Neuropathogenesis of Simian Immunodeficiency Virus in Neonatal Rhesus Macaques

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Neonatal human immunodeficiency virus (HIV) infection usually occurs intrapartum or postpartum and results in a higher incidence of neurological dysfunction than is seen in adults. To explore the neuropathogenesis of neonatal HIV infection, we infected neonatal macaques with simian immunodeficiency virus (SIV) and followed the course of infection focusing on early time points. Infected neonates had decreased brain growth and mild histological changes in brain that resembled those seen in pediatric AIDS, including perivascular infiltrates of mononuclear cells, mineralization of vessels in the basal ganglia, and gliosis. The perivascular lesions and gliosis were associated with the presence of occasional infected cells that required *in situ* hybridization with radiolabeled riboprobes for detection. Using this technique, SIV-infected cells were detected in the brain parenchyma within 7 days of infection. These findings were confirmed by nested PCR for SIVgag DNA in brain and RT-PCR for viral RNA in cerebrospinal fluid. Together, these techniques revealed SIV infection of the CNS in 12 of 13 neonates infected with SIVmac239, 3 of 3 infected with SIVmac251, and 2 of 2 infected with SIVmac239/316. The prevalence of CNS infection was indistinguishable from that of older animals infected with the same dose and stock of virus, but neonates appeared to have fewer infected cells in the CNS and detecting them required more sensitive techniques. This observation was true regardless of inoculum and despite the fact that neonates had equal or greater viral loads in the periphery compared with older animals. These data suggest that maturation-dependent host factors have a major impact on the neuropathogenesis of pediatric AIDS. (*Am J Pathol* 1999, 155:1217-1228)

Pediatric human immunodeficiency virus (HIV) infection usually occurs intrapartum or immediately postpartum

and results in a higher incidence of neurological disease than is seen in adults.¹⁻³ The central nervous system (CNS) disease in children is also considered to be more severe than in adults but has a different spectrum of manifestations due to the developmental state of the brain. In children, the neurological disease is characterized by progressive or static loss of previously acquired neurodevelopmental milestones, impaired brain growth with cortical atrophy, and progressive motor dysfunction.³⁻⁶ Histopathological findings are similar to those seen in adults and include perivascular mononuclear cell infiltrates with occasional multinucleated giant cells (MNGCs) and gliosis.^{4,7,8} These lesions have a predilection for specific regions of the CNS, including basal ganglia, central white matter in cerebral hemispheres, and brain stem. In contrast to the neurological disease in adults, infants have a lower incidence of CNS opportunistic infections, peripheral neuropathies, and vacuolar myelopathy, but an increased incidence of vessel-associated mineralization.^{3,7} In the CNS of pediatric AIDS patients, HIV has been detected primarily within macrophages, microglia, and MNGCs, similar to what has been described in adults.^{7,9-12} Unique to pediatric AIDS is the possibility of restricted infection of astrocytes.^{13,14}

Early events in neonatal HIV-1 infection, including the timing of neuroinvasion, the distribution of virus in the CNS, and host and viral factors that contribute to neurological disease, are poorly understood due to the difficulty of obtaining appropriate samples. Nevertheless, current data suggest that the increased severity of CNS disease in young children compared to adults is related to the time of infection (*in utero*, intrapartum, or postpartum) and the immaturity of the host immune system.¹ To further examine the neuropathogenesis of pediatric AIDS with a focus on early events, we have used the neonatal rhesus macaque infected with simian immunodeficiency virus (SIV) as a model of pediatric AIDS.¹⁵⁻¹⁷

In this study, we examined the neuropathogenesis of SIV infection in neonatal macaques, focusing on the first 2 months of infection. This is the time period when SIV infection of the CNS has been shown to occur in older

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Table 1. Animals, Viral Inoculum, and Major Pathological Findings in Neonates Infected with SIV

