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Low Levels of Human Immunodeficiency Virus Replication in the Brain Tissue of Children with Severe Acquired Immunodeficiency Syndrome Encephalopathy

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The authors examined the autopsy brain samples of nine children infected with human immunodeficiency virus (HIV) at birth by histology, immunologic staining, and* in situ *hybridization. Surprisingly, although seven of these children presented with typical AIDS encephalopathy, the authors could detect a multifocal HIV infection in the brains of only three of these patients. The authors could not detect any significant HIV replication in the brain of four other children despite severe neurologic disease. However, HIV DNA was detected by polymerase chain reaction (PCR) in the central nervous system (CNS) of all patients. In addition, the authors found associated lesions in the brains of three of these four patients. This study shows that severe AIDS encephalopathy exists in children and therefore might exist in adults with few signs or without any signs of HIV replication or inflammation in the CNS. Understanding the pathogenesis of this neurologic disease and the kinetics of HIV replication in brain tissue of children with AIDS encephalopathy is essential to determine the best therapeutic strategy. (Am J Pathol 1992, 140:137–144)

About 35% of children born to human immunodeficiency virus (HIV)-infected mothers are HIV-infected.[1–3] A clinical disease appears in most of these children before the age of 2 years. A third of HIV-infected children develop an early and severe immunosuppression revealed by opportunistic infections and/or by AIDS encephalopathy usually during the first year of life. AIDS encephalopathy occurs almost exclusively in children with severe immunosuppression[4,5] and is characterized by a loss of developmental milestones that evolve to severe intellectual deficiency and severe deficiency associated with impaired brain growth. The course of this encephalopathy most often progresses rapidly to a plateau that is stable for several months or years, and is associated with periods of marked neurologic deterioration.[6–13] AIDS encephalopathy in children is characteristically associated with calcifications in the basal ganglia and the white matter, and with large areas of white matter pallor.[7,9] The presence of HIV infection is revealed in those lesions by multinucleated cells, viral particles, viral antigens, HIV RNA, and proviral DNA.[6–16] The association with other pathogens is rare.[8,10] The high incidence of AIDS encephalopathy in children with severe immunosuppression, the presence of HIV brain infection, and the usual absence of other pathogens strongly suggest that HIV itself is responsible for this neurologic disease. However, several cases of AIDS encephalopathy in children with isolated brain calcifications and white matter lesions without evidence for any etiologic agent on formalin-fixed tissue have also been described and remain to be explained.[6–9,13,15]

To better understand the relationship between HIV central nervous system (CNS) infection and AIDS encephalopathy, we examined autopsy brain samples of nine children found to be infected at birth. Seven of these children presented with typical AIDS encephalopathy. We looked for HIV, for other pathogens, and for brain damage in CNS using histology on formalin-fixed tissues.

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Because HIV is related to lentiviruses,[17] which can induce encephalopathy with low levels of viral replication in the CNS,[18] we used an extensive and more sensitive search for HIV and other pathogens by immunohistochemistry and *in situ* hybridization on subjacent frozen tissue sections. We also used polymerase chain reaction (PCR) to detect HIV DNA. In addition, to look for an ongoing infectious process, we characterized inflammatory infiltrates using immunohistologic staining for monocyte/macrophages and lymphocytes. Since it may be possible to improve the neurologic conditions of patients with antiviral drugs,[19] the pathogenesis of this encephalopathy and the kinetics of HIV replication in the brain of children with AIDS must be understood to determine the best therapeutic strategy.

