Brain Pathology 7: 851-861 (1997)

RESEARCH ARTICLE

Neuropathogenesis of Chimeric Simian/Human Immunodeficiency Virus Infection In Pig-tailed and Rhesus Macaques.

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We recently reported that a chimeric simian/human immunodeficiency virus ($SHIV_{KU-1}$) developed in our laboratory caused progressive depletion of $CD4^+$ T lymphocytes and AIDS within 6 months of inoculation into pig-tailed macaques (M.nemestrina). None of the pig-tailed macaques showed productive SHIV infection in the central nervous system (CNS). In this report, we show that by further passage of the pathogenic virus in rhesus macaques [M. mulatta], we have derived a new strain of SHIV ($SHIV_{KU-2}$) that has caused AIDS and productive CNS infection in 3 of 5 rhesus macaques infected with the virus. Productive replication of SHIV in the CNS was clearly shown by high infectivity titers and p27 protein levels in brain homogenates, and in 2 of the 3 rhesus macaques this was associated with disseminated, nodular, demyelinating lesions, including focal multinucleated giant cell reaction, largely confined to the white matter. These findings were reminiscent of HIV-1 associated neurological disease, and our immunohistochemical and in situ hybridization data indicated that the neuropathological lesions were associated with the presence of SHIV-specific viral antigens and nucleic acid respectively. However, the concomitant reactivation of opportunistic infections in these macaques suggested that such pathogens may have influenced the replication of SHIV in the CNS, or modified the neuropathological sequelae of SHIV infection in the rhesus species, but not in pig-tailed macaques. Our findings in the two species of macaques highlight the complexities of lentiviral neuropathogenesis, the precise mechanisms of which are still elusive.

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Introduction

Neurological disease is a well documented complication of HIV-1 infection, especially in the terminal stages of AIDS (2,3). The spectrum of neuropathological abnormalities ranges from lesions considered to be the direct consequence of HIV infection, such as aseptic meningitis, vacuolar myelopathy, peripheral neuromuscular syndromes and HIV encephalitis, to opportunistic infections and neoplasms in the brain precipitated by the profound immunodeficiency caused by the virus (2). A constellation of neuropsychiatric symptoms (HIV-1 associated cognitive/motor complex or HIV dementia) develops in 15-20% of AIDS victims, and is associated with worsening of prognosis and death within a year after diagnosis (17). In about 3% of HIV-infected individuals, neurological disease is the earliest presentation of AIDS (8). The exact mechanisms by which infected blood derived monocyte/macrophages and microglia, perhaps the only cells in the nervous system capable of sustaining productive HIV infection, lead to a progressive deterioration of neuropsychiatric function are largely unknown. In this context, animal models of HIV-related neurological disease play key roles in providing further insights into the neuropathogenesis, treatment and prevention of this complication.

Simian immunodeficiency virus (SIV) encephalitis in macaques is a useful model of HIV-related neurological disease, but it does not provide an opportunity to study the effects of the HIV envelope glycoprotein which is thought to be important in the pathogenesis of HIV-associated neurological disease (20,25). Recently,

we derived a pathogenic SIV-HIV (SHIV) chimera containing the *tat*, *rev*, *vpu*, and *env* genes of HIV-1 HXB2, and the LTR, *gag*, *pol*, *vif*, *vpr*, *vpx* and *nef* genes of SIVmac239, by serial passaging of the agent in pig-tailed macaques (11). Although this virus, $SHIV_{KU-1}$, caused rapid depletion of $CD4^+$ T lymphocytes and AIDS in pigtailed macaques, none of these animals developed lentiviral encephalitis or productive SHIV infection in the CNS (11). In a further attempt to develop neurovirulent SHIV, we made an addtional passage of $SHIV_{KU-1}$ in rhesus macaques and used infectious cerebrospinal fluid from one of these animals to prepare stock virus, $SHIV_{KU-2}$. We inoculated this virus into 5 rhesus macaques and all developed loss of $CD4^+$ T cells and AIDS. However, unlike pig-tailed macaques infected with $SHIV_{KU-1}$, 4 of these 5 rhesus macaques developed CNS lesions. In this report, we have characterized the neuropathological lesions in both pig-tailed and rhesus macaques, and used in situ hybridization and immunohistochemistry to understand the etiological factors underlying these lesions. Our data show that, of the 5 pig-tailed animals dying with AIDS caused by infection with $SHIV_{KU-1}$, all developed latent SHIV infection in the brain. All rhesus macaques dying with AIDS caused by $SHIV_{KU-2}$, also developed infection in the CNS, but 3 animals had productive SHIV infection in the CNS. In 2 of these, replicating SHIV in the CNS was associated with morphological changes of lentivirus-induced abnormalities. However, both of these cases were also associated with latent CNS infection with opportunistic viruses, SV40 and CMV, respectively. Two rhesus macaques, one with productive SHIV infection in the CNS and another with latent SHIV infection, developed progressive multifocal leucoencephalopathy (PML). One of the rhesus macaques had latent SHIV infection in the CNS that was not accompanied by neuropathological abnormalities or opportunistic infection. In this paper we have discussed the implications of these findings to the neuropathogenesis of SHIV infection in the two species of animals.