Virus	Animal no.	Days pi	Major pathological findings*
SIVmac239	68-97	3	NSL
	69-97	3	Hepatitis, periportal minimal
	102-97	7	Thymic dysinvolution, mild; lymphoid hyperplasia, moderate
	103-97	7	Thymic dysinvolution, moderate; lymphoid hyperplasia, moderate
	136-97	14	Lymphoid hyperplasia, moderate; thymic dysinvolution, mild
			Hepatitis, typhlocolitis, myocarditis (<i>Clostridium pilliforme</i>)
	137-97	14	Lymphoid hyperplasia, mild; thymic dysinvolution, marked
			Hepatitis, typhlocolitis, myocarditis (<i>Clostridium pilliforme</i>)
	190-97	21	Lymphoid hyperplasia, moderate
			Typhlocolitis (<i>Clostridium pilliforme</i>)
	191-97	21	Lymphoid hyperplasia, moderate
			Typhlocolitis (<i>Clostridium pilliforme</i>)
	296-97	50	Lymphoid hyperplasia, moderate
	297-97	50	Lymphoid hyperplasia, moderate
SIVmac251	415-97 [†]	79	Lymphoid depletion, severe
			Adenoviral enteritis
	484-97 [†]	141	Lymphoid depletion, severe
			Adenoviral enteritis, severe
	412-97 [†]	209	Lymphoid depletion, severe
			Disseminated adenoviral infection
SIVmac239/316			<i>Pneumocystis carinii</i> pneumonia
SIVmac251	163-98	21	Lymphoid hyperplasia, moderate; thymic dysinvolution, mild
	162-98 [†]	35	Lymphoid depletion, moderate
			Disseminated adenoviral infection
SIVmac239/316	160-98	50	Lymphoid depletion, moderate
			Adenoviral enteritis
SIVmac239/316	109-98	50	Lymphoid hyperplasia, mild
	110-98	50	Adenoviral enteritis
			Lymphoid hyperplasia, mild
			Adenoviral enterocolitis

*Excludes the CNS.

[†]Animals euthanized when moribund with AIDS.

NSL, no significant lesions.

macaques.¹⁸⁻²⁰ For these studies animals were infected with equal doses of the pathogenic molecular clone SIVmac239 ($n = 13$), the macrophage-tropic derivative of SIVmac239 known as SIVmac239/316 ($n = 2$), or uncloned SIVmac251 ($n = 3$). Although the prevalence of CNS infection was indistinguishable from that of older animals infected with the same dose and stock of virus, neonates appeared to have fewer infected cells in the CNS and detecting them required more sensitive techniques. This was true regardless of inoculum and despite high viral loads in peripheral blood and peripheral lymphoid organs. Thus, although neuroinvasion by SIV occurred rapidly in neonatal macaques, viral replication and neuropathology were limited. This suggests that maturation-dependent host factors have a major impact on the neuropathogenesis of pediatric AIDS.

Materials and Methods

Animals and Viral Inocula

A total of 18 rhesus macaque (*Macaca mulatta*) neonates were obtained by cesarean section at 155 ± 5 days of gestation and inoculated intravenously within 24 hours of birth with 20 ng p27/kg (approximately 10^3 50% tissue culture infectious doses/kg) of one of three isolates of

SIV: SIVmac239 and SIVmac239/316, which are molecular clones, and uncloned SIVmac251 (Table 1). These are the same stocks and doses of virus (per kilogram) that have been used previously in our juvenile macaque studies.¹⁹⁻²³ The origin, gene sequence, and biological behavior of these viruses have been described extensively.²⁴⁻²⁸ Briefly, SIVmac239 is the prototypical pathogenic molecular clone; it replicates poorly in monocyte/macrophages *in vitro*. SIVmac239/316 is a macrophage-competent derivative of SIVmac239 that differs by eight amino acids in envelope. SIVmac251 is a highly pathogenic uncloned isolate that replicates well in both lymphocytes and monocyte/macrophages.

Thirteen animals were inoculated with SIVmac239 and two animals were euthanized at 3, 7, 14, 21, and 50 days postinfection (dpi). Three additional animals were similarly infected and allowed to progress until they became moribund with AIDS, at which time they were euthanized at 79, 141, and 209 dpi. Two animals were inoculated with SIVmac239/316 and euthanized at 50 dpi. Three animals were inoculated with SIVmac251 and euthanized at 21, 35, and 50 dpi. The SIVmac251-infected infant euthanized at 35 dpi was moribund with AIDS.

All animals were housed in accordance with standards of the American Association for Accreditation of Laboratory Animal Care. The investigators adhered to the *Guide*

for the Care and Use of Laboratory Animals prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council. All dams were negative for antibodies to HIV-2, SIV, Type D retrovirus, and Simian T-cell leukemia virus type 1 before cesarean section.

Determination of Viral Load by Limiting Dilution Culture of Peripheral Blood Mononuclear Cells (PBMC) and Quantitation of Viral RNA in Plasma and Cerebrospinal Fluid (CSF)

Peripheral blood was collected from all animals before inoculation, at days 3, 7, 14, 21, 35, and 50, monthly thereafter, and terminally. CSF was collected at similar intervals, but only from animals inoculated with SIVmac251, SIVmac239/316, and two of the three animals inoculated with SIVmac239 that survived more than 50 days. Peripheral blood was used for quantitation of cell-associated viral loads and determination of plasma SIV RNA levels. Quantitative viral cultures were performed on each blood sample as described previously.²⁹ Briefly, serial threefold dilutions were performed in duplicate beginning with 10⁶ PBMC. PBMC dilutions were cocultured with 10⁵ CEMX174 cells in a volume of 1 ml. Cultures were split 1:2 twice weekly until day 21, when the cultures were assayed for virus production by enzyme-linked immunosorbent assay for SIV p27 (Coulter Corp., Hialeah, FL). Results are expressed as the number of SIV-infected cells/10⁶ PBMC.

