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Simian Immunodeficiency Virus SIV_{mac} Chimeric Virus Whose *env* Gene Was Derived from SIV-Encephalitic Brain Is Macrophage-Tropic but Not Neurovirulent

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We inoculated four rhesus macaques with molecularly cloned simian immunodeficiency virus SIV_{mac}239/17E *env*, a chimeric virus whose *env* gene was derived from the brain of an SIV-encephalitic macaque. Blood and lymphoid tissues had high frequencies of infected cells. The virus was neuroinvasive, but productive virus replication did not occur in the brain, and animals did not develop encephalitis.

Simian immunodeficiency virus SIV_{mac} infection in macaques results in virus persistence and immunosuppression that leads to the development of AIDS and in some cases, neurological disease associated with virus replication in brain macrophages (3, 9). Molecularly cloned SIV_{mac}239 is T-lymphocyte-tropic and causes immunosuppression. Most animals develop AIDS within 2 years (8, 11, 13). SIV_{mac}239 does not replicate productively in any cells in the brain, including microglia, and cannot be rescued from an inoculated brain by explant procedures (14). Neuroadaptation of virus 239 in macaques resulted in the development of encephalitis in some animals (14). In these cases, neurological disease was associated with productive virus replication in macrophages in the brain, giving rise to a high degree of infectivity in cell-free homogenate of the brain. Neuroadapted virus was macrophage-tropic (M-tropic) in cell cultures (14).

To investigate whether an M-tropic virus alone could cause neurological disease, we constructed a chimeric virus in which bp 6351 to 8742 of SIV_{mac}239 were replaced with the predominant *env* gene in the encephalitic brain of macaque 17E, as described previously (1). The resulting virus, SIV_{mac}239/17E *env*, was M-tropic, replicating efficiently in both lymphocyte cultures and primary macrophage cultures (1). DNA of SIV_{mac}239/17E *env* was transfected into CEMx174 cells by the lipofectin procedure. The cells developed fusion cytopathic effects, and supernatant fluids were stored as stock virus. CEMx174 cells were cultured in RPMI containing 10% fetal bovine serum (FBS) (RPMI/10% FBS) as described previously (7).

Four juvenile rhesus macaques (*Macaca mulatta*), 1 to 2 years of age were obtained from the Wisconsin Primate Research Center. To prevent the generation of variants on passaging the virus in CEMx174 cells, we aspirated bone marrow from the animals and fractionated cells on Ficoll-Hypaque gradients. Mononuclear bone marrow cells (10^7) from each animal were suspended in RPMI, transfected with 25 μg of infectious plasmid DNA of 239/17E *env* mixed with lipofectin (Gibco/BRL), and reinfused into the animals.

Serial dilutions of plasma, obtained at weeks 1 to 4 and monthly thereafter, were cultured with CEMx174 cells, but neither infectious virus nor p27 antigen was recovered at any time (Table 1). Positive-control plasma from SIV_{mac}239-infected macaques had both infectivity and p27. Peripheral blood mononuclear cells (PBMC) were cultured in supplemented RPMI (sRPMI)/10% FBS plus 100 U of recombinant interleukin 2 (IL-2) for 3 days after which supernatant fluids were assayed for infectivity in CEMx174 cells. Under these conditions, cells that are activated in vivo will produce virus, but quiescent, latently infected cells will not produce virus (15). Production of virus under these culture conditions is a sensitive indicator of the presence of activated cells in vivo (4). PBMC from the infected animals repeatedly failed to yield infectious virus under these conditions (Table 1, IL-2), suggesting that activated infected cells were not present in peripheral blood.

