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Characteristics of a Pathogenic Molecular Clone of an End-Stage Serum-Derived Variant of Simian Immunodeficiency Virus (SIV_{F359})

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End-stage simian immunodeficiency virus (SIV) isolates are suggested to be the most fit of the evolved virulent variants that precipitate the progression to AIDS. To determine if there were common characteristics of end-stage variants which emerge from accelerated cases of AIDS, a molecular clone was derived directly from serum following in vivo selection of a highly virulent SIV isolate obtained by serial end-stage passage in rhesus monkeys (*Macaca mulatta*). This dominant variant caused a marked cytopathic effect and replicated to very high levels in activated but not resting peripheral blood lymphocytes. Furthermore, although this clone infected but did not replicate to detectable levels in rhesus monocyte-derived macrophages, these cells were able to transmit infection to autologous T cells upon contact. Interestingly, although at low doses this end-stage variant did not use any of the known coreceptors except CCR5, it was able to infect and replicate in human peripheral blood mononuclear cells homozygous for the Δ32 deletion of CCR5, suggesting the use of a novel coreceptor. It represents the first pathogenic molecular clone of SIV derived from viral RNA in serum and provides evidence that not only the genetic but also the biological characteristics acquired by highly fit late-stage disease variants may be distinct in different hosts.

Simian immunodeficiency virus (SIV) of sooty mangabeys causes AIDS in macaques, providing an important animal model for human immunodeficiency virus (HIV)-induced AIDS in humans (13, 19, 21, 31, 41). Molecular clones of HIV and SIV have been valuable for addressing specific questions in AIDS pathogenesis (29, 45, 53), vaccine development (6, 9, 10, 13, 30, 56, 60), and the evaluation of antiviral drugs (2, 63). To date, molecular clones of SIV have been derived from proviral DNA rather than viral RNA, and most proviral clones have been obtained from cultured cells and frequently from infected human cell lines (14, 23, 31, 36, 45, 50, 52). It has been demonstrated that by in vitro propagation certain viral variants are selected (20, 65). In particular, growth of virus in human cell lines results in major changes in the SIV genome, such as deletions leading to truncation of the transmembrane envelope protein (7, 24, 34, 35). This has resulted in important biological differences between the derived clones and the original pathogenic virus population in the host. In addition, proviral DNA frequently contains a high proportion of defective proviruses (37, 42, 43, 47, 66).

Recently it has been demonstrated that late-stage SIV_{mne} variants in pigtail macaques are highly fit, having acquired multiple mutations encoded at several genetic loci that facilitate immune escape and increase replication and cytopathic properties (32). These observations have recently been supported by another line of evidence. A series of in vivo passage studies were performed in which blood samples taken at the time of AIDS development were subsequently used to infect naive rhesus macaques. End-stage blood samples were taken from the most rapidly progressing animal and passaged again in vivo. This in vivo passage of end-stage variants resulted in a progressively accelerated disease course with each successive passage until the fourth passage, by which time AIDS had developed in as little time as 2 weeks (28). Taken together, the results of these two independent lines of investigation suggested that the passage of primate lentiviruses late in disease could result in the transmission of highly virulent variants capable of causing rapid progression to AIDS. The data suggested that highly fit end-stage or late-stage fitness variants had common biological properties (32).

The provirus population in mononuclear cells in vivo is generally considered to be a sanctuary of biological variants which have accumulated in such intracellular reservoirs as a consequence of previous host immune pressures and/or defective viral replication (66). Alternatively, we reasoned that extracellular virions represented the most actively replicating, dominant virus population, having become the most predominant in the host at a particular stage in disease development. SIV and HIV clones that are derived directly from extracellular virus populations in biological fluids such as serum have not been characterized or evaluated for virulence in vivo. A key feature of lentivirus pathogenesis is a persistent high-level cell-free viremia. Since the predominance of certain extracellular lenti-

