Correlation of Acute Humoral Response with Brain Virus Burden and Survival Time in Pig-Tailed Macaques Infected with the Neurovirulent Simian Immunodeficiency Virus SIVsmmFGB

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Infection of pig-tailed macaques with the simian immunodeficiency virus (SIV) isolate SIVsmmFGB frequently results in SIV encephalitis (SIVE) in addition to immunodeficiency and acquired immune deficiency syndrome. We used *in situ* hybridization to quantitate the number of SIV-infected cells in brain parenchyma, choroid plexus, and meninges from 17 macaques that developed acquired immune deficiency syndrome after infection with SIVsmmFGB. SIV-infected cells and histopathological lesions of SIVE were identified in 15 of 17 animals (88.2%), including 12 of 12 rapid progressors (RP) and 3 of 5 slow progressors (SP). The parenchymal virus burden was much greater in RP macaques than in the three SP macaques with SIVE (median values of 24.3 versus 0.3 infected cells/mm², respectively; $P < 0.05$). Viral load differences between RP and SP with SIVE were less marked in choroid plexus (29.6 versus 12.8 infected cells/mm², respectively) and meninges (133.0 versus 34.2 infected cells/mm², respectively). A significant negative correlation was observed between the magnitude of the anti-SIV antibody titer at 1 month after inoculation and brain virus burden at necropsy ($r = -0.614$; $P < 0.01$). The close association between immune response and SIVE in this model should prove useful for identifying correlates of immune protection against primate lentiviral encephalitis. (Am J Pathol 2004, 164:1157–1172)

HIV-1-associated dementia (HAD) is a frequent and devastating complication of HIV infection, affecting up to 25% of all untreated individuals.^{1–5} The advent of highly active anti-retroviral therapy has led to a reduction in the

prevalence of HAD and its pathophysiological correlate, HIV encephalitis (HIVE).^{6–9} Moreover, improvements in neuropsychological, cognitive, and psychomotor function in demented patients after the initiation of highly active anti-retroviral therapy suggest that treatment may be associated with resolution of neurological disease.^{10–12} Despite clinical evidence of anti-retroviral efficacy for HAD, the prevalence of encephalitic and neurodegenerative lesions reported from postmortem studies of AIDS patients that received highly active anti-retroviral therapy remains quite high.^{13–16} Furthermore, recent reports suggest a resurgence in the frequency of HIVE in response to the increased prevalence of anti-retroviral-resistant HIV strains, as well as the emergence of new variants of HIVE, such as severe leukoencephalopathy.^{17,18}

The central nervous system (CNS) is an important anatomical reservoir for HIV.^{19,20} Primate lentiviruses invade the CNS within the first days of infection,^{21–23} and studies using simian immunodeficiency virus (SIV)-infected macaques have shown that a proviral reservoir is established in the parenchyma of the brain during acute infection and maintained throughout the disease course, despite postacute suppression of active virus replication in the brain.^{24,25} In addition to inciting the cascade of pathogenic processes that culminate in neurological impairment, virus sequestered in brain tissue could pose a serious challenge to the therapeutic eradication of HIV from infected individuals, because many anti-retroviral agents exhibit poor penetration across the blood-brain barrier and therapeutic levels of these drugs are often not achieved in the extracellular space of the brain.^{19,20,26} A better understanding of the kinetics of target cell turnover

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in the CNS and identification of latently infected resident cell populations in the brain would expedite the development of treatment strategies to prevent the neurological consequences of HIV infection.

Infection of macaques with SIV is a useful animal model for investigating the neuropathogenesis of primate lentiviral infections.²⁷⁻²⁹ However, the incidence of SIVE in macaques infected with conventional isolates and clones of SIV is low, ranging from 18 to 32%,^{30,31} which has limited the utility of this model for studying the effect of anti-retroviral agents on CNS virus burden. We have isolated a macrophage tropic SIV (SIVsmmFGB) from a sooty mangabey monkey that is consistently and profoundly neurovirulent in pig-tailed macaques.³² Here, we describe the anatomical distribution of productively infected cells within the brain parenchyma of 17 pig-tailed macaques infected with the PGM isolate of SIVsmmFGB, and correlate CNS virus burden with plasma viral load and systemic antibody titers during infection. Neurovirulent infection was observed in 15 of 17 macaques, with lesions typical of lentiviral encephalitis. The SIV-binding antibody titer was predictive of brain virus burden and rate of disease progression by 1 month after inoculation, providing a strategy for identifying macaques likely to develop SIVE.

Materials and Methods

Animals and Virus

This retrospective study used tissues collected from 17 pig-tailed macaques (*Macaca nemestrina*). This cohort included all animals infected intravenously with the PGM/mln isolate of SIVsmmFGB and euthanized because of the development of simian acquired immune deficiency syndrome (SAIDS) without receiving anti-retroviral therapy. All macaques were SIV-, STLV-, and SRV-negative before inoculation and housed at the Yerkes National Primate Research Center, in accordance with Animal Welfare Act guidelines. The PGM/mln isolate was obtained from the mesenteric lymph node of a pig-tailed macaque (PGM) that developed SIVE and SAIDS 4 months after a transfusion with whole blood from a SIV-positive sooty mangabey monkey (FGB), as previously described.³² Virus was isolated by co-culture of lymph node cells with PHA-stimulated human peripheral blood mononuclear cells. Cell-free virus stocks were prepared by collecting expanded culture supernatants at peak reverse transcriptase activity and centrifuging to clarify cell debris. The infectious titer of the inoculum, 2.5×10^3 TCID₅₀/ml, was determined by limiting dilution in CEM $\times$ 174 cells. Macaques were inoculated with infectious doses of virus ranging from 10 through 10,000 TCID₅₀.

Necropsy, Tissue Collection, and Histopathological Evaluation

All animals were euthanized because of the development of SAIDS or severe SIV-related diseases. Complete necropsies were performed, collecting and fixing tissues in

10% neutral buffered formalin before routine processing and embedding in paraffin. Hematoxylin and eosin (H&E)-stained sections of all major organ systems were examined for histopathological lesions. Brain sections from each of the SIVsmmFGB-infected macaques were evaluated independently by two pathologists (DCA, SPO) and scored for the presence of multinucleated giant cells, microglial nodules, and perivascular cuffs of inflammatory cells. Twelve regions of the brain were examined: midfrontal cerebral cortex (gray and white matter), cerebellum (gray and white matter), caudate nucleus, putamen, thalamus, hippocampus, midbrain, medulla, meninges, and choroid plexus.

Lymphocyte Subset Analysis

Specimens of whole blood containing ethylenediaminetetraacetic acid (EDTA) anti-coagulant were obtained from each macaque before inoculation and at necropsy and a complete blood count with differential and lymphocyte phenotype analysis was performed. Percentages of CD3⁺/CD4⁺ and CD3⁺/CD8⁺ T lymphocytes were measured using two- and three-color flow cytometry and gating on the lymphocyte population. The fluorochrome-conjugated monoclonal antibodies used recognize the CD3 (clone SP34; BD Pharmingen, San Diego, CA), CD4, and CD8 (clones SK3 and SK1, respectively; BD Immunocytometry Systems, San Jose, CA) T lymphocyte surface antigens. Isotype-matched irrelevant antibodies were used as controls.