Materials and methods:

Animals

5 rhesus and 5 pig-tailed macaques of varying ages and either sex were used in this study. They had been individually housed in American Association for Laboratory Animal Care-accredited facilities of the University of Kansas Medical Center. All animals had been serologically tested and declared free of SIV, simian retrovirus type D and simian herpes B virus infection.

Virus inoculation and animal passages for the derivation of neurovirulent SHIV

Two pig-tailed macaques, PPc and PNb, had developed AIDS in the course of our initial derivation of pathogenic SHIV, as described (12). Pooled bone marrow cells from both animals, and cell-free virus isolated from macaque PNb (designated as $SHIV_{KU-1}$) were inoculated into rhesus macaques, 14A and 14B, by the bone marrow and intravenous routes. Macaque 14A developed transient loss of $CD4^+$ T cells, and infectious cells were recovered from the CSF at 7 weeks after inoculation. Macaque 14B did not show any loss of $CD4^+$ T cells (11). Neither animal developed AIDS or CNS disease during a follow-up period of 21 months. The 14A CSF virus was amplified in macaque PBMC cultures and a cell-free virus stock (designated as $SHIV_{KU-2}$) was prepared in mitogen-stimulated normal rhesus peripheral blood mononuclear cells (PBMC) and frozen in aliquots at -80 °C. This virus has a titer of 10^4 $TCID_{50}$ in C8166 cell cultures. Undiluted stocks of this virus ($SHIV_{KU-2}$) were inoculated into 5 rhesus macaques. Macaques 16A and 16B received 0.1 ml of the inoculum by the bone marrow and intracerebral routes, whereas macaques 23A, 23D and 23E received the same dose by the subcutaneous, intravenous and oral routes respectively.

Details of inoculation of $SHIV_{KU-1}$ into pig-tailed macaques 18A,18D and 18E have already been published (10). Pig-tailed macaques 24B and 24C received 0.1 ml. of undiluted $SHIV_{KU-1}$ by the intravenous route, and were followed up in the same manner as the above three animals. Material from these 5 macaques was mainly used to investigate in detail the CNS events after SHIV inoculation, and the prevalence of opportunistic CNS infections (by PCR analysis for SV40 and CMV sequences; see below) in the pig-tailed species.

Necropsy

Infected animals were euthanized when they became anorexic and too weak or ataxic to move about in their cages. They were sedated with ketamine and deeply anesthetized by an intravenous injection of phenobarbital sodium. Following slow exanguination through the abdominal aorta, we performed saline perfusion to flush out excess blood from the vascular compartments of the animals, so as to minimize the risks of viral DNA contamination of solid tissues from the blood. After collection of relevant thoracic, abdominal and pelvic viscera

	SHIV-KU2 infected macaques (rhesus)					SHIV-KU1 infected macaques (pig-tailed)				
Central Nervous System:	16A	16B	23A	23D	23E	18A	18D	18E	24B	24C
Neuropathological findings	PML	SHIV-L	SHIV-L	PML	-	Tox	-	-	-	PML
Regions positive for SIVgag DNA sequences by PCR (no. of regions tested in parentheses)	9(16)	13(16)	16(16)	11(11)	13(13)	17(17)	5(15)	15(16)	12(16)	12(16)
In-situ hybridization for SIVgagmRNA	-	++	++	-	-	ND	ND	ND	ND	ND
Immunohistochemistry for SIVGag protein	-	++	++	-	-	ND	ND	ND	ND	ND
Infectivity for SHIV in brain homogenates (logTCID50)	3.4	2.3	3	-	-	-	-	-	-	-
SIVp27 concentration (pg/ml)	158	604	608*	-	-	-	-	-	-	-
Lungs:										
Interstitial Pneumonia	++	++	++	-	-	-	-	-	++	++
Infectivity for SHIV in lung homogenate (logTCID50)	5.6	4.3	4.3	1	-	2.3	3	2.7	2.1	2
SIVp27 concentration (pg/ml)	6460	7788	3681	-	3	2637	5074	395	113	305
Lymphoid Tissue:										
Infectivity for SHIV in spleen homogenate (logTCID50)	2.9	3.7	3.1	3.5	3.5	1.5	3.1	3.2	2.3	2.4
SIVp27 concentration (pg/ml)	5074	2038	3686	1157	104	1555	6685	1677	189	144
Infectivity for SHIV in lymph node (logTCID50)	2.9	3.3	3.5	2.5	2.7	2	3.8	3.1	3.6	2.6
SIVp27 concentration (pg/ml)	5611	3416	3023	189	23	4345	7012	1339	1716	1233
Opportunistic Infections in the CNS:										
Regions positive for CMV-IE DNA by PCR (no. of regions tested in parentheses)	7(14)	-	14(15)	-	-	-	-	6(15)	-	-
Regions positive for SV40-VP1 DNA by PCR (no. of regions tested in parentheses)	12(16)	16(16)	-	6(13)	-	-	-	-	16(16)	15(16)
Other pathogens	-	-	-	-	-	Tox	-	-	-	-

*Infectivity and SIVp27 concentration (parentheses) in CNS tissues, after sub-dissection of macaque 23A brain are as follows :
Cortical Grey matter = 0, Subcortical White matter= 2.4(192), Brain stem= 3(608), Deep Grey matter= 1(0), Spinal Cord=2.2 (262)
PML : Progressive Multifocal Leucoencephalopathy SHIV-L: SHIV- associated Leucoencephalitis Tox: CNS Toxoplasmosis
Present (++); Absent (-); Not Done (ND)

Table 1. Summarized neurovirological and neuropathological data correlated with productive SHIV infection in systemic tissues and opportunistic infections in rhesus macaques infected with SHIV-KU2 and pig-tailed macaques infected with SHIV-KU1.

for histopathological, virological and immunological studies, we removed the brain and spinal cord aseptically, rinsed them in normal saline and transferred them immediately to Hanks balanced salt solution.