Patients and Methods

Patients

All children were infected by perinatal transmission except for patient 3 who was contaminated in the neonatal period by transfusion. HIV infection was diagnosed according to CDC guidelines.[20] Patients 1 to 9 presented with symptoms of HIV infection included in CDC class P-2. The clinical statuses of the patients are summarized in Table I. The severity of AIDS encephalopathy was evaluated by the same neurologist according to a published disability status scale,[5] and was classified as severe, moderate, or absent. Neurologic development of patient 1 had been normal for at least 1 year when she was lost to follow-up until death, and neurologic development of patient 2 was normal throughout his life. Seven children developed AIDS encephalopathy between 4 and 17 months of life. AIDS encephalopathy was moderate in patient 3 and consisted in a progressive delay in cognitive development associated to a moderate spasticity in the lower limbs. In six other patients, a gross impairment of cognitive development and severe loss of motor function appeared progressively within a few months. Eventually, a cerebral atrophy developed in patients 4 to 9 as judged by serial measurements of head circumference and by CT scan. A severe systemic CMV infection developed in patient eight 6 months after the appearance of typical AIDS encephalopathy and seizures during the last 3 months of life. Patients 2 to 9 had biologic signs of profound immunosuppression.[5] We repeatedly detected HIV p24 antigen in sera of all nine children. HIV p24 antigen was looked for in cerebrospinal fluid (CSF) of all patients except patient 3 and was detected only in patient 7. We found a high level of anti-HIV antibody titer in sera and CSF of only patients 1 and 4, whereas this titer was low in sera and CSF of other patients. Six patients (patient 2, patients 5–9) had been treated with Azidothymidine (AZT) orally, 5 mg/kg every 6 hours at first signs of severe immunodeficiency (onset of neurologic symptoms or opportunistic infections). AZT was pursued at the same doses until death except for patients 6 and 9 for whom treatment was discontinued 3 months before they died. In patients 5 to 9, treatment with AZT was pursued for respectively 13, 16, 18, 27, and 27 months without any improvement of their neurologic condition.

Table 1. *Clinical Status of the Nine Children*

Patients			Symptomatic infections		Neurologic disease				
Identification	Age at death (months)	Maternal risk factor	Age at appearance (months)	Type	Age at appearance (months)	Severity	HC at death (SD)	CT scan	AZT
1	33	Heterosexual (Haitian)	9	Polyadenopathy Hepatomegaly	Absent?	Absent?	+1	Not done	0
2	24	Drug addict	18	Cryptosporidiosis	Absent	Absent	−1	Normal	+
3	42	Transfusion at birth	11	PCP	28	Moderate	−2	Not done	0
4	24	Drug addict	22	PCP	17	Severe	0	Atrophy	0
5	18	Heterosexual (African)	3 4 14	PCP CMV pneumopathy Oesophagal candidiasis	9	Severe	−2	Calcifications	+
6	23	Drug addict	4	PCP	8	Severe	−3	Calcifications	±
7	28	Drug addict	26	PCP	4	Severe	0	Atrophy	+
8	42	Heterosexual (French)	10 15	Oesophagal candidiasis CMV pneumopathy	9	Severe	+1	Atrophy	+
9	39	Drug addict	36	Disseminated candidiasis	9	Severe	−2	Atrophy	±

PCP = pneumocystis carinii pneumonitis; CMV = cytomegalovirus, HC = head circumference, SD = standard deviation. AZT: 0 = no treatment; + = treatment until death; ± = treatment discontinued 3 months before death.

Neuropathologic Study

Patients underwent autopsy between 24 hours and 72 hours after death. Neuropathologic examination was completed in each patient with formalin-fixed tissues and sometimes with celloidin-embedded sections after usual staining (hematoxylin-eosin, Nissl, Wolcke, Bodian, Grocott, Perls, Gram). For each patient, samples of hemispheres, basal ganglia, brain stem, cerebellum, and when possible spinal cord, were subdivided, frozen in isopentane, cooled in liquid nitrogen, and stored at −80°C. As controls for the techniques of *in situ* hybridization and immunohistochemistry, we used CNS samples of HIV-1 uninfected patients and CNS samples of 28 adult AIDS patients with severe HIV encephalitis. For PCR, samples were rinsed in phosphate-buffered saline (PBS), separated in individual sterile bags, and stored directly at −80°C. All preparations were performed under a hood with disposable material.