PBMC were also cultured in sRPMI/10% FBS plus 1 μg of phytohemagglutinin (PHA) (phytohemagglutinin-P; Wellcome) per ml. Cultures were centrifuged after 2 days, and cell pellets were resuspended in sRPMI/10% FBS plus 100 U of recombinant IL-2 per ml for 5 days, after which supernatant fluids were assayed for virus infectivity and p27. Under these conditions, PBMC from all four animals yielded infectious virus and p27 readily during the first 2 to 3 months postinoculation (PI) (Table 1, PHA/IL-2). Thereafter, infectious virus was not recovered from any of the four animals, although p27 was sometimes recovered.

Coculture of PBMC with CEMx174 cells yielded virus during the first 2 to 3 months, but not thereafter (Table 1). However, quantitative analyses, using the PCR/ICA procedure at months 3, 6, 9, and 12 showed consistently high frequencies of infected cells in PBMC (1/1,000 for M6 and M7 and 1/10,000 for M4 and M5). For PCR/ICA, cell suspensions with 10^7 to 10^2 cells per ml were lysed, digested, and PCR amplified, using nested primers in two rounds (35 cycles in each round) as described previously (7). After 2 months, the maintenance of a high level of infection in mononuclear cells, despite the failure to obtain cytopathic virus (for CEMx174 cells), suggested that the agent had undergone a phenotypic change in vivo. The high frequencies of infected cells are difficult to reconcile with the presence of a defective virus. A possible explanation is that the virus is capable of replication in some as yet unidentified cell

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TABLE 1. Recovery of infectious virus and SIV p27 from plasma samples and PBMC

Material and assay	No. of animals (of 4) from which virus was recovered at the following mo. PI:									
	0.5	1	1.5	2	3	4	5–6	9–10	11	12
Plasma[a]										
Infectious virus	0	0	0	0	0	0	0	0	0	0
p27	0	0	0	0	0	0	0	0	0	0
IL-2[a]										
Infectious virus	0	0	0	0	0	0	0	0	0	0
PHA/IL-2[a]										
Infectious virus	0	4	4	0	0	0	0	0	0	0
p27	0	4	4	3	1	2	3	1	1	1
Coculture[b]										
Infectious virus	0	4	1	0	0	0	0	0	0	0
p27	0	4	1	1	3	2	0	0	0	0
Macrophage[a]										
Infectious virus	0	0	0	0	ND[c]	ND	0	0	0	0
p27	0	4	4	0	ND	ND	0	3	2	1

[a] Plasma samples and supernatant fluids of IL-2, PHA/IL-2, and macrophage cultures were titrated in CEMx174 cells. SIV p27 was assayed with an antigen capture enzyme-linked immunosorbent assay kit (Coulter) which has a sensitivity of 20 pg/ml.
[b] PBMC were cocultured with CEMx174 cells.
[c] ND, not done.

TABLE 2. Recovery of infectious virus and SIV p27 from lymphoid tissues

Tissue and test[a]	No. of animals positive/no. of animals tested at the following mo. PI:		
	2	9	12
Lymph node			
Homogenate			
Infectious virus	0/4	ND[b]	0/1
p27	4/4	ND	1/1
PHA/IL-2			
Infectious virus	4/4	0/4	0/1
p27	4/4	4/4	1/1
Macrophage			
Infectious virus	0/2	0/4	0/1
p27	2/2	4/4	0/1
Spleen			
Homogenate			
Infectious virus	0/4	ND	0/1
p27	4/4	ND	1/1
PHA/IL-2			
Infectious virus	3/4	0/4	0/1
p27	3/4	3/4	1/1
Macrophage			
Infectious virus	0/2	0/4	0/1
p27	2/2	4/4	0/1

[a] Homogenates and supernatant fluids of PHA/IL-2 and macrophage cultures were titrated in CEMx174 cells. SIV p27 assays were done with an antigen capture enzyme-linked immunosorbent assay kit.
[b] ND, not done.

type in vivo, ensuring continuous infection of fresh lymphocytes.