Virion-associated SIV RNA in plasma and CSF was quantified by using a real-time reverse transcription-polymerase chain reaction (RT-PCR) assay on an Applied Biosystems (Foster City, CA) Prism 7700 sequence detection system as described previously.^{30,31} Results shown are averages of duplicate determinations. Analyses of viral RNA levels were performed by Drs. Jeffrey Lifson and Michael Piatak at Scientific Applications International Corporation (Frederick, MD).

Tissue Collection and Processing

Animals were sacrificed at intervals described above and as shown in Table 1. At necropsy animals were exsanguinated and body and organ weights were recorded. A complete set of tissues, including frontal cortex, basal nuclei, thalamus, and brain stem, were collected and fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 6 μ m, and stained with hematoxylin and eosin by routine techniques. *In situ* hybridization was performed on serial sections. Adjacent blocks of fresh tissue were snap-frozen for immunohistochemistry in optimum cutting temperature compound (O.C.T., Miles Inc., Elkhart, IN) by immersion in 2-methylbutane cooled in dry ice.

Localization of Virus in Tissues

Viral localization was examined in peripheral lymphoid tissues and at least three different regions of the brain,

including cerebral cortex, basal ganglia, and brain stem, by immunohistochemistry for viral antigens and two different *in situ* hybridization techniques to detect RNA. The *in situ* hybridization techniques used either a digoxigenin-labeled, random primed DNA probe or a ³⁵S-labeled riboprobe. The DNA probe was a combination of two plasmids: a subclone of p239SpE3' in pBS⁻, which contains *tat*, *rev*, *env*, *nef*, and small part of the 3'LTR, and p239SpSp5', which contains *gag*, *pol*, *vif*, *vpx*, *vpr*, and the 5'LTR in pBS⁺. This combination provides essentially the entire SIVmac genome. The probe was labeled with digoxigenin-11dUTP by random priming (Boehringer Mannheim, Indianapolis, IN) as previously described.²⁹ Controls consisted of hybridizing sections with plasmid pUC19, which had been labeled with digoxigenin at the same time as the probe and matched tissues from uninfected, age-matched macaques. Labeled cells were detected using a digoxigenin-specific antibody in a standard avidin-biotin-horseradish peroxidase complex (ABC) technique as previously described.²⁹

The second *in situ* hybridization technique used radio-labeled RNA probes synthesized from five DNA templates, covering 90% of the SIV genome, subcloned into pGEM4.³² Controls consisted of hybridizing sections with sense probes and matched tissues from uninfected, age-matched macaques. The radiolabeled *in situ* hybridization was performed by Dr. Cecil Fox at Molecular Histology, Inc. (Montgomery Village, MD).

To localize viral antigen, snap-frozen tissues were used in immunohistochemical procedures as previously described.²¹ Briefly, frozen tissue sections were fixed in 2% paraformaldehyde for 10 minutes at 4°C and immunostained using an ABC technique with diaminobenzidine (DAB) as the chromogen. The primary antibody used was Senv71.1 (provided by C. Colignon, C. Thiriart, SmithKline Beecham, Rikensart, Belgium), which recognizes SIV gp120. Negative controls included serial sections processed identically, using equivalent concentrations of irrelevant primary antibodies of the same isotype and matched tissues from uninfected macaques.

Detection of Viral DNA by PCR

To confirm the results of *in situ* hybridization for viral RNA and to detect viral DNA we performed nested PCR for SIVgag. Tissue sections were collected at necropsy from selected brain regions, including the frontal cortex, basal ganglia, and brain stem, in 50- to 100-mg pieces, frozen in microcentrifuge tubes on dry ice, and stored at -70°C for future use. DNA was isolated from frozen tissues using the Qiagen (Valencia, CA) DNA isolation kit as per manufacturer's recommendations with an additional overnight incubation with proteinase K at 55°C. Two hundred nanograms of genomic DNA were amplified using nested primers (10 pmol/50 μ l reaction) for SIVgag (outer 5': 5'-CTA CGA CCC AAC GGC AAG-3'; outer 3': 5'-TTG CTT CCT CAG TGT GTT TC-3'; inner 5': 5'-GAA AGC CTG TTG GAG AAC AAA GAA GGA-3'; inner 3': 5'-AGT GTG TTT CAC TTT CTC TTC TGC GTG-3') with 2 mmol/L MgCl₂ and denatured at 92°C for 2 minutes. The ampli-

fication profile consisted of 30 seconds at 92°C, 30 seconds at 61°C, and 30 seconds at 72°C for 40 cycles followed by an extension time of 10 minutes at 72°C. PCR products were visualized on an ethidium bromide-impregnated agarose gel.