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viruses after seroconversion is most likely the result of escape from immune surveillance or escape from drug therapy during treatment, such viral variants are of particular biological interest. Recently we developed a strategy to generate pathogenic clones directly from extracellular virions present in the circulation, in serum or plasma (25, 26). Using this strategy, we derived a full-length infectious molecular clone of SIV_{8980}, an end-stage isolate from a macaque which had progressed rapidly to AIDS following serial end-stage passage of SIV_{B670} in vivo (27). Sequence analysis revealed a unique relationship, placing this virus between the two groups of SIV_{sm} and SIV_{mac} primate lentiviruses. During in vivo passage the variability of the V1 envelope region decreased as virulence increased. The SIV_{F359} molecular clone represented the most dominant variant that had emerged during end-stage passage. This variant was highly cytopathic and replicated to high titers in vivo. It was predominantly T-cell tropic, infecting macrophages but not productively replicating in macrophages at detectable levels. Interestingly, if autologous lymphocytes were placed in direct contact with macrophages exposed to this clone, a very high level of viral production was found in the culture supernatants. Of all the known coreceptors, SIV_{F359} was highly selective for CCR5. However, it could replicate in human peripheral blood mononuclear cells (PBMC) homozygous for the Δ32 deletion, suggesting the possible use of a novel coreceptor.

MATERIALS AND METHODS

Molecular clone derived from serum. SIV_{8980} was derived from SIV_{B670} by four in vivo passages in Indian rhesus macaques. Monkey 8980 rapidly progressed to AIDS following the fourth in vivo passage (27). Serum from this animal was, without culture, directly used to derive the F359 molecular clone of SIV (25). Since the synthesis of full-length (10-kb) SIV cDNA molecules from small amounts of RNA templates proved to be very difficult, a modified reverse transcription-PCR technique was developed to separately generate 5′ and 3′ halves of the SIV genome (26). By ligating these two 5-kb fragments, we were able to reconstitute the SIV_{F359} infectious molecular clone directly from serum as we have previously described (25).

Infectivity in vitro. Primary rhesus PBMC cultures were maintained with medium changes every 2 days and were observed regularly for cytopathic effect (CPE). Infection was confirmed by immunocytochemistry for the expression of SIV Gag antigen. Single-cell preparations for immunocytochemistry were prepared on acetone-cleaned glass slides which had been air dried for 30 min. Cells were fixed in acetone-methanol (1:1) and in ethanol (70%) for 15 and 30 min, respectively. Slides were washed in 0.05 M Tris-HCl (pH 7.6)–0.1 M NaCl for 5 min and incubated with 20 μl (1:25 dilution) of anti-Gag monoclonal antibody (51). Cells were washed for 5 min and incubated with goat anti-mouse immunoglobulin G (IgG) antibody for 30 min at room temperature. To amplify the signal, the cells were then washed and incubated with mouse anti-alkaline phosphatase (APAAP complex; Boehringer). Cells were washed for 5 min and were incubated with 20 μl of substrate solution (0.1 M Tris-HCl [pH 9.5], nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate, 5 mM levamisole [10:1:1]) for 30 min at room temperature. Cells were washed for 5 min in tap water, 1 drop of 50% glycerol–phosphate-buffered saline (PBS) was added per slide, and the cells was covered with a coverslip. The preparations were examined at a magnification of ×40,and CPE was quantified. In cell culture supernatants virus was quantified by measuring p27 concentrations (SIV p27 antigen capture enzyme-linked immunosorbent assay; Coulter Corp., Hialeah, Fla.).

Pathogenicity. Adult rhesus macaques (*Macaca mulatta*) used in this study were housed at the animal facility of the Biomedical Primate Research Centre, Rijswijk, The Netherlands. Animals were negative for SIV, simian T-cell leukemia virus, and simian D type retroviruses. Two outbred Indian rhesus macaques were inoculated intravenously with 50 50% tissue culture infective doses ($TCID_{50}$) of the SIV_{F359} stock grown on rhesus PBMC. EDTA-treated blood samples were collected every 2 weeks postinfection for quantitative virus isolations (QVI) from PBMC and for determination of SIV p27 antigen in plasma. Rhesus monkeys that developed clinical evidence of AIDS were euthanized, and full pathological analysis was performed to confirm the diagnosis. For histological examination, tissues were formalin fixed and paraplast embedded. Four-micrometer-thick sections were stained with hematoxylin and eosin. For the detection of microsporidia, Gram staining was applied on gall bladder sections.

For QVI, PBMC were prepared from EDTA-treated blood by lymphocyte separation medium density gradient centrifugation. Cells at the interface were collected and washed twice with RPMI. Twofold dilutions of PBMC (starting with 10^6 cells) were cocultured with 2.5×10^5 cells of the human T-cell line C8166 in a 24-well plate (Greiner Labortechnik, Alphen a/d Rijn, The Netherlands) in duplicate. Cell culture medium (RPMI with 10% fetal calf serum [FCS]) was partly changed twice a week. The cell cultures were screened regularly for the presence of CPE.

