

Chimeric Simian/Human Immunodeficiency Virus That Causes Progressive Loss of CD4⁺ T Cells and AIDS in Pig-Tailed Macaques

SANJAY V. JOAG,¹ ZHUANG LI,¹ LARRY FORESMAN,² EDWARD B. STEPHENS,¹ LING-JUN ZHAO,¹ ISTVAN ADANY,¹ DAVID M. PINSON,³ HAROLD M. McCLURE,⁴ AND O. NARAYAN^{1*}

Marion Merrell Dow Laboratory of Viral Pathogenesis and Department of Microbiology,¹ Laboratory Animal Resources² and Department of Pathology and Laboratory Medicine,³ University of Kansas Medical Center, Kansas City, Kansas 66160-7420, and Yerkes Regional Primate Center, Emory University, Atlanta, Georgia 30322

Received 30 August 1995/Accepted 31 January 1996

By animal-to-animal passage of simian/human immunodeficiency virus (SHIV) in pig-tailed macaques, we have developed a macaque model of human immunodeficiency virus type 1 (HIV-1) disease in humans. Passaging was begun with a chimeric virus containing the *env* gene of HIV-1 HXBc2 and the *gag* and *pol* genes of simian immunodeficiency virus SIVmac239. SHIV was passaged serially in cohorts of two macaques each, using bone marrow-to-bone marrow transfers at 5, 5, and 16 weeks for passages 2, 3, and 4, respectively. The fifth passage was done by using cell-free virus isolated from cerebrospinal fluid of a passage 4 macaque. The virus became more virulent with each passage. Virus replication was restricted in all three animals in passages 1 and 2 but not in five of the six animals in passages 3, 4, and 5. In these animals, intense virus replication in the lymphoid tissues resulted in almost total elimination of CD4⁺ T cells within weeks of inoculation, and three of these animals developed AIDS in less than 1 year. The more uniform virus-host interaction initiated by the cell-free virus in the passage 5 animals contrasted with a more variable pattern of disease initiated by infectious bone marrow cells during earlier passages. The virulent cell-free SHIV can now be used to screen the efficacy of vaccines directed against the envelope of HIV-1.

Human immunodeficiency virus type 1 (HIV-1) can infect nonhuman primates such as chimpanzees but does not cause disease in these animals (16). This is a major impediment to understanding the mechanisms of pathogenesis of HIV-1 infection and has also retarded efforts to evaluate the efficacy of anti-HIV drugs and vaccines. At present, simian immunodeficiency virus SIVmac infection in macaques is the best-characterized model of HIV-1 disease (3). HIV-2 infection in baboons represents another useful model (1). SIVmac and HIV-2 are closely related viruses, but both have genetic, antigenic, and structural features in their *env* genes that clearly distinguish them from HIV-1.

Chimeric simian/human immunodeficiency viruses (SHIVs) that bear the envelope of HIV-1 and are infectious in macaques potentially offer a solution to this problem. However, SHIVs have so far proven avirulent in macaques (2, 9, 13, 15, 17). Since the bone marrow (BM) of SIVmac-infected macaques has high virus burdens (7) and since specific pathogenic viral genotypes can be selected by serial passage of infectious BM in animals (14), we hypothesized that similar passaging of SHIV-infected BM cells would also result in selection of pathogenic genotypes of this virus. We report here on derivation of a pathogenic SHIV by using this procedure and the initial characterization of the disease process caused by this virus.

MATERIALS AND METHODS

Passage in macaques. SHIV, obtained as described below, was used to begin passaging in young (7 to 19 months old) rhesus (*Macaca mulatta*) and pig-tailed (*Macaca nemestrina*) macaques (Fig. 1). Macaques were housed in American Association for Laboratory Animal Care-accredited facilities and were sedated with ketamine for all procedures. Virus was inoculated either in the BM or by the intravenous route. BM inocula immediately reach the systemic circulation and are thus equivalent to intravenous inocula. The rationale for BM inoculation was to guarantee exposure of precursor cells in the BM to virus in the inoculum.

As shown in Fig. 1, we inoculated 10⁴ 50% tissue culture infective doses (TCID₅₀) of SHIV into the BM of rhesus macaque 8A. Four weeks later, heparinized BM was obtained from this animal, mononuclear cells were purified over Ficoll-Hypaque gradients, and 5 × 10⁷ cells were inoculated into the femoral BM of two pig-tailed macaques, PLc and PRc. Five weeks later, BM was aspirated from PLc and PRc, pooled, and inoculated into the BM of macaques Ppc and Pqc. Sixteen weeks later, bone marrow and splenic biopsies were obtained from macaques Ppc and Pqc, and mixtures of splenocytes and BM cells from both animals were pooled and inoculated into two new pig-tailed macaques, Pfb and Pnb. Cell-free virus isolated from macaque Pnb was used for inoculation of macaques 15A and 15B in passage 5.

Viruses. (i) **Original SHIV.** We obtained a SHIV DNA encoding the *env*, *tat*, *rev*, and *vpu* genes of HIV-1 HXBc2 on a background of SIVmac239 (9) from Joseph Sodroski, Harvard University. Viral DNA was transfected into CEMx174 cells to produce a virus stock, which had a titer of 1 × 10⁴ TCID₅₀/ml. The virus stock was stored at -80°C. This virus was inoculated into macaque 8A, whose BM cells were then used to begin passaging as described above.

(ii) **Virulent SHIV.** Virus was obtained by cocultivation of cerebrospinal fluid cells from macaque Pnb with indicator C8166 cells. Supernatant fluid was inoculated into phytohemagglutinin (PHA)-activated peripheral blood mononuclear cells (PBMC) from a healthy pig-tailed macaque. The culture developed syncytial cytopathic effects (CPE) by day 5, and supernatant fluid was harvested on day 10. This fluid was clarified at 5,000 × g for 10 min, and 1-ml aliquots were stored in liquid nitrogen. This virus was inoculated intravenously into two pig-tailed macaques, 15A and 15B, for passage 5.

(iii) **HIV-1 HXBc3.** HIV-1 HXBc3 was obtained from P. Nara (National Cancer Institute), and a stock was prepared in CEMx174 cells. The virus stock was stored at -80°C.

Cell cultures. Human T-cell lines CEMx174 and C8166 were used as indicators to measure virus infectivity. Cells were cultured at a concentration of 10⁶/ml in RPMI supplemented with 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (pH 7.3), 50 μg of gentamicin per ml, 5 × 10⁻⁵ M

* Corresponding author. Mailing address: Marion Merrell Dow Laboratory of Viral Pathogenesis, Department of Microbiology, University of Kansas Medical Center, 3901 Rainbow Blvd., Kansas City, KS 66160-7420. Phone: (913) 588-5575. Fax: (913) 588-5599. Electronic mail address: bnarayan@kumc.edu.

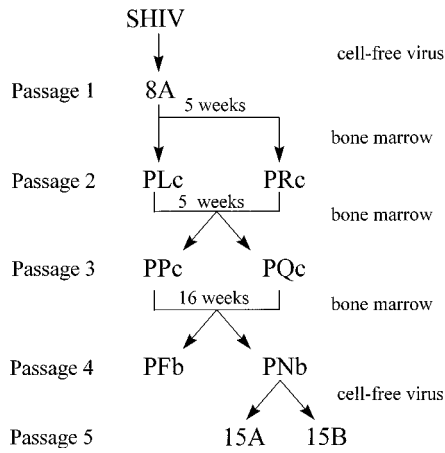


FIG. 1. Passage of SHIV in macaques.

2-mercaptoethanol, 2 mM glutamine, and 10% fetal bovine serum (supplemented RPMI).

