

Pathogenesis of lymphocyte-tropic and macrophage-tropic SIV_{mac} infection in the brain

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SIV_{mac} 239 replicates productively in activated CD4⁺ T lymphocytes, but inefficiently in macrophages from rhesus macrophages. Inoculation of the virus into animals results in an acute, highly productive burst of virus replication in activated T lymphocytes in lymphoid tissues and infected cells invade the central nervous system (CNS). This phase lasts a few weeks and is eventually followed by development of immunosuppression of different degrees of severity, opportunistic infections, and tumors related to the loss of T lymphocytes. On rare occasions, infected immunosuppressed animals develop encephalitis and/or interstitial pneumonia, syndromes that are associated with selection of mutant viruses that replicate efficiently in macrophages of these tissues. Usually, however, brains of animals dying with AIDS caused by SIV_{mac} 239 appear histologically normal. Is the brain infected with virus? We report here on a macaque dying with AIDS, a neuroinvasive tumor and interstitial pneumonia associated with macrophage-tropic virus. Except for focal infiltration of tumor cells, the brain was normal histologically. We examined the virus and viral DNA from different tissues and found that lymphocytes but not macrophages from lymph nodes and spleen yielded virus, whereas macrophages but not lymphocytes from the lung produced virus. No virus was recovered from the brain but small amounts of viral p27 were present in the brain homogenate. Viral sequences were present in the brain as determined by PCR from tissue DNA. Comparison showed that the viral sequences in the brain closely resembled those from the spleen. Presumably, the virus caused a minimally productive infection detectable by production of small amounts of p27, but was not accompanied by any histopathological changes. It is unclear why the macrophage-tropic virus in the lung failed to 'take-off' in the brain of this animal. To determine whether this virus had encephalitic potential, we inoculated the lung homogenate containing cell-free, macrophage tropic virus into a young pigtail macaque, a species known to be sensitive to primate lentiviral infections. This animal developed severe encephalitis 10 weeks later. Virus from the brain was very similar to the inoculum virus, proving its encephalitic potential. Possible reasons for the differences in neurovirulence of this virus between the two animals remain speculative.

Keywords: viral tropism; encephalitis; silent infection

Introduction

Human immunodeficiency type 1 (HIV-1) is best known for causing loss of CD4⁺ T lymphocytes and the resultant profound immunosuppression that precedes development of AIDS. In addition to causing pathological effects in the lymphoid system, the

virus invades the brain during the early acute phase of infection and more than 60% of infected individuals develop neurological syndromes that vary from mild to severe symptomatology (Price, 1994). Approximately 10% of these individuals develop encephalitis after establishment of immunosuppression (Griffin *et al*, 1994). Thus, many of the individuals who develop mild types of dementia do not develop obvious lesions in the brain.

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in rhesus macaques (Kestler *et al*, 1990). This virus is highly lymphocyte-tropic but replicates inefficiently in macaque macrophages (Mori *et al*, 1993; Stephens *et al*, 1995). In the animal, the virus causes activation and productive infection in CD4⁺ T lymphocytes which cross the blood-brain barrier and cause meningitis (Sharma *et al*, 1992a). After developing anti-viral CD8⁺ T lymphocytes, this phase of productive replication ceases and viral-infected cells can no longer be isolated from cerebrospinal fluid (CSF). In a study on macaques inoculated with SIV_{mac} 239 and killed at different times postinfection, Lackner *et al*, 1994 showed using *in situ* hybridization and histopathological techniques that virus expression and histological changes were much more intense at a few weeks following inoculation than months later. Thus, virus gene expression apparently subsides after the acute phase of infection is over, but whether virus persists in the brain is not known. In rare cases, infected animals that are severely immunosuppressed develop frank encephalitis and/or interstitial pneumonia. These syndromes are associated with selection of mutant viruses that replicate productively in macrophages of the respective tissues (Letvin and King, 1990; Sharma *et al*, 1992b; Anderson *et al*, 1993; Brinkmann *et al*, 1993; Clements *et al*, 1994).

A salient feature of lentivirus infections is the ability of the virus to undergo extensive variation within the host (Clements *et al*, 1988). This variation is most probably a result of the error-prone reverse transcription of RNA to DNA resulting in changes in the nucleotide sequence, and the selection of antigenic and tissue specific variants that escape humoral and cellular immune surveillance mechanisms. While nucleotide sequences of the *gag* and *pol* genes usually remain relatively constant among isolates, the envelope glycoprotein gene (*env*) has been shown to undergo extensive variation. The greatest amount of variation is confined to certain domains of the glycoprotein gene with five hypervariable domains (V1-V5) described for the gp120 of HIV and SIV (Starcich *et al*, 1986; Johnson *et al*, 1991; Johnson and Hirsch, 1992; Almond *et al*, 1993; Kodama *et al*, 1993). In addition, two variable regions (V6-V7) within the gp41 glycoprotein gene of SIV_{mac} have also been described (Burns and Desrosiers, 1991).

In this report, we describe genetic and biological characteristics of viruses obtained from tissues of a rhesus macaque, inoculated with molecularly cloned SIV_{mac} 239 that died of AIDS characterized by severe interstitial pneumonia and lymphomas which originated in the kidney and urinary bladder and had metastasized to the brain. Brain sections showed evidence of tumor metastasis but lacked evidence of viral encephalitis. Biological characterization of viruses from these tissues revealed that infectious cytopathic virus was present in all tissues but the brain. Virus isolated from the spleen

and lymph nodes was lymphocyte-tropic whereas virus from the lung was macrophage-tropic. Viral DNA was present in the lung, lymph node, spleen, and brain, but viruses had undergone variation in