Brain dissection and other Neuropathological methods

Within an hour after necropsy, the brain was bisected sagittally inside a biosafety hood. The right half was transferred immediately into 10% buffered formalin. The left half of the brain and spinal cord were subdissected into 16 anatomically distinct regions based on a brain map generated in our laboratory from serial slices of normal macaque brains of the same size. Care was taken to strip off leptomeninges and choroid plexus to minimise contamination with residual viral DNA from the meningeal or choroidal vasculature. All samples were snap-frozen over dry ice and saved at -70 °C for PCR analysis of viral DNA. Portions of the deep white matter and overlying cortical regions were explanted into culture, and supernatant fluids examined for virus content. Other portions were homogenized and the cell-free extracts assayed for infectivity and p27. The remaining portions were fixed in 10% buffered formalin for 3-4 days. Blocks taken from areas adjacent to the previously sampled parts of the fixed left half of the brain, and from identical parts of the right half were embedded in molten paraffin wax and processed in the routine fashion. A preliminary screening for morphological abnormalities was performed on paraffin sections stained with hematoxylin and eosin (H&E). Serial sections were stained with Luxol Fast Blue (LFB) and Sevier Munger (SM) stains respectively, for the assessment of suspected myelin and axonal abnormalities. The remaining sections were set aside for immunostains and in situ hybridization procedures.

Immunohistochemistry

Paraffin sections were reacted overnight with primary antibodies, anti-CD3 (Dako) rabbit polyclonal to detect T lymphocytes, anti-CD68 ([KP1]Dako) mouse monoclonal to detect monocytes/macrophages, anti-GFAP (Dako) rabbit polyclonal to detect astrocytes (all used at 1:50 dilution) and anti-SIV *Gag* ([FA2]NIH) mouse monoclonal (used at 1:100 dilution). After repeated washing, the sections were incubated with biotinylated goat anti-mouse [Dako] or anti-rabbit [Vector] antibody, which in turn was detected using avidin-biotin-peroxidase enzyme complex [Vector] followed by the appropriate substrate, AEC [Biomeda]. Appropriate controls were used with each test antibody.

In situ hybridization

Probe generation and labelling: A PCR amplified

CD4+T (cells/ul)

Wk	16A	16B	23A	23D	23E
0	2550	3100	942	3058	1391
1	1432	1468	158		1916
2	55	75	2	8	3
4	121	73	43	7	35
6	35	11	3	5	2
8	533	12	7	7	7
10	19	3	1033		6
11				59	
12			4		4
13		1		5	
14			12		3
15	5			359	
17	3		111	4	4
19			32	708	4
20	5				
21			83	4	73
23				0	2
24	428				
25					4
27	8				3
28	4				
29					93

CD8+T (cells/ul)

Wk	16A	16B	23A	23D	23E
0	4657	2600	459	2839	266
1	1203	1137	1471		668
2	2497	3502	886	2943	1420
4	1889	2119	1832	2771	875
6	1182	1160	1581	1693	478
8	2506	3214	1376	2419	972
10	1234	1305	1218		819
11				1777	
12			1747		529
13		82		187	
14			1671		692
15	2088			1476	
17	1526		554	2597	431
19			1380	259	1247
20	1926				
21			1289	1906	818
23				2365	389
24	2354				
25					48
27	3076				1682
28	482				
29					1568

*PBMC Infectivity for SHIV

Wk	16A	16B	23A	23D	23E
1	100	100	10		100
2	100	100	1000	1000	10000
4	100	100	100	100	100
6	100	10	10	100	10
8	100	100	10	100	100
10	100	10	10		100
11				10	
12			10		100
13		100		10	
14			10		10
15	10			10	
17	100		100	10	100
19			10	1	5
20	10				
21			10	10	10
23				10	10
24	10				
25					100
27	5				5
28	3				
29					10

**Plasma Infectivity for SHIV

Wk	16A	16B	23A	23D	23E
1	0	0	0		0
2	0	0	0	0	0
4	0	0	0	0	0
6	0	0	0	100	0
8	0	0	0	100	0
10	0	10	0		0
11				0	
12		10	10		0
13		0		100	
14			0		0
15	0			0	
17	0		0	0	0
19			100	0	0
20	0				
21			0	0	0
23				0	0
24	10				
25					0
27	0				0
28	0				
29					0

Plasma SIVp27 (pg/ml)