The phenotype of rhesus PBMC was assessed by two-color fluorescence-activated cell sorter (FACS) analysis. Briefly, heparinized blood (100 μl) was incubated with 10 μl of monoclonal antibody mix at room temperature. After incubation, 2.5 ml of lysing solution (Becton Dickinson, Etten-Leur, The Netherlands) was added, followed by an incubation at room temperature for 10 min and then centrifugation for 10 min at 200 × *g*. Four milliliters of PBS with 2% formaldehyde was added, and the tubes were centrifuged for 10 min at 200 × *g*. The supernatant was aspirated, and the cells were resuspended in 5 ml of PBS with 2% formaldehyde and stored overnight at 4°C. Flow cytometry was performed on a FACScan using the CellQuest software (Becton Dickinson), with 5,000 events analyzed. To assess CD4 T-cell levels in peripheral blood, the following monoclonal antibodies were used: an anti-CD3 monoclonal antibody (FN18; Biomedical Primate Research Centre) covalently coupled to fluorescent isothiocyanate-phycoerythrin and an anti-CD4 monoclonal antibody (SK3; Becton Dickinson) covalently linked to phycoerythrin conjugate. Once it was determined that this molecular clone was pathogenic and caused AIDS in two animals, an additional eight rhesus monkeys were infected and monitored for the time to development of AIDS.

Cell tropism. To assess susceptibility to infection of resting and activated lymphocytes, blood was taken from healthy rhesus monkeys which were negative for SIV, simian T-cell leukemia virus, and type D retroviruses. PBMC were isolated by lymphocyte separation medium density gradient centrifugation and were washed twice with RPMI. Activated lymphocytes were prepared by concanavalin A mitogen stimulation (5 μg/ml; 48 h) and interleukin-2 (IL-2) treatment (50 U/ml, starting after virus adsorption and continuing throughout the experiment). Resting lymphocytes were cultured in RPMI (plus 10% FCS) without phytohemagglutinin and IL-2. Resting and stimulated lymphocytes were distinguished by double labeling with anti-CD3 antibody specific for T cells and anti-MIB-1 antibody specific for the cellular proliferation marker K_i-67 (3, 15, 40). Resting and stimulated cell cultures (5×10^6 cells) were simultaneously infected with 100 $TCID_{50}$ of $SIV_{mac239/YEnef}$ (a molecular clone capable of proliferating in resting cells) (15) or of the SIV_{F359} clone at 37°C for 18 h. Unbound virus particles were removed by washing the cell pellets five times with 5 ml of RPMI (plus 10% FCS), and the cells were cultured for 12 days in RPMI (plus 10% FCS) either with or without IL-2 for stimulated or resting PBMC, respectively. Supernatants were monitored for the production of virus p27 by antigen capture enzyme-linked immunosorbent assay. The absence of K_i-67 staining was used to confirm that resting lymphocyte cultures remained in a quiescent state.

SIV p27 *gag* expression in monocyte-derived macrophage (MDM) and PBMC cultures was studied by double-staining immunocytochemistry. Briefly, cells were incubated with a mixture of the mouse anti-Gag monoclonal antibody 2E4 (IgG2a; kindly provided by M. Niedrig [51]) and mouse anti-CD68 monoclonal antibody KP1 (IgG1; DAKO, Glostrup, Denmark), which was used to costain macrophages. Subsequently, slides were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG2a subclass-specific antibody (Southern Biotechnology Inc., Birmingham, Ala.) and horseradish peroxidase-conjugated goat anti-mouse IgG1 subclass-specific antibody (Southern Biotechnology). All incubation steps were performed at 20°C for 30 min. Endogenous peroxidases were blocked with 0.1% NaN_3 plus 0.3% H_2O_2 in PBS after the incubation with the first antibody. Alkaline phosphatase activity was detected with naphthol-AS-MX phosphate (Sigma Chemical Co., St. Louis, Mo.) and Fast Blue BB (Sigma) in 0.1 M Tris-HCl (pH 8.5) (20 min in the dark), yielding a blue color. Horseradish peroxidase activity was detected using H_2O_2 (0.03%) and 3-amino-9-ethylcarbazole (Sigma), yielding a red color.