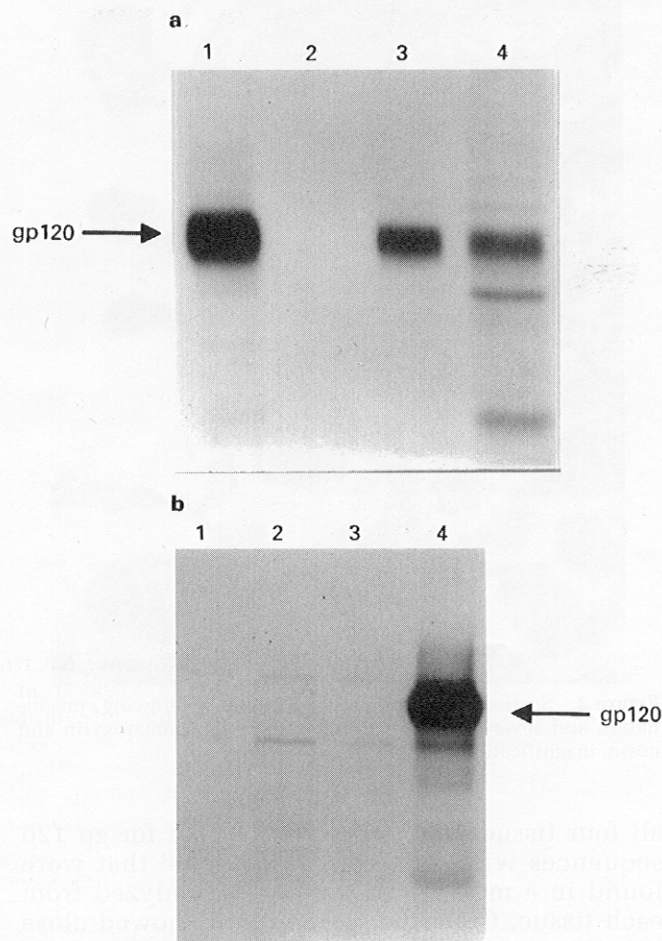


Figure 1 Immunoprecipitation of SIV_{mac} gp120 using plasma from macaques 7F (a) and pHt (b). CV-1 cells were infected with a recombinant vaccinia virus vector expressing the SIV_{mac} 239/17E gp120 and radiolabeled as described in the Material and methods section. Lysates were prepared and SIV specific proteins immune precipitated using plasma from infected animals as described in the text. Proteins were analyzed by electrophoresis through 8.5% gels and visualized by standard fluorographic techniques. (a) Lane 1: gp120 immunoprecipitated using a positive control serum from a macaque infected with SIV_{mac} for 1 year without signs of immunosuppression. Lane 2: inability to immunoprecipitate gp120 using a negative control plasma from an uninfected rhesus macaque. Lane 3: gp120 immune precipitated using plasma from macaque 7F at 12 weeks postinfection. Lane 4: gp120 immunoprecipitated using plasma from macaque 7F at 51 weeks postinoculation. (b) Lane 1: inability to immunoprecipitate gp120 using a negative control plasma from an uninfected rhesus macaque. Lane 2: inability to immunoprecipitate gp120 using plasma from macaque pHt at 7 weeks postinoculation. Lane 3: inability to immunoprecipitate gp120 using plasma from macaque pHt at 10 weeks postinoculation. Lane 4: gp120 immunoprecipitated using a positive control serum from a macaque infected with SIV_{mac} for 1 year without signs of immunosuppression.

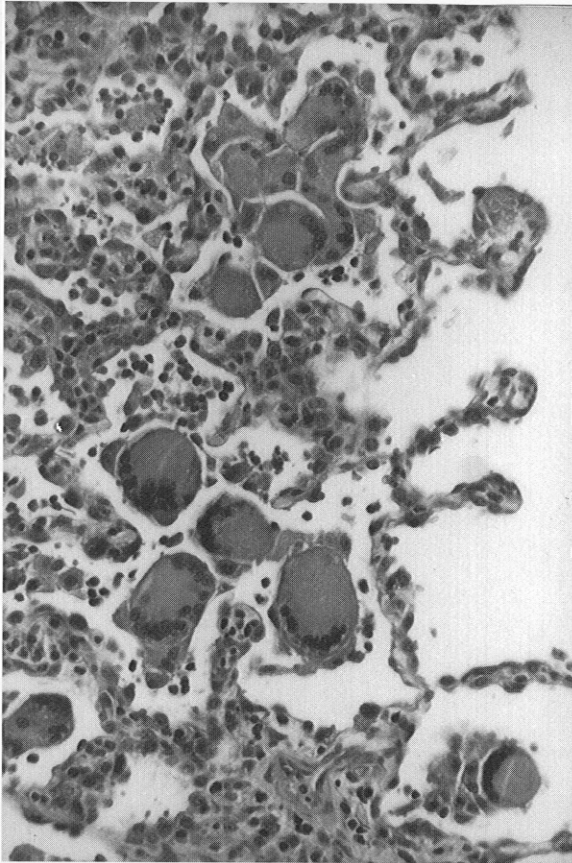


Figure 2 Section of the lung from macaque 7F showing consolidation and several multinucleated giant cells. Hematoxylin and eosin; magnification, $\times 200$.

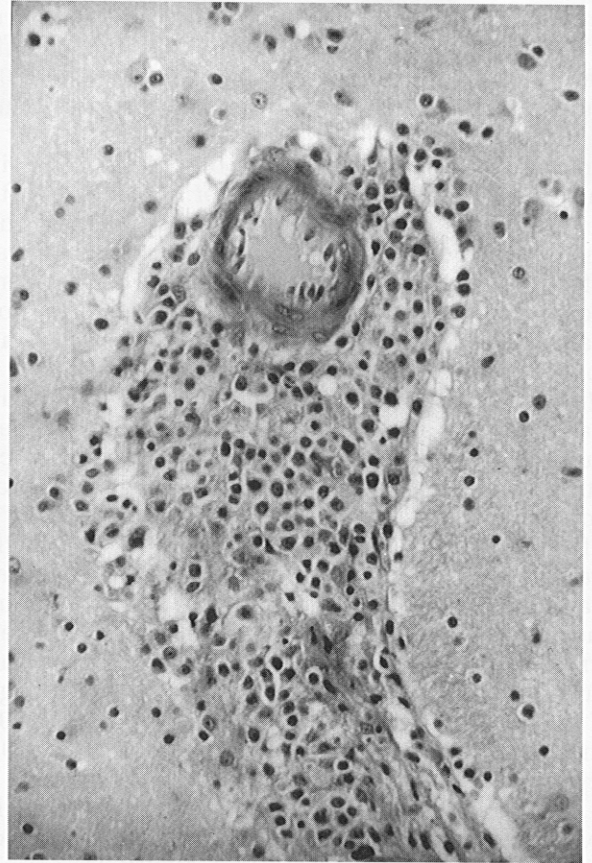


Figure 3 Section of the brain from macaque 7F showing infiltration of tumor cells in an otherwise normal neuropil. Hematoxylin and eosin; magnification, $\times 200$.

all four tissues and each had selected for gp 120 sequences with 'signature variations' that were found in a majority of the clones analyzed from each tissue. Clones from the brain showed close homology with those from the spleen.

To determine whether the macrophage-tropic variants present in the lung homogenate had neuro-invasive properties and could cause encephalitis,

we inoculated this material intratracheally into a young pigtail macaque, a species of macaque that is highly sensitive to primate lentiviral infections (Dewhurst *et al*, 1990; Agy *et al*, 1992). This macaque became severely immunosuppressed and developed fulminant encephalomyelitis. These findings suggest that both lymphocyte-tropic and macrophage-tropic viruses could cause infection in

Table 1 Evaluation of virus burden in macaque 7F

Tissue	Titer of virus in homogenates ^a	Titer of virus from PHA/IL-2 cultures	PCR ^b	ICA ^c	PCR/ICA ^d
Brain	Neg	ND	Pos.	ND	ND
PBMC	ND ^b	2.4	Pos.	10	100
Lung	5.2	ND	Pos.	500	5000
Lymph node	4.0	3.3	Pos.	70	500
Spleen	3.6	3.8	Pos.	100	500

^aExpressed as the log₁₀ TCID₅₀ per ml

^bNot determined

^cPositive or negative for SIV sequences on DNA extracted from DNA extracted from tissues as determined by polymerase chain reaction