Wk	16A	16B	23A	23D	23E
1	0	0	0		160
2	1032	841	2351	2124	3061
4	0	51	128	28	0
6	0	60	29	0	0
8	0	215	84	0	0
10	0	529	132		0
11				0	
12			160		0
13		121		0	
14			103		0
15	0			0	
17	0		267	0	0
19			536	0	0
20	0				
21			445	0	0
23					0
24	0				
25					0
27	0				0
28	0				
29					

***CSF Infectivity for SHIV

Wk	16A	16B	23A	23D	23E
1	+	+	-		-
2	+	+	-	+	+
4	+	-	-	-	-
6	+	+	-	-	-
8	-	-	-	-	+
10	-	-	-		-
11			-		
12			-		-
13		+		+	
14			-		+
15					
17	-		-		-
19			-		-
20	+				
21			-	-	-
23				-	
24	-				
25					-
27	-				-
28	-				
29					-

* Infectivity measured as number of infectious cells per million PBMC
** Infectivity measured as TCID50 per ml of plasma
*** CSF infectivity indicated as present (+) or absent (-)

Table 2. CD4+ and CD8+ T cell counts; Infectivity for SHIV in PBMCs, plasma and CSF; and plasma SIVp27 concentration in rhesus macaques infected with SHIV-KU2.

Anatomical Region	Macaque 16A	Macaque 16B	Macaque 23A	Macaque 23D	Macaque 23E
Pre-Frontal Cortex	+	+	+	+	+
Parietal Cortex	+	-	+	+	+
Temporal Cortex	-	+	+	+	+
Occipital Cortex	+	+	+	+	+
Motor Cortex	+	+	+	ND	ND
Basal Ganglia	-	+	+	+	+
Thalamus	+	+	+	ND	ND
Deep White Matter	+	+	+	+	+
Corpus Callosum	+	+	+	ND	ND
Midbrain	+	+	+	ND	+
Pons	-	+	+	ND	+
Medulla	+	+	+	+	+
Cerebellum	-	+	+	+	+
Cervical Cord	-	-	+	+	+
Thoracic Cord	-	-	+	+	+
Lumbar Cord	-	+	+	+	+

+ : Present; - : Absent; ND: Not Done (due to insufficiency of tissue samples)

Table 3. Regional distribution of SIV-*gag* sequences in the central nervous system of rhesus macaques inoculated with $SHIV_{KU-2}$

SIVgag DNA fragment (approximately 350 bp long) was cloned into a plasmid containing the SP6 and T7 promoter sites. Digoxigenin-labelled sense and antisense riboprobes were generated from this plasmid by in vitro transcription using SP6 and T7 RNA polymerases, respectively [DIG RNA Labeling Kit (SP6/T7), Boehringer Mannheim], according to the manufacturer's instructions. After the labeling reaction the probe was purified by ethanol precipitation and dissolved in DEPC treated water. Hybridization: Paraffin sections were digested with pepsin [Boehringer] or proteinase K, and hybridized overnight with the digoxigenin labeled SIV-*gag* sense and antisense RNA probes. Following hybridization and extensive washing of sections, the hybridization product was detected by reacting the sections with alkaline phosphatase conjugated anti-digoxigenin fragments [Boehringer] followed by incubation with NBT/BCIP alkaline phosphatase substrate [Pierce] to obtain a blue-black product. Appropriate controls were included in these reactions.

Virological methods

Tissue processing methods, cell culture, assessment of infectivity, SIVp27 assay and FACS analysis techniques (for CD4/CD8 cell counts) have already been described in detail in earlier reports (9-13).

PCR amplification

SHIV. For detection of SHIV, total cellular genomic DNA was extracted from 16 regions of the CNS. Two rounds of PCR amplification were performed to detect SIV *gag* sequences as described (9).

CMV. For the detection of cytomegalovirus, the oligonucleotides used in the first round were 5'-AGATTCTATAGGCTATATATGACGTAATGG-3'(sense) and 5'-GCAGACAAAA TAAGGTGTTG-TATGCC-3' (antisense) based on the sequence of the rhesus cytomegalovirus immediate early gene (RhCMV-IE) (1). One μl of the above PCR product was used in a nested PCR under the same reaction conditions using the primers 5'-ATGCATGCTATATATGGGA GGAGG-3' (sense) and 5'-GAATAGCGTCAC-CACTTGGCAAGGG-3' (antisense) which yielded a 248 base pair product. To confirm the specificity of the PCR product, a Southern blot was performed using ^{32}P-labeled RhCMV-IE sequences internal to the nested PCR primers.

SV40. For the detection of SV40 virus, the oligonucleotides used in the first round were 5'-ATGGGTGCT-GCTTTAACACTGTTGGG-3' (sense) and 5'-AGC-CATTCCTGGTTGTTGATATAAAA-3' (antisense) based on the sequence of the rhesus SV40VP1 gene (7). One μl of the above PCR product was used in a nested PCR under the same reaction conditions using the primers 5'-CCTAATTGCTACTGTGTCTGAAGCT-GC-3' (sense) and 5'-GTAGAAACTTTGTGATC-CCAGTCACTA-3' (antisense) which yielded a 250 base pair product. To confirm the specificity of the PCR product, a Southern blot was performed using ^{32}P labeled SV40VP1 sequences internal to the nested PCR primers.

Appropriate controls were included (13,18) with each procedure.