To determine if SIV_{F359} was able to infect rhesus MDM cultures, lymphocyte separation medium-isolated PBMC were seeded at a concentration of 5×10^6 cells per ml in 24-well plates in RPMI with 10% FCS. Adherence was allowed to continue for 5 days. Prior to infection, nonadherent cells were separated from adherent cells by rigorous washing with culture medium. Adherent cells were checked for purity (>98%) and for being macrophages by demonstrating the presence of the cell surface marker CD68 and by microscopic examination of their characteristic morphology. After infection with SIV_{mac316} (a macrophage-tropic molecular clone of SIV used as a positive control) (48), SIV_{8980} (the

parental strain of SIV_{F359}), or SIV_{F359}, unbound virus was removed by washing the cells twice. MDM cultures were maintained for 12 days in RPMI 1640 medium supplemented with 20% FCS, penicillin, and streptomycin with medium changes once per week. At day 12 samples were analyzed for intracellular *gag* expression and the presence of SIV p27 Gag antigen in supernatants.

Studies were subsequently undertaken to determine if cell-cell contact between MDM previously exposed to virus could result in productive infection of autologous lymphocytes. MDM cultures (prepared as described above) were exposed to either the parental SIV_{8980} or the molecular clone variant SIV_{F359} (both at 3,000 $TCID_{50}$). On the following day residual virus was removed from cultures by vigorous washing at least three times. Specific infection was determined by double staining of MDM for the specific marker CD68 as well as viral p27. Virus replication in these cultures was determined by the amount of p27 present in the culture supernatants at days 0, 6, and 12 postinfection. On day 12 macrophage cultures were extensively washed, and autologous concanavalin A-stimulated rhesus lymphocytes (2×10^6) were added to the SIV_{8980}- and SIV_{F359}-exposed MDM cultures. Virus replication and production in autologous lymphocytes after cocultivation with MDM cell interaction was measured by the amount of p27 which accumulated in the culture supernatants at days 0, 2, 6, 12, and 14 following cocultivation of the two different cell populations.

Coreceptor studies. The coreceptor usage of the molecularly cloned virus was determined by three different assays. The first assay involved the use of HOS-$CD4^+$ cell lines expressing either the macaque or the human CCR5 and was based on immunostaining of SIV-infected cells. The HOS-$CD4^+$ cell lines were infected with SIV_{F359} by adding 10^4 $TCID_{50}$ of virus per ml to the adherent cells in 3 ml of medium. After 72 h, cells were analyzed for syncytium formation, washed once in serum-free medium, fixed in methanol-acetone (50:50) for 2 min at −20°C, and washed twice in PBS supplemented with 1% FCS. Anti-Gag mouse monoclonal antibody (0.6 ml/well) was added, and the cells were incubated for 1 h at room temperature and washed three times in PBS supplemented with 1% FCS. Goat anti-mouse β-galactosidase-conjugated polyclonal antibodies (0.6 ml/well) were added, and the cells were incubated for 1 h at room temperature and washed three times in serum-free PBS. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) substrate (0.6 ml/well) was added, and the preparation was incubated in a sealed box for 30 min at 37°C. For quantification the stained cells were washed three times in PBS.

The second assay used the astroglia cell line U87 stably expressing human CD4 and one of the chemokine receptors CCR2b, CCR3, CCR5, or CXCR4. These cells were seeded in 24-well plates at 2×10^4 cells per well in 1 ml of medium. Infection was performed overnight at 37°C with 10-fold serial dilutions of virus (1-ml final volume) beginning with a 1:8 dilution of the SIV_{F359} stock. After infection, the cultures were washed three times with Dulbecco's modified Eagle medium (DMEM) (Gibco) and cultured for 13 days. Medium was changed twice a week. Cultures were examined microscopically for CPE, and supernatants, collected at several time points after infection, were tested for p27 concentration.

In the third assay several CD4-transformed human osteosarcoma HOS cell lines were used, expressing the chemokine receptors CCR1, CCR2b, CCR3, CCR4, CCR5, CXCR4, BOB/GPR15, Bonzo/STRL33, CXCR1 (V28), CCR8, APJ, GPR1, and US28 (the reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, from V. N. KewalRamáni and D. R. Littman). The CD4-transformed (under neomycin selection) HOS parental cells containing the HIV type 2 (HIV-2) long terminal repeat driving green fluorescent protein introduced via cotransfection with the cytomegalovirus promoter driving a hygromycin-resistant construct were maintained in DMEM supplemented with 10% FCS under selection with neomycin (G418 [0.5 mg/ml]; Gibco) and hygromycin (100 μg/ml). Coreceptor genes were introduced via retroviral infection with the pBABE-puro vector (11, 39) under selection with puromycin (1 μg/ml; Calbiochem, La Jolla, Calif.). For cell-free infection experiments, HOS-CD4 cells expressing the different coreceptors were seeded at 2×10^4 cells per well (2 ml) in 12-well plates and cultured in DMEM with 10% FCS. The next day, infection with the virus stocks (500 μl/well) was performed in the presence of Polybrene (20 μg/ml) overnight at 37°C. After infection, the cultures were washed and cultured for another day. Forty-eight hours after infection, cells were analyzed for green fluorescent protein fluorescence by FACS.