^dNumber of infected cells per 100,000 by cytopathology assay

^eNumber of infected cells per 100,000 as determined by PCR/ICA

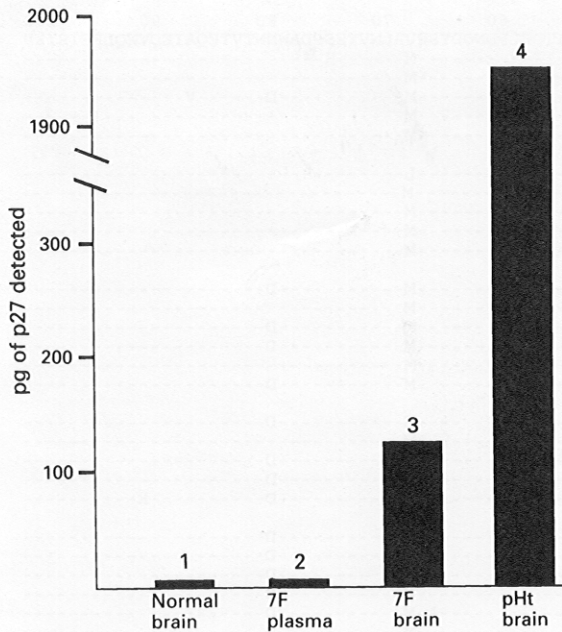


Figure 4 Levels of p27 antigen detected in normal brain, in the plasma and brain from macaque 7F, and in the brain tissue of macaque pHt. The level of p27 in the brain tissue of macaque pHt was greater than the indicated 1950 pg and represents the upper limit of the assay.

the brain, the former being histologically silent and the latter encephalitic, but this may be predicated on prior immunosuppression of the animal.

Results

Macaque 7F

Virus burden and pathology Rhesus macaque 7F was euthanized after 1 week of increasing respiratory distress, 51 weeks after inoculation with SIV_{mac} 239. This animal had developed binding antibodies to the gp120 of SIV_{mac} early during infection (12 weeks) as shown in Figure 1a. However, by 51 weeks, the levels of binding antibodies to gp120 had decreased. Histologic examination revealed lesions in several organs including: (1) severe interstitial pneumonia characterized by dense infiltra-

tion of interalveolar spaces with macrophages, neutrophils and giant cells (syncytia) (Figure 2); (2) enterocolitis; (3) lymphomas of a kidney and the urinary bladder; and (4) brain sections were normal except for a few small foci in the caudate nucleus where tumor cells had accumulated around blood vessels (Figure 3). Immunocytochemical staining of frozen tissue sections detected p27 antigen in the peri-follicular areas of the spleen and lymph node and in alveolar macrophages, many of which had fused into multinucleated giant cells (syncytia). However, p27 antigen was not detected in brain sections tissue using this method (data not shown).

The virus burden in the PBMC, brain, lung, lymph node and spleen were evaluated by several procedures and the data are summarized in Table 1. The PBMC, lung, spleen, lymph node and brain cells were positive for SIV *gag* and *env* sequences as determined by PCR. Homogenates of the lung, lymph node and spleen yielded high virus titers but no infectious virus was recovered from brain homogenates. However, the brain homogenate did contain low levels of p27, although p27 was not detected in the plasma (Figure 4). In addition, explant cultures of the brain were shown to yield virus when co-cultured with CEMx174 cells. These data suggested that limited virus replication occurred in macrophages in the brain of this animal. A similar type of replication had been observed after SIV_{mac} 239 was inoculated into cultures of macrophages (Stephens *et al*, 1995). The number of infectious cells ranged from 10/10⁵ cells among the PBMC to 500/10⁵ cells from the lung. Supernatant fluids of PHA/IL-2 cultures from PBMC, lymph node, and spleen cultures had infectivity titers varying from 2.5 × 10² to 6.5 × 10³ TCID₅₀ ml⁻¹, suggesting the presence of virus in lymphocytes. Mononuclear cells teased from the densely infiltrated lung responded poorly to treatment with PHA/IL-2 suggesting the presence of few lymphocytes in the cell suspensions. Supernatant fluids from these cultures did not have infectious virus as was obtained from the spleen and lymph node. Instead, culture of lung cells in MDM yielded macrophages which did produce infectious virus. Further, in explant cultures of the lung,

Table 2 Genetic changes in the gp120 isolated from macaque 7F^a

Origin of clones	No. of clones	Total no. of nucleotides sequenced	Total no. of substitutions (deletions)	Average nucleotide substitution rate (%)	Mutation rate (%)	% nonsilent changes
Brain	5	7935	135(91)	1.7	2.9	76
Lung	5	7935	63(48)	0.80	1.4	80
Spleen	5	7935	46(63)	0.58	1.37	78
Lymph node	6	9522	73(15)	0.77	0.9	74

^aViral DNA from the gp120 of *env* were derived from macaque 7F by PCR, cloned and sequenced compared to parental SIV_{mac}239



	10	20	30	40	50	60	70	80	90	100
239	MGCLGNQLLIAILL	SVYGIYCTLYVT	VFYGVPAWRNAT	IPFCATKNRDT	WGTTQCLPDNG	DYSEVALNVTES	FDANNNTVTEQ	AIEDVWQLFETS	IKP	
7FBE1				G			M	R		
7FBE2							M			
7FBE3		R					M	D	V	
7FBE4		R					M			
7FBE5							M			
7FSE1				E			L			
7FSE2							M			
7FSE3		T					M			
7FSE4		C					M			
7FSE5							M			
7FLN1							M	D		
7FLN2							M			
7FLN3			S				M	D		
7FLN4							M	D		
7FLN5							M			
7FLN6							M	D		
7FLG1							M	D		
7FLG2							M			
7FLG3							M	D		
7FLG4					*		M	D		
7FLG5							M	D	E	
pHtBE1							M	D		
pHtBE2		V					M	D		
pHtBE3					A		M	D		
pHtBE4		V					M	D		
pHtBE5				Y			A	M	D	
pHtBE6		V					M	D		
pHtBE7							M	D		
pHtLG1							M	D		
pHtLG2							M			
pHtLG3							M	D		
pHtLG4							M			
pHtLG5							M			

	110	120	130	140	150	160	170	180	190	200
239	CVKLSPLCITMRCN	KSETDRWGLTKS	ITTTASTTSTTAS	AKVDMVNETSSC	IAQDNCTGLEQE	QMISCKFNMTGL	KRDKKKEYNETW	YSADLVCEQGNNT		
7FBE1		R		P	E	SM*	G	I		
7FBE2		R		P	E		I			
7FBE3			*	Kxx	E		Y	V	R	QK
7FBE4			x	T	xx					K
7FBE5				P	E		I			R
7FSE1		S		T	E					E
7FSE2		R		P		G				S
7FSE3		R		P	A					
7FSE4				P	A					
7FSE5				P						
7FLN1		R		P	E					
7FLN2		R		P	A					
7FLN3				T	xxxP	E				
7FLN4		R		P	M					
7FLN5		R		P	A					
7FLN6				I	A	xx				I
7FLG1				Pxxx	T	S				D
7FLG2				xxxP	xx				E	
7FLG3				xxxP	E				T	
7FLG4				T	xxxP				I	
7FLG5				L	P	xx			E	
pHtBE1				XX	P				G	
pHtBE2				P	A	P			N	
pHtBE3				P	A	P			N	
pHtBE4				XXXNI			G			
pHtBE5				P	A	P			N	
pHtBE6				T	XXXP	E		Y	Q	N
pHtBE7				T	XXXP	E			K	
pHtLG1				L	XX		G	V	E	
pHtLG2							G	V	I	D
pHtLG3				P	A	E			N	
pHtLG4				R	P	A	E		N	
pHtLG5				R	P	E			I	