Immunophenotype of Infected Cells

To examine the immunophenotype of infected cells we combined nonradiolabeled *in situ* hybridization for viral RNA with immunohistochemistry for monocyte/macrophages. This entailed performing nonradiolabeled *in situ* hybridization for SIV as described above using nickel cobalt-enhanced DAB (black), followed by immunohistochemistry for monocyte/macrophages (HAM56) using DAB (brown) as previously described.²² These double labels were performed on peripheral lymphoid tissues of rhesus neonates. The scarcity of infected cells in the brain precluded effective use of this technique in the CNS.

Results

Infant Macaques Infected with SIV Have High Viral Loads and Rapid Disease Course

All 18 animals inoculated with SIV were viremic within 3 days of inoculation and remained persistently viremic throughout the course of infection. PBMC viral loads and plasma SIV RNA levels rose quickly, peaked at 7 to 14 dpi, and remained high for the life of the animal (Figure 1). This pattern of viral infection is similar to that of adult rapid progressors.²⁰ Notably, viral loads in neonates were as high as or higher than those seen in juveniles or adults inoculated with the same stock and dose of virus at the same time points, but the difference did not reach statistical significance (data not shown). Robust viral replication was also demonstrated by immunohistochemistry and *in situ* hybridization in spleen, lymph nodes, and thymus as early as 7 dpi in animals infected with SIVmac239, SIVmac251, and SIVmac239/316 (Figure 2). Overall, disease progression was rapid as evidenced by the early deaths of animals that were allowed to progress to terminal disease: 35 dpi with SIVmac251 and 79, 141, and 209 dpi with SIVmac239. Similar rapid disease progression in SIV-infected neonates has been described previously.¹⁵

In addition to high viral loads and rapid disease course, there was a high incidence of opportunistic infections, which affected 10 of 18 animals (56%, Table 1), including all of the animals allowed to progress to terminal disease. The most common opportunistic infection in SIV-infected rhesus neonates was adenovirus, which was detected in 3/13 SIVmac239-, 2/3 SIVmac251-, and 2/2 SIVmac239/316-infected neonates. Other lesions caused by opportunists included hepatitis and/or typhlocolitis, caused by *Clostridium pilliforme*, in four SIVmac239-infected neonates at 14 and 21 dpi, and pneumonia, caused by *Pneumocystis carinii* in one SIVmac239-infected infant at 209 dpi. None of the opportunistic infections involved the CNS.

Infant Macaques Infected with SIV Have Decreased Brain Growth

Maximum growth rate for body weight, brain weight, and head size in normal infant rhesus macaques is from birth to 6 months of age with continued gradual increases in these parameters until maturity.^{33,34} In contrast, in SIV-infected neonates maximum brain weights occurred at 50 dpi, with decreased brain weights thereafter (Figure 3A). Brain/body weight ratios in neonates infected with SIVmac239 also decreased over the course of infection (Figure 3B), indicating that the decrease in brain weight was not simply a reflection of an overall decrease in growth in SIV-infected infants.¹⁵ One animal (number 484-97) had a higher brain/body weight ratio due to severe wasting associated with adenovirus infection. These data indicate that the brains of infected neonates did not grow normally and that the effect is more severe than can be accounted for by generalized growth retardation.

CNS Lesions Occur in Most Infant Macaques Infected with SIV

Histological lesions of the brain were observed in 12/18 (66%) infected neonates as early as 14 dpi and in all animals infected for >21 days (Tables 2 and 3, Figure 4). Common lesions included perivascular aggregates of histiocytes and lymphocytes, vascular and perivascular mineralization in the basal ganglia and rostral thalamus, and gliosis. The perivascular aggregates of histiocytes and lymphocytes were most frequent within the cortical gray and white matter and the basal ganglia. Surprisingly, none of the neonates had classical SIV encephalitis (SIVE) characterized by the presence of MNGCs.

An unusual CNS lesion, characterized by severe lymphohistiocytic and neutrophilic meningoencephalomyelitis, was observed in one of the day 50 SIVmac239-infected neonates (Mm 296-97). This animal exhibited frequent SIV-positive cells within lymphohistiocytic and neutrophilic perivascular cuffs in the cerebral cortex, putamen, and caudal thalamus, suggesting that there was increased trafficking of virus-infected cells associated with another pathogenic process. Bacterial culture and nested PCR revealed the presence of *Streptococcus* sp. It is unclear, however, whether these bacteria were the cause of this unusual lesion, because they were not detected within the tissues.