Anti-SIV Serology

SIV-binding antibody responses were measured in plasma specimens collected from each animal at 1 and 2 months after inoculation and at necropsy. The relative magnitude of the humoral response was determined using a commercial enzyme immunoassay (HIV-2 EIA; Genetic Systems, Redmond, WA). Anti-SIV reactivity was measured in twofold serial dilutions of plasma, beginning with a 1:400 dilution and titrating specimens to endpoint.

Plasma Virus Load

Virion-associated RNA was measured in plasma specimens from each animal at 1 and 2 months after inoculation and at necropsy, using the branched chain DNA signal amplification (bDNA) assay, as previously described.³³ All bDNA assays were performed by the Bayer Reference Testing Laboratory, in Emeryville, CA.

In Situ Hybridization

Productively infected cells were localized in formalin-fixed, paraffin-embedded sections of brain by *in situ* hybridization for SIV RNA, using methods described in detail elsewhere.³² Tissue sections were deparaffinized in xylene and rehydrated in graded ethanol to diethyl pyrocarbonate (Sigma Chemical Co., St. Louis, MO)-treated

water. Endogenous alkaline-phosphatase activity was blocked in 5 mmol/L levamisole (Sigma). Tissue sections were hydrolyzed in 0.2 N HCl (Sigma), digested with proteinase K (Roche Diagnostics Corp., Indianapolis, IN), acetylated in acetic anhydride (Sigma), and hybridized overnight at 50°C with a digoxigenin-labeled anti-sense riboprobe that spans the entire genome of the SIVsmmPGm5.3 molecular clone of SIVsmmFGb (Lofstrand Laboratories, Gaithersburg, MD). After hybridization, sections were washed extensively and bound probe was detected by immunohistochemistry (IHC), using alkaline phosphatase-conjugated sheep anti-digoxigenin F(ab) fragments (Roche) and the chromogen nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP, Roche). Slides were then washed in 10 mmol/L Tris-HCl containing 10 mmol/L EDTA, and counterstained with nuclear fast red (Vector Laboratories, Burlingame, CA). Sections of brain from a pig-tailed macaque with SIVsmmFGb encephalitis served as both positive control (hybridized with SIV anti-sense probe) and negative control (reacted with SIV sense probe). Additional negative controls included sections of brain from uninfected macaques reacted with SIV anti-sense probe.

IHC

Macrophages were localized in the white matter tracts of the midfrontal cortex by IHC, using anti-CD68 monoclonal antibody KP1 (DAKO Corp., Carpinteria, CA) and a commercial kit (LSAB plus, DAKO Corp.). Formalin-fixed, paraffin-embedded sections of midfrontal cerebral cortex from the 17 SIV-infected animals and anatomically matched sections from three uninfected controls were deparaffinized in xylene, and rehydrated through graded ethanol to distilled water (dH₂O). Antigen retrieval was accomplished by heating sections at 95°C for 20 minutes in citrate buffer (DAKO Corp.), and endogenous peroxidase activity was blocked by incubation in 3% H₂O₂ in dH₂O. Sections were first incubated for 45 minutes at room temperature with KP1 antibody, and then reacted sequentially with biotinylated secondary antibody and horseradish peroxidase-conjugated streptavidin. Antigen-antibody complex formation was localized by development in the chromogenic substrate 3, 3'-diaminobenzidine (DAB, DAKO Corp.). Tissue sections were counterstained in Gill's hematoxylin (Sigma), cleared, and coverslipped with permanent mounting medium (Cytoseal XYL; Richard-Allan Scientific, Kalamazoo, MI).

Computer Image Quantitation

SIV-infected cells and macrophages were enumerated in sections of brain from each SIV-infected macaque by computer image analysis as described elsewhere.³⁴ Images of tissue sections were captured without manipulation using a Dage DC 330 3-CCD color video camera (Dage-MTI Inc., Michigan City, IN) mounted on a Zeiss Axioskop II microscope (Carl Zeiss Inc., Oberkochen, Germany) and analyzed using NIH Scion Image 1.62C software.

Quantitation of Tissue Virus Burden

The relative cell-associated virus load was measured in the meninges, choroid plexus, and five regions of brain parenchyma (midfrontal cortical gray and white matter, caudate nucleus, putamen, and hippocampus) from each infected macaque. For each anatomical compartment, virus burden was measured by computer image analysis, counting the number of chromogen-positive cells per unit area (mm²) on tissue sections subjected to a carefully controlled *in situ* hybridization assay for SIV RNA. Particle size and threshold parameters were established on control sections of brain from infected and uninfected macaques that had been incubated with SIVsmmFGb anti-sense probe and processed in parallel with experimental sections. The mean virus burden for each anatomical compartment of brain (regional mean) was determined for each SIV-infected macaque by dividing the number of chromogen-positive cells in 10 contiguous fields at ×100 magnification by the total area analyzed (reported as SIV-infected cells per mm²). Global CNS means were calculated for each macaque as the average of the five parenchymal regional means.

Quantitation of Macrophage Population Density

The relative population density of macrophages was determined in midfrontal cortical white matter by computer image analysis. Threshold and particle gating were established using SIV-infected and uninfected control sections of brain, as described above. The number of chromogen-positive cells per mm² was measured at ×200 magnification from 10 contiguous fields of brain parenchyma for each of the 17 SIV-infected macaques and 3 uninfected controls, and the mean and SD were calculated.

Determination of the Phenotype of Infected Cells

A dual *in situ* hybridization/IHC assay (*in situ* hybridization for SIV RNA, IHC for cell phenotype) was used to identify the phenotype of productively infected cells in the CNS. *In situ* hybridization was performed as described above, using NBT/BCIP as the chromogen. After chromogen development and washing, sections were blocked in 10% normal goat serum and the primary antibody was applied for 1 hour at room temperature. The antibodies and antisera used included HAM-56 (DAKO) to label macrophages, rabbit anti-human CD3 (DAKO) to label T lymphocytes, rabbit anti-cow GFAP (DAKO) to label astrocytes, rabbit anti-human vonWillebrand factor (DAKO) to label endothelial cells, and anti-NF200 (Sigma) to label neurons. After washing, sections were incubated in appropriate secondary antibodies conjugated to 5 nm gold particles (Accurate Chemical and Scientific Corp., Westbury, NY). Gold particles were complexed with silver to enhance resolution (Silver Enhancing kit; ICN Pharmaceuticals, Inc., Costa Mesa, CA). Counterstaining was omitted to facilitate visualization of the end products.