DNA sequencing and phylogenetic analyses. Double-stranded plasmid DNA containing the 10-kb SIV_{F359} insert was used as a template for sequencing. DNA-sequencing reactions were carried out using dye-primer chemistry and were executed on a LiCor automated DNA sequencer. The entire insert was sequenced from both directions. Nucleotide sequences were aligned using ClustalW version 1.7 (61). Alignments were examined and adjusted as necessary using the Genetic Data Environment program (59). Regions of sequences that could not be unambiguously aligned were removed from subsequent analyses. Neighbor-joining phylogenetic analyses were conducted using the DNADIST and NEIGHBOR programs from version 3.5c of the PHYLIP package (17). Maximum-likelihood analyses of *env* sequences from selected SIVs were performed using the PAUP* program (version 4.0.0d60; D. Swofford) as follows. Initial maximum-likelihood estimates for the *env* tree were produced using a two-substitution-type model (HKY model) without rate variation among sites. The topology of this tree was used as a starting topology for subsequent analyses. The shape parameter (alpha) for the gamma distribution describing rate variation among sites was estimated using the maximum-likelihood method to be 0.22966. This value was used for the estimation of the parameters for the six-substitution-type model (general time reversible model). The values estimated ($a = 2.499$, $b = 10.46$, $c = 1.278$, $d = 1.746$, $e = 9.962$, and $f = 1$) were then in turn used to refine estimates. Final estimates for the parameters were as follows: $a = 2.499$, $b = 10.44$, $c = 1.278$, $d = 1.745$, $e = 9.95$, $f = 1$, and alpha = 0.23156.

RESULTS

Cell tropism in vitro. To determine the replicative properties and cell tropism of SIV_{F359}, a series of in vitro assays were performed to compare the properties of this molecular clone with the well-established characteristics of two other well-defined SIV_{mac} molecular clones. Mitogen-stimulated PBMC cultures were used to assess virus replication in rhesus lymphocytes. All viruses tested (SIV_{F359}, $SIV_{mac239/YEnef}$, and SIV_{mac239}) grew well in stimulated rhesus lymphocyte cultures as measured by p27 concentrations in the supernatants (Fig. 1A). These data indicate that all viruses, including SIV_{F359}, were able to infect and replicate in stimulated rhesus lymphocytes. Subsequently we determined the ability of SIV_{F359} to replicate in resting rhesus lymphocytes, a capability that has been reported for SIV_{smmPBj} (18) and $SIV_{mac239/YEnef}$ (15). Virus production was observed only in resting cell cultures infected with $SIV_{mac239/YEnef}$ at day 6 postinfection, producing 1.6 ng of p27 per ml in the supernatant, which increased to 12 ng of p27/ml by day 12 postinfection (Fig. 1B).

Infection of MDM cultures with the parental SIV_{8980}, the serum RNA-derived clone SIV_{F359}, or the macrophage-tropic molecular clone SIV_{mac316} yielded detectable virus production only upon infection with SIV_{mac316}, with p27 concentrations in cell supernatants increasing from 0.8 to 7.8 ng of p27/ml from day 6 to day 12, respectively. During the entire experimental period virus production in supernatants could not be detected in cultures infected with either the parental SIV_{8980} or the serum RNA-derived clone SIV_{F359} (Fig. 1C). The absence of SIV_{F359} in the supernatants of MDM cultures was concluded to be due to the inability to either infect or replicate efficiently in this cell type. MDM cultures were double stained immunocytochemically for the presence of both the macrophage marker CD68 and the viral antigen p27. Analysis of stained cells indicated that SIV_{8980} as well as SIV_{F359}, although not able to produce detectable amounts of p27 in the supernatant, did infect macrophages and resulted in the expression of Gag protein (Fig. 2A). In contrast, SIV_{mac316} not only infected MDM (Fig. 2B) but also replicated well, producing substantial p27 concentrations in macrophage cultures (Fig. 1C).