Histochemistry

Each CNS sample was checked for the presence of HIV antigens using monoclonal antibodies directed against the p18 and p24/p25 antigens (Genetic systems, B.S. Parekh, Biorad) and in some cases with antibodies directed against the gp41 and gp120 viral proteins (Genetic systems). Toxoplasma, cytomegalovirus (CMV), herpes simplex virus 1 and 2, JC virus antigens were examined in each frozen CNS sample by immunologic staining as previously described.[21,22] Antibodies directed against the EBNA1 antigen of Epstein–Barr virus (EBV, Biosoft) and the internal p19 antigens of HTLV1 (C. Desgranges) were used in some cases. For each patient, all tissue blocks were screened for the presence of monocyte/macrophage/microglial lesions using antibodies directed to CD68 antigen (KiM7, Boehring). In addition, samples of white matter, basal ganglia, and pathologic areas were tested for the presence of monocytes, and B and T lymphocytes with antibodies directed to the CD14, CD19, CD3, CD8, CD4 molecules (Becton-Dickinson). In patient 1, we used antibodies directed to CD22 antigen, and to kappa- and lambda-light chains of immunoglobulins (Becton-Dickinson). We identified CNS cells using antibodies directed to astrocytes (GFAP, Amersham), neurones (neurofilaments, D. Paulin, Pasteur Institute), and endothelial cells (Factor VIII, Dakopatts).

In Situ *Hybridization*

HIV 1 RNA probes were labeled by in vitro transcription with ^{35}S UTP and ^{35}S ATP (Amersham). Sections were hybridized according to published procedure,[22] except that they were denatured in 70% formamide, 2XSSC at 70°C for 2 min and dehydrated in ice-cold alcohol before being incubated with the probe.[23] We used "anti-sense" HIV RNA probes complementary to HIV mRNA to detect both viral mRNA and proviral DNA in denatured tissue sections. We used also "sense" HIV ^{35}S RNA probes which had the same sense as transcription and therefore would be unable to hybridize to HIV mRNA but which could hybridize with HIV DNA in denatured tissue sections. This selective DNA hybridization was used to estimate the sensitivity of our technique of hybridization. We checked the specificity of our results using "sense" and "anti-sense" HIV 1 probes on uninfected brain tissue sections and on HIV-infected tissue sections that had not been denatured.

Polymerase Chain Reaction

For each patient we extracted the total DNA from three to five samples of white matter and basal ganglia subjacent to the areas where white matter lesions and calcifications were detected. Tissues were lysed in guanidinium thiocyanate. Cesium chloride was added to a final concentration of 1 g/ml and centrifuged overnight at 50,000 rpm.[24] The DNA was extracted from the middle of the gradient and precipitated with ethanol. All of the extraction steps were performed in a laminar air-flow hood set aside for PCR work. Each set of six to eight HIV-infected CNS samples were extracted at the same time as two HIV1-uninfected control brain samples, which were amplified during the same assay. PCR was performed according to published procedure using the gag specific primers SK38/39.[25] PCR products were analyzed by Southern blotting with an internal SK19P^{32} end-labeled probe. The presence of HIV DNA in CNS samples was checked using SK29/30 and SK68/69 primers,[25] that amplified DNA in the LTR and the Env gene of HIV1. For each series of extraction, the absence of contamination during the enzymatic reaction was verified by amplification of the reagents alone.

Results

Brain tissue was examined for HIV by immunostaining, histology, *in situ* hybridization, and PCR. Because the virion-proteins gag and envelope are produced late in the viral life cycle, the detection of these antigens by immunologic staining indicate cells in which the virus is replicating. Likewise the presence of multinucleated cells arises from the end step of viral replication. *In situ* hybridization and PCR were used for the detection of HIV RNA and DNA that might be present without detectable levels of HIV antigens.

Three groups of patients could be distinguished according to their neurologic status and to the presence of HIV replication in the CNS (Table 2). Group 1 comprised two patients without encephalopathy (patients 1 and 2). In patient 1, we found one multinucleated cell in the spinal cord without any other signs of HIV replication in the CNS. We did not detect any neuropathologic lesions, any sign of HIV replication, and any other pathogen in patient 2. In both patients, HIV DNA was detected by PCR in three of the five tested CNS samples. The negative samples were found in both the white matter and basal ganglia. In addition, we found perivascular infiltrates of polytypic small lymphoplasmocytic $CD19^+$, $CD22^+$ B cells associated with $CD4^+$ and $CD8^+$T lymphocytes and with macrophages in one patient. There was not morphologic or immunologic evidence for a malignant lymphoma. We could not detect any etiologic agent, especially any EBNA-1 or JC virus T antigens in these infiltrates.