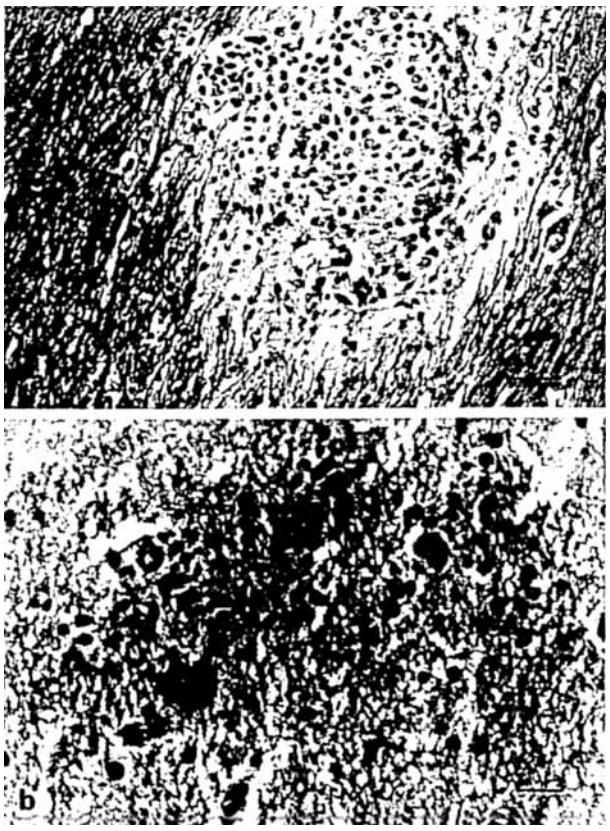

Figure 1 a. Perilesional demyelination associated with a nodular infiltrate of mononuclear inflammatory cells in the anterior commissure of macaque 16B (Luxol Fast Blue stain. 266 x). *Scale bar: 1cm = 38μ* **b.** White matter lesion composed of mononuclear inflammatory cells, microglia and a multinucleated giant cell in the occipital region of macaque 16B (H&E stain. 532 x). *Scale bar: 1cm = 19μ*

Results

$SHIV_{KU-1}$ in pig-tailed macaques. Earlier reports from our laboratory had shown that all pig-tailed macques inoculated with $SHIV_{KU-1}$ developed a typical syndrome of massive virus replication in $CD4^+$ T cells in lymphoid tissues, and this was accompanied by viremia, p27 antigenemia and loss of $CD4^+$ T cells within 2 weeks following inoculation (11,12). The macaques became virtually depleted of $CD4^+$ T cells and developed fatal AIDS within the following 8 months. The CNS abnormalities from five such macaques are discussed in this study. Data from 3 of these, macaques 18A, 18D, and 18E have been reported previously (10,11). The other two macaques 24B and 24C are new, and were added to this study to investigate in detail the CNS events after SHIV inoculation. Since the sequence of events leading up to AIDS in these 2 animals was identical to that in other pig-tailed macaques (10), the data are not shown here. All 5 pig-tailed macaques had developed transient infection of the cerebrospinal fluid (CSF) in the first week after inoculation, and at necropsy on these animals, we found SHIV DNA in most regions of the CNS (Table 1). However, none of these brains had lesions typical of lentiviral encephalitis. Homogenates of brain tissue lacked infectivity and p27 (Table 1).

Two of the 5 animals, macaques 18A and 24C, had encephalitic lesions, but these were clearly due to activation of Toxoplasma sp and SV40 respectively, as typical morphological changes of CNS Toxoplasmosis and progressive multifocal leucoencephalopathy (PML) were present (Table 1). PCR analysis of tissues from different regions of the CNS from the 5 animals (Table 1) revealed SV40 sequences in 24B and 24C, although as mentioned above, only the latter had lesions of PML. A search for CMV showed sequences in the CNS in only one (18E) of the 5 macaques (Table 1), but this macaque's brain was histologically normal. Thus, despite the presence of SHIV-DNA in the CNS of these 5 pig-tailed macaques, and the fact that they were immunosuppresed and had concomitant opportunistic infections, there was no activation of SHIV in any of these animals, and none had typical lentivirus-induced lesions.

$SHIV_{KU-2}$ in rhesus macaques. The 5 rhesus macaques inoculated with $SHIV_{KU-2}$ also developed the acute systemic infection in $CD4^+$ T cells (Table 2) that was characteristic of $SHIV_{KU-1}$ infection in pig-tailed macaques. All five of the rhesus macaques became virtually depleted of $CD4^+$ T cells within 2 weeks of inoculation and during this period the animals developed high levels of infectivity and SIV p27 concentrations in the blood (Table 2). During the first week following inoculation, all five animals had developed infection in the CSF, but beyond this period virus isolation from the CSF was rare (Table 2). Within the next 3 to 5 months, they had developed typical signs of AIDS, including anemia (associated with simian Parvovirus infection in macaque 16A), protracted diarrhea and progressive weight loss. At the time of euthanasia, none of the animals showed neurological signs except macaque 23A, which had mild loss of muscle power on one side. Examination of the brain from these 5 animals showed that SHIV DNA was present in virtually every region that was analysed (Table 3). However, unlike the pig-