Table 1. Baseline Characteristics of the Cohort *versus* Rate of Disease Progression*

Group	Animal	Survival (weeks)	Sex	Virus dose (TCID ₅₀)	Age (months)	Weight (kg)	CD4+ T cells		CD8+ T cells		CD4:CD8 ratio
							%	(cells/ μ l)	%	(cells/ μ l)	
Rapid progressors <30 weeks survival (<i>n</i> = 12)	12058	12	M	10,000	81	11.8	31.0	1,170	48.0	1,800	0.65
	PEs	14	F	10,000	95	7.7	37.0	700	37.0	700	1.00
	PBt	22	F	10,000	107	6.9	47.0	1,100	36.0	840	1.31
	PUf1	11	F	10,000	21	3.2	30.0	1,392	40.0	1,856	0.75
	PSc1	23	F	10,000	34	5.0	33.0	1,964	46.0	2,737	0.72
	PBf1	13	M	10,000	40	4.6	29.0	1,176	51.0	2,069	0.57
	PZg1	14	M	1,000	22	3.1	32.0	2,195	31.0	2,127	1.03
	PMil	26	M	1,000	19	2.5	21.0	1,085	36.0	1,860	0.58
	PNfl	12	M	100	38	5.0	28.0	2,119	34.0	2,573	0.82
	PKe1	13	M	100	43	6.0	35.0	1,971	42.0	2,365	0.83
	PDf1	20	M	50	64	8.7	38.0	851	39.0	874	0.97
	PEj1	16	M	10	26	3.5	41.0	1,093	20.0	533	2.05
	Median	14.0		5,500	39.0	5.0	32.5	1,173	38.0	1,858	0.83
sIQR		3.9		4,950	21.6	1.8	3.8	437	3.8	661	0.15
Slow progressors >30 weeks survival (<i>n</i> = 5)	PJf1	65	M	10,000	37	4.1	15.0	1,173	54.0	4,223	0.28
	POf1	161	M	10,000	36	4.6	31.0	1,426	44.0	2,024	0.70
	PKg1	63	M	1,000	25	3.7	26.0	1,573	44.0	2,662	0.59
	PJd1	89	M	100	46	4.9	27.0	2,189	50.0	4,054	0.54
	PTf1	55	M	50	60	9.1	28.0	1,004	34.0	1,219	0.82
Median		65.0		1,000	37.0	4.6	27.0	1,426	44.0	2,662	0.59
sIQR		13.0		4,950	5.0	0.4	1.0	200	3.0	1,015	0.08
Significance, <i>P</i> <		0.002		0.792	0.916	0.874	0.020	0.598	0.155	0.092	0.040

*Survival, in weeks after inoculation; age, weight, and lymphocyte subset data before inoculation; sIQR, semi-interquartile range.

Statistical Analysis

The Mann-Whitney *U*-test was used to compare data sets between rapid (*n* = 12) and slow (*n* = 5) progressor macaques. The statistical significance of differences between baseline and necropsy CD4 and CD8 counts within progression groups was determined by the paired *t*-test, and the relationships between survival time and baseline parameters and between numbers of CD68⁺ cells and viral load in the brain were examined by simple least-squares linear regression. Significant differences were assumed for probability values of *P* ≤ 0.05. Unless otherwise specified, group data are reported as median values ± semi-interquartile range.

Results

Rate of Disease Progression

The study cohort was composed of 13 male and 4 female macaques that ranged from 19 to 107 months of age (median, 38.0 months), and from 2.5 to 11.8 kg (median, 4.9 kg) at inoculation (Table 1). All infected macaques developed AIDS; survival times ranged from 11 through 161 weeks after inoculation, with a median survival of 20.0 weeks after inoculation. There was no correlation between survival time and any of the baseline parameters, including virus dose, age, sex, or weight of macaques, the number or percentage of CD4⁺ or CD8⁺ T lymphocytes, or the CD4:CD8 ratio.

Macaques could be discretely segregated into rapid progressors (RP, *n* = 12), those surviving less than 30 weeks after inoculation (median, 14 weeks), and slow progressors (SP, *n* = 5), those surviving longer than 30

weeks after inoculation (median, 65 weeks) (Table 1). There was no difference in age or body weight of RP *versus* SP; however, the percentage of CD4⁺ T lymphocytes at baseline was significantly higher for RP as opposed to SP (32.5 *versus* 27.0%, *P* < 0.02). There was also a trend toward lower numbers and percentages of baseline CD8⁺ T cells in RP, which cumulatively resulted in a significantly higher baseline CD4:CD8 ratio for RP as opposed to SP macaques (0.83 *versus* 0.59, respectively, *P* < 0.04).

Necropsy Findings

All macaques experienced marked, progressive decreases in numbers and percentages of CD4⁺ T cells during the course of infection. Median values for number and percent CD4⁺ T cells for all macaques at necropsy were 230 cells/ μ l and 20.0%, respectively, as compared with baseline values of 1176 cells/ μ l and 31.0% (*P* < 0.002). Both RP and SP groups showed statistically significant decreases (*P* < 0.015) from baseline for percent and absolute CD4⁺ T cells (Figure 1, A and B). RP had significantly lower numbers (Figure 1D) and percentages (Figure 1C) of CD8⁺ T cells at necropsy (median, 519 cells/ μ l; 46.5%) than SP (median, 1240 cells/ μ l; 67.0%; *P* < 0.05).

The plasma virus burden was significantly greater in RP as opposed to SP macaques (median values of 8.11 *versus* 5.68 log₁₀ SIV RNA copies/ml, respectively; *P* < 0.005) (Figure 2A and Table 2). A strong inverse correlation was observed between the plasma virus burden and the magnitude of the anti-viral humoral immune response at necropsy for all animals (*R* = − 0.90, *P* < 0.01). Further evidence of this inverse relationship was

Figure 1. Group median values for percentages and absolute numbers of CD3⁺/CD4⁺ and CD3⁺/CD8⁺ T lymphocytes in RP ($n = 12$) versus SP ($n = 5$) pig-tailed macaques before inoculation (baseline) with SIVmmFGB and at necropsy after the development of AIDS. In each panel, columns depict the group median value and error bars show the semi-interquartile range for the group. Percentages (A) and absolute numbers, in T cells per μ l (B), of CD3⁺/CD4⁺ T lymphocytes in RP and SP macaques. Percentages (C) and absolute numbers, in T cells per μ l (D), of CD3⁺/CD8⁺ T lymphocytes in RP and SP macaques.

apparent when anti-SIV antibody responses were compared between progression groups, as SP macaques had much stronger anti-SIV antibody responses at necropsy than RP animals (5.6 versus 2.3 log₁₀ titer⁻¹, respectively; $P < 0.002$) (Figure 2B and Table 2).

All animals developed AIDS or lethal SIV-related disorders (Table 2). Surprisingly, 11 of 12 RP macaques (91.7%) had opportunistic infections despite relatively high terminal CD4⁺ T-cell counts (median value of 366 cells/ μ l) and a median survival time of only 14 weeks after inoculation. Opportunistic infections were diagnosed in all SP macaques at necropsy. In addition to opportunistic infections, other SIV-specific (eg, giant cell inflammation of the lungs, intestine, and colon) or SIV-related (eg, wasting, thrombocytopenia) disorders were observed at necropsy of both RP and SP macaques.

Neuropathological Lesions

Neuropathological lesions reminiscent of those described previously for both HIVE and SIVE^{27,35-37} were observed in all 12 RP macaques and in 3 of 5 SP macaques (Table 2). Lesions in the brain parenchyma included perivascular accumulations of macrophages, multinucleated giant cells, and lymphocytes (Figure 3A), parenchymal giant cells (Figure 3B), microglial nodules (Figure 3C), parenchymal granulomas (Figure 3D), and vacuolation of the white matter tracts of the cerebrum and cerebellum.