Group 2 comprised three patients with AIDS encephalopathy and consisted of patients 3, 5, and 9. In these patients we found signs of multifocal HIV replication in the CNS. However, the number of infected cells was much lower than in adults with severe HIV encephalitis and similar postmortem timeframe that we have studied in parallel using immunohistology.[21,22,26] In patient 5 and in patient 3, HIV-infected cells were detectable only in half or even less of the CNS areas tested. Only in patient 9 did we detect extensive HIV CNS infection similar to severe encephalitis observed in adult AIDS patients. In this patient, HIV-infected cells were present in almost every tissue block examined, and were clustered in microglial nodules, perivascular infiltrates, or disseminated in the parenchyma (Figure 1). They were mainly located in the white matter, the basal ganglia and the brain stem, and were associated with calcifications and large areas of gliosis and white matter pallor. Using double immunologic staining for viral and cellular antigens, we found that cells expressing HIV proteins were always labeled with monocyte/macrophage/microglial markers (Figure 2a). We could not detect any HIV infection in astrocytes, neurones, or endothelial cells (Figure 2b). We looked for HIV infected cells using *in situ* hybridization with sense and anti-sense HIV S^{35} RNA probes in the white matter and basal ganglia of all three patients. By comparison between immunohistologic staining and *in situ* hybridization on serial tissue sections, we detected HIV mRNA and HIV proviral DNA only in tissue sections in which HIV–antigen-expressing cells were present. HIV-infected cells revealed by *in situ* hybridization and immunohistologic staining on subjacent tissue section expressed the same pattern of labelling (Figure 3). In addition, we found microglial nodules, perivascular infiltrates of CD8 T lymphocytes, and monocytes only in areas where HIV replication was detectable. In these patients, PCR detected HIV DNA in all CNS samples. We did not find any other pathogen in these three children.

Group 3 was the most interesting one. This group consisted of four patients (patients 4, 6, 7, 8), who despite severe AIDS encephalopathy and typical neuropathologic lesions (intraparenchymal calcifications (patients 4,6,8) or gliosis and white matter pallor (patient 4), showed few or no signs of HIV replication in the CNS. Signs of HIV replication were limited to a single multinucleated cell in patient 4 and to one cluster of HIV-infected cells in the cerebellum of patient 6. However, except for patient 7 who did not present any inflammatory infiltrates nor any other pathogen, these patients showed additional CNS lesions. We found cortical foci of ischemic necrosis in patient 4 and foci of Aspergillus infection in the cerebral cortex of patient 6. Both findings appeared

Table 2. *Summary of Neuropathologic and Virologic Findings*

	Neuropathology			Signs of HIV infection					
Patients	Calcif.	Gliosis	White matter lesions	Multinucleated cells	HIV antigens	*In situ* hybridization	HIV DNA PCR	Inflammation	Associated CNS lesions
1	+	0	±	+*	0/14	0	3/5	+ + +	Inflammatory infiltrates
2	0	0	0	0	0/19	0	3/5	0	0
3	0	0	0	+*	4/14	+	5/5	+	0
4	+ + +	+ +	+ +	+*	0/12	0	5/5	+	Ischemic necrosis
5	+ +	+ +	+ +	+	7/14	+	5/5	+	0
6	+ + +	+	±	0	1/14	+	5/5	±	Aspergillosis
7	0	0	0	0	0/14	0	4/5	0	0
8	+ + +	+ +	+	0	0/16	0	5/5	Peri-ependymal infiltrates + +	Cytomegalovirus encephalitis
9	+ +	+ +	+ +	+	17/18	+	3/3	+ +	0

Calcif = calcifications, HIV antigens = number of brain tissue blocks with detectable HIV antigens/number of CNS tissue blocks tested. Signs of HIV infection: 0 = absence of HIV-infected cells; + = presence of HIV-infected cells; +* = only 1 multinucleated cell found at the whole neuropathologic examination. HIV DNA PCR = number of brain samples positive for the presence of HIV DNA/number of brain samples tested (we tested areas of white matter and of basal ganglia in all patients).