tailed macaques, three of these animals, macaques 16A, 16B and 23A also had productive lentiviral infection in the CNS as shown by the presence of infectious virus and high concentrations of p27 in brain homogenates (Table 1). Two of these animals, macaques 16B and 23A had features of a disseminated multifocal encephalitis, largely involving the white matter (Fig 1a). The lesions were characterized by perivascular or nodular parenchymal infiltrates of mononuclear inflammatory cells, mainly composed of $CD68^+$ monocyte-macrophages and microglia, and few $CD3^+$ T cells (Figs. 1d-e). Besides the perivascular orientation of inflammatory cells, typical multinucleated giant cells (MGC) of lentiviral encephalitis were clearly present in brain sections from macaque 16B (Fig.1b). Macaque 23A had fewer MGCs, but had disseminated demyelinating lesions involving the cerebral and cerebellar white matter, brain stem, spinal cord and cranial nerves. In situ hybridization showed SIV *gag* mRNA (Fig.1f), and immunohistochemical analysis showed SIV Gag antigen (Fig.1g) in inflammatory cells associated with the lesions of both macaques, thereby confirming that replicating SHIV was indeed present within the lesions seen in these animals. However, on PCR analysis, we found that macaque 16B also had SV40 sequences in several brain regions (Table 1), although typical morphological features of PML were absent in this animal. Similarly, in macaque 23A, PCR analysis revealed CMV sequences in several brain regions (Table 1), although the characteristic neuropathological abnormalities of CMV encephalitis such as necrotising periventricular lesions, ependymitis or typical viral inclusions were not detected. It would also have been very unusual for CMV to induce lesions in the white matter alone, and spare cortical grey matter in the presence of such fulminant encephalitis. The third animal with productive SHIV infection in the CNS, macaque 16A, had small rare lesions, morphologically consistent with PML. This macaque had SV40 sequences in the brain (Table 1) in keeping with this diagnosis. Thus, among the three animals that had productive SHIV infection in the brain, two macaques, 16A and 16B had disseminated infection with SV40, and one macaque, 23A, with CMV. SHIV genome was present, but was not activated in the fourth animal, macaque 23D, which had focal lesions of PML and SV40 sequences (Table 1) in the brain. The CNS of the fifth macaque, 23E, was histologically normal. This animal had latent SHIV infection in the brain, but had no opportunistic pathogens on PCR analysis (Table 1).

Examination of CNS tissues from macaque 23A that had disseminated white matter lesions revealed two

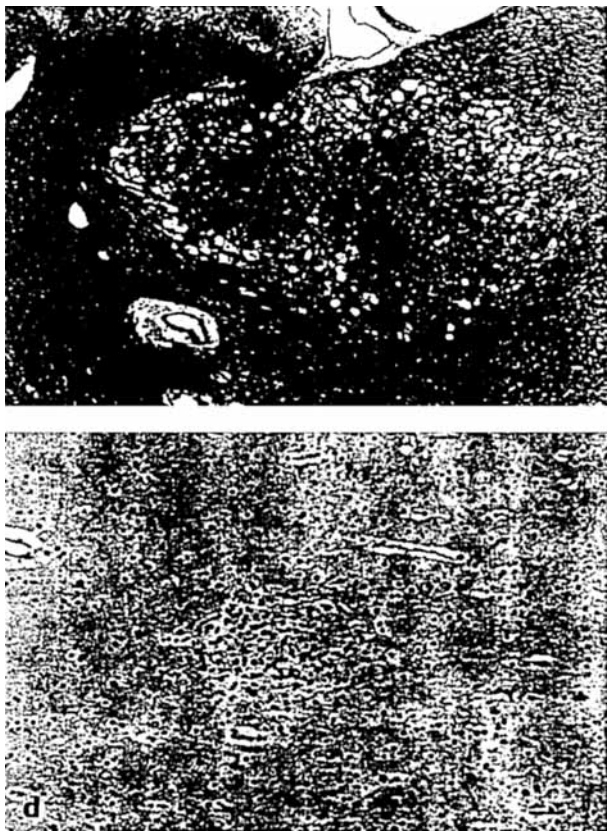

Figure 1 c. A large 'plaque-like' lesion causing white matter destruction and prominent vacuolation (arrow) of myelinated fibres in the region of the middle cerebellar peduncle of macaque 23A (H&E stain. 53 x). *Scale bar: 1cm = 190μ* **d.** A small compact lesion infiltrated by T cells in the deep white matter of macaque 23A (immunostained with anti-CD3 antibody; red reaction product; 133x) *Scale bar: 1cm = 76μ*

basic types of abnormalities: a) small focal accumulations of $CD3^+$ T cells (Fig.1d), associated with minimal tissue damage or evidence of SHIV replication, and b) larger, 'plaque-like lesions' mainly made up of macrophages (Fig.1e) and reactive astrocytes. The latter lesions were associated with more extensive damage in the underlying white matter (Fig.1c) and SIV gag mRNA and *Gag* proteins (Fig.1g) were readily demonstrable in the infiltrating macrophages. The peculiar localization of lesions to the white matter prompted the question whether SHIV genome had become activated only in the white matter. Examination of pooled cortical grey matter and subcortical white matter carefully subdissected from the frontal, temporal, parietal and occipital regions of the freshly removed brain from this animal showed that the grey matter lacked infectious virus and p27, whereas the adjacent white matter had high concentrations of p27 and infectivity (see footnote