Infection of MDM cultures from different animals revealed the expression of p27 in macrophages but undetectable production of p27 in supernatants with either the parental SIV_{8980} isolate or the SIV_{F359} variant. To determine if these antigen-positive nonproductively infected MDM were capable of transmitting the infection to other cell types, we cocultured autologous lymphocytes with these MDM cultures weeks after their exposure to virus. Importantly, the results revealed transfer to

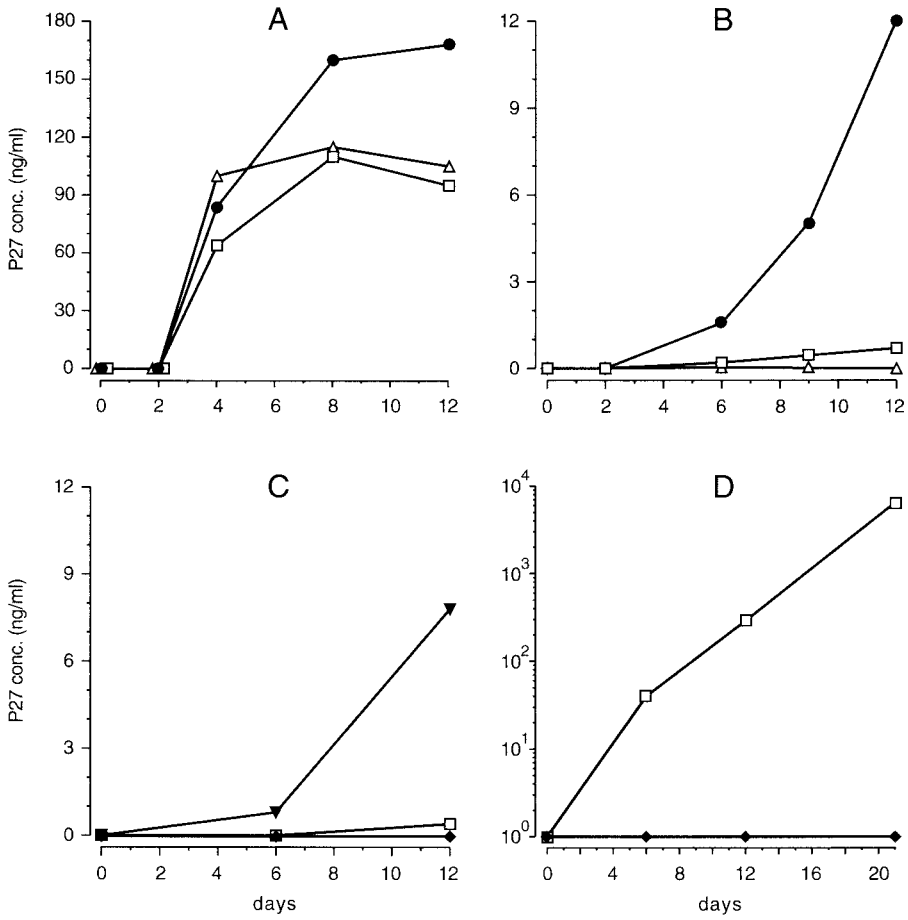

FIG. 1. (A to C) Production of SIV p27 antigen in stimulated (A) and resting (nonstimulated) (B) rhesus lymphocyte cultures and in MDM cultures (C). Cultures were infected with equivalent amounts of either SIV_{F359} (□), $SIV_{mac239/YEnef}$ (●), SIV_{mac239} (△), or SIV_{mac316} (▲). (D) Production of p27 in culture supernatants of lymphocytes following cocultivation with MDM. Virus production by the parental SIV_{8980} (◆) was compared to that by the end-stage variant SIV_{F359} (□).

and productive infection of lymphocytes by the SIV_{F359} variant but not the parental SIV_{8980} (Fig. 1D). Transmission to and infection by lymphocytes in this fashion by this late-stage variant resulted in production of virus at magnitudes greater than detected in the three previous assays (Fig. 1A, B, and C). In summary, these results add further insight to previous studies of late-stage variants in humans and macaques (32), indicating that the molecular clone SIV_{F359} was similar to cytopathic (syncytium-inducing), rapidly replicating, and predominantly T-cell-tropic variants but retained the ability to infect monocytes/macrophages, which upon contact with naive autologous T cells resulted in productive infection.