Detection and Localization of Virus in the CNS

Although abundant virus was detected in peripheral lymphoid tissues of SIV-infected neonates by 7 dpi using immunohistochemistry or nonradiolabeled *in situ* hybridization (Figure 2), relatively little virus was detected in the CNS, regardless of viral inoculum. Of the 18 SIV-infected neonates, only one animal 50 days after infection with SIVmac239 had detectable viral protein (gp120) or RNA in the CNS by immunohistochemistry or nonradiolabeled *in situ* hybridization (Figure 5). These observations differed from our previous experience with juvenile and

Figure 1. Viral loads in rhesus neonates infected with SIV determined by limiting dilution culture of peripheral blood mononuclear cells (A and C) or quantitation of plasma viral RNA (B and D). A and B show viral loads in groups of animals inoculated with SIVmac239, SIVmac239/316, or SIVmac251 through 50 days of infection. Each bar represents the mean $\pm$ SD. C and D show viral loads at multiple time points throughout infection for the three SIVmac239-infected neonates that were allowed to progress to terminal AIDS.

adult rhesus macaques infected with the same stocks and dose of virus on a per-kilogram basis, in which productive viral infection was readily detectable in the brain by 14 dpi.^{19,20,23} Detection of virus in the brain of SIV-infected rhesus neonates required the more sensitive techniques of nested PCR and radiolabeled riboprobe *in situ* hybridization to detect virus (Tables 2 and 3).

Viral DNA was detected in multiple brain regions by nested PCR for SIVgag in 17 of 18 (94%) infected neonates, regardless of inoculum (Tables 2 and 3). With this technique, virus was found to be present as early as 3 dpi and continued to be present at each time point thereafter (Table 2). These data were supported by radiolabeled

riboprobe *in situ* hybridization of multiple brain sections that revealed viral RNA at 7 dpi and at each subsequent time point (Tables 2 and 3 and Figure 6). SIV-infected cells were present in the CNS of 8/13 SIVmac239-, 2/3 SIVmac251-, and 1/2 SIVmac239/316-infected rhesus neonates (61% total) detected by radiolabeled riboprobe *in situ* hybridization (Table 3). Although virus was present in most of the animals, the number of infected cells detected was less than that previously observed in older macaques.¹⁹

In addition to being more difficult to detect, SIV-infected cells in the CNS of neonates tended to have a different distribution from that previously described in older macaques inoculated with these same viruses.^{20,35}

Figure 2. Detection of SIV by nonradiolabeled *in situ* hybridization in the spleen of a rhesus neonate 7 dpi. Note the large number of infected cells. Original magnification, $\times 80$

In neonates, SIV-infected cells were most frequently located in cortical gray matter and basal ganglia (Figure 6), whereas infected cells in adult and juvenile macaques were most often present in the cortical white matter and

basal ganglia.^{18,20,35} Furthermore, in contrast to older macaques where most infected cells are around vessels, in neonates scattered infected cells were also present within the parenchyma unassociated with vessels (Figure 6). Thus, although SIV could be detected within the CNS of infant macaques soon after infection, the number of infected cells and the histopathological sequelae appear to be decreased compared to older macaques inoculated with these same viruses.

Detection and Quantitation of Virus in the CSF

Samples of CSF were collected from infants infected with SIVmac251, SIVmac239/316, and two of three animals inoculated with SIVmac239 that survived more than 50 days for quantitation of viral RNA. As seen in Table 4, viral RNA could first be detected in CSF from two animals at 7 dpi and from all animals examined by 14 dpi. This is similar to observations in older macaques, where virus could be isolated from CSF of all animals inoculated with either SIVmac239 or SIVmac251 by 14 dpi.²⁰ All CSF samples from neonates examined after 14 dpi were positive for viral RNA. However, the amount of virus in CSF was generally several logs less than what was present in matched samples of plasma. These data support the idea that neuroinvasion occurs early but that there is relatively little virus in the CNS of these infant macaques.

Figure 3. Brain weights (A) and brain/body weight ratios (B) of SIV-infected rhesus neonates at death. Note that maximum brain weights were recorded at 50 dpi. Normal animals show continued rapid growth of the brain through 6 months of age. Symbols are blue for animals infected with SIVmac239, red for SIVmac239/316, and green for SIVmac251.