Processing of blood samples. Heparinized blood obtained from the femoral vein was centrifuged to separate plasma and buffy coats. Plasma infectivity was assayed immediately, and aliquots of the plasma were stored at -80°C . PBMC were separated from buffy coat cells by centrifugation through Ficoll-Hypaque density gradients.

Assessment of virus infectivity. The frequency of infectious cells was measured by inoculation of serial 10-fold dilutions of PBMC or other cells onto indicator cells in 24-well tissue culture plates, which were then observed for development of syncytial CPE during a 7-day period. Then 100 μl from each well was transferred to another plate, fresh indicator cells were added, and the plates were observed for a further 7 days. Routinely, 10^6 cells from each animal were used for estimation of infectious cell frequency. Results were expressed as the number of infectious cells per 10^6 PBMC or other cells.

For quantitation of infectivity in plasma or culture supernatant fluids, serial 10-fold dilutions of test material were inoculated into freshly prepared CEMx174 or C8166 cell cultures. The cells were cultivated in 24-well tissue culture plates and examined for CPE for 7 days, after which 100 μl from each well was

transferred to another plate, fresh indicator cells were added, and the plates were observed for CPE for a further 7 days.

Virus recovery from PBMC cultured with PHA and IL-2. A total of 2×10^6 PBMC were cultured in medium containing 1 μg of PHA-P (Wellcome) for 2 days. Cultures were then rinsed once, the cell pellets were resuspended in medium with 100 U of recombinant human interleukin-2 (IL-2; Cetus) per ml, cultured for 5 days, and then centrifuged again, and the cell-free supernatant fluids were assayed for virus infectivity. In some cases, the supernatant fluids were also assayed for SIV p27 concentration. Virus infectivity was assayed by development of CPE in C8166 cells as described above, and SIV p27 was measured by using a capture enzyme-linked immunosorbent assay (ELISA) kit from Coulter Laboratories (Hialeah, Fla.).

Virus recovery from CD8⁺-depleted PBMC. A total of 2×10^6 PBMC were incubated with a monoclonal antibody to CD8 (Dako-T8; Dako) for 1 h at 4°C and washed once, and sheep anti-mouse immunoglobulin G-coated paramagnetic beads (Dynal Laboratories) were added. The beads were removed with a magnet. This procedure routinely resulted in removal of 75 to 80% of the CD8⁺ T cells (6). Aliquots (2×10^6 cells each) of CD8⁺-depleted and control undepleted PBMC were cultured in PHA medium for 2 days and in IL-2 medium for 5 days as described above, after which the supernatant fluids were assayed for virus infectivity and SIVmac p27 content.

SIV p27 assay. Plasma and culture fluids were assayed for p27 by using a capture ELISA kit from Coulter. For each assay, a standard curve was prepared according to manufacturer's instructions, and p27 concentrations were calculated from the optical density at 450 nm, using linear regression analysis. The sensitivity of detection of p27 in these assays varied between 20 and 30 pg/ml.

PCR amplification of DNA. PCR was performed as follows. For amplification of SIVmac gp120 sequences, the oligonucleotide primers used in the first round were 5'-GGCTAAGGCTAATACATCTTCTGCATC-3' (sense) and 5'-ACCC AAGAACCCTAGCACAAAGACCCC-3' (antisense), which are complementary to bases 6565 to 6591 and 8179 and 8205, respectively, of SIVmac239 (12). One microgram of genomic DNA was used in the PCR, which was performed with a mixture containing 2.0 mM MgCl_2 , 200 μM each of the four deoxynucleotide triphosphates, 100 pM each oligonucleotide primer, and 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). The template was denatured at 92°C for 3 min, and PCR amplification was 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 55°C for 1 min, and primer extension at 72°C for 3 min. Amplification was completed by incubation of the PCR mixture for 10 min at 72°C . One microliter of the PCR product was used in a nested PCR performed as described above. For the second round of amplification, the nested primers were 5'-GTAAGTATGGGATGTCTTGGGAATC AG-3' (sense) and 5'-GACCCCTCTTTTATTCTTGAGGTGCC-3' (antisense),

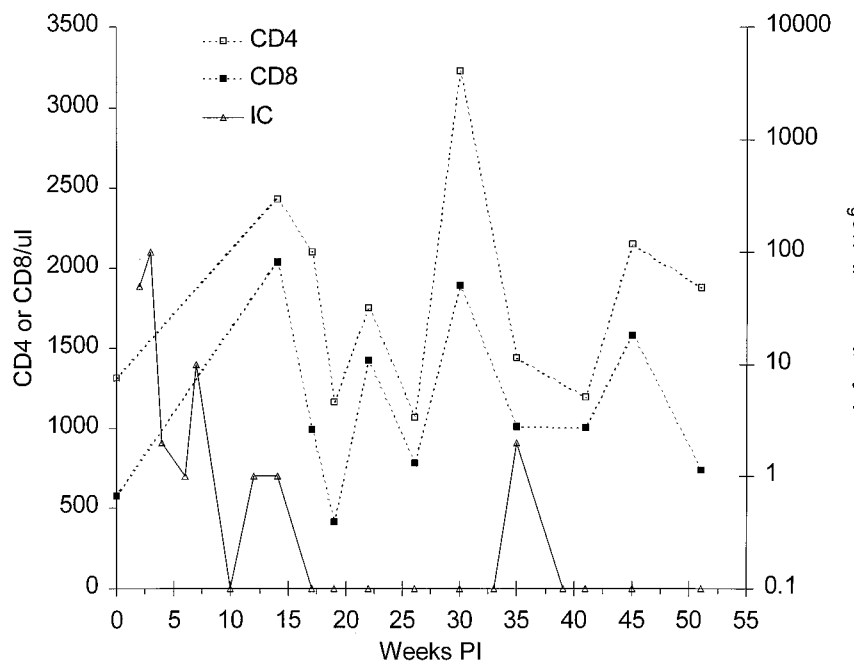


FIG. 2. Frequencies of infectious cells and of CD4⁺ and CD8⁺ cells in peripheral blood from rhesus macaque 8A. The infectious cell (IC) frequency was measured by inoculation of serial dilutions of PBMC into indicator cells and examining for CPE as described in the text; CD4⁺ and CD8⁺ frequencies were assessed by FACS analysis.

TABLE 1. CD4⁺, CD8⁺, and infectious cell frequencies in lymphoid tissues^a

Macaque	Tissue	Time (wk) p.i.	% CD4 ⁺	% CD8 ⁺	No. of infected cells/ 10 ⁶ examined	
8A	Lymph node	19	50	7	5	
	Spleen		22	10	1	
PQc	Spleen	16	13	13	1,000	
PPc	Spleen	16	6	13	1,000	
	Spleen	26 (necropsy)	1	8	100	
	Lymph node		1	12	10	
PNb	Thymus	Preinoculation	8	35	10,000	
	Spleen		12	17		
	Spleen		24 (necropsy)	<1	9	10
	Inguinal lymph node		<1	24	10	
	Mesenteric lymph node		<1	17	10	
15A	Thymus	4	2	33	10,000	
	Inguinal lymph node		2	42	1,000	
	Mesenteric lymph node		24	1	30	100
15B	Spleen	4	3	9	10	
	Inguinal lymph node		<1	36	100	
	Mesenteric lymph node		24	<1	15	10
	Spleen	31 (necropsy)	1	14	10	
	Mesenteric lymph node		1	8	10	
	Spleen		1	9	10	
	Spleen		1	9	10	

^a CD4⁺ and CD8⁺ frequencies were assessed by FACS analysis. The frequency of infectious cells was assayed by coculture with C8166 cells and examination for development of CPE.

which are complementary to bases 6598 to 6624 and 8158 to 8184, respectively, of the SIVmac239 genome.

