietal cortical and parietal white matter areas.

Immunohistochemical investigation showed some HIV-1 p24 antigen perivascularly and in oligodendroglial elements *(data not shown)*.

## *Discussion*

The early diagnosis of HIV infection in infants born from HIV-infected or AIDS diseased mothers is essential for the introduction of therapy as soon as possible. This may be problematic since routine serological methods (anti-HIV antibody assays) do not give reliable results in the first 18 months of life due to the passively transferred maternal antibodies. Therefore the diagnosis hinges upon the demonstration of the presence of the virus.[2,5,14,16]

In the case of a newborn child from a mother with AIDS we have succesfully isolated the virus. We found that the virus was a syncytium inducing strain by the use of PCR, heteroduplex formation assay and DNA sequencing and belongs to the B subtype, which is the dominant subtype in Europe and in USA Presence of the virus in the infant was also proven by p24 antigen and reverse transcriptase assay. The virus was AZT sensitive. Although AZT-therapy was started at the patient's 7$^{th}$ month of age, the clinical and laboratory (decreased CD4 cell count) symptoms continuously progressed, resulting in the child's death at the age of 20 months.

The histological studies of the brain and nervous system provided an explanation for the neurological signs and symptoms (including CT), indicating involvement of the brain and nervous system in the HIV-induced pathological process. HIV infection of the central nervous system (CNS) results in clinical disorders ranging from acute encephalopathies to the AIDS dementia complex.[11] Productive HIV replication occurs predominantly in macrophages and microglia, suggesting that neurological disfunction may be a consequence of the altered interaction of neurons with infected microglia. Toxic cellular factors induced by HIV infection and toxic viral proteins may contribute to this process (see 11 for review), although one may also consider the possibility

that neurological disfunction may result from disturbances in the function, independent of cell killing.[10] The fact that AIDS related dementia is reversible in patients receiving antiviral treatment suggests that – at least initially – HIV proteins or the mediators induced by the virus are noncytotoxic.[11] We suggest that the neurological symptoms of the infant and certain pathological findings (retarded development of hemispheres, brain stem and spinal cord as well as delayed myelinization) are probably due to the presence of HIV-1 in the CNS. Thus, HIV-1 infection may interfere with the development of the CNS either by affecting cell-cell interactions or by causing cell death in a direct or indirect way. This case is the first in Hungary, when a mother to child transmission of HIV was proven.

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