Variable degrees of choriomeningitis were also observed in all macaques with SIVE. The meninges, choroid plexus, and tela choroidea of encephalitic macaques typically contained multinucleated giant cells and perivascular mononuclear cell infiltrates (Figure 3, E and F). Mild choriomeningitis was observed in one of two SP

Figure 2. Plasma viral load (A) and anti-SIV-binding antibody response (B) at necropsy of RP ($n = 12$) and SP ($n = 5$) pig-tailed macaques infected with SIVmmFGB. **A:** Scatter plot showing the range of values for plasma viral load at necropsy, reported as log₁₀ SIV RNA copies/ml, for RP (●) and SP (○) groups. **B:** Scatter plot showing the range of anti-viral antibody titers at necropsy, reported as the reciprocal log of the last positive serum dilution, for RP (◆) and SP (◇) groups. Horizontal bars indicate the group median value in each plot (A, B).

Table 2. Necropsy Results

Group	Animal	Anti-SIV antibodies*	Plasma viral load†	SIVE‡	Histopathologic/etiologic diagnoses§
Rapid progressors	PBf1	2.3	8.54	+	Cryptosporidial enteritis; CMV pneumonitis; SIV giant cell enteritis
	PNf1	2.3	8.37	+	CMV pneumonitis; SIV giant cell enterocolitis and pneumonia; adenoviral enteritis
	PEj1	2.3	7.84	+	Cryptosporidial enteritis
	PZg1	2.3	8.64	+	CMV pneumonitis and myelitis; cryptosporidial enteritis; SIV giant cell pneumonia; adenoviral enteritis
	PUf1	2.3	8.36	+	Cryptosporidial enteritis; SIV giant cell gastroenteritis and pneumonia; CMV pneumonitis
	12058	2.3	8.15	+	CMV enteritis; SIV giant cell pneumonia
	PKe1	2.3	8.13	+	CMV pneumonitis
	PMil	3.2	7.35	+	Cryptosporidial enteritis; oro-esophageal candidiasis; SIV giant cell enterocolitis and pneumonia; pneumocystis pneumonia
	PBt	3.5	7.34	+	Pneumocystis pneumonia; SIV giant cell enterocolitis
	PDF1	2.9	7.62	+	Cryptosporidial enteritis; CMV enteritis; oro-esophageal candidiasis
	PEs	2.3	8.08	+	SIV giant cell enterocolitis; granulomatous hepatitis
	PSc1	2.3	7.14	+	Cryptosporidial enteritis; mycobacteriosis; SIV giant cell colitis
	RP median	2.3	8.11		
	sIQR	0.1	0.40		
Slow progressors	PJf1	5.9	5.68	+	Mycobacteriosis; CMV myelitis
	POf1	5.6	5.66	+	SIV giant cell gastroenterocolitis; cryptosporidial pharyngitis
	PJd1	5.3	7.46	+	CMV vasculitis and colitis; SIV giant cell enterocolitis; thrombocytopenia
	PKg1	5.6	6.00	–	Mycobacterial colitis; thrombocytopenia
	PTf1	5.9	5.29	–	Cryptosporidial enteritis; SIV giant cell colitis; granulomatous hepatitis
	SP median	5.6	5.68		
	sIQR	0.2	0.17		
<i>P</i> <		0.002	0.005		

*Humoral immune responses to whole, disrupted SIV, as measured by ELISA performed on plasma specimens collected at necropsy. Results are reported as the reciprocal log of the last positive plasma dilution. Specimens that were unreactive at 1:400 dilution were arbitrarily assigned a value of 2.3 log₁₀ (i.e. 1:200 titer).

†Plasma viral load, as measured by bDNA assay (Bayer Reference Testing Laboratory, Emeryville, CA) performed on plasma specimens collected at necropsy. Results are reported as log₁₀ RNA copies/ml.

‡SIVE, presence (+) or absence (–) of SIV encephalitis, as determined by histopathological examination.

§Excludes the brain.

macaques without SIVE (PTf1), whereas small perivascular cuffs of inflammatory cells were present within the choroid plexus of the remaining SP macaque without SIVE (PKg1). Opportunistic agents were not found in the brains of any of the animals in the study; however, CMV myelitis was diagnosed in one RP (PZg1) and one SP (PJf1) macaque at necropsy.

The anatomical distribution of neuropathological lesions was mapped by scoring 12 morphological compartments of the brain for the presence of multinucleated giant cells, microglial nodules, and perivascular cuffs of inflammatory cells (summarized in Table 3; complete results available in Supplemental Table 1 at <http://ajp.amjpathol.org>). Lesions were diffusely distributed throughout the brains of RP macaques, occurring in 134 of the 143 anatomical compartments evaluated (93.7%). Among RP, lesions were most frequent in the meninges (23 of 24; 95.8%), followed by midbrain (34 of 36; 94.4%), choroid plexus (21 of 24; 87.5%), and medulla (30 of 36; 83.3%). Brain lesions were far less abundant and not as

diffusely distributed (15 of 36 compartments; 41.7%) in the three SP macaques with SIVE (SP/SIVE). Among SP macaques, inflammatory lesions were most commonly observed in the choroid plexus and meninges (five of five and four of five macaques, respectively); parenchymal lesions were most often observed in the cerebral white matter and putamen (three of five and two of five animals, respectively).

To compare the severity of SIVE among infected monkeys, brain sections were scored for the presence of three histopathological lesions characteristic of retroviral encephalitis (multinucleated giant cells, microglial nodules, and perivascular cuffs of inflammatory cells). The cumulative frequency of these lesions within the anatomical compartments of brain tissue examined for each macaque is reported as the SIVE index (summarized in Supplemental Table 1 at <http://ajp.amjpathol.org>). The SIVE index ranged from 0.35 to 1.00 among RP macaques, from 0.26 to 0.32 for SP/SIVE, and was less than 0.10 for SP macaques without SIVE.

Figure 3. Neuropathological lesions in pig-tailed macaques infected with SIVmmFgB. **A:** *In situ* hybridization for SIV RNA combined with H&E counterstain (*in situ* hybridization-SIV/H&E), demonstrating several SIV-infected cells (dark blue) within a perivascular cuff in the midfrontal cortex of macaque PM11. **Arrows** identify two SIV-positive multinucleated giant cells. **B:** H&E-stained section showing a multinucleated giant cell in the globus pallidus of macaque PDF1. **Inset a:** Multinucleated giant cells are macrophage phenotype, confirmed here by IHC for CD68 (IHC-CD68, brown), and are universally infected with SIV, as shown by *in situ* hybridization for viral RNA in **inset b** (ISH-SIV, dark blue). **C:** H&E-stained section of midbrain from macaque PEJ1, showing a microglial nodule (MGN, **arrow**) within the trochlear nucleus. **Inset a:** MGNs are focal, noncircumscribed lesions composed of heterogeneous populations of macrophage-lineage cells, as demonstrated on this serial section by IHC for CD68 (IHC-CD68, brown). **Inset b:** Many of the cells within MGNs are infected with SIV, as shown by *in situ* hybridization for SIV RNA on this serial section (ISH-SIV, dark blue). **D:** H&E-stained section showing a granuloma within the midfrontal cortical gray matter of macaque PSc1. In contrast to MGNs, granulomas are well circumscribed and are composed of relatively homogeneous populations of large, CD68-positive, epithelioid macrophages with abundant foamy cytoplasm, as shown in **inset a** (IHC-CD68, brown). Most of the macrophages within granulomas are SIV-positive, as shown by *in situ* hybridization for SIV RNA in **inset b** (ISH-SIV, dark blue). **E:** Choriomeningitis was observed in all SIVmmFgB-infected macaques with SIVE. This H&E-stained section of prefrontal cortex from SP macaque POF1 shows a cellular infiltrate in the leptomeninges that includes numerous multinucleated giant cells. **F:** *In situ* hybridization for SIV RNA in a section of choroid plexus from macaque PEJ1. SIV-infected cells (dark blue) can be seen within the stroma of the choroid plexus and tela choroidea. Scale bars: 50 μ m (**A**, **B**, **D**, **E**, and insets in **B** and **D**); 100 μ m (**C**, **F**, and insets in **C**).