Table 2. Neuropathology and Localization of Virus in SIV-Infected Rhesus Neonates

Virus	Animal no.	Days pi	Neuropathological findings	<i>In situ</i> hybridization*				
				CG	CW	BN	Other	PCR
SIVmac239	68-97	3	NSL	-	-	-	-	-
	69-97	3	NSL	-	-	-	-	+
	102-97	7	NSL	++	-	+++	-	+
	103-97	7	NSL	++	+	+++	-	+
	136-97	14	Choroid plexitis, histiocytic, mild	-	-	-	-	+
	137-97	14	NSL	++	-	-	+	+
	190-97	21	NSL	-	-	++	-	+
	191-97	21	Vascular mineralization, basal nuclei and thalamus	-	-	-	-	+
	296-97	50	Meningoencephalomyelitis, multifocal, neutrophilic and histiocytic, moderate to severe	+	-	+++	-	+
			Vascular mineralization, globus pallidus					
	297-97	50	Encephalitis, multifocal with lymphohistiocytic, perivascular cuffs and glial nodules	+	-	+	-	+
	415-97†	79	Encephalitis, lymphohistiocytic, multifocal, mild	+	-	N/A	+	+
	484-97†	141	Encephalitis, histiocytic, multifocal, mild with glial nodules	-	-	+	-	+
			Vascular mineralization, basal nuclei					
	412-97†	209	Meningoencephalomyelitis, mild, multifocal with lymphohistiocytic, perivascular cuffs	-	-	-	-	+
			Vascular mineralization, caudate nucleus					
SIVmac251	163-98	21	Encephalitis, mild with glial nodules	-	-	-	+	+
			Vascular mineralization, temporal cortex					
	162-98†	35	Encephalitis, lymphohistiocytic, multifocal, mild	-	-	-	-	+
			Vascular mineralization, cerebral meninges					
	160-98	50	Encephalitis, lymphohistiocytic, multifocal, mild	-	-	++	-	+
SIVmac239/316	109-98	50	Encephalitis, lymphohistiocytic, multifocal, mild	-	-	-	-	+
	110-98	50	Encephalitis, lymphohistiocytic, multifocal, mild	-	-	-	+	+
			Vascular mineralization, caudate nucleus					

*Results of *in situ* hybridization experiments were scored from negative to +++ as follows: -, no positive cells; +, 1-2 positive cells/section; ++, 3-5 positive cells/section; +++, more than 5 positive cells/section.

†Animals euthanized when moribund with AIDS.

NSL, no significant lesions; N/A, Not available for examination.

CG, cortical gray; CW, cortical white; BN, basal nuclei; other, any other region of the brain.

Examination of the *in Vivo* Macrophage-Tropism of the Viral Inocula

Because the ability of SIV to replicate well in monocyte/macrophages has been shown to be associated with the development of CNS disease,^{26,36} we examined tissues from infants infected with each stock of SIV for the presence of infected monocyte/macrophages. This was done using *in situ* hybridization for SIV nucleic acid combined with immunohistochemistry for monocyte/macrophages (HAM-56). Because so few virus-positive cells were present in the CNS, we analyzed macrophage infection in the spleen. In neonates infected with SIVmac251 and SIVmac239/316, SIV-infected monocyte/macrophages were readily apparent (Figure 7A). In contrast, macrophages were rarely infected in SIVmac239-infected neonates (Figure 7B). In fact, only one SIVmac239-infected

macrophage was detected in over 50 sections examined from 11 animals (animals 68-97 and 69-97 were not examined). This is in contrast to previous observations in older macaques infected with SIVmac239, where infected macrophages were easily detected in multiple tissues, including the brain, soon after infection.²¹⁻²³ Although the absence of macrophage infection in SIVmac239-infected neonates could explain the relatively mild infection of the CNS of those animals, SIV infection of the CNS was limited in rhesus neonates regardless of macrophage-tropism and viral inoculum.

Discussion

In this study, we have demonstrated that infection of rhesus neonates with SIV results in rapid neuroinvasion

Table 3. Neurologic Lesions and Virus in the CNS of Neonatal Macaques Infected with SIV

Viral inoculum	CNS lesions (# positive/# tested)	SIV in the CNS by ISH (# positive/# tested)	SIV in the CNS by PCR (# positive/# tested)
SIVmac239	7/13	8/13	12/13
SIVmac251	3/3	2/3	3/3
SIVmac239/316	2/2	1/2	2/2
Totals	12/18 (66%)	11/18 (61%)	17/18 (94%)

ISH, *in situ* hybridization.