Figure 5

	210	220	230	240	250	260	270	280	290	300
239	GNESRCYMNHCNTSVIQESCDKHYWDAIRFRYCAPPGYALLRCNDTNYSGFMPKCSKVVSSTRMMETQTSTWFGFNGTRAENRTYIYWHGRDNRTIIS									
7FBE1	D							S		
7FBE2				*	D			S		
7FBE3	D		*					D	K	
7FBE4										
7FBE5							T			
7FSE1					R			D		
7FSE2										
7FSE3										
7FSE4										
7FSE5										
7FLN1										
7FLN2										
7FLN3										
7FLN4										
7FLN5										
7FLN6										
7FLG1										
7FLG2										
7FLG3	D									
7FLG4					E					
7FLG5	D									
pHtBE1										
pHtBE2					S					
pHtBE3	D									
pHtBE4	D								N	
pHtBE5	D		S							
pHtBE6										
pHtBE7	D									
pHtLG1										
pHtLG2										
pHtLG3					M			K		
pHtLG4										N
pHtLG5			*		D					
	-----V3-----									
	310	320	330	340	350	360	370	380	390	400
239	LNKYYNLTMKCRRPGNKTIVLPVTIMSGLVFHSQPINDRPKQAWCWFGGKWKDAIKEVKQTIIVKHPRYTGTNNTDKINLTAPGGGDPEVTFMWTNCRGEFL									
7FBE1					R	*	K			
7FBE2					R	E	*	K		
7FBE3					R	E	*	K		
7FBE4					R	E	*	K		
7FBE5					R	E	*	K		
7FSE1										
7FSE2		R								
7FSE3										
7FSE4				R						
7FSE5										
7FLN1		I						R		
7FLN2										
7FLN3		L						R		
7FLN4		L		M				R		
7FLN5		L						R		
7FLN6		L						R		
7FLG1								R		
7FLG2				D				R		
7FLG3		L								
7FLG4					E			R	G	
7FLG5				N				R		
pHtBE1		V		M				R		
pHtBE2								R		
pHtBE3		V		M				R		
pHtBE4		I			ER					
pHtBE5		L						R		
pHtBE6										
pHtBE7							T			I
pHtLG1		I	K		N			R	N	
pHtLG2		I			E				N	
pHtLG3			P		E			R	I	I
pHtLG4				G	E			R	N	
pHtLG5					R	E	*	K		

Figure 5 (contd)

	410	420	430	440	450	460	470	480	490	500
239	YCKMNVFLNWVEDRNTANQKPKQHKRNYVPC	HIRQIIINTWHKVGKNVYLP	PPREGDLTCNSTVTSLIANIDWIDGNQTNITMSAEVAEL	YRLELGDYKLV						
7FBE1	--I-----KN-----xxxxx--K-----									
7FBE2	--I-----KN-----xxxxx--K-----									
7FBE3	-----K-----K-----									
7FBE4	--I-----KN-----xxxxx--K--A-----									
7FBE5	--I-----KN-----xxxxx--K-----									
7FSE1	-----G-----xxxxx--K-----									
7FSE2	-----xxxxxx-----									
7FSE3	-----xxxxxx-----									
7FSE4	-----S-----									
7FSE5	-----xxxxx--K-----									
7FLN1	-----N-----									
7FLN2	-----T-----									
7FLN3	-*------T-----									
7FLN4	-----N-----									
7FLN5	-----T-----T-----									
7FLN6	-----T-----									
7FLG1	-----N-----H-----									
7FLG2	-----T-----									
7FLG3	-----N-----									
7FLG4	-----E-----									
7FLG5	-----E-----									
pHtBE1	-----QA-----									
pHtBE2	-----A-----									
pHtBE3	-----A-----									
pHtBE4	-----									
pHtBE5	-----T-----D-----									
pHtBE6	-----T-----									
pHtBE7	-----A-----G-----									
pHtLG1	-----XXXXX--K-----									
pHtLG2	-----G-----									
pHtLG3	-----XXXXX--K-----									
pHtLG4	-----N-----									
pHtLG5	--I-----KN-----XXXXX--K-----									
	510	520								
239	EITPIGLAPTDVKRYTTGGTSRNRK									
7FBE1	-----N-----A-----									
7FBE2	-----N-----A-----									
7FBE3	-----N-----A-----									
7FBE4	-----N-----A-----									
7FBE5	-----N-----A-----									
7FSE1	-----N-----									
7FSE2	-----N-----A-----									
7FSE3	-----A-----A-----									
7FSE4	-----N-----									
7FSE5	-----N-----									
7FLN1	-----N-----A-----									
7FLN2	-----N-----A-----									
7FLN3	-----N-----									
7FLN4	-----N-----									
7FLN5	-----N-----									
7FLN6	-----N-----D-----									
7FLG1	-----G-----									
7FLG2	-----N-----									
7FLG3	-----N-----									
7FLG4	-----N-----									
7FLG5	-----N-----									
pHtBE1	-----N-----									
pHtBE2	-----N-----									
pHtBE3	-----N-----									
pHtBE4	-----N-----									
pHtBE5	-----N-----A-----									
pHtBE6	-----N-----									
pHtBE7	-----N-----									
pHtLG1	-----S-----A-----									
pHtLG2	-----L-S-----A-----									
pHtLG3	-----S-----A-----									
pHtLG4	-----S-----A-----									
pHtLG5	-----N-----A-----									

Figure 5 Deduced amino acid sequence of gp120 clones from various tissues of macaque 7F and from the brain and lung of macaque pHt at necropsy. BE refers to brain, SE to spleen, LN to lymph node and LG to lung as the tissue source of DNA for PCR amplification and cloning. Shown above the sequences of the 33 clones is the sequence of SIV_{mac} 239 clone (Price, 1994). Dashes (-) represent amino acid identity; crosses (x) represent deletions and asterisks (*) represent premature termination codons. For comparative purposes, brackets corresponding to the variable regions V1, V2, V3, V4 and V5 as described previously (Kodama *et al*, 1993) were placed above the SIV_{mac} 239 sequence.

macrophages migrating from the explants produced cell-free virus which was easily detected in CEMx174 cultures inoculated with supernatant fluids. This virus also replicated productively after inoculation into fresh cultures of normal macaque macrophage cultures. In contrast, macrophages migrating from explant cultures of the spleen did not produce infectious virus, suggesting the absence of virus in this population of cells. From these data, we concluded that lymphocyte-tropic virus which could replicate efficiently in lymphocytes but not macrophages was present in lymphoid tissues but that replication-competent macrophage-tropic variant(s) which could replicate efficiently in both lymphocyte and macrophage populations had evolved from the parental SIV_{mac} 239 in the lung during the course of infection. The heavy virus burden in the lung of this animal was atypical when compared to the other monkeys infected with this strain of SIV (unpublished observations).