Infectious and pathogenic properties of SIV_{F359}. To investigate if the molecular clone SIV_{F359} was infectious and pathogenic in vivo, two rhesus macaques (L52 and WT) were intravenously infected with 100 $TCID_{50}$ of SIV_{F359} propagated in rhesus PBMC. $CD4^+$ T lymphocytes were monitored by FACS analysis, and fluctuations in virus loads were measured by QVI (Fig. 3). For animal L52, these parameters were determined at 2-week intervals. The same measurements were performed for WT at weekly intervals during the first month and subsequently at 2-week and monthly intervals. Animal L52 developed a virus load of 1,024 virus-producing cells/10^6 PBMC at 2 weeks after infection. Decreased values (524 virus-producing cells/10^6 PBMC) were detected at 4 and 6 weeks after infection, and the load dropped further to 128 virus-producing cells/10^6 PBMC at weeks 8, 10, and 14 postinfection A second peak of viremia, which reached 514 virus-producing cells/10^6 PBMC, was observed 15 and 17 weeks after infection. At weeks 19 and 21 virus loads decreased again to 256 and 48 infected cells per 10^6 PBMC, respectively. A third peak, which was higher than the previous ones, was observed at the end of the experimental period, from week 23 until week 37; virus loads of as high as 2,024 infected cells per 10^6 PBMC at week 32 were detected (Fig. 3A). The presence of this peak coincided with the development of severe anemia, diarrhea, and the start of extensive weight loss. L52 was euthanized after 37 weeks of infection.

The first positive virus isolation from monkey WT was observed already at 1 week postinfection; 512 virus-producing cells per 10^6 PBMC were measured. This value increased to 1,024 virus-producing cells/10^6 PBMC at weeks 2 and 3 postinfection but dropped over time to 64 virus-producing cells/10^6 PBMC at week 8 after infection. Relatively low virus loads, fluctuating between 256, 64, 128, and 16 virus-producing cells/10^6 PBMC, were found at 10, 12, 14, 16, and 19 weeks postinfection. An increase in virus loads from 700 to 4,048 virus-

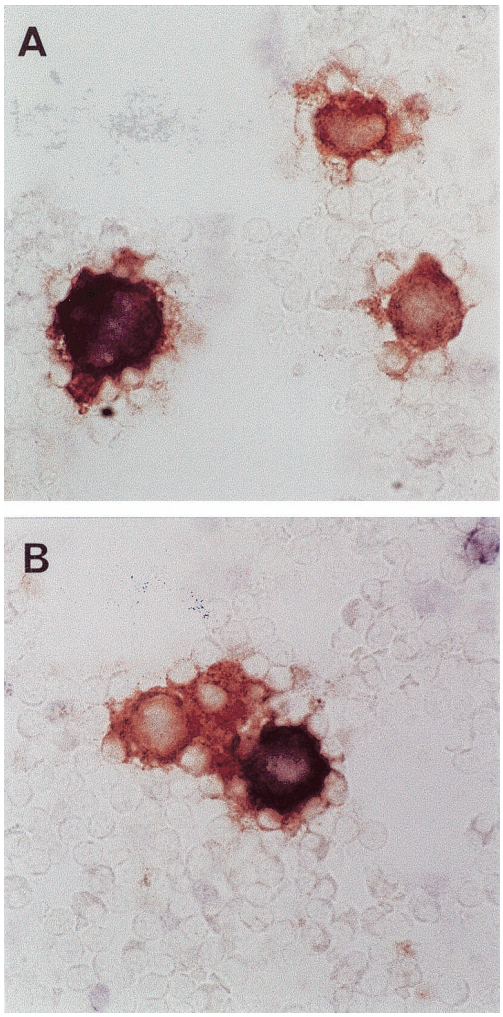

FIG. 2. Photomicrographs of MDM cultures infected with SIV_{mac316} (A) and SIV_{F359} (B); infection is demonstrated by $CD68^+$-$p27^+$ double staining. Magnification, ×660.

producing cells/10^6 PBMC was then detected from week 23 to week 41, respectively. During the last 22 weeks (until week 68), virus loads stayed at a constant level of 1,012 virus-producing cells/10^6 PBMC (Fig. 3B). WT was euthanized after 68 weeks.