For amplification of HIV-1 HXBc2 gp120 sequences, the oligonucleotide primers used in the first round were 5'-CAAAGAAAATAGACAGGTTAAT TGAT-3' (sense) and 5'-AGTGCTTCTGCTGCTCCCAAGAACC-3' (antisense), which are complementary to bases 6166 to 6192 and 7810 to 7784, respectively, of the HXBc2 genome (4). For the second round of amplification, the nested primers were 5'-GACTAATAGAAAGAGCAGAAGACAGTGG CA-3' (sense) and 5'-GAACAAAGCTCCTATTCCCACTGCTCT-3' (antisense), which are complementary to bases 6194 to 6223 and 7780 to 7754, respectively, of the HXBc2 genome. The conditions for amplification of the HXBc2 gp120 sequence were the same as described for the SIVmac sequence. A 10- μ l aliquot from each of the second-round amplifications was run on a 0.8% agarose gel, and bands were visualized by staining with ethidium bromide. To confirm the specificity of the PCR products, the DNA in the gel was transferred onto nitrocellulose and hybridized with ³²P-labeled gp120 probes generated from the gp120 gene of either SIVmac239 or HIV-1 HXBc2 (data not shown).

PCR/infected cell assay. The assay was performed as described previously (6). In the first round, oligonucleotide primers used were 5'-GATGGCGTGAGA AACTCCGCTCTT-3' and 5'-CCTCCTGCGCTAGATGGTGCTGTTG-3', which are complementary to bases 1052 to 1075 and 1423 to 1450, respectively, of the SIVmac239 gag gene (12). To standardize cell numbers, the fourth exon of β -actin was amplified with oligonucleotide primers 5'-TCATGTTTGAGACCT TCAACACCCAG-3' and 5'-CCAGGAAGGAAGGCTGGAAGAGTGCC-3' (noncoding), complementary to the published sequence (11). The PCR amplification conditions were as specified above. To increase the sensitivity of the reaction, 1 μ l of the first PCR product was used as a template for a second amplification performed under the same conditions. The nested SIV primers used were 5'-GTTGAAGCATGTAGTATGGGCAGC-3' and 5'-GCCTCAGG GCAGCGAACCCTCA-3', which are complementary to bases 1142 to 1165 and 1356 to 1382, respectively, of SIVmac239. The nested β -actin primers used were 5'-CCCCAGCCATGTACGTTGCTATCC-3' and 5'-GCCTCAGGGCAG CGGAACCCGCTCA-3'. Following the second round of amplification, a 10- μ l aliquot was removed and run on a 1.5% agarose gel, and bands were visualized by staining with ethidium bromide.

Fluorescence-activated cell sorting (FACS) analysis. Cells were reacted with a monoclonal antibody to CD4 (SIM.4; NIH AIDS Research and Reagents Repository) or CD8 (Dako-T8; Dako). After washing, the cells were stained with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Dako), fixed in 1% buffered formalin, and analyzed on an EPICS fluorescence-activated cell counter (Coulter).

Neutralizing antibody assays. We performed the assays as described previously (5). Briefly, serial doubling dilutions of plasma in RPMI were prepared in duplicate or quadruplicate in 96-well plates, 10 to 20 TCID₅₀ of the virus was added to each well, plates were incubated for 1 h at 37°C, and 10⁴ indicator cells were added to each well. Plates were observed for CPE 7 days later, wells were scored individually, and the 50% neutralization endpoint was calculated by the Kärber method (8).

Raji cell cocultures. Raji is a human B-lymphocyte cell line in which simian

retrovirus type D produces a characteristic CPE (10). Raji cells were maintained at a concentration of 5×10^5 /ml in RPMI containing 5% fetal bovine serum. PBMC or other mononuclear cells (2×10^6) were cultured with 2×10^6 Raji cells in a 25-cm² culture flask and examined for CPE for 7 days; 500 μ l was then transferred to another flask, fresh Raji cells were added, and the flasks were observed for CPE for a further 7 days.

RESULTS

A summary of the passage history is outlined in Fig. 1.

Passage 1. We inoculated 10⁴ TCID₅₀ of the chimeric SHIV into the BM of rhesus macaque 8A. Virus was readily isolated by cocultivation with indicator cells during the first 12 weeks postinoculation (p.i.). Rhesus macaque 8A had infectious cell frequencies of 10 to 100/10⁶ early in infection, but the frequency declined to <1/10⁶ PBMC by week 14 p.i., after which infectious cells were detected only once, at week 35 p.i. (Fig.

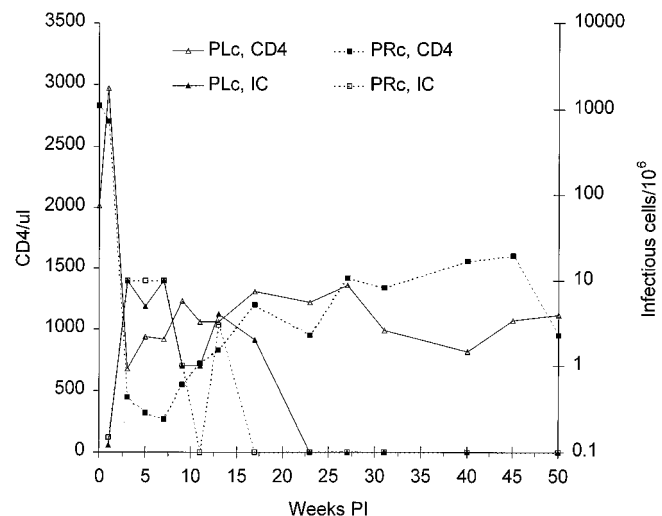


FIG. 3. Frequencies of infectious cells (IC) and CD4⁺ cells in peripheral blood from passage 2 animals. For details, see the legend to Fig. 2.

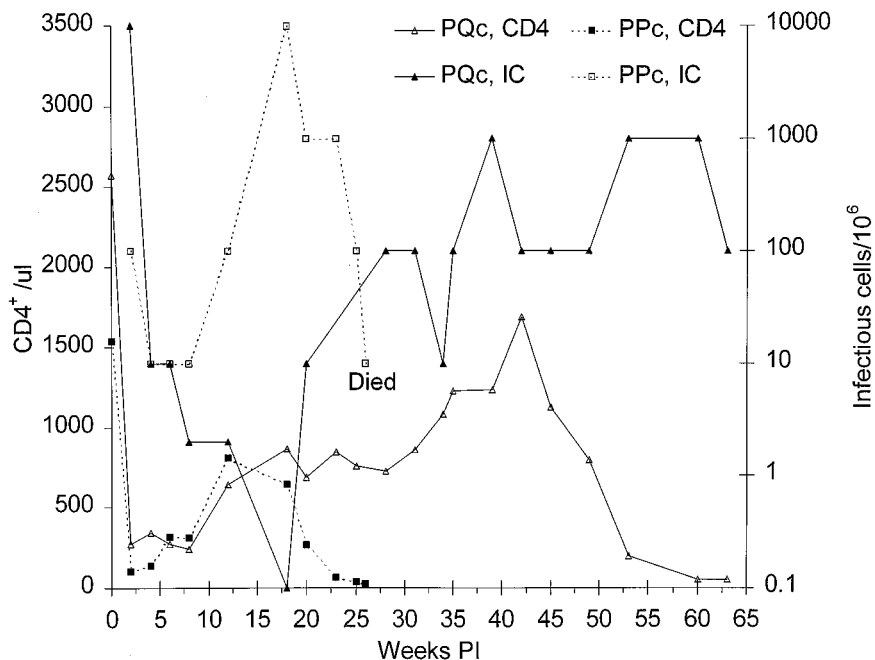


FIG. 4. Frequencies of infectious cells (IC) and CD4⁺ cells in peripheral blood from passage 3 animals. For details, see the legend to Fig. 2.