Construction of 7F gp120 clones and sequence analyses

Since the viral genome in the brain was expressed poorly in comparison to other tissues, we next examined the sequence of the glycoprotein gene (*env*) for comparative purposes. Based on previous studies that showed very little variation in the gene gp41 (Johnson *et al*, 1991; Overbaugh *et al*, 1991; Johnson and Hirsch, 1992), we chose to analyze only the gp120 sequences from the four tissues of the animal. Total genomic DNA prepared from portions of brain, lung, lymph node and spleen were used as templates in PCR to amplify the gene encoding gp120. Five or six clones of gp120 were selected at random from separate PCR of each tissue and analyzed. The predicted protein sequences of the gp120 clones are shown in Figure 5 and a summary of the genetic changes is shown in Table 2.

Sequence analyses of 21 PCR amplified gp120 products from the four tissues indicated that mutations had occurred most frequently within the V1 and the V4 regions. Surprisingly, only a few changes were noted within the V2 region, the 'pseudo V3' region corresponding structurally to the V3 region of HIV, and the V5 region. Certain amino acid changes were common to gp120 clones isolated from all tissues. These included the valine to methionine change at amino acid position #67 (20/21 clones examined) and the aspartic acid to asparagine or glycine change at position #511 (19/21 clones examined). Interestingly, while both changes have been reported previously (Burns and Desrosiers, 1991), in our study the aspartic acid at position #511 had changed to asparagine in 18/21 clones whereas in the previous study by Burns and Desrosiers (1991), the aspartic acid had changed to a glycine residue in 14/16 clones examined.

Four of five gp120 clones sequenced from 7F lung tissue contained an open reading frame whereas one had premature stop codon at nucleotide residue 156. A finding common to all five gp120 lung clones were deletions within the V1 region that ranged from two to five amino acids. In four of the five clones (LG1–4), two threonine and one serine residues, located amino acid positions 133–135, were deleted. One of these clones (LG2) had another deletion of alanine and lysine residues at amino acid positions 140–141. Clone LG5 also had the same two amino acids deleted from the V1 region.

The five lymph node gp120 clones were most closely related to the gp120 sequence of parental SIV_{mac} 239, with an average nucleotide substitution rate of 0.77% and an overall mutation rate of 0.9% (Table 2). Only one of the clones had a deletion within the V1 region and a premature termination codon (LN3).

All five gp120 clones from the spleen had an open reading frame but four of five clones (SE1,2,3 and 5) had five to six amino acid deletions in the V4 region in addition to the substitutions in V1.

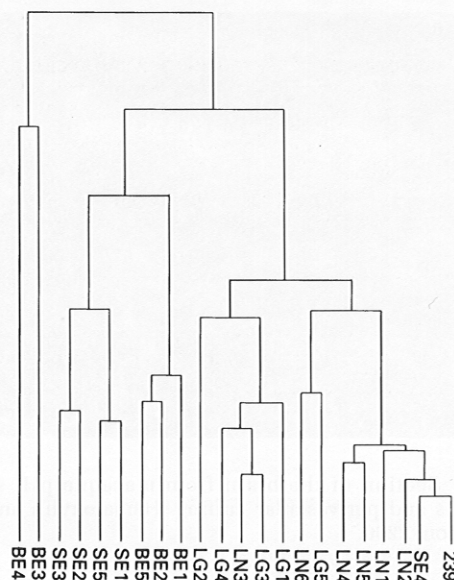


Figure 6 Phylogenetic tree depicting the relationships among the gp120 genes of the input virus (SIV_{mac}239) and the 21 gp120 genes sequenced from macaque 7F.

The V4 deletions were located at amino acid positions 417–424 (Figure 5). The spleen had the lowest overall nucleotide substitution rate of 0.58% of the four tissues examined but due to the deletions observed, had an overall mutation rate of 1.37%.

All five gp120 clones sequenced from the brain had premature termination codons. In four of five clones, premature termination was due to in-frame nucleotide substitutions to stop codons whereas one clone (BE-1) was prematurely terminated because of a single base pair deletion at nucleotide position 439 (Figure 5). These results are consistent with biological data discussed above suggesting a lack of infectious virus in the brain homogenate. Also unique to all five brain clones were the following changes: (1) a five amino acid deletion within the V4 region (amino acid positions #420–425); (2) a tryptophan to arginine change at position #345; (3) a glutamic acid to lysine change at position #356; and (4) a glutamic acid to lysine change at position #412.

Two of five clones from the spleen (SE1,5) had deletions located at the same position as brain clones whereas two others (SE2,3) had six amino acid deletions. While the average nucleotide substitution rate for the spleen, lymph node and lung was between 0.58–0.80%, the brain had an average nucleotide substitution rate of 1.7% and an overall mutation rate of 2.9%. This was twice as high as the mutation rate among clones from the other tissues and may account for the presence of premature termination codons in all of the gp120 clones sequenced from macaque 7F brain.

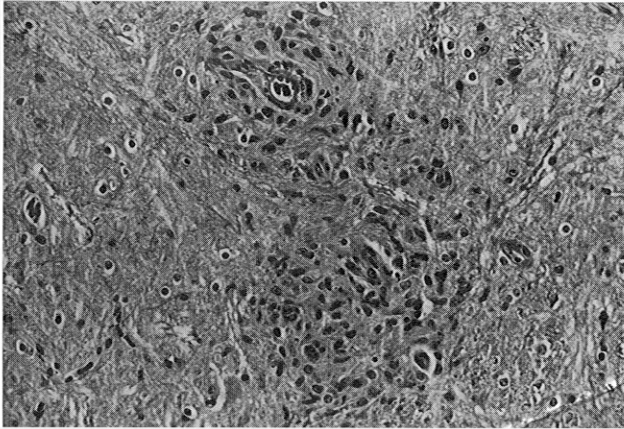


Figure 7 Section of the brain from macaque pHit showing microgliosis and perivascular cuffing. Hematoxylin and eosin; magnification, $\times 200$.

Relationships among the various gp120 genes

A phylogenetic tree in which the sequences of the 21 gp120 clones sequenced in this study were aligned to the input virus (SIV_{mac} 239) is shown in Figure 6. This alignment indicates that in general, gp120 clones of the lymph node and lung were clustered together as were the clones from the brain and spleen. Sequence analyses also revealed the presence of amino acid substitutions commonly found in lung and lymph node clones, suggestive of a common origin. For example, aspartic acid was substituted for asparagine at amino acid #80 (8/11 of clones), arginine substituted for glycine at position #382 (9/11 clones), and serine for threonine at position #481 (9/11 clones). Finally, a consistent finding among all lung and lymph node clones was a lysine substitution at position #422 (11/11 clones).

The common deletions in the V4 region in all of the clones from the brain and spleen suggested that the viruses in brain and spleen had a common origin. All five brain clones had the same five amino acid deletion within the V4 region and four of five

spleen clones also had five or six amino acids deleted from V4. Interestingly, those brain and spleen clones with the same five amino acid deletion (7/10 clones) also had an arginine to lysine substitution at amino acid position #427.