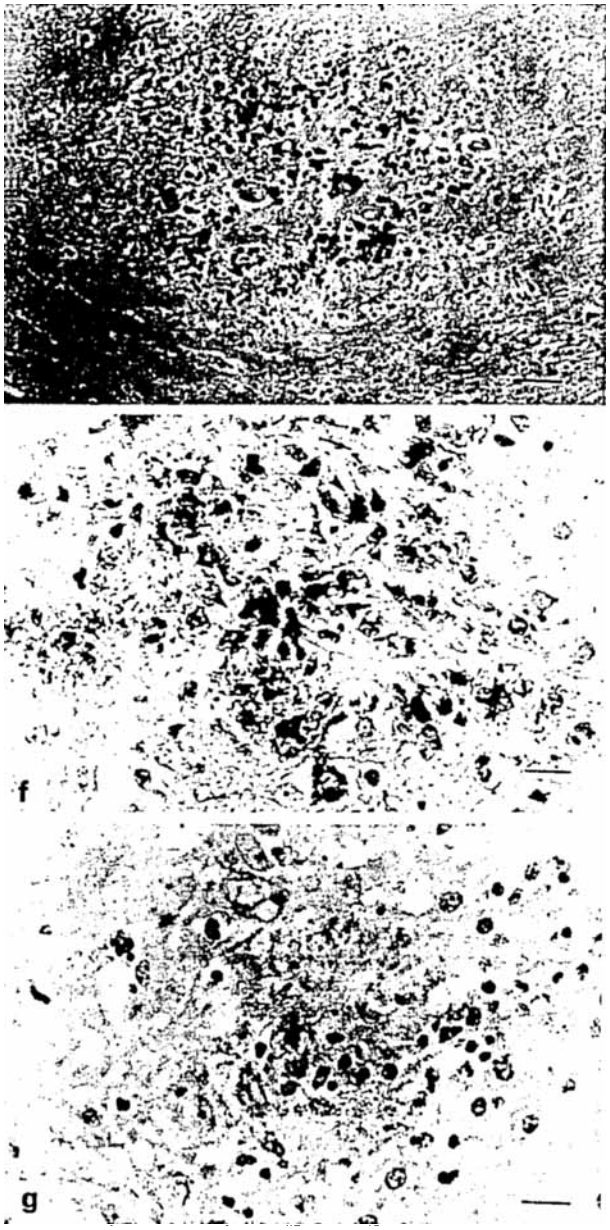

Figure 1 e. An established 'plaque-like' lesion in the deep white matter of macaque 23A showing intense staining for monocyte-macrophages (immunostained with anti-CD68 antibody; red reaction product; 53 x). *Scale bar: 1cm = 190μ* **f.** In-situ hybridization of lesion shown in Fig.1e using a riboprobe against SIVgagmRNA showing positive hybridization product (arrows) within mononuclear inflammatory cells (532 x). *Scale bar: 1cm = 19μ* **g.** Immunohistochemical staining of lesion in Fig.1e showing *Gag* antigen within mononuclear cells (anti-SIVGag [FA2] antibody; red reaction product; 532 x). *Scale bar: 1cm = 19μ*

in Table 1). Thus, SHIV genome had indeed been activated only in the white matter of this animal.

Discussion

This is the first description of neurological disease in macaques infected b· ı chimeric lentivirus bearing the envelope of HIV-1. C · data show a clear contrast in the neuropathogenesis of SHIV infection in pig-tailed and rhesus macaques. In both species the virus had a common pathogenesis in causing acute infection and elimination of $CD4^+$ T cells cells and concurrent development of viremia and antigenemia. This acute systemic infection, accompanied by loss of $CD4^+$ T cells, set the stage for development of AIDS in both species of animals. However, there was a clear difference in the neuropathogenesis of this infection in the two species of macaques. SHIV was neuroinvasive in both species and the viral genome was found in several regions of the brains of both pig-tailed and rhesus macaques. However, in the pig-tailed macaques, the virus failed to become activated and there was no evidence of lentivirus replication. Nevertheless, other neurotropic opportunistic agents replicated efficiently in the CNS of these animals. In rhesus macaques in contrast, the lentiviral genome became activated in three of the five animals. Two of these developed multifocal leucoencephalitis which was accompanied by productive replication of the lentivirus in brain lesions, as demonstrated by immunohistochemical and in situ hybridization techniques.In the rhesus macaque with disseminated leucoencephalitis, the small compact T-cell rich lesions with minimal macrophage infiltration showed very little morphological evidence of CNS damage. Definite, but minimal SHIV replication was identified in these sites. On the other hand, the larger 'plaque-like' macrophage-rich lesions with more damage to the neuropil and reactive astrocytosis, had readily identifiable SIV *gag* mRNA and antigens in the macrophages. Although purely speculative without access to time-course data, the morphological differences suggested that the destructive 'plaque-like' lesions rich in macrophages and astrocytes may have evolved over a period of time from the T-cell rich foci. It is therefore conceivable that T lymphocytes responding to a few infected cells, i.e. activated microglial cells in the neuropil, could have precipitated the early abnormalities, and that macrophages which gradually migrated into the lesions may have become productively infected and extended the white matter damage.