Figure 4. Representative histological lesions in the CNS of SIV-infected rhesus macaque neonates. **A:** A typical lesion consisting of perivascular aggregates of mononuclear cells in the cortical white matter. Similar lesions are commonly seen in older macaques infected with SIV. **B:** Mineralization within a vessel wall in the basal ganglia. **C:** A glial nodule in the basal ganglia. Original magnifications, $\times 100$ (A and B), $\times 180$ (C).

with virus consistently present in the CNS within 7 days of infection. Most infected animals had histopathological lesions in the brain, including perivascular infiltrates of mononuclear cells, mineralization of vessels in the basal ganglia, and gliosis. These lesions, which closely resemble those described in HIV-1-infected children, were observed as early as 14 dpi coincident with peak viremia and were present in all animals infected for more than 21 days. Infected infant macaques also had evidence of decreased brain growth, particularly in animals infected for more than 50 days. Decreased brain growth is a common feature of pediatric AIDS.^{3,4}

Figure 5. Immunohistochemistry for SIV gp120 in the CNS of a neonatal macaque infected with SIVmac239 50 days after infection. Several positive cells (arrowheads), including one with a distinct cellular process (arrow), are present in the cellular infiltrate. Original magnification, $\times 80$.

Although it is known that HIV causes severe neurological disease in human infants, it has not been possible to determine when neuroinvasion occurs or how the presence of virus is related to the development of CNS lesions over time. The data from this time course study clearly show that neuroinvasion occurs within days of inoculation and is associated with consistent development of histopathological lesions by 3 weeks and decreased brain growth within 2 months. The similarity of the CNS lesions in pediatric AIDS patients and in infant macaques infected with SIV suggests that this is an excellent model for further examination of the time course and mechanisms of the neuropathogenesis of AIDS in the immature host.

Rapid and consistent neuroinvasion by pathogenic strains of SIV has also been observed in older macaques.^{18–20} However, CNS infection of neonatal macaques differed from that of older animals in several respects. These differences include the relative difficulty of detecting virus, a slightly different distribution of infected cells, and the absence of MNGCs, which are the hallmark of SIVE.^{28,35} The absence of SIVE may reflect the small number of animals that were followed to terminal disease. However, many infant macaques have been infected with SIV in other studies and followed to terminal disease, but few if any reports of SIVE exist.^{15,17,37,38} This implies that age-related host factors may limit CNS viral infection and development of SIVE. The data suggest this occurs independently of the ability of the virus to replicate in the periphery and invade the CNS, because all viruses used in this study replicated to high levels in the periphery and rapidly invaded the CNS.

Despite an abundance of virus in peripheral blood and lymphoid tissues, detecting virus in the CNS of most animals required the sensitive techniques of radiolabeled *in situ* hybridization with riboprobes and nested PCR. This contrasts with prior work in older macaques, where immunohistochemistry for viral antigens was often sufficient to detect infected cells in the CNS.³⁵ These data, in conjunction with much lower viral loads in CSF than in

Figure 6. Radiolabeled *in situ* hybridization for SIV in brain of neonatal rhesus macaques. **A:** Scattered positive cells were intimately associated with small vessels. In addition, scattered positive cells without any obvious vascular association were present in the white matter (**B**) and gray matter (**C**). **D:** An unusual perivascular lesion consisting of neutrophils and histiocytes containing several positive cells. Original magnifications, $\times 125$ (**A**), $\times 180$ (**B**), $\times 100$ (**C**), $\times 80$ (**D**).

plasma, indicate that SIV-infected infants had considerably less virus in the CNS than older macaques inoculated with the same dose and stock of virus at the same time points. Though virus was difficult to detect in the CNS of neonates, it was generally associated with histological lesions, particularly perivascular cuffs and glial

nodules. This is similar to what has been described in older macaques. However, in contrast to older animals, virus was more prevalent in cortical gray matter than white matter. In addition, scattered infected cells were present with no apparent vascular association. Similar observations have been made in fetal macaques inocu-

Table 4. Quantitation of Viral RNA in Paired Samples of Plasma and CSF

Virus	Animal no.	Days pi	Viral RNA copy Eq/ml	
			Plasma	CSF
SIVmac251	160-98	7	5.2×10^6	$<2 \times 10^3$
		14	3.1×10^8	2.6×10^4
		50	6.7×10^8	2.1×10^5
	162-98	7	1.5×10^8	$<2 \times 10^3$
		14	3.6×10^8	5.3×10^4
	163-98	7	1.9×10^4	$<2 \times 10^3$
		14	5.5×10^8	2.5×10^4
SIVmac239/316	109-98	7	1.3×10^6	3.2×10^5
		21	2.4×10^8	3.5×10^3
	110-98	7	8.1×10^5	3.2×10^4
		21	1.9×10^7	3.2×10^3
		35	7.4×10^7	8.1×10^3
SIVmac239	412-97	167	1.1×10^7	7.7×10^4
		209	6.0×10^7	2.5×10^4
	484-97	126	4.9×10^8	1.2×10^4

Eq/ml, equivalents/milliliter.