Table 3. Histopathologic Lesions and Virus Distribution in the Brains of SIVsmmFGB-Infected Macaques

Anatomic compartment	RP (n = 12)		SP (n = 5)	
	Lesions*	SIV+†	Lesions	SIV+
Midfrontal cortex				
Gray	29/36	12/12	2/15	0/5
White	28/36	12/12	5/15	1/5
Cerebellum				
Gray	25/36	12/12	0/15	0/5
White	25/36	12/12	3/15	2/5
Caudate nucleus	24/36	12/12	0/15	1/5
Putamen	26/36	12/12	5/15	2/5
Thalamus	27/33	11/11	0/15	0/5
Hippocampus	27/36	11/11	0/15	0/5
Midbrain	34/36	10/10	1/15	1/5
Medulla	30/36	12/12	1/15	1/5
Meninges	23/24	12/12	7/10	4/5
Choroid plexus	21/24	12/12	8/10	3/5

*Lesions, cumulative sum of the fractions of: (compartments containing multinucleated giant cells) + (compartments containing microglial nodules) + (compartments containing perivascular cuffs).

†SIV+, fraction of compartments containing productively infected cells, as determined by *in situ* hybridization for SIV RNA.

Virus Distribution within the Brain

We used *in situ* hybridization to localize viral RNA within productively infected cells in brain sections of infected macaques (summarized in Table 3; complete results available in Supplemental Table 1 at <http://ajp.amjpathol.org>). We found excellent correlation between the presence of neuropathological lesions and viral RNA, in agreement with other studies of SIV neuropathogenesis.^{38,39} SIV-infected cells were found within the leptomeninges, within the stroma of the choroid plexus and tela choroidea, and within every parenchymal CNS compartment evaluated for RP macaques (140 of 140 sites; 100%), including nine sites in which histopathological lesions were not observed. For all RP macaques, *in situ* hybridization revealed large numbers of multinucleated giant cells, macrophages, and

microglial nodules that were not readily apparent on serial sections stained with H&E.

In contrast, SIV-infected cells were localized in only 14 of 36 brain compartments from SP/SIVE (38.9%) and in only 1 of 24 sites (PTf1, meninges) examined from the two SP macaques without SIVE (4.2%). In SP macaques, infected cells were most frequently observed within the meninges (four of five) and choroid plexus (three of five), whereas the most common parenchymal sites of productive infection were the putamen and cerebellar white matter (two of five animals each). Mild neuropathological lesions were observed in the absence of detectable SIV RNA in 6 of 60 brain compartments evaluated from SP macaques.

Two patterns of virus distribution were observed in the brain parenchyma of macaques with SIVE. In animals with low parenchymal virus burden, the majority of productively infected cells were macrophages and giant cells that were localized in a multifocal, predominantly perivascular pattern (Figure 4A), although infected cells were also observed in rare parenchymal granulomas and microglial nodules that did not have an obvious angio-centric orientation. In contrast, a combined perivascular and diffuse pattern of virus distribution was typical of macaques with high CNS virus burden. In addition to perivascular accumulations of SIV-positive macrophages and giant cells, productively infected cells occurred diffusely throughout the neuropil in these animals (Figure 4B).

Brain Virus Burden

The relative cell-associated virus burden was measured in the meninges, choroid plexus, and five regions of brain parenchyma (midfrontal cortical gray and white matter, caudate nucleus, putamen, and hippocampus) by semi-quantitative *in situ* hybridization (sQISH). The mean virus burden in each anatomical compartment of brain (regional mean) is shown for each animal (Table 4). With one

Figure 4. *In situ* hybridization for SIV RNA, showing the distribution pattern of productively infected cells (dark blue) in the frontal cortex of two SIVsmmFGB-infected RP pig-tailed macaques. SIV-positive cells occur primarily in perivascular locations in SP macaques and RP macaques with low brain virus burdens (A: macaque PBf1). In contrast, infected cells were distributed diffusely throughout the parenchyma as well as around vessels in the brains of RP macaques with high brain virus burdens (B: macaque PDF1). Arrows identify blood vessels. Scale bars, 100 μ m.

Table 4. Virus Burden in CNS Tissues of SIVsmmFGB-Infected Pig-Tailed Macaques

Group	Animal	Midfrontal cortex*		Caudate nucleus	Putamen	Hippocampus	Global mean†	Choroid plexus	Meninges
		Gray matter	White matter						
Rapid progressors	PBf1	10.7	6.5	3.4	2.1	9.7	6.5	19.6	103.1
	PNf1	38.1	9.4	15.7	17.7	22.1	20.6	39.3	148.5
	PEj1	44.4	29.5	8.6	21.6	40.4	28.9	42.9	207.3
	PZg1	73.2	66.5	32.7	33.9	100.1	61.3	ND	191.4
	PUf1	47.3	69.4	41.2	ND	33.7	47.9	41.6	194.9
	12058	52.9	18.2	15.5	17.1	22.7	25.3	33.0	78.2
	PKe1	6.6	11.1	0.7	0.5	22.6	8.3	14.4	11.8
	PMi1	59.3	23.1	24.1	23.6	23.8	30.8	6.0	185.7
	PBt	0.5	1.0	0.0	0.0	ND	0.4	ND	30.4
	PDF1	34.5	85.7	53.5	41.6	50.0	53.1	46.9	251.3
	PEs	27.0	15.2	14.7	12.7	15.4	17.0	26.1	117.5
	PSc1	23.3	42.3	18.4	6.5	26.1	23.3	5.0	75.7
	Median	36.3	20.7	15.6	17.1	23.8	24.3	29.6	133.0
	sIQR	14.3	18.8	9.5	9.2	7.4	10.1	12.7	57.4
Slow progressors	PJf1	0.4	0.1	0.4	2.7	0.0	0.7	15.7	70.5
	POf1	0.0	0.0	0.1	1.3	0.0	0.3	12.8	34.2
	PJd1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	PKg1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	PTf1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Median	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	sIQR	0.0	0.0	0.1	0.7	0.0	0.2	6.4	17.1
Mann-Whitney test		<i>P</i> <	0.002	0.002	0.006	0.018	0.002	0.003	0.005

*Virus burden, as determined by *in situ* hybridization for SIV RNA, and reported as SIV infected cells per mm². Individual values in *bold italics* identify the compartment of CNS parenchyma with highest virus load for each animal.