PBMC were also cultured for 10 days in macrophage differentiation medium that consisted of sRPMI containing 10% human serum supplemented with granulocyte-macrophage colony-stimulating factor and macrophage colony-stimulating factor as described previously (5), after which supernatant fluids were assayed for p27 and virus infectivity in CEMx174 cells. Under these culture conditions, monocyte-derived macrophages from all four animals failed to produce virus at all time points tested, although p27 production was seen sporadically (Table 1, macrophage).

Mesenteric lymph nodes and biopsies of the spleen were obtained from each animal at 2 and 9 months and at necropsy of M6 at 12 months. Cell-free homogenates of these tissues (4) were inoculated into CEMx174 cells, but infectious virus was not recovered although p27 was detected (Table 2). Single-cell suspensions of lymph node cells (LNC) and spleen cells (SPC) were obtained as previously described (13), and IL-2, PHA and then IL-2 (PHA/IL-2), and macrophage cultures were done as for PBMC. LNC and SPC cultured in IL-2-containing medium failed to yield infectious virus at all times, whereas LNC and SPC cultured in PHA/IL-2 yielded infectious virus at 2 months but yielded only p27 at 9 months (Table 2). Macrophage cultures of LNC and SPC did not yield infectious virus at any time, although p27 was recovered occasionally. Thus, the genome of a virus with cytopathic potential for CEMx174 cells was present in resting lymphocytes and monocytes during the first 2 months of infection. This virus was subsequently replaced with one that maintained infectiousness in vivo but not for CEMx174 cells. Mononuclear cells carrying this viral genome nevertheless expressed p27.

Open biopsies of brain were obtained from the animals at 7 months PI. Tissues of M4, M5, and M6 were histologically normal, while M7 had mild meningitis but no encephalitis. Portions of finely minced tissue were cultured in macrophage differentiation medium to obtain macrophage cultures (5). Supernatant fluids were assayed for p27 and infectivity in CEMx174 cells. Brain macrophages from M7 yielded p27, although infectious virus was not recovered by passage in normal macaque macrophage cultures. Brain macrophage cultures from the other three animals were negative for both p27 and infectious virus. PCR techniques showed the presence of viral DNA in brain biopsy material from M4, M6, and M7 but not M5. Thus, although viral DNA was present in the brain in three of four animals, productive virus replication did not occur.

The frequency of $CD4^+$ cells in PBMC and LNC was assessed by fluorescence-activated cell sorting analysis at 6 months PI and monthly thereafter as previously described (7). PBMC from macaque M6 consistently had a $CD4^+$ frequency of 2% or less. In contrast, $CD4^+$ frequencies in PBMC from the other three animals were consistently higher (12 to 24% for M4 and M7 and 20 to 34% for M5). Similarly, the $CD4^+$ frequency in LNC was only 3% for M6, but the $CD4^+$ frequencies were 23% in M4, 32% in M5, and 17% in M7.

All four animals responded to the virus with potent antivirus immune responses. Neutralizing antibodies to virus 239/17E *env*, assayed as previously described (6), were detected at one month PI and rose steadily to >1:10,000 by 6 months (Fig. 1) but failed to neutralize virus 239, even at dilutions of 1:20. Despite these powerful immune responses, the frequencies of infected cells by PCR/ICA remained high. Whether the change in viral phenotype 2 months into the infection was the result of selective pressure by these antibodies is not known. This type of change had been observed previously in another macaque infected with virus $SIV_{mac}239$ (6).

Macaques M4, M5, and M7 have remained clinically well, with no signs of neurological disease for over 15 months PI, although these animals have failed to gain weight normally. Macaque M6 developed chronic diarrhea and weight loss and was euthanized. Gastroenteritis associated with *Cryptosporidium* infection, pancreatitis, myocardial myositis, and fibrosis