Figure 7. Combined *in situ* hybridization (black) for SIV nucleic acid and immunohistochemistry for macrophages (brown, HAM56) in spleen. In neonates infected with SIVmac251 or SIVmac239/316 (A), infected macrophages with brown cytoplasm and black nuclei (arrow and inset) were readily apparent. Numerous other infected cells (black) morphologically compatible with lymphocytes are also present. In contrast, in infant macaques infected with SIVmac239 (B), infected macrophages were not evident. Original magnifications, $\times 100$ (A) and $\times 80$ (B).

lated *in utero*.³⁹ Whether some of these infected cells could be astrocytes, as has been described in HIV-infected infants, is unclear.^{13,14}

Although the histopathological lesions were fairly mild in neonates, it is important to note that they were present in all animals infected for more than 21 days regardless of inoculum. Furthermore, the low brain weights suggest that brain development was adversely affected and that there is subtle, diffuse damage to the brain. Delayed brain growth is a common observation in HIV-infected children, but its pathogenesis is unclear. Although indirect mechanisms are generally considered to be responsible for neuronal dysfunction and loss, these mechanisms appear to be particularly relevant in those neonatal macaques with little virus in the CNS. Careful quantitative neuropathological analyses of additional animals in conjunction with techniques such as magnetic resonance spectroscopy should enable us to address this issue.^{40–43}

Limited HIV infection has also been observed in the CNS of children.⁴⁴ Despite reports of a high incidence of neurological impairment in HIV-1-infected children, several studies have revealed little or no virus in the brain and fewer MNGCs, the hallmark of HIV encephalitis, than in adults.⁴⁴ In fact, Kure reported that only 8/20 children with AIDS encephalitis had HIV gp41 immunoreactive

cells in the CNS and only 5/31 cases of pediatric AIDS had MNGCs in the brain.⁴⁵ In another small study, 4/9 HIV-infected children had no detectable virus in the brain and 5/9 had no MNGCs.⁴⁴ Similarly, Sharer et al reported that only 3/11 HIV-infected children with encephalitis were positive by *in situ* hybridization for HIV in the CNS.⁷ Thus, our observation of relatively less virus and less severe lesions in the CNS of SIV-infected neonates compared to older animals is consistent with observations in HIV-infected infants and children. The major issue then becomes determining why CNS infection of neonates is attenuated compared to older macaques and humans.

A likely starting point for examination is the monocyte/macrophage. All pathogenic isolates of SIV studied to date are neuroinvasive, but the ability to cause significant neurological lesions such as SIVE is closely linked to the ability to replicate in monocyte/macrophages.^{19,20,23,25,26,36,46–49} Similarly, the neurovirulence of HIV has been linked to macrophage tropism.^{50,51} Furthermore, a recent study has shown that CCR5, the major coreceptor for HIV and SIV infection, is expressed at lower levels in monocytes obtained from human infants than adults and that some primary HIV-1 isolates do not infect neonatal macrophages.⁵² Therefore, we examined the ability of the viruses used in this study to infect monocyte/macrophages *in vivo*. Due to the small number of infected cells in the CNS, we were limited to performing this experiment in the spleen. In infant macaques infected with SIVmac239, essentially no evidence of macrophage infection was found. This is in marked contrast to infection of older macaques with the same dose and stock of virus, where infection of macrophages is easily demonstrated *in vivo* using these same techniques within 3 weeks of inoculation.^{21,22} This observation supported the hypothesis that CNS disease was attenuated due to limited infection of macrophages. However, equally attenuated CNS disease was observed in animals infected with SIVmac251 and SIVmac239/316, and these viruses were easily detected *in vivo* within macrophages. These observations make it less likely that differences in macrophage tropism are responsible for the attenuated CNS disease. However, we did not directly examine brain macrophages. It is possible that the key factor is the ability to infect brain macrophages/microglia, not macrophages in general. Future studies will need to examine the level of cellular activation, chemokine receptor expression, and susceptibility to SIV infection of neonatal brain macrophages and microglia compared to those obtained from adults.

In summary, we have shown that SIV infection of neonatal macaques results in rapid neuroinvasion and persistent infection of the CNS. Infection of the CNS was associated with a spectrum of histopathological lesions and decreased brain growth similar to that described in pediatric AIDS. Significant differences were also observed compared to older macaques inoculated with the same stock and dose of virus, indicating that the pathogenesis of CNS infection of neonates differs from that of adults. The SIV-infected macaque provides an excellent model to examine maturation-dependent factors that affect the neuropathogenesis of AIDS.

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