2). Plasma infectivity was not detected at any time. Plasma had 325 pg of SIV p27 per ml at week 2, but p27 was not present in later samples up to week 51. Supernatant fluids from PBMC cultured in PHA and then IL-2 yielded p27 at week 2 but not at week 4, 6, or 19 and did not have infectivity. PBMC at weeks 4 and 6 were depleted of CD8⁺ cells and cultured in PHA and IL-2. On both occasions, the supernatant fluids lacked p27 and infectivity.

CD4⁺ and CD8⁺ T-cell frequencies in PBMC remained normal in this animal during a follow-up period of 1 year p.i., and the CD4/CD8 ratio remained >1 at all times (Fig. 2). Biopsies of mesenteric lymph nodes and spleen were obtained surgically at 19 weeks p.i. Separated lymph node and spleen cells had low infectious cell frequencies (Table 1), although these levels were higher than in PBMC obtained at the same time (<1/10⁶; Fig. 2). FACS analysis of separated lymph node cells showed normal CD4⁺ and CD8⁺ frequencies. PBMC obtained at the same time had 28% CD4⁺ cells and 10% CD8⁺ cells. The remaining mononuclear cells from the lymph nodes included, among others, B cells, macrophages, and dendritic cells.

Passage 2. BM cells from rhesus macaque 8A were inoculated into two pig-tailed macaques, PLC and PRc, for passage 2, each animal receiving an inoculum of 5 × 10⁷ cells. Like rhesus macaque 8A, these two animals had infectious cell frequencies of 10 to 100/10⁶ early in infection, but the frequency declined to <1/10⁶ PBMC by week 17 p.i., after which infectious cells were not detected (Fig. 3). Plasma infectivity was not detected at any time. SIV p27 was detected in plasma from PRc only at week 3, while plasma from PLC was always negative. Supernatant fluids from PBMC cultured in PHA and then IL-2 yielded infectivity and p27 up to week 23 (PLc) or week 27 (PRc). However, CD8⁺-depleted and control undepleted PBMC obtained at weeks 31 and 36 from both animals did not yield either infectivity or p27.

In contrast to rhesus macaque 8A, a steep decline in CD4⁺ T-cell counts was seen in both PLC and PRc during the first 3

to 4 weeks p.i. (Fig. 3). Thereafter the counts increased gradually in both animals but remained at 1,000 to 1,500/μl, lower than the preinoculation CD4⁺ counts. These animals have maintained control of virus replication for more than a year and have remained healthy.

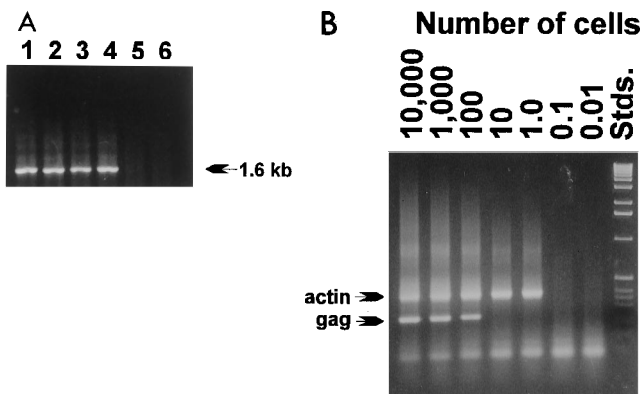


FIG. 5. PCR amplification of DNA from infected macaques. (A) Total cellular genomic DNA was extracted from splenic tissue of pig-tailed macaques PPc and PQc and used as a template in a nested PCR to amplify HIV-1 HXBc2 and SIVmac239 gp120 sequences. Lanes 1 to 3, amplification using oligonucleotide primers specific for HIV-1 HXBc2 gp120 sequences from a plasmid containing the SHIV genome (lane 1), PPc spleen DNA (lane 2), and PQc spleen DNA (lane 3). Lanes 4 to 6, amplification using oligonucleotide primers specific for SIVmac239 gp120 sequences from a plasmid containing the complete SIVmac239 genome DNA (lane 4), PPc spleen DNA (lane 5), and PQc spleen DNA (lane 6). Amplifications of gp120 sequences from uninfected animals, using either set of oligonucleotide primers, were negative (data not shown). (B) A PCR/infected cell assay was used to determine the number of virus-infected cells in PBMC from macaque PPc. Cell suspensions were diluted to 10⁷, 10⁶, 10⁵, 10⁴, 10³, and 10² cells per ml, and cells were then lysed and digested. Two rounds of PCR amplification were used to detect SIV gag and, as an internal control, β-actin sequences. After the second of amplification, the β-actin yielded a 393-bp band, whereas the amplified SIV gag sequence yielded a 240-bp band. Stds., standards.