†Global mean, average value for all anatomic compartments measured in brain parenchyma for each animal.

ND, not done; sIQR, semi-interquartile range.

exception (PKe1), greater numbers of infected cells were observed per unit area of meninges than for any of the parenchymal compartments examined among RP macaques. Similarly, among SP/SIVE, higher viral burdens were observed in the meninges and choroid plexus than in the brain parenchyma. The compartments of brain parenchyma with the highest virus burdens in RP macaques were the cerebral cortical gray matter, cerebral cortical white matter, and hippocampus (6, 4, and 2 of 12 animals, respectively); whereas the putamen contained

the highest virus burden among parenchymal sites in two of three SP/SIVE.

The median and semi-interquartile range for each brain compartment is shown for RP versus SP macaques (Table 4). Small numbers of SIV-infected cells were found in the cerebral and cerebellar white matter of SP macaque PJd1 (Supplemental Table 1); however, infected cells were not observed in the sections used for virus quantitation, resulting in median values of 0.0 infected cells/mm² for all brain compartments in SP macaques.

Table 5. Correlation of Systemic Virologic and Immunologic Measurements with Simian AIDS Progression and Markers of SIV Encephalitis

Disease correlate	1 month after inoculation		2 months after inoculation		Necropsy	
	<i>r</i> *	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Plasma viral RNA <i>versus</i> :						
SAIDS progression: survival time	-0.22	NS†	-0.58	<0.05	-0.72	<0.01
SIV encephalitis:						
CNS global mean viral load	0.17	NS	0.52	<0.05	0.58	<0.05
Choroid plexus viral load	0.44	NS	0.59	<0.05	0.60	<0.05
Meninges viral load	0.32	NS	0.58	<0.05	0.54	<0.05
SIVE index	0.10	NS	0.70	<0.01	0.71	<0.01
Anti-SIV antibody titer <i>versus</i> :						
SAIDS progression: survival time	0.68	<0.01	0.82	<0.01	0.81	<0.01
SIV encephalitis:						
CNS global mean viral load	-0.61	<0.01	-0.62	<0.01	-0.63	<0.01
Choroid plexus viral load	-0.76	<0.01	-0.68	<0.01	-0.63	<0.05
Meninges viral load	-0.62	<0.01	-0.58	<0.05	-0.61	<0.05
SIVE index	-0.77	<0.01	-0.77	<0.01	-0.83	<0.01

**r*, correlation coefficient. Negative values denote inverse correlations.

†NS, not significant.

The median virus burden for each brain compartment evaluated, including meninges and choroid plexus, was significantly greater in RP as opposed to SP macaques ($P < 0.02$). When the comparison to RP macaques was limited to SP/SIVE, significant differences were still apparent for all compartments except choroid plexus ($P < 0.09$) and putamen ($P < 0.09$) (data not shown).

The average of all parenchymal compartments for each animal is reported in Table 4 as the global mean. Regional means for meninges and choroid plexus virus burden were excluded from calculations of global mean because of the differences in morphology and vascularity of these tissues as compared with that of the brain parenchyma. Global means were significantly greater for RP as compared to SP macaques (24.3 ± 10.1 versus 0.0 ± 0.2 infected cells/mm², respectively; $P < 0.005$) and SP/SIVE (0.3 ± 0.2 infected cells/mm², $P < 0.02$). There was a strong correlation between the CNS virus burden, measured as the global mean, and the severity of SIVE, as measured by the SIVE index ($r = 0.75$, $P < 0.01$). Global virus burden and severity of SIVE were inversely correlated with survival time ($r = -0.52$ and -0.60 , respectively; $P < 0.05$).

Immunological and Virological Correlates of Encephalitis

We compared survival time, a marker of SIV disease progression, with plasma virus loads at 1 and 2 months after inoculation and at necropsy (Table 5). As expected, there was a strong inverse correlation between terminal plasma virus burden and survival time ($P < 0.01$). Plasma virus load at 2 months after inoculation was also inversely

correlated with survival time ($P < 0.05$); however, there was no association between plasma virus load at 1 month after inoculation and survival. In contrast, we observed a much stronger association between survival time and the anti-SIV antibody titer, with significant correlations detectable as early as 1 month after inoculation ($r = 0.68$, $P < 0.01$) and persisting at 2 months after inoculation ($r = 0.82$, $P < 0.01$) and at necropsy ($r = 0.81$, $P < 0.01$).

To investigate potential associations among systemic virus burden, host anti-viral immune response, and SIVE in the SIVsmmFGB model, we compared plasma virus load and anti-SIV antibody titers at 1 month after inoculation, 2 months after inoculation, and necropsy, with virus burdens in the CNS parenchyma, meninges, and choroid plexus. Terminal plasma virus load was significantly correlated with global parenchymal virus burden ($r = 0.58$, $P < 0.05$), and with virus loads in both meninges ($r = 0.54$, $P < 0.05$) and choroid plexus ($r = 0.60$, $P < 0.05$) (Table 5). Surprisingly, we found a similar level of correlation between plasma virus load at 2 months after inoculation and virus burdens in the CNS parenchyma ($r = 0.52$, $P < 0.05$), meninges ($r = 0.58$, $P < 0.05$), and choroid plexus ($r = 0.59$, $P < 0.05$) at necropsy; however, there was no correlation between plasma virus load at 1 month after inoculation and CNS virus load. These data suggested that plasma virus load at 2 months after inoculation was predictive of SIVE and CNS virus load at necropsy of SIVsmmFGB-infected macaques.

An even stronger, but inverse correlation was observed between the magnitude of the anti-SIV antibody titer and the CNS virus burden in SIVsmmFGB-infected macaques. The anti-SIV antibody titers at 1 month after

Figure 5. Longitudinal plots of plasma viral load (A) and anti-SIV-binding antibody response (B) in RP ($n = 12$), SP macaques with SIVE (SP + SIVE; $n = 3$), and SP macaques without SIVE (SP - SIVE; $n = 2$). Plasma viral load and anti-SIV antibody titer were measured at 1 and 2 months after inoculation and at necropsy. **A:** Plasma viral load, reported as log₁₀ SIV RNA copies/ml, for RPs (black line), SPs with SIVE (SP + SIVE; red line), and SP without SIVE (SP - SIVE; red dashed line). **B:** Anti-SIV-binding antibody titers, reported as the reciprocal log of the last positive serum dilution for RP (black line), SP + SIVE (red line), and SP - SIVE (red dashed line) macaques. The horizontal dashed line represents the lower limit of detection for the assay (1:400 dilution of plasma; $2.6 \log_{10}$ titer⁻¹).

inoculation, 2 months after inoculation, and necropsy were negatively correlated with global virus burden in the CNS parenchyma ($r = -0.61$, -0.62 , and -0.63 , respectively; $P < 0.01$). Furthermore, the anti-SIV titer was inversely correlated with both meningeal and choroid plexus virus burdens at 1 month after inoculation ($P < 0.01$), 2 months after inoculation ($P < 0.05$ and $P < 0.01$, respectively), and at necropsy ($P < 0.05$). These findings show that the anti-SIV-binding antibody titer is highly predictive of SIVE and CNS virus burden as early as 1 month after inoculation in the SIVsmmFgb/pig-tailed macaque model.