Macaque pHit

Virus burden and pathology To determine if the macrophage-tropic viruses selected in the lungs of macaque 7F had encephalitic properties, a homogenate of this tissue was inoculated intratracheally into pigtail macaque pHit. This animal began to lose weight at approximately 8 weeks later, developed motor difficulties and was euthanized at 10 weeks postinoculation. This animal failed to develop any antibodies to the gp120 of SIV_{mac} as determined by immunoprecipitation analyses (Figure 1b). In addition, between weeks 6 and 10, four separate blood samples had CD4⁺T cell counts of < 500 cells per μ l accompanied by reversal of CD4/CD8 ratios in the blood. Taken together, this indicated that the animal had become immunosuppressed in both arms of the immune response. Histologic examination revealed a diffuse encephalomyelitis characterized by multifocal microgliosis and modest cuffing of the microvasculature (Figure 7). Aggregates of microglia were observed randomly throughout the gray and white matter of the brain. Microglial nodules were also observed in the white and gray matter of the spinal cord. There was prominent astrogliosis in the brain sections. Selected tissues and PBMCs were assessed for virus burden (Table 3). There was no evidence of opportunistic infections in the brain of pHit or activation of latent cytomegalovirus. Unlike macaque 7F, in which virus was not recovered from brain homogenates, $10^{3.0}$ TCID₅₀ of virus per ml were recovered from homogenates of pHit brain tissue. Explant cultures derived from the brain tissue were also positive for the presence of virus. PBMC, lymph node and spleen cells yielded virus after culture in the PHA/IL-2 protocol, indicating the presence of virus in the lymphocytes. Explant cultures

Table 3 Evaluation of virus burden in macaque pHit

Tissue	Titer of virus in homogenates ^a	Titer of virus from PHA/IL-2 cultures	PCR ^c	ICA ^d	Virus from explant cultures
Brain	3.0	ND	Pos.	ND	+
PBMC	ND ^b	2.1	Pos.	Neg.	ND
Lung	3.0	ND	Pos.	ND	+
Lymph node	3.0	3.8	Pos.	16	ND
Spleen	2.0	1.4	Pos.	Neg.	+

^aExpressed as the log₁₀ TCID₅₀ per ml

^bNot determined

^cPositive or negative for SIV sequences on DNA extracted from DNA extracted from tissues as determined by polymerase chain reaction

^dNumber of infected cells per 100,000 by cytopathology assay

derived from lung and spleen tissues were positive for virus indicating the presence of virus in macrophages that migrated from these tissues. From these data, we conclude that the viruses in the lung homogenate of macaque 7F were macrophage-tropic and were capable of causing both encephalitis and immunosuppression.

Sequence analyses of gp 120 clones from macaque pHt

We sequenced gp120 clones isolated from both the lung and the brain tissue of pHt. The predicted protein sequences of the gp120 clones are shown in Figure 5. All clones from the brain and lung retained a characteristic methionine at amino acid position 67. Most pHt clones (8/11) retained the glycine to arginine substitution at position 382, shown previously to be associated with macrophage tropism (Mori *et al*, 1992), and all clones retained an amino acid substitution at position 511. Interestingly, while four of five clones sequenced from macaque 7F lung had a three amino acid deletion within the V1 region, only two of seven gp120 clones sequenced from pHt brain tissue and none from pHt lung retained this deletion. Three of five pHt gp120 clones from lung tissue exhibited a five amino acid deletion in the V4 region and an arginine to lysine substitution at position 427. Interestingly, all gp120 clones from the brain and two of five gp120 clones from the spleen of macaque 7F had the same deletions observed in gp120 sequences isolated from pHt lung. Thus, while macaque pHt was not inoculated with a molecularly cloned virus (like SIV_{mac}239), it appears that by the 10th week post inoculation, some tissue specific amino acid substitutions in the gp120 were beginning to occur in the brain and lung.

Discussion

While many of the mechanisms underlying the pathogenesis of SIV (and HIV) induced encephalopathy remain to be elucidated, it has been firmly established that the replication of macrophage-tropic viruses in the brain is central to this disease process (Spencer and Price, 1992). In a previous study, we had shown that during the early phase of the infection in rhesus macaques with SIV_{mac}239, activated infected T cells had crossed the blood-brain barrier (Joag *et al*, 1994a) correlating with and resulting in transient meningitis, but not encephalitis (Lackner *et al*, 1994). The spleen was the major source of activated, infected T cells (Joag *et al*, 1994a). This established a precedent for the spleen-blood brain conduit of this virus. Based on these observations, we asked two questions regarding the pathogenesis of SIV_{mac}239 infection of macaques. First, can lymphocyte-tropic viruses such as SIV_{mac}239 establish a silent infection in the brain? Second, is the selection of macrophage-tropic

variants in macaques inoculated with SIV_{mac}239 predictive of the onset of encephalitis?

To address these questions, we examined the tissues from a rhesus macaque that had been inoculated with lymphocyte-tropic SIV_{mac}239 and had become moribund with pneumonia 1 year later. The main findings were that the viruses obtained from the lung were macrophage-tropic variants of the virus, whereas the viruses in the spleen and lymph node were primarily CD4⁺ T-cell tropic in character (Table 1). Viruses in all three tissues were in a state of productive replication in the respective organs at the time of death of the animal. While cell free homogenate from the brain tissue lacked infectious virus, antigen capture assay revealed the presence of p27 in the brain tissue. Since the macrophages from explant cultures of the brain showed the presence of virus, these cells may have been the source of p27. This type of replication has been observed earlier in cultured macrophages infected with SIV_{mac}239 (Stephens *et al*, 1995). These results suggest that virus replication, albeit at low levels, was occurring in brain tissue up to the time of death of the animal.

Comparisons of sequence analyses of gp120 genes obtained from different tissues provided insights into selection of particular viral genotypes by different tissues and into possible trafficking patterns of these genotypes in the tissues during the persistent viral infection in the animal. Selection was contingent on the well known mutation rate of the virus and in this study we found that the nucleotide substitution frequency of gp120 clones in lung, lymph node and spleen were 0.58 to 0.8%, agreeing with those reported during studies in other rhesus macaques inoculated with SIV_{mac}239 (Kodama *et al*, 1993). The intriguing novel findings however were the clustering of similar sequences in clones between the lung and lymph node and between spleen and the brain. The phylogenetic analyses suggested that the lymph node virus most closely resembled the inoculum SIV_{mac}239 but nevertheless had many similarities in common with those from the lung. The lymph node viruses may have given rise to macrophage-tropic viruses in the lung. The clustering of gp120 clones between the spleen and the brain was not anticipated, but suggests that the gp120 (and hence virus) in the brain may have been derived from virus in the spleen. The observation that the viruses derived from the spleen appeared to be lymphocyte-tropic in nature (explant cultures were negative for virus whereas PHA/IL-2 cultures were positive) provides indirect evidence that the viral genomes present in the brain were lymphocyte-tropic in nature. However, the lack of infectious virus in homogenates of the brain precluded any biological characterization.