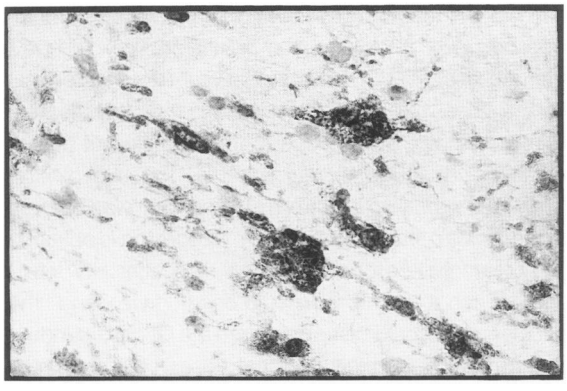

Figure 1. *HIV-infected mononucleated and multinucleated cells detected by immunoperoxydase staining in the white matter of patient 9 using a monoclonal antibody directed to HIV internal proteins, ×630.*

to be recent and active, and we found a severe periependymal CMV encephalitis in patient 8.

We looked for an underlying latent HIV encephalitis in the basal ganglia and the white matter of these four patients. We could not detect any HIV mRNA, or any HIV proviral DNA by *in situ* hybridization. We could not detect any significant inflammatory infiltrate in those areas using immunohistochemistry for monocytes and B and T lymphocyte markers. Perivascular infiltrations with CD8 T lymphocytes and monocytes, and microglial proliferation were detected only when associated lesions were present, i.e., in cerebral cortex or periependymal area. However, in these patients, HIV DNA was detected by PCR in all five CNS samples tested for patients 4, 6, 8 and in four of five CNS tissue block tested for patient 7 (Figure 4). The amplification of control DNA extracted from uninfected brain samples was always negative as well as the amplification of PCR reagents alone.

Discussion

We have examined the brain tissue sections of seven children infected at birth with typical AIDS encephalopathy. Surprisingly, using immunologic staining and *in situ* hybridization, we detected only minor signs of HIV replication (or no signs) in the brain of four of these patients. In addition, even in patients with multifocal HIV CNS infection, the number of HIV-infected cells was strikingly lower than in adults with severe HIV CNS infection. Furthermore, we detected associated lesions in the brains of three of the four patients with AIDS encephalopathy but with only minimal signs, or no signs at all, of HIV CNS infection. The ischemic necrosis in patient 4 and the Aspergillus-induced lesions in patient 6 appeared to be recent and active and could not explain the neurologic disease that had been progressing for 7 and 15 months. In patient 8, a systemic CMV infection and seizures occurred several months after the onset of typical AIDS encephalopathy. We concluded that in the seven patients, AIDS encephalopathy was related to HIV infection, and that associated CNS lesions contributed to the children's secondary neurologic deterioration. Therefore, we have to consider that AIDS encephalopathy in children is related to HIV but not necessarily associated with viral replication in the CNS at the time of death.

AIDS encephalopathy in children is characteristically associated with calcifications in the basal ganglia and the white matter, and with large areas of gliosis and white matter pallor[6,7,9] which suggest a previous insult to the brain. Those lesions could be related to soluble factors (HIV gp120 surface glycoprotein,[27–29] or cytokines[30,31]). However, in adults, severe HIV-related dementia appears only in a subgroup of AIDS patients and is usually correlated with the detection of extensive HIV CNS infection.[32] This suggests that viral replication in the brain is required to produce those factors and induce neuropathologic lesions.

In four children, despite severe AIDS encephalopathy, we detected few or no signs of HIV replication in the CNS using neuropathologic examination or immunologic staining. Using *in situ* hybridization, we could not detect