It was of interest that SHIV replication in the brain was largely restricted to the white matter regions of the

CNS. This was reminiscent of the encephalitis of HIV-1 disease (2,3), although features such as diffuse white matter pallor and gliosis, vacuolar myelopathy, and diffuse poliodystrophy were not observed in these animals. The absence of the full spectrum of HIV-1 associated neuropathologic changes may be related to the acute nature of SHIV-induced illness or the severe depletion of $CD4^+$ T cells. The CNS lesions in SHIV-infected animals differed from those typically caused by SIVmac (15) by the virtual absence of meningeal inflammation or lesions in the cortical grey matter. These features have been observed consistently in macaques which develop fulminant encephalitis following inoculation with macrophage-tropic neurovirulent SIV in ours (data not shown), and other laboratories (15). It was also intriguing that activation of SHIV in the CNS in all 3 instances was associated with productive SHIV infection in the lungs and interstitial pneumonia (see data in Table 2). We have previously reported the invariable association of productive lentiviral infection in the CNS with pneumovirulence in SIV disease of macaques (14,24) and visna-maedi of sheep (14,19). Whether the two phenomena are causally related is not known, but in addition to SIV-infected macaques and visna-maedi in infected sheep, the syndrome of encephalitis and lymphoid interstitial pneumonia is also relatively common in HIV-1 infected children (23).

The two rhesus macaques developing SHIV-associated encephalitis duplicated events of neuro-AIDS in human beings in terms of productive replication of the virus in macrophages in the brain and development of histological changes typical of lentivirus replication. However, the other animals that failed to develop these lesions illustrated how poorly understood are the mechanisms involved in activation of the virus in the brain, the event that is fundamental for subsequent development of lesions. Criteria thought to be important for the neurovirulence of HIV are that the virus must be macrophage-tropic and it must invade the brain. The fact that most people developing HIV encephalitis are profoundly immunosuppressed suggests that immunosuppression of the host is a prerequisite also. In the pig-tailed macaques reported in this study, the SHIV genome was present in the brain; the virus has been shown to be macrophage-tropic (26); and the animals were profoundly immunosuppressed to the point that even opportunistic infections with other pathogens had occurred in the brain. Yet, the latent SHIV did not become activated in the brains of these animals. Possibly, although this virus was capable of replicating in blood-derived macrophages in culture (26), it could not replicate productively in microglia in brain. In the rhesus macaques, the problem was equally complex. One animal that did develop productive replication of SHIV in brain only developed lesions of PML, in concert with productive replication of SV40. Whether this animal would have eventually succumbed to SHIV encephalitis is a moot point. The encephalitic brains of the other two animals that did develop productive SHIV infection in brain with pathognomonic lentiviral lesions, were associated with infection due to SV40 and CMV respectively. Whether these infections had a role in activating replication of the latent SHIV genome is unknown. However, it is well known from in vitro studies that pathogens such as CMV are capable of reactivating SIV (4) and HIV (4-6,16). It is of interest that these opportunistic infections were only detectable in the CNS by PCR and not by classical histopathological criteria. Whether similar events occur in humans who appear to develop exclusively HIV replication in brain in the "absence" of other opportunistic infections is not known.

In conclusion, this is the first report of neuropathological and neurovirological findings in macaques infected by a chimeric simian human immunodeficiency virus containing HIV-1 genes including the HIV-1 *env*. Our experiments have shown that productive SHIV infection and lesions can be induced in the CNS and the lungs of rhesus macaques within a few months after inoculation of $SHIV_{KU-2}$, but not in pig-tailed macaques inoculated with $SHIV_{KU-1}$. The clear demonstration of productive infection of SHIV in the CNS lesions of 2 of the animals indicated that $SHIV_{KU-2}$ may have provoked a lentiviral encephalitis in these rhesus macaques, and therefore is more neurovirulent than $SHIV_{KU-1}$. However, the complexities of SHIV neuropathogenesis were highlighted by the discordant findings in relation to activation of SHIV in the brains of the two species of macaques, and the possible synergistic effects of reactivated opportunistic infections and SHIV in the rhesus species. While this study has emphasised some of the remarkable similarities between the CNS findings in the SHIV- macaque model and human neuro-AIDS, it has also raised questions regarding the specific determinants of lentiviral neurovirulence.

Acknowledgements

This work was supported by grants AI-38492, RR-06753, NS-32203, A1- 40372 and DK49516 from the National Institutes of Health, and with partial support from the Max Planck Society of Germany, and a gift

from the Hoechst Marion Roussel Corporation. Monoclonal antibodies to CD4 (SIM.4) and SIVmac p27 were obtained from the AIDS Reference and Reagents Program, NIAID, NIH. We are grateful to Professor John Kepes, Department of Pathology, University of Kansas Medical Center, Kansas City, KS, for critical appraisal of some of the histological material. We also thank Dr. Kevin Brown (National Heart, Lung and Blood Institute, NIH) for identification of Parvovirus sequences in plasma, and Dr M Zucker (Department of Pathology, St. Luke's Hospital, Kansas City) for her valuable opinion on hematological preparations from the anemic rhesus monkeys.

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