Previous studies on the genetic characterization of variants of SIV_{mac}239 had shown that a glycine to arginine change at position 382 correlated with

acquisition of macrophage tropic properties of the viruses (Mori *et al*, 1992). Examination of the sequences from the four tissues showed that those from the lymph node and lung had this mutation while those from the spleen and brain lacked this change. This provided further indirect evidence of the lymphocyte-tropic nature of the spleen and brain clones. One possible reason for the lack of infectious virus from the brain was that the brain contains macrophages and not lymphocytes. The fact that the brain clones did not have all the genetic markers associated with macrophage tropism (Mori *et al*, 1992) and that the latter replicates very poorly in macrophages (Mori *et al*, 1993; Stephens *et al*, 1995) suggests that the brain of macaque 7F had become persistently infected with lymphocyte tropic viruses. This infection was not accompanied by the classical hallmarks of lentiviral encephalitis. Examination of brains from seven other macaques inoculated with SIV_{mac} 239 has shown a similar lack of histological lesions although viral DNA has been identified in the brain (Stephens *et al*, manuscript in preparation). Thus, the findings reported here do not represent a single case. Lymphocyte-tropic viruses can cause a histologically silent infection in the brain. Whether this type of infection could cause any of the neurological problems associated with AIDS cognitive/motor complex is not known.

Neither the high mutation rate of the gp120 genes isolated from the brain (2.9%) nor the presence of truncated *env* genes was anticipated. The latter could explain the failure to isolate infectious cytopathic viruses from the brain. However, even if the truncated *env* genes are representative of the predominant species in the brain tissue, *env* genes with open reading frames must have been present in the brain at low percentage to account for the low levels of p27 in the tissue. We determined whether the macrophage-tropic viruses in the 7F lung homogenate had encephalitic potential by experimental inoculation into a pigtail macaque. This animal died 10 weeks later with severe encephalitis. Virus was easily cultured from cell free homogenates of brain tissue and cultures of brain macrophages. Sequence analyses of five brain clones from this animal revealed that they all had open reading frames and had many similarities to the sequences isolated from 7F lung tissue. Thus, the viruses in the inoculum were neuroinvasive and encephalitic in character. These results contrasted dramatically with the truncated *env* genes found in 7F brain.

The reason for the failure of the macrophage-tropic variants in the lung tissue to cause encephalitis in macaque 7F is not understood. One possibility is that the full expression of viral neuroinvasiveness and neurovirulence can occur only in animals that are severely immunosuppressed. Our data indicate that macaque 7F was only partially immunosuppressed because serum from this animal examined

at both 12 and 51 weeks postinoculation had binding antibodies to gp120 and that the animal produced anti-viral CD8⁺ T cells early in infection as reported earlier (Joag *et al*, 1993). Whether these cells were still present at the time of death is not known. Low levels of humoral and cellular immune responses could have been enough to suppress the replication of the macrophage-tropic virus in the brain (although interestingly, it was not enough to suppress the metastasis of the lymphoma to the brain). In contrast, the infected pigtail macaque became more severely immunosuppressed since no anti-gp120 antibodies were detected at any point in the infection while the CD4⁺ T cell counts in the animal had dropped to < 500 cells per μ l. Use of this highly sensitive animal illustrated that the lung homogenate contained a swarm of viruses that could cause CD4⁺ dependent immunosuppression as well as macrophage-dependent encephalitis and that only the former was expressed in rhesus macaque 7F. Taken together, these results suggest that CD4⁺ T cell tropic virus caused a persistent, minimally productive and histologically silent infection in the brain of immunocompetent animals and that selection of macrophage-tropic variants is obviously important for the development of encephalitis. We speculate that the immune status of the animal is an important factor in determining whether the encephalitic potential of the macrophage-tropic viruses can be expressed.

Materials and methods

Cell cultures, virus stocks and infection

The CEMx174 cell line was used as the indicator cell line to measure infectivity of SIV_{mac} 239. CEMx174 is a human B-T hybrid cell line (Salter *et al*, 1985) and was maintained in RPMI-1640, supplemented with 10mM Hepes buffer pH 7.3, 5×10^{-5} β -mercaptoethanol, 2 mM glutamine, 50 μ g per ml gentamicin and 10% fetal bovine serum (FBS). CV-1 cells were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 50 μ g per ml of gentamicin and 10% FBS.

The molecular clone of the lymphotropic SIV_{mac} 239 was used to inoculate rhesus macaque 7F (Regier and Desrosiers, 1990). λ DNA encoding the viral genome was transfected into normal mitogen-activated rhesus PBMC and the progeny virus stock frozen at -80°C. One ml of this material was inoculated into the bone marrow of rhesus macaque 7F. The animal subsequently developed AIDS and was euthanized at 51 weeks postinoculation (Joag *et al*, 1994b). A lung homogenate derived from macaque 7F was shown to contain a high titer of macrophage-tropic viruses (discussed below) and was used to inoculate a pigtail macaque (pHt) by the intratracheal route. This animal was euthanized at 10 weeks post inoculation after continued weight

loss, and development of motor difficulties.

Processing of blood and tissue samples

Heparinized blood was centrifuged to separate the plasma from the buffy coat. Plasma was assayed directly for infectivity in CEMx174 cells and the cells were centrifuged through Ficoll-Hypaque density gradients to isolate peripheral blood mononuclear cells (PBMC). Portions of tissue specimens obtained at necropsy were processed as follows: (1) frozen at -80°C and used as the source of genomic DNA; (2) used to prepare cell-free, 10% homogenates (wt vol⁻¹) which were assayed for infectivity in CEMx174 cells; (3) used to prepare single cell suspensions by passing through a fine mesh (spleen, lymph node, and lung) and the number of infected cells assessed by both a polymerase chain reaction/infected cell assay (PCR-ICA) and co-cultivation with CEMx174 cells; (4) aliquots of cells cultivated in macrophage differentiation medium (MDM) and in sRPM1 containing phytohemagglutinin/IL-2 to test for infectious virus in macrophages and lymphocytes; (5) used to prepare tissue explants in MDM to test for productive virus replication in tissue macrophages; (6) fixed in 10% buffered formalin embedded in paraffin, sectioned and stained with hematoxylin and eosin for histopathological examination; and (7) embedded and frozen in OCT for immunohistochemical analyses on frozen sections.

Assessment of virus burden

To determine the number of cells actively producing virus, infectious cell assays (ICA) were performed. Serial two-fold dilutions of PBMC, lymph node, lung, or spleen cells were inoculated onto CEMx174 cells, which were then observed for development of syncytial cytopathic effects (CPE) during a 7 day period. The results were expressed as the number of infectious cells per 10⁵ cells (Joag *et al*, 1994).

To determine if activated T-lymphocytes would produce infectious virus, PBMCs were cultured in the presence of 1 µg ml⁻¹ phytohemagglutinin (PHA-P, Wellcome) for 2 days. The cells were centrifuged, and the cell pellets resuspended in sRPMI + 100 U ml⁻¹ rhuIL-2 (Cetus) and cultured for an additional 5 days, after which they were centrifuged again and the cell-free culture medium assayed for infectivity in CEMx174 cultures.