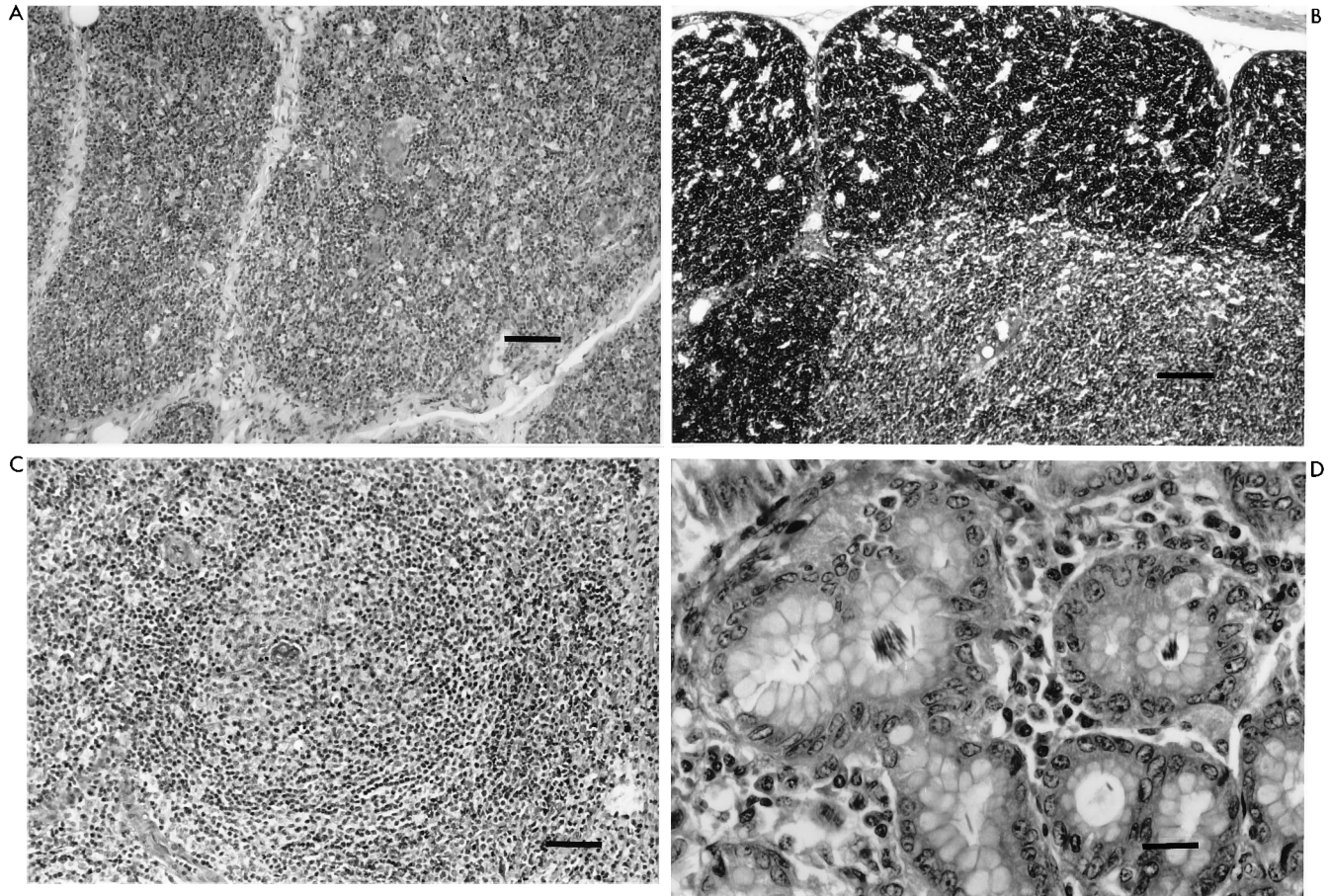


FIG. 6. (A) Photomicrograph of macaque PPc thymus, showing Hassall's corpuscles and severe depletion of cortical thymocytes. Hematoxylin-and-eosin stain; bar = 253 μ m. (B) Photomicrograph of a histologically normal thymus, showing clear distinction of cortex and medulla. Hematoxylin-and-eosin stain; bar = 253 μ m. (C) Photomicrograph of PPc spleen biopsy at 16 weeks p.i. Hematoxylin-and-eosin stain; bar = 63 μ m. (D) Photomicrograph of PPc stomach. Mycotic hyphae are visible in the glands. The lamina propria contains lymphocytes, plasma cells, and a few macrophages. Hematoxylin-and-eosin stain; bar = 63 μ m.

Passage 3. Five weeks after PLc and PRc were inoculated, 2 ml of BM was aspirated from both animals, pooled, and inoculated into the BM of two new pig-tailed macaques, PPc and PQc, for passage 3. PQc had a high infectious cell frequency ($10^4/10^6$ PBMC) at week 2, but the frequency declined rapidly to $10/10^6$ by week 4 and further to $<1/10^6$ at week 16 (Fig. 4). Thereafter, the infectious cell frequency showed a steady increase, to 10^2 to $10^3/10^6$ PBMC (Fig. 4). Plasma infectivity was never detected in plasma from PQc, and p27 was present only at week 2. As in the passage 2 animals, $CD4^+$ T-cell counts fell rapidly in PBMC from PQc during the first 2 weeks p.i. and remained low until week 8, after which a steady increase was seen till week 42, after which the $CD4^+$ count fell again to $50/\mu$ l by week 63.

The course of infection was more acute in PPc than in PQc. The infectious cell frequency in PBMC from PPc was initially low ($10^2/10^6$ PBMC) but decreased only slightly, to $10/10^6$ during weeks 4 to 8, after which it increased again to a peak of $10^4/10^6$ PBMC at week 18 and remained at 10^2 to $10^3/10^6$ until the animal was euthanized (Fig. 4). Plasma infectivity was detected only at week 23. However, p27 was detected in plasma at week 2 and again at weeks 24 and 26. As in PQc and the passage 2 animals, a steep decline in $CD4^+$ T-cell counts was seen in PPc during the first 2 weeks p.i. (Fig. 4). Thereafter, the $CD4^+$ count increased till week 12 and then fell to only $28/\mu$ l

at week 26, when the animal was euthanized in a moribund condition.

Biopsies of the spleen were obtained surgically from both PPc and PQc at 16 weeks p.i. The infectious cell frequency in separated spleen cells from both animals was $10^3/10^6$ cells. Spleen cells from PPc had 6% $CD4^+$ cells and 13% $CD8^+$ cells, while PQc spleen cells had 13% $CD4^+$ cells and 13% $CD8^+$ T cells. The remaining mononuclear spleen cells were mainly B cells, along with some macrophages and dendritic cells. Histological examination showed massive loss of cells in the T-cell-rich areas around the germinal centers. This loss was noted histologically as a depletion of the mantle zone lymphocytes, with relative sparing of the follicular centers.

The identity of virus isolated from PPc and PQc was confirmed by amplification of a 1.6-kb DNA fragment from PBMC DNA, using PCR with oligonucleotide primers specific for sequences encoding the gp120 of HIV-1 HXBc2 but not SIV-mac239 (Fig. 5A). Examination of splenocytes from a portion of spleen biopsied from macaques PPc and PQc at 16 weeks p.i. showed 10^3 infectious cells per 10^6 splenocytes, similar to the burden in PBMC at that time. Use of a PCR/infected cell assay confirmed the infected cell frequency of 10^3 to 10^4 cells per 10^6 splenocytes (Fig. 5B).

Macaque PPc developed progressive anemia and loss of weight and was euthanized at 26 weeks p.i. in a moribund

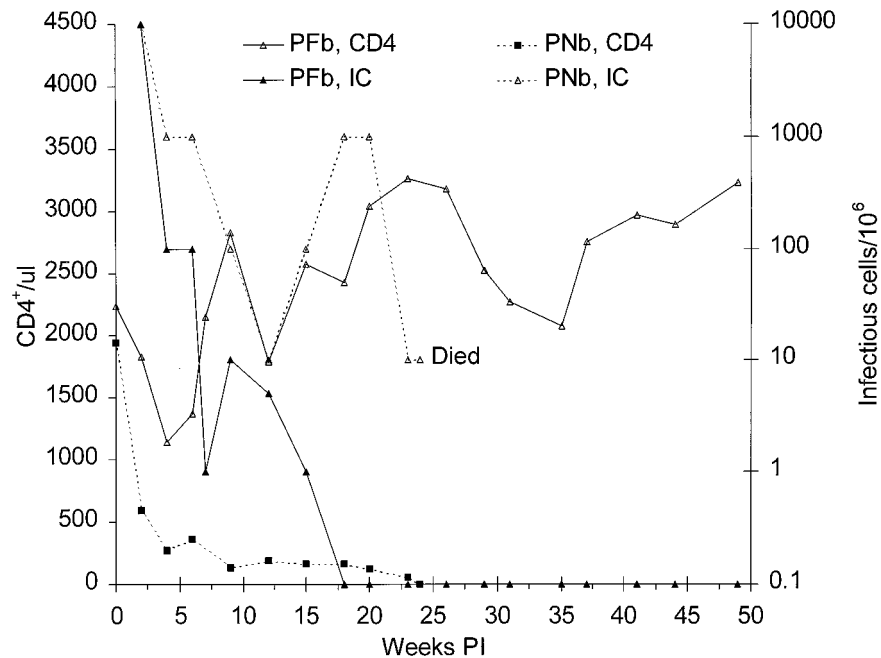


FIG. 7. Frequencies of infectious cells (IC) and $CD4^+$ cells in peripheral blood from passage 4 animals. For details, see the legend to Fig. 2.

condition. The $CD4^+$ count had fallen rapidly during the last 4 weeks, from $164/\mu\text{l}$ at week 22 to $64/\mu\text{l}$ at week 23 to $28/\mu\text{l}$ at week 25, and was only $28/\mu\text{l}$ at necropsy. This animal had whipworm (*Trichuris* sp.) infestation, which responded to treatment, although some parasites were still present at necropsy. Histological examination of the thymus revealed that the cortex and medulla were indistinguishable, but Hassall's corpuscles were visible in all areas, suggesting severe depletion of cortical thymocytes (Fig. 6A). Examination of the lymph nodes showed that cortices were very cellular with small lymphocytes, with minimal or no follicular development. Mantle zones and germinal centers were absent. The spleen contained numerous follicles, but germinal centers were poorly developed and mantle zones had reduced cellularity (Fig. 6C). The stomach contained microscopic mixed mononuclear cell inflammation in the lamina propria and mycotic hyphae in surface debris and in glands (Fig. 6D). The hyphae were morphologically consistent with *Candida* sp.