Histopathology revealed moderate catharrhalic enteritis and severe lymphoid hyperplasia in the spleen and lymph nodes, with moderate atrophy of splenic follicles. CD4 cell numbers in inoculated animals were compared to those in two uninfected rhesus monkeys of the same sex, age, and body weight. The two infected animals showed a decrease in $CD4^+$-T-cell lymphocytes during the course of the experiment (25% for L52 [Fig. 3C] and 21% for WT [Fig. 3D]). $CD4^+$ T lymphocytes cultured from these animals were positive by immunocytochemical staining for p27 antigen expression (data not shown). The uninfected animals had stable CD4 cell counts, as expected.

In both animals histopathological findings were consistent with the diagnosis of AIDS. Cryptosporidial enteritis without remission as seen in WT is observed in advanced immunodeficiency (46) and is one of the criteria for the diagnosis of AIDS in humans. In L52, a cholecystitis due to a microsporidial infection was identified. The etiologic organism has only recently been detected in SIV-infected rhesus monkeys and was classified as an *Enterocytozoon bieneusi*-like microsporidial protozoan. *E. bieneusi* is a common opportunistic pathogen of AIDS patients, causing significant morbidity.

Coreceptor usage. To further correlate the observations of cell tropism, the coreceptor use of SIV_{F359} was characterized. Infection experiments with SIV_{F359} virus were performed on HOS-$CD4^+$ cell lines expressing either the macaque or human CCR5 coreceptor. Both cell lines were highly susceptible to infection with SIV_{F359}, and almost 90% of the cells formed syncytia, which stained positive for SIV p27 antigen. To investigate the ability of SIV_{F359} to use additional coreceptors, the extent of its replication in GHOST cells and U87MG-CD4 cells expressing CCR1, CCR2b, CCR3, CCR4, CCR5, CXCR4, BOB/GPR15, Bonzo/STRL33, CX3Cr1 (V28), CCR8, APJ, GPR1, and US28 coreceptors (12, 22) was assayed in parallel with that of the well-characterized SIV_{mac239} and SIV_{mac316} molecular clones. All three viruses were found to use CCR5 for infection (Table 1) as determined by FACS analysis, while SIV_{F359} proved to be unable to use any coreceptor other than CCR5 at standard doses. Particular effort was made to determine if CXCR4 was used by this apparent T-cell-tropic variant; however, all assays were negative, corroborating several other reports that this group of viruses do not use this coreceptor (8, 16, 33). The observation that only CCR5 appeared to be utilized taken together with the ability of SIV_{F359} to infect T cells and macrophages was in agreement with previous reports that the CCR5 receptor is commonly used by both macrophage-tropic and T-cell-tropic primate lentiviruses (8, 16). Interestingly, both SIV_{mac239} and SIV_{mac316} used multiple coreceptors, including BOB/GPR15 and Bonzo/STPR, in contrast to the end-stage variant SIV_{F359}. Furthermore, the macrophage-tropic molecular clone SIV_{mac316} used as a fourth coreceptor CCR3 (Table 1). Finally, to determine if the SIV_{F359} clone was truly restricted to only CCR5 coreceptor use, we compared the ability of the CCR5-dependent $HIV\text{-}1_{Ba\text{-}L}$ strain to productively infect human PBMC homozygous for the 32-bp deletion of CCR5 receptor gene, affecting CCR5 receptor expression (44), to those of the parental SIV_{8980} and variant SIV_{F359}. These Δ32 cells are resistant to infection with viruses such as $HIV\text{-}1_{Ba\text{-}L,}$ which exclusively use the CCR5 coreceptor. As shown in Fig. 4, although the $HIV\text{-}1_{Ba\text{-}L}$ strain is completely blocked by the Δ32 CCR5 deletion, SIV_{8980} and SIV_{F359} grow to a slightly reduced but significant extent on the CCR5-defective cells. These data suggested that the SIV_{F359} clone may additionally utilize a novel, previously undescribed coreceptor for entry.

Is SIV_{F359} representative of the predominant variant following in vivo passage? The first hypervariable region (V1) represents the most variable region of the SIV genome, and therefore it provides a sensitive indicator of the genotypic variation present within a quasispecies of an infected animal. Trichel et al. identified 12 different genotypes in the SIV_{B670} inoculum based on sequence analyses of the V1 region (62). The V1 regions derived from the viral variants emerging during in vivo passage (27) were determined (64) and compared with the V1 sequences of the viral variants that were present in the original SIV_{B670} inoculum (62). Homologies were quantified by using software that calculated the actual alignment fit as the fraction of the optimal fit. SIV_{B670}-CL12 emerged as the predominant