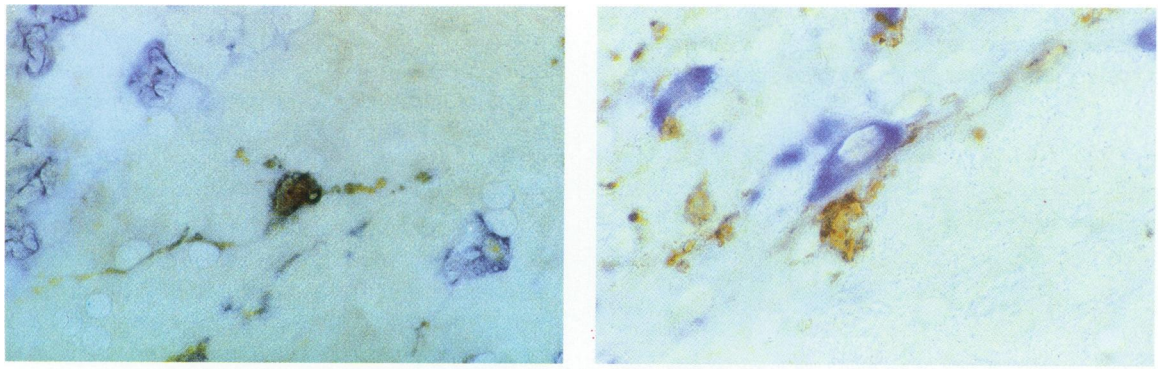

Figure 2. *Identification of HIV-infected cells by double immunostaining for HIV internal proteins (peroxidase/brown) and cellular antigens (alkaline phosphatase/blue) in the brain stem of patient 9.* **A:** *HIV-infected macrophages/microglial cells are labeled for viral ($p25^+$) and macrophage ($CD68^+$) markers (×630).* **B:** *Neurones ($neurofilament^+$) are uninfected and HIV-infected cells ($p25^+$) are neurofilament. Note the close relationship, which is usual in patients with HIV encephalitis, between HIV-infected cells and uninfected neurones, ×630.*

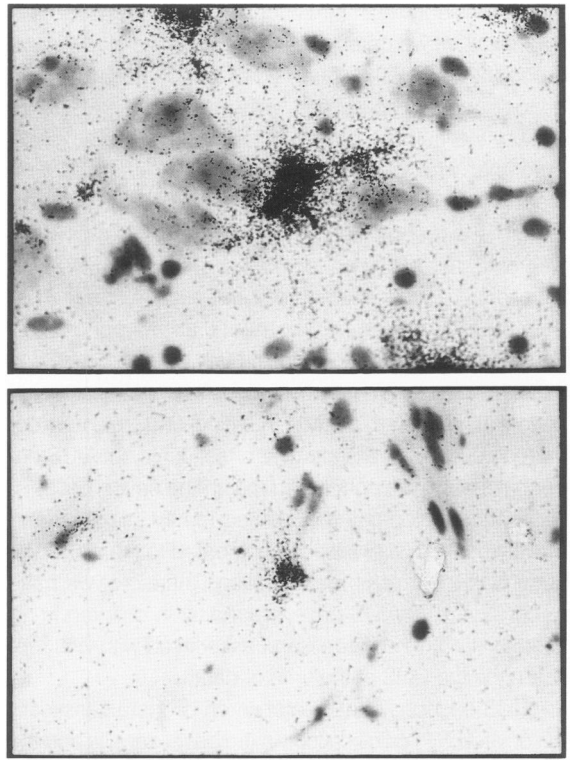

Figure 3. *HIV-infected cells detected by in situ hybridization in the brain stem of patient 9. These cells were detected on tissue sections subjacent to those of Figure 2.* **A:** *HIV-infected cell labelled for viral mRNA and proviral DNA with HIV "anti-sense" probe after a three weeks exposure. This cell was surrounded by uninfected neurons.* **B:** *In a subjacent tissue section HIV-infected cells were selectively labelled for HIV proviral DNA with a "sense" probe after the same exposure time, autoradiography and H&E ×630.*

any HIV mRNA or HIV DNA in cells that did not express HIV antigens. Based on reported copy number of HIV DNA in the brain of adult AIDS patients,[33] and our ability to detect HIV DNA on tissue sections (Figure 2B), we estimated that we could detect at least 200 copies of HIV RNA and DNA per infected cell using *in situ* hybridization. However, we detected HIV DNA using PCR in almost all the CNS samples tested. The presence of HIV DNA alone detected by PCR does not necessarily indicate an active replication. It could be related to a latent HIV infection or to levels of viral replication too low to be detectable by the other techniques. However, we do not favor this hypothesis because of the absence of inflammatory infiltrates that are described in another lentiviral latent encephalitis.[18] Rather, we believe that it is more likely that the neurologic symptoms were due to the sequelae of a prior acute or subacute HIV encephalitis. The fate of HIV-infected cells *in vivo* is currently unknown. It was demonstrated *in vitro* that the majority of microglial cells obtained at primary culture of adult brains died after HIV infection.[34] Such a mechanism could occur *in vivo,* and HIV-infected cells could disappear from the CNS after inducing irreversible sequelae. In that case, the virus would have replicated in the CNS during earlier stages of HIV infection before or at the onset of immunosuppression. The high incidence of AIDS encephalopathy in children perinatally infected might be explained by a transient HIV infection of monocytes/macrophages/microgliae which differentiate in the developing brain.[35]