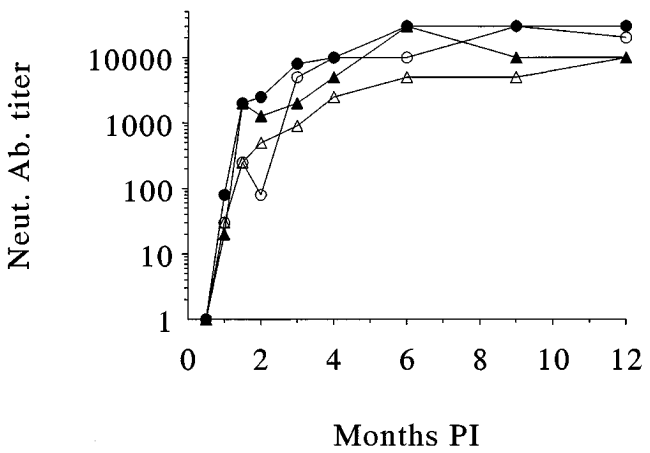

FIG. 1. Neutralizing antibody (Neut. Ab.) titers to SIV 239/17E *env* in serum samples from macaques M4 (△), M5 (▲), M6 (○), and M7 (●).

were observed at necropsy. These lesions were accompanied by lymphoid hyperplasia, with lymphocytic infiltration of the lungs, pancreas, and bone marrow. Encephalitis was not present. At necropsy, infectious virus was not recovered from the blood, lymph nodes, spleen, or brain by all culture techniques mentioned above.

Since it was not possible to recover infectious virus from tissues of macaque M6 at necropsy, PCR was used to examine tissues for SIV DNA. Using nested PCR, we readily amplified gp120 sequences from all non-central nervous system tissues (lymph nodes, spleen, kidney, lung, pancreas, and intestine) except the liver. In contrast, viral gp120 sequences were not amplified by PCR in portions obtained from the spinal cord, brain stem, cerebellum, and parietal and occipital cerebral cortex. The frontal cortex was the only portion of the brain from which gp120 sequences were amplified. Since viral DNA was also detected 5 months earlier in the brain biopsy, it is clear that neuroinvasion occurred but was not followed by virus replication and spread throughout the central nervous system.

In previous studies of five macaques inoculated with brain homogenates or bone marrow cells containing neuroadapted M-tropic SIV_{mac}, three animals developed immunosuppression and all three developed encephalitis; the other two animals that mounted immune responses did not develop encephalitis (4, 5, 12, 14). The association between encephalitis and poor antibody responses to SIV has also been reported by other investigators (2, 10, 16). Of six SIV_{mac}239-inoculated macaques, all developed immunosuppression, but M-tropic virus was not present, and none developed encephalitis. In this study, four macaques were inoculated with M-tropic virus, and three did not develop either immunosuppression or encephalitis. The fourth animal (macaque M6) developed immunosuppression and AIDS but not encephalitis. Two explanations why M6 did not develop encephalitis are (i) that although M6 developed AIDS, the high levels of neutralizing antibodies to the virus (Fig. 1) may have prevented spread of virus in the brain and/or (ii) that the virus had changed phenotype and was now incapable of replicating in the brain. Other unspecified viral or host factors may also be involved.

These studies showed that the chimeric virus failed to cause encephalitis despite its predicted neuropathogenic potential as identified by its tropism for macrophages and the fact that its *env* gene was derived from an SIV-encephalitic brain. The virus caused a type of infection that was distinct from that caused by either SIV_{mac}239 or virus in tissue material (4). There was no activation of T lymphocytes, and the animals did not develop lymphadenopathy, splenomegaly, or plasma viremia. Although persistent infection was clearly demonstrable in both T cells and macrophages, productive virus replication in these cells occurred only transiently. Thus, the infection was characterized by a restricted type of virus replication accompanied by the development of a potent neutralizing antibody response. Despite the restricted replication, PCR showed that the brain became infected in three of the four animals, indicating that the virus was neuroinvasive although the agent did not spread in the brain. Further, typical of the restricted type of replication in other tissues, no productive replication occurred in the brain, and the animals failed to develop encephalitis.

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