Explant cultures yielded florid cultures of macrophages that migrated from the tissue fragments. Supernatant fluids from these cultures were inoculated into cultures of CEMx174 cells to test for cytopathic virus and the indicator cells were also added to macrophage cultures to test for the development of fusion.

Tissue homogenates were prepared by dounce homogenization (10% wt vol[A-]1), clarified by centrifugation to remove cell debris and assayed for

virus infectivity in CEMx174 cells and for p27 levels.

A polymerase chain reaction infected cell assay (PCR-ICA) was used to determine the number of virus-infected cells in various cell suspensions. This assay was similar to a procedure described previously (Pozansky *et al*, 1991) with certain modifications. Cell suspensions were diluted to 1–2 × 10⁷, 1–2 × 10⁶, 1–2 × 10⁵, 1–2 × 10⁴, 1–2 × 10³, and 1–2 × 10² cells per ml. Cells in the various dilutions were then lysed and digested as described previously (Joag *et al*, 1994a). Two rounds of PCR amplification were used to detect SIV gag sequences. In the first round, SIV oligonucleotide primers used were 5'-GATGGGCGTGAGAACTCCGTCTT-3' and 5'-CCTCCTCTGCCGCTAGATGCTGCTGTTG-3' which are complementary to bases 1052 to 1075 and 1423 to 1450 of the SIV_{mac}239 gag gene respectively (Regier and Desrosiers, 1990). To standardize cell numbers, the fourth exon of β-actin was amplified with oligonucleotide primers 5'-TCATGTTTGACCTTCAACACCCCAG-5' and 5'-CCAGGAAGGAAGGCTGGAAGAGTGCC-5' (non-coding) complementary to the published sequence (Nakajima-Iijima *et al*, 1985). The PCR amplification was performed using the following conditions: denaturation at 92°C for 1 min; annealing at 55°C for 1 min; and primer extension at 72°C for 3 min. To increase the sensitivity of the reaction, 1 µl of the first PCR product was used as a template for a second amplification using the same conditions. The nested SIV primers used were 5'-GTTGAAGCATGTAGTATGGGCAGC-3' and 5'-GCCTCAGGGCAGCGGAACCGCTCA-3' which are complementary to bases 1142 to 1165 and 1356 to 1382 of SIV_{mac}239, respectively. The nested β-actin primers used were 5'-CCCCAGCCATGTACGTTGCTATCC-3' and 5'-GCCTCAGGGCAGCGGAACCGCTCA-3'. Samples were amplified for another 35 cycles as described above. Following the second round of amplification, a 10 µl aliquot was removed and run on a 1.5% agarose gel and bands visualized by staining with ethidium bromide. The results also were calculated as the number of infected cells 10⁻⁵.

PCR amplification of SIV gp120 sequences

Total cellular genomic DNA was extracted from frozen tissues and used as a template in the polymerase chain reaction (PCR) to amplify SIV gp120 sequences (Saiki *et al*, 1985; Saiki *et al*, 1988). Two rounds of amplification were used to generate a sufficient amount of product for cloning. The oligonucleotide primers used in the first round were ES12: 5'-GGCTAAGGCTAATACATCTTCTGCATC-3' and ES14: 5'-ACCCAAGAACCCTAGCACAAAGACCCC-3', which are complementary to bases 6565 to 6591 and 8179 and 8205 of SIV_{mac}239, respectively (Regier and Desrosiers, 1990). For the second round of amplification a nested set of primers were ES11: 5'-GTAAGTATGGGATGTCTTGGGAATCAG-3' and ES13: 5'-GACCCCTCTTTTATTCTTGAGGTGCC-3'

which are complementary to bases 6598 to 6624 and 8158 to 8184 of the SIV_{mac}239 genome, respectively (Regier and Desrosiers, 1990). One µg of genomic DNA was used in the PCR containing 4.0 mM MgCl₂, 200 µM each of the four dinucleotide triphosphates, 100 pM each oligonucleotide primer and 2.5 U of Taq polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). The template was denatured at 95°C for 3 min and PCR amplification performed with an automated DNA Thermal Cycler (Perkin-Elmer Cetus) for 35 cycles using the following profile: denaturation at 92°C for 1 min, annealing at 60°C for 1 min and primer extension at 72°C for 3 min. Amplification was completed by a final extension at 72°C for 10 min. In order to minimize the effects of variation in each PCR, several PCR amplifications were performed with each sample.

Molecular cloning and sequence analyses

Amplified SIV DNA fragments were gel purified and molecularly cloned into the Sma I site of the pGEM3Zf(-) vector using standard cloning techniques (Sambrook *et al*, 1989). Recombinant plasmids containing gp120 sequences were identified by the presence of a 1.6 kilobase insert and confirmed by hybridization with ³²P-labeled gp120 prepared by random primer labeling (Feinberg and Vogelstein, 1983). Recombinant plasmids containing gp120 sequences were sequenced by primer directed dideoxy chain termination method using Sequenase (United States Biochemicals, Cleveland, Ohio) and ³⁵S-dATP. Sequence analyses were performed using PCGENE sequence analysis software programs. For comparative purposes, the designation of the variable regions (V1–V5) of the SIV gp120 gene were based on results published previously (Kodama *et al*, 1993). The nucleotide sequences of the PCR-derived clones have been filed with GenBank under accession numbers U18019–U18051.

p27 assays

Levels of p27 core antigen in the brain tissue and

plasma of macaques inoculated with SIV_{mac} were determined using an antigen capture assay (Coulter Corp, Hialeah, Florida).

Assessment of immune status of animal

We used several criteria to assess the immune status of the inoculated animals including the determination of the number of CD4⁺ T lymphocytes in the PBMCs and the CD4/CD8. In addition, we used immunoprecipitation analyses (Stephens *et al*, 1992; Stephens *et al*, 1991) to determine whether infected animals produced antibodies against the gp120 of the virus. CV-1 cells grown to confluence in 35 mm dishes were inoculated with a recombinant vaccinia virus expressing the SIV_{mac}239/17E gp120 (vvSIV17Egp120) at a multiplicity of infection of 1. Four hours later, the virus inoculum was removed and infected cells incubated in EMEM without methionine and cysteine for 2 h. Cells were then radiolabeled with 200 µCi of ³⁵S-methionine and cysteine (Translabel, ICN Radiochemicals) for 18 h. The medium was removed and the cells lysed in 1X RIPA buffer (50 mM Tris-HCl, pH 7.5: 100 mM NaCl; 0.1% SDS, 0.5% Triton X-100, 10 mM EDTA). Lysates were clarified in a microcentrifuge and incubated with 20 µl of plasma from macaque 7F, macaque pHt, normal plasma (-control), or plasma from animals infected previously with SIV_{mac}239 for over 1 year (+control). Immunoprecipitates were collected on protein A Sepharose, washed four times with RIPA buffer and boiled in SDS-PAGE sample buffer. Proteins were analyzed by electrophoresis (Laemmli, 1970) in SDS-polyacrylamide gels (7.5%) and proteins visualized by standard autoradiographic techniques.

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