Plasma virus load and anti-SIV antibody titer at 1 and 2 months after inoculation and at necropsy were plotted longitudinally for each macaque to gain a better understanding of the interrelationships between virus kinetics, host humoral immunity, and SIV disease progression, particularly SIVE (Figure 5). There was considerable overlap between RP and SP values for plasma viral load at 1 month after inoculation (Figure 5A). By 2 months after inoculation, RP and SP populations began to segregate with respect to plasma virus burden; however, an individual measurement for plasma virus load at 2 months after inoculation would not necessarily be predictive of either the rate of disease progression or the presence of SIVE for an individual macaque at the time it developed SAIDS. In contrast, RP and SP populations were discretely segregated with respect to anti-SIV antibody titer by 1 month after inoculation and remained distinct throughout the course of infection (Figure 5B). SP/SIVE could not be discerned from SP without SIVE based on either plasma viral load or anti-SIV antibody response.

Correlation of CNS Macrophages, CNS Virus Burden, and SIVE

Cells of macrophage lineage are thought to play a central role in HIV neuroinvasion, in the kinetics of HIV replication in the CNS, and in the pathophysiology of HIV-induced encephalitic and neurodegenerative lesions. To further validate the SIVsmmFgb model of SIVE for studying pathophysiological mechanisms of HIV, we sought to examine the relationships among CNS macrophage population density, CNS virus burden, and severity of SIVE in SIVsmmFgb-infected pig-tailed macaques. To this end, cells of macrophage lineage were enumerated in sections of cerebral cortical white matter by immunohistochemical localization of CD68 antigen and computer image quantitation. The number of CD68⁺ cells in the cortical white matter of RP was significantly higher than that of SP macaques (medians of 207.3 ± 80.4 versus 17.4 ± 1.9 , respectively; $P < 0.005$), SP/SIVE (median of 17.4 ± 6.7 , $P < 0.04$), and SIV-negative controls (median of 10.2 ± 1.4 , $P < 0.01$) (Figure 6A and Figure 7; A to D). A trend toward greater numbers of CD68⁺ cells in the cortical white matter of SP macaques versus uninfected controls (median values of 17.4 versus 10.2 cells/mm², $P < 0.11$), was statistically significant when the comparison was restricted to SP/SIVE versus uninfected animals ($P < 0.05$). The number of CD68⁺ cells in the cortical

Figure 6. Number of CD68⁺ cells in the white matter tracts of the midfrontal cortex is positively correlated with brain viral load and severity of encephalitis. **A:** The mean number of macrophages in midfrontal cortical white matter was determined for each SIVsmmFgb-infected macaque and three SIV-negative controls by IHC for CD68 followed by computer image analysis of 10 contiguous sections of tissue at $\times 200$. Columns depict the mean number of CD68⁺ cells per mm² for individual macaques and error bars show the SD. **Black columns** represent macaques with SIVE, as defined by the presence of viral nucleic acids by *in situ* hybridization; **gray columns** represent macaques without SIVE. **B:** The number of CD68⁺ cells in the white matter tracts of the midfrontal cortex of RP and SP SIVsmmFgb-infected macaques is positively correlated with both brain virus burden, measured as SIV⁺ cells per mm² ($P < 0.01$), and the severity of SIVE, as determined by SIVE index ($P < 0.01$). **C:** The positive correlations observed between number of CD68⁺ cells in the cortical white matter and both brain virus burden and severity of SIVE are maintained when the analysis is restricted to RP macaques alone ($P < 0.01$).

white matter of infected macaques was significantly correlated with global CNS virus burden ($r = 0.83$; $P < 0.01$) and SIVE index ($r = 0.86$; $P < 0.01$) (Figure 6B), and inversely correlated with terminal antibody titer ($r = -0.61$; $P < 0.01$), but was not correlated with terminal plasma virus burden ($r = 0.46$). The correlation between the population density of CD68⁺ cells in cortical white matter, CNS global virus burden ($r = 0.71$; $P < 0.01$), and SIVE index ($r = 0.79$; $P < 0.01$) was maintained when the analysis was restricted to RP macaques (Figure 6C).

Phenotype of Infected Cells in the CNS

The number, diffuse distribution, and heterogeneous cytomorphology of infected cells in the brain parenchyma of RP macaques led us to question whether the markedly neuropathogenic phenotype of SIVsmmFgb might be correlated with broader permissivity for productive infec-

Figure 7. Macrophages were identified by IHC for CD68 (IHC-CD68) in the white matter tracts of the midfrontal cerebral cortex in SIVsmmFGB-infected and uninfected pig-tailed macaques. Large numbers of CD68-positive cells (dark brown) were found throughout the brain parenchyma as well as in perivascular locations in RP macaques (**A**: RP macaque PDF1). CD68-reactive cells were cytomorphologically diverse, and included club-shaped cells, multinucleated giant cells, and cells with extensive cytoplasmic processes. In contrast, cells expressing CD68 were far fewer in number and were predominantly confined to perivascular locations in SP macaques with SIVE (**B**: SP/SIVE macaque POF1) and SP macaques without SIVE (**C**: SP macaque PKG1). CD68-positive cells were infrequently observed in SIV-negative controls (**D**: SIV-negative macaque PSs). **Arrows** identify blood vessels (**B**, **C**). Scale bars, 50 μ m.

tion of target cells in the CNS. To identify the phenotype of productively infected cells in the brain parenchyma, we simultaneously localized viral RNA by *in situ* hybridization and cellular antigens by IHC in sections of cerebrum, cerebellum, and medulla from three pig-tailed macaques. RP macaques with high brain virus burden (PUf1, PSc1, and PZg1) were selected for these assays to maximize the number of target cells available for dual labeling. Productive infection of the CNS was restricted to cells of macrophage phenotype in all three animals examined, although adequate immunohistochemical labeling was achieved for other cellular markers (Figure 8; A to F). Cells that contained SIV hybridization signal but were unlabeled with respect to phenotype were observed in all CNS sections dual-labeled for SIV and macrophage antigens.

Discussion

Virus that is sequestered in anatomical reservoirs such as the CNS or male genital tract may present a serious

challenge to the development of therapeutic strategies aimed at eradicating HIV from infected individuals.^{19,20,26,40,41} SIV-infected macaques provide a powerful animal model for investigating the neuropathogenesis of lentivirus infections; however, use of this model to measure lentiviral replication dynamics in the CNS or to evaluate therapies designed to treat or prevent HIVE have been limited by the inconsistent presence of SIVE in macaques at the time that they develop immunodeficiency. Although a few isolates and passage-adapted strains of SIV and SIV/HIV chimeric viruses (SHIVs) have shown a capacity for enhanced neurovirulence,^{42–44} conventional isolates and clones of SIV induce neuropathological lesions in fewer than 50% of infected macaques.^{27–30,45} Other SIV models associated with a greater incidence of neuropathogenic infections involve manipulations of the host immune system, such as depletion of CD8⁺ T lymphocytes during acute infection,^{46,47} or use inocula composed of mixtures of both immunosuppressive and neurovirulent virus strains.⁴⁸ In the present study, we observed SIVE in 15 of 17 pig-