Separated lymph node, spleen, and thymus cells were used for FACS analysis and assessed for infectious cell frequency. Few $CD4^+$ cells were seen in the thymus, lymph nodes, and spleen (Table 1). Infectious cell frequencies in the thymus were 10 to 100 times higher than those in the lymph nodes and spleen. Interestingly, both the infectious cell frequency and the percentage of $CD4^+$ cells in the spleen were lower at necropsy than in the biopsy sample obtained at week 16 (Table 1). The B-cell and macrophage frequencies in the lymphoid tissues are not shown.

PBMC, splenocytes, lymph node cells, and BM cells from macaque PPc were cultured with Raji cells for 2 weeks to detect the presence of simian type D retroviruses. None of these cultures developed CPE. All animals were serologically negative for simian retroviruses.

Passage 4. BM cells and spleen cells obtained at 16 weeks p.i. from macaques PPc and PQc were pooled and inoculated into two new pig-tailed macaques, PFb and PNb. Infectious cell frequencies in PBMC from PFb were $10^4/10^6$ at week 2, but the virus burden decreased rapidly and by week 18 had fallen

to $<1/10^6$, a level that was maintained for the next 30 weeks. Plasma infectivity was not present at any time, while p27 was detected only at week 2. Supernatant fluids from PBMC cultured in PHA and then IL-2 yielded infectivity and p27 during the first 8 weeks, but neither $CD8^+$ -depleted nor control undepleted PBMC obtained at weeks 18 and 23 yielded either infectivity or p27. $CD4^+$ T-cell counts in blood from PFb declined during weeks 2 to 4 p.i. and then increased steadily to higher than the preinoculation level (Fig. 6).

In contrast to data from PFb, infectious cell frequencies in PNb remained high during the entire infection (Fig. 6). Plasma infectivity and SIVmac p27 were detected during the first 4 weeks and also during weeks 18 and 20. $CD4^+$ T-cell counts in PBMC from PNb fell dramatically during the first 2 weeks and remained low thereafter (Fig. 7). The animal developed severe progressive anemia and weakness and was euthanized at week 24 p.i. to prevent suffering. At necropsy, the blood hemoglobin concentration was only 5.2 g/dl, having declined from 11.4 g/dl at week 12 to 8.9 g/dl at week 20 to 6.2 g/dl at week 23. The $CD4^+$ T-cell count in peripheral blood of PNb was less than $10/\mu\text{l}$ at necropsy and had fallen steadily during the final 6 weeks, from $161/\mu\text{l}$ at week 18 to $122/\mu\text{l}$ at week 20 to $56/\mu\text{l}$ at week 23 (Fig. 6).

Histopathological lesions in the thymus, lymph nodes, spleen, and stomach of PNb were almost identical to those in PPc. In the thymus, severe depletion of cortical thymocytes was seen and the cortex and medulla were indistinguishable, although Hassall's corpuscles were visible in all areas. Lymph node cortices were very cellular, but with minimal or no follicular development, and germinal centers were absent. In the spleen, germinal centers were poorly developed and mantle zones had reduced cellularity in comparison with the normal histological appearance of a biopsy of the organ obtained prior to inoculation (Fig. 8). Histological examination of the gastrointestinal tract showed severe necrotizing mycotic gastritis, similar to that seen in PPc.

Separated lymph node, spleen, and thymus cells were used for FACS analysis and assessed for infectious cell frequency.

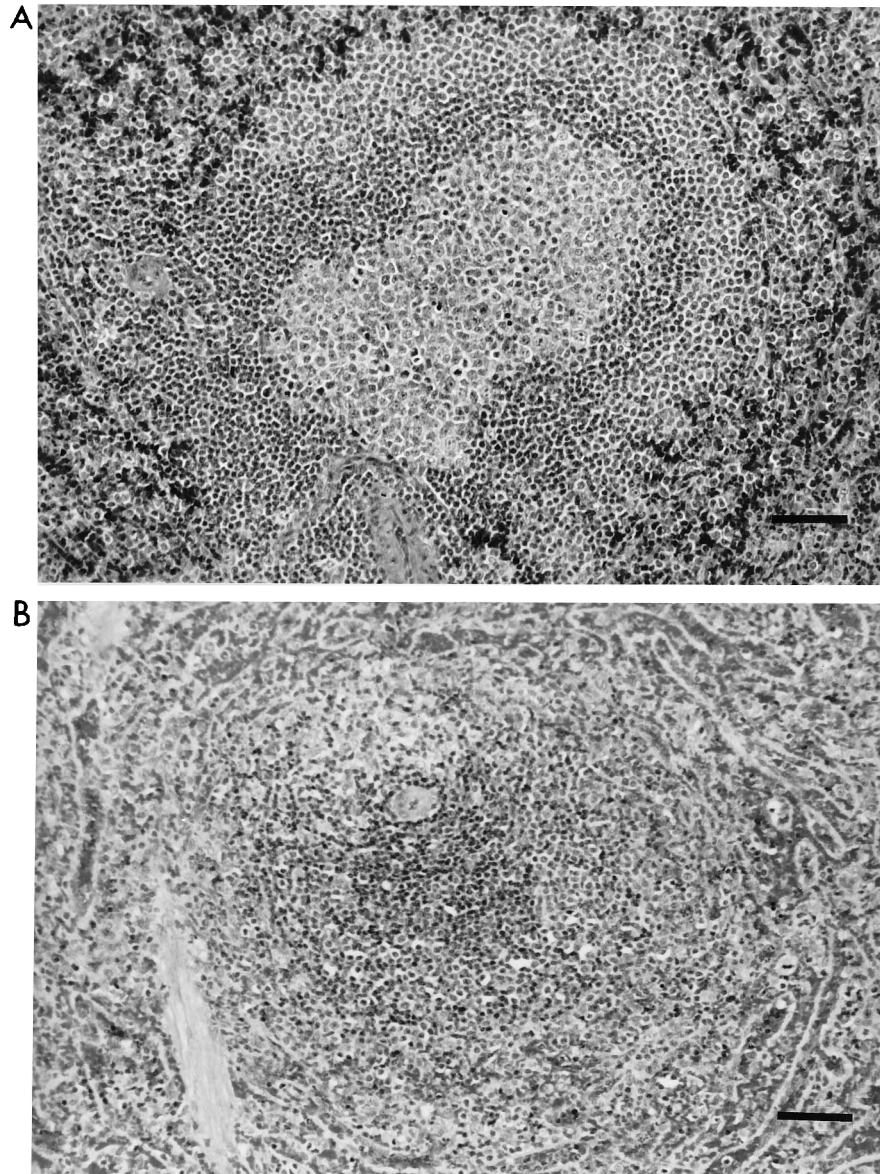


FIG. 8. Photomicrographs of macaque PNB spleen obtained preinoculation (A) or at necropsy (B). Note the diffuse loss of cells from the mantle zone and marginal zone and the absence of germinal centers in panel B. Hematoxylin-and-eosin stain; bars = 63 μ m.

The lymph nodes and spleen had <1% CD4⁺ cells, while only 1% of thymocytes were CD4⁺ (Table 1). Infectious cell frequencies in the thymus were 1,000 times higher ($10^4/10^6$) than in the lymph nodes and spleen ($10^1/10^6$).

PBMC, thymocytes, splenocytes, and lymph node cells from macaque PNB were cultured with Raji cells for 3 weeks (passed weekly) to detect the presence of simian type D retroviruses. None of these cultures developed CPE.