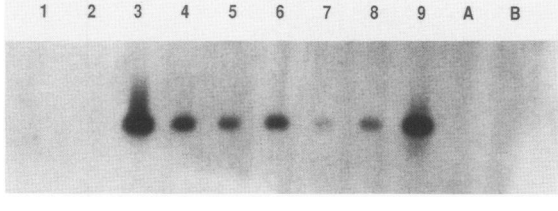

Figure 4. *Analysis by PCR of the total DNA extracted from basal ganglia of the nine patients, of one uninfected control* **(A)** *and of the reagents alone* **(B).** *DNA samples were amplified with SK38/39 primers and amplification products hybridized with SK19 P^{32} probe.*

Because of the extreme sensitivity of PCR, it is also possible that the positive results by PCR were due to HIV-infected cells present in circulating blood rather than in brain parenchyma. Regardless of which cells gave rise to the positive PCR result, because this HIV brain infection could only be detected by PCR, the level of HIV replication in the CNS of these children must be minimal or absent, in spite of severe AIDS encephalopathy.

The low levels of viral replication detected in the CNS of some of our patients could also be related to antiviral treatment. However, treatment with AZT did not improve the neurologic condition and did not decrease the level of HIV antigenemia of our patients. In addition, AZT cannot be the only way to explain the absence of detectable HIV CNS infection in our patients since we detected minimal signs of HIV replication in the brain of the one patient with severe AIDS encephalopathy who had not been treated with antiviral drugs at all (patient 4). In previous reports, several cases of AIDS encephalopathy in children associated with isolated brain calcifications and white matter lesions, without signs of inflammation or multinucleated cells, were described in the absence of any treatment with AZT.[6–9,13,15] Although immunocytochemistry and *in situ* hybridization were not used in these studies, the absence of multinucleated cells and of inflammatory infiltrates was similar to that of our patients. Therefore, the low level of HIV replication observed in the brains of these children could reflect the usual fate of HIV-infected cells in infected brains. In addition, the evolution of AIDS encephalopathy, which is often episodic,[6,11] and the variable response to treatment in children[19] are consistent with the hypothesis that bursts of HIV encephalitis were followed by periods of low turnover or cessation of HIV replication in the CNS.

In adults, a severe dementia occurs in about 20% of AIDS patients[36,37] and is usually associated with a mas-

sive and multifocal HIV brain infection and/or signs of inflammation in the CNS.[21,32,38] Interestingly, some cases of dementia were described in adults with AIDS, with few or no signs of HIV replication and no signs of inflammation in the CNS.[38,39] Because our work demonstrates that severe AIDS encephalopathy can be found in children with few or without any signs of HIV replication or inflammation in the CNS it is possible that similar situation occurred in adults with dementia, and that HIV might replicate transiently and induce sequelae in the brains of adult patients with ARC or AIDS. In that case, the presence of HIV antigens in severe dementia could merely reflect the persistence of viral dissemination in the brain, possibly triggered by associated pathogens.

In conclusion, our work demonstrates that severe HIV-related neurologic symptoms and associated calcifications and white matter lesions can occur in children with minor signs of HIV replication in the CNS at death. Assuming that these lesions were the result of prior HIV replication in the CNS, this suggests that HIV replicates early in the disease and that infected children must be treated before the appearance of immunosuppression to avoid HIV dissemination in the CNS and irreversible sequelae. Further large prospective studies and further experiments on animal models will certainly be required to understand the pathogenesis of AIDS encephalopathy and the kinetics of HIV replication on the brains of children and adults. This is essential to determine the best therapeutic strategy and improve the neurologic condition of these patients.

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