Figure 8. The phenotype of productively infected cells was determined in brain tissue from SIVsmmFGB-infected pig-tailed macaques by dual-label histological assays that combined *in situ* hybridization for SIV RNA (dark blue) with IHC using antibodies that recognize antigens specific for immune cells or resident cells of the CNS parenchyma (dark brown to black). **A:** SIV/Ham-56: **Arrows** identify three SIV-infected macrophages (co-localized blue and black chromogens) in the white matter tracts of the midfrontal cortex of RP macaque PZg1, using the antibody Ham-56. Uninfected macrophages (**arrowhead**) are also present. **B:** SIV/Ham-56: **Arrows** identify three cells out of a group of several SIV-infected macrophages (co-localized blue and black chromogens) in the cerebral gray matter of macaque PZg1. **Arrowheads** identify uninfected macrophages. **C:** SIV/CD3: Even small SIV-positive cells (**arrows**, blue chromogen) are generally larger than CD3-expressing cells (**arrowheads**, brown chromogen) and do not co-localize CD3 antigen. **D:** SIV/VWF: **Arrows** identify two SIV-infected cells (dark blue) within the perivascular space of a blood vessel in the cerebral cortex of RP macaque PUF1. The infected cells are immediately adjacent to capillary endothelial cells (**arrowheads**), but do not express the endothelial cell antigen, von Willebrand factor (black). **E:** SIV/GFAP: **Arrows** identify three SIV-positive cells (dark blue) that are immediately adjacent to GFAP-positive astrocytes (**arrowheads**, brown-black chromogen) in the medulla of macaque PUF1. Infected cells often appear cradled between astrocyte processes, but GFAP-expressing cells (astrocytes) do not co-express viral RNA. **F:** SIV/NF: **Arrows** identify two SIV-infected cells (dark blue) adjacent to the apical dendrites of pyramidal neurons (**arrowheads**) in the cerebral cortex of RP macaque PZg1. Neurons are labeled with an antibody that recognizes a 200-kd neurofilament polypeptide (brown chromogen). Neurons do not co-localize hybridization signal, showing that they are not productively infected with SIV. Scale bars, 50 μ m.

tailed macaques inoculated intravenously with a single cell-free virus stock of SIVsmmFGB, without experimentally modifying the host immune system.

To further characterize the SIVsmmFGB model of SIVE, we performed a comprehensive semiquantitative evaluation of productive virus burden throughout the brain parenchyma of untreated, SIVsmmFGB-infected pig-tailed macaques. Encephalitic lesions occur diffusely throughout the brains of HIV-infected individuals, however, postmortem studies that have analyzed the distribution of productively infected cells in brain tissue from demented patients have generally found that subcortical white matter, basal ganglia, and hippocampus harbor the greatest viral burdens.^{49–55} In contrast to the predominant pattern of virus distribution in the brains of HIV-infected humans, surveys of brain tissue from SIV-infected monkeys have generally revealed that virus burden is highest in cerebral and cerebellar cortical gray and white matter rather than in the basal nuclei.^{29,38,39,56,57} As reported for other models of SIVE, viral loads were higher in midfrontal cortex and hippocampus than in caudate nucleus and putamen of RP macaques infected with SIVsmmFGB. Interestingly, the distribution of brain virus burden in SP/SIVE macaques was similar to that described for HIV-infected humans, with the highest viral loads in the putamen and caudate nucleus.

Brain virus burdens were much higher in RP than SP/SIVE macaques, which confirms the importance of host immunity in containing viral replication in the CNS as well as in peripheral lymphoid tissues. In agreement with other models of SIVE pathogenesis, dual-label histological assays showed that productively infected cells in the brains of SIVsmmFGB-infected pig-tailed macaques are primarily of macrophage lineage,³⁹ and both brain virus burden and lesion severity were strongly correlated with macrophage population density in the CNS.³⁸ Infected macrophages were distributed in a perivascular pattern in the brain parenchyma of those animals with low brain virus burden (including RP as well as SP/SIVE macaques), consistent with the observations of Williams and colleagues,²⁴ who reported that productive infection in the brains of rhesus macaques infected with either SIVmac239 or SIVmac251 was confined predominantly to blood-derived, perivascular macrophages and not parenchymal microglia. In contrast, productively infected macrophages were distributed throughout the parenchyma of the brain as well as perivascular locations in RP macaques with high brain virus burdens. The cytomorphological characteristics and parenchymal location of many of the infected macrophages in SIVsmmFGB-infected macaques are highly reminiscent of HIV-positive parenchymal microglia in HIVE,⁵⁸ leading us to speculate that initial infection of perivascular macrophages may extend to include parenchymal microglia in the setting of catastrophic immune failure, as seen with RP macaques. Additional studies are needed to subphenotype infected macrophage populations in both RP and SP/SIVE groups of SIVsmmFGB-infected macaques.

The correlates of immune protection against retroviral encephalitis have not been identified, and the role of the

host immune system in the pathogenesis of HIVE and HAD remains unclear. The onset of HAD typically occurs late in the course of HIV infection, after the development of profound immunodeficiency, which argues that a competent immune system protects against neuropathogenic progression.^{59–61} Moreover, recent evidence suggests that patients with impaired neutralizing antibody responses may be at increased risk for the development of HAD.⁶² SIV studies have established that early anti-viral immunity is critical for protecting against severe encephalitis and rapid disease progression, because catastrophic immune failure, as determined by a weak or undetectable anti-SIV antibody response, has been associated with rapid disease progression and SIVE.^{23,30,63–65} Here, we provide further proof that immune failure during acute SIV infection results in severe and rapidly fatal SIVE; indeed, the anti-SIV-binding antibody response at 1 month after inoculation was predictive of brain virus burden and severity of encephalitis, as well as survival time of SIVsmmFGB-infected macaques.

In this study it is uncertain whether anti-viral antibodies played a role in protecting macaques against SIVE, or simply served as a surrogate marker of overall immunological health. The quantity of antibodies elicited against SIV may reflect the magnitude and quality of cell-mediated immunity, because vigorous innate (ie, natural killer cell) and/or antigen-specific (ie, CD8⁺ CTL) cellular responses during acute infection may preserve memory CD4⁺ T cells essential for generating anti-viral antibodies. Depletion studies in the SIV/macaque model have shown that the absence of CD8⁺ cells during acute infection increases the incidence and severity of SIVE in rhesus macaques, confirming an integral role for cell-mediated immunity in protection against lentiviral encephalitis.⁴⁶ Recent demonstration of potent neutralizing antibody responses during early HIV-1 infection^{66,67} contradict the longstanding notion that neutralizing antibodies directed against viruses isolated during acute HIV infection are undetectable for months to years after infection.^{68,69} Furthermore, macrophage-tropic strains of SIV appear to be particularly sensitive to antibody-mediated neutralization.^{70,71} Taken together, these findings suggest a more significant role for antibodies in containing virus burden in anatomical compartments such as the CNS, where macrophages account for nearly all of the productive virus infection as opposed to peripheral lymphoid tissues, where virus is produced predominantly by infected CD4⁺ T lymphocytes. The SIVsmmFGB model should be a valuable resource for investigating the impact of binding and functional antibodies on the neuropathogenic outcome of lentivirus infections.

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