Passage 5. The cell-free virus stock prepared from macaque PNB had an infectivity titer of 10^4 TCID₅₀/ml in C8166 cells. Two 3-year-old pig-tailed macaques, 15A and 15B, were inoculated intravenously with 0.5 ml of this material. The animals had a uniform response, developing high infectious cell frequencies in PBMC and rapid CD4⁺ loss (Fig. 9). Plasma infectivity and p27 were detected in both animals (not shown). The loss of CD4⁺ cells was more rapid in both animals than in PNB. CD4⁺ counts in blood from 15A varied between 20/ μ l

and 60/ μ l during weeks 4 to 31. In contrast, CD4⁺ counts in blood from 15B were consistently <15/ μ l from weeks 2 to 31.

Biopsies of inguinal lymph nodes were obtained surgically at 4 weeks, while biopsies of the spleen and mesenteric lymph nodes were obtained at 24 weeks. FACS analysis of separated lymph node and spleen cells showed less than 2% CD4⁺ cells, establishing that the loss of CD4⁺ cells had occurred in lymphoid tissues as well as in peripheral blood and that this loss had taken place as early as 4 weeks p.i. The lymph nodes and spleen had minimal or no follicular development, and germinal centers were absent.

Macaque 15B developed uncontrollable diarrhea at week 27 and became progressively cachectic; the animal was euthanized at 31 weeks in an emaciated condition. Histological changes at necropsy in the thymus, lymph nodes, and spleen were similar to those seen in PPc and PNB. Both lungs showed areas of patchy interstitial pneumonia characteristic of *Pneumocystis*

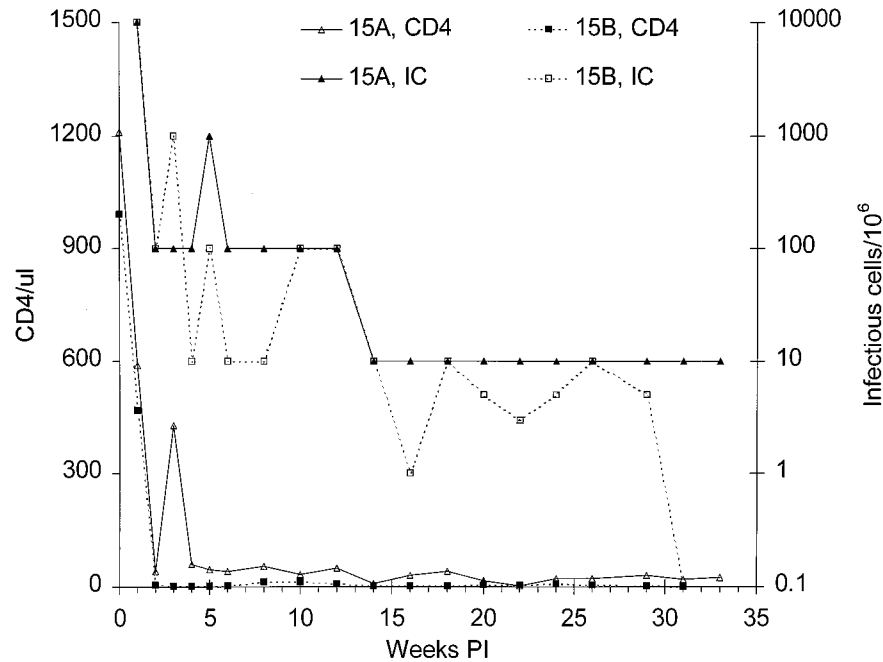


FIG. 9. Frequencies of infectious cells (IC) and CD4⁺ cells in peripheral blood from passage 5 animals. For details, see the legend to Fig. 2.

carinii infection, the presence of which was confirmed by Gomori methenamine silver staining.

Neutralizing antibodies. All animals in passages 1 to 4 developed neutralizing antibodies to SHIV and to HIV-1 (Table 2). Antibodies appeared as early as 8 weeks p.i., and neutralizing antibody titers remained steady for a prolonged period. Plasmas from all animals neutralized not only the original SHIV virus but also SHIVs isolated from macaques PPc and PQc with similar efficiencies (data not shown). Preinoculation plasmas from all animals did not neutralize any of these viruses. None of these plasmas neutralized SIVmac239.

DISCUSSION

We have established a nonhuman primate model of severe CD4⁺ T-cell loss and AIDS in pig-tailed macaques infected with animal-passaged SHIV. This is the first demonstration of a lentivirus bearing the HIV-1 envelope that is pathogenic in nonhuman primates. Disease became evident in pig-tailed macaques during passage 3, using BM cell inocula. Passage 5, with cell-free virus, resulted in rapid and severe CD4⁺ T-cell loss. Three animals (PPc, PNB, and 15B) developed AIDS and were euthanized in a moribund condition with low CD4⁺ T-cell counts (<50/ μ l) and opportunistic infections (candidiasis in PPc and PNB and *P. carinii* pneumonia in 15B). Two other animals, PQc and 15A, had CD4⁺ T-cell counts of <50/ μ l at 63 and 33 weeks, respectively, p.i. We have shown that cell-free virus caused disease in macaque 15B. Thus, this virus, which was isolated from infected animals and cultured in vitro, reproduced the disease upon reinoculation into another animal, from which the virus was reisolated.

All animals in this study were screened for antibodies to simian type D retrovirus, and PBMC, lymph node, spleen, BM, and thymus cells from the animals that developed AIDS were cocultured with Raji cells. The absence of antibodies to type D retrovirus and the absence of CPE in Raji cell cocultures rule out the possibility that the disease was due to simian type D retroviruses. Moreover, pathogenic type D retroviruses infect

and deplete T and B cells; in contrast, animals in this study developed loss of only CD4⁺ T cells.

Inoculation of the original SHIV in rhesus macaque 8A resulted in sustained infection but not in disease. The animal did not develop loss of CD4⁺ T cells, and virus burdens in peripheral blood and lymphoid tissues subsided within the first 12 weeks to barely detectable levels. This pattern is similar to the results obtained by other investigators, who inoculated rhesus or cynomolgus macaques with various strains of SHIV (2, 9, 13, 15, 17). During passage 2, the pig-tailed macaques PRc and PLc, which were inoculated with BM cells from rhesus macaque 8A, developed initial loss of CD4⁺ T cells, but the cell count recovered to approximately 50% of preinoculation values. This correlated with effective control over virus replication, since by week 25 virus could not be obtained from PBMC, even after depletion of CD8⁺ T cells. The end result was similar to that observed in rhesus macaque 8A. Evidence of increased virulence of SHIV emerged during passage 3, when macaques PPc and PQc were inoculated with BM cells from PRc and PLc. These animals developed a more severe

TABLE 2. Neutralizing antibody titers in SHIV-infected animals^a

Macaque	Neutralizing antibody titer vs:	
	SHIV	HIV-1 HXBc3
8A	64	130
PLc	80	64
PRc	130	320
PPc	64	160
PQc	64	80
PFb	64	130
PNb	32	32

^a Serial dilutions of heat-inactivated plasma from infected animals were incubated with 10 to 20 TCID₅₀ of stock virus for 1 h, after which indicator cells were added, and plates were observed for development of CPE. Plasma samples from all animals neutralized viruses isolated from PPc and PQc as effectively as the original SHIV. In all macaques, titers against SIVmac239 were <10.

initial loss of CD4⁺ T cells. In both animals, the cell count recovered, but in PPc, the cell count dropped precipitously again, ending in development of severe AIDS at 25 weeks. This second phase of CD4⁺ T-cell loss correlated with increased virus burdens. PQc developed a similar pattern of disease extended over a longer time period. This animal was still healthy at 63 weeks, but as can be seen in Fig. 4, the prognosis appears to be poor. The virus appeared more virulent in passage 4. The CD4⁺ T-cell count in one of the two macaques, PNB, fell rapidly during the first 2 weeks and never recovered. Macaque PFb became infected, but this animal appeared to control the infection and did not develop CD4⁺ T-cell loss, possibly reflecting variation among individual animals or the virus selected from its BM inoculum.

In contrast to the original cell-free SHIV, which caused no serious pathological effects during two successive passages in macaques, the cell-free virus prepared from passage 4 was extremely virulent. Both animals that were inoculated intravenously with this stock, 15A and 15B, developed severe sustained loss of CD4⁺ T cells and high virus burdens in the blood and lymphoid tissues. One of the two, 15B, developed diarrhea, cachexia, and *P. carinii* pneumonia at week 29 and had to be euthanized. The other, 15A, was still healthy at 33 weeks, but the prognosis appears to be poor.

Analysis of tissues from the three animals that developed AIDS showed that the CD4⁺ T-cell count seen in the PBMC was reflected by similar changes in the lymphoid tissues, and this correlated with the intensity of virus replication. The loss of CD4⁺ cells in blood represented a global loss rather than new regional localization patterns. It is interesting that in both PPc and PNB, thymocytes had a higher frequency of infectious cells than mononuclear cells from the secondary lymphoid tissues (lymph nodes and spleen). Whether this reflects sequential pattern of infection by this virus in primary and secondary lymphoid tissues or a more prolonged infection in the thymus is not clear. Thymocytes were not obtained from 15B, since this animal had severe thymic atrophy.

A characteristic feature of human AIDS is an increase in virus burden during the terminal phase. This increase was seen in two of the macaques (PPc and PNB) that developed terminal disease. The upsurge in virus replication was manifested as increases in plasma infectivity, plasma p27, and infectious cell frequencies in PBMC.

The development of neutralizing antibodies to HIV-1 in the inoculated animals is similar to observations of HIV infection in humans. Similar to the infection in humans, the presence of neutralizing antibodies did not have any apparent protective effects. Whether such antibodies would have protective effects in passive immunization experiments will be tested in the future. A number of potential vaccines against HIV-1 infections rely on the induction of a neutralizing antibody response. The SHIV/pig-tailed macaque system thus offers a model in which such vaccines can be evaluated.

In summary, our studies have shown that an SHIV whose *tat*, *rev*, *vpu*, and *env* genes were derived from a laboratory strain of HIV-1 increased in virulence upon passage of infectious BM cells from animal to animal, with the final derivation of a cell-free stock containing pathogenic virus. Increasing virus burdens in PBMC and lymphoid tissues, development of plasma viremia, progressive loss of CD4⁺ T cells from the

blood, lymph nodes, and spleen, severe infection in the thymus, and development of AIDS have all been documented. The pathogenic SHIV model utilizing a cell-free virus stock, described here for the first time, now provides the means for studying pathogenesis of HIV-1 infection in so far as the viral envelope contributes to the disease. It also provides a disease-causing challenge virus for evaluating the efficacy of HIV-1 envelope vaccines and anti-HIV-1 drugs.

ACKNOWLEDGMENTS

We thank J. Sodroski for providing SHIV DNA and Peter Nara for providing HIV-1 HXBc3.

This work was supported by grants AI-29382, NS-32203, and RR-06753 from the National Institutes of Health.

REFERENCES

- Barnett, S. W., K. K. Murthy, B. G. Herndier, and J. A. Levy. 1994. An AIDS-like condition induced in baboons by HIV-2. *Science* **266**:642-646.
- Cheng-Mayer, C., J. A. Levy, and P. A. Luciw. 1994. Infection of rhesus macaques with T-cell-line tropic and macrophage-tropic SHIVs. *Int. Conf. AIDS* **10**:61.
- Desrosiers, R. C. 1990. The simian immunodeficiency viruses. *Annu. Rev. Immunol.* **8**:557-578.
- Fisher, A. G., E. Collalti, L. Ratner, R. C. Gallo, and F. Wong-Staal. 1985. A molecular clone of HTLV-III with biological activity. *Nature (London)* **316**:262-265.
- Joag, S. V., M. G. Anderson, J. E. Clements, M. F. McEntee, D. P. Sharma, R. J. Adams, and O. Narayan. 1993. Antigenic variation of molecularly cloned SIV_{mac}239 during persistent infection in a rhesus macaque. *Virology* **195**:406-412.
- Joag, S. V., E. B. Stephens, R. J. Adams, L. Foresman, and O. Narayan. 1994. Pathogenesis of SIV_{mac} infection in Chinese and Indian rhesus macaques: effects of splenectomy on virus burden. *Virology* **200**:436-446.
- Joag, S. V., E. B. Stephens, and O. Narayan. 1995. Lentiviruses, p. 1977-1996. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), *Virology*. Raven Press, New York.
- Lenette, E. H. 1969. General principles underlying laboratory diagnosis of viral and rickettsial infections, p. 1-65. *In* E. H. Lenette and N. J. Schmidt (ed.), *Diagnostic procedures for viral and rickettsial infections*. American Public Health Association, New York.
- Li, J., C. I. Lord, W. Haseltine, N. L. Letvin, and J. Sodroski. 1992. Infection of cynomolgus monkeys with a chimeric HIV-1/SIV_{mac}X virus that expresses the HIV-1 envelope glycoproteins. *J. Acquired Immune Defic. Syndr.* **5**:639-646.
- Maul, D. H., C. P. Zaiss, M. R. MacKenzie, S. M. Shiigi, P. A. Marx, and M. B. Gardner. 1988. Simian retrovirus D serogroup 1 has a broad cellular tropism for lymphoid and nonlymphoid cells. *J. Virol.* **62**:1768-1773.
- Nakajima-Iijima, S., H. Hamada, P. Reddy, and T. Kakunaga. 1985. Molecular structure of the human cytoplasmic beta-actin gene: interspecies homology of sequences in the intron. *Proc. Natl. Acad. Sci. USA* **82**:6133-6137.
- Regier, D. A., and R. C. Desrosiers. 1990. The complete nucleotide sequence of a pathogenic molecular clone of simian immunodeficiency virus. *AIDS Res. Hum. Retroviruses* **6**:1221-1231.
- Sakuragi, S., R. Shibata, R. Mukai, T. Komatsu, M. Fukasawa, H. Sakai, J. Sakuragi, M. Kawamura, K. Ibuki, M. Hayami, et al. 1992. Infection of macaque monkeys with a chimeric human and simian immunodeficiency virus. *J. Gen. Virol.* **73**:2983-2987.
- Sharma, D. P., M. C. Zink, M. Anderson, R. Adams, J. E. Clements, S. V. Joag, and O. Narayan. 1992. Derivation of neurotropic simian immunodeficiency virus from exclusively lymphocytotropic parental virus: pathogenesis of infection in macaques. *J. Virol.* **66**:3550-3556.
- Shibata, R., and A. Adachi. 1992. SIV/HIV recombinants and their use in studying biological properties. *AIDS Res. Hum. Retroviruses* **8**:403-409.
- Spertzel, R. O. 1989. Animal models of human immunodeficiency virus infection. *Public Health Service Animal Models Committee. Antiviral Res.* **12**:223-230.
- Yamamoto, H., T. Igarashi, Y. Ami, T. Komatsu, R. Shibata, T. Kuwata, A. Adachi, H. Shida, M. Tanaka, and M. Hayami. 1994. HIV-1 env specific killer lymphocytes in macaque monkeys infected with SIV_{mac} HIV-1 chimeric viruses. *Int. Conf. AIDS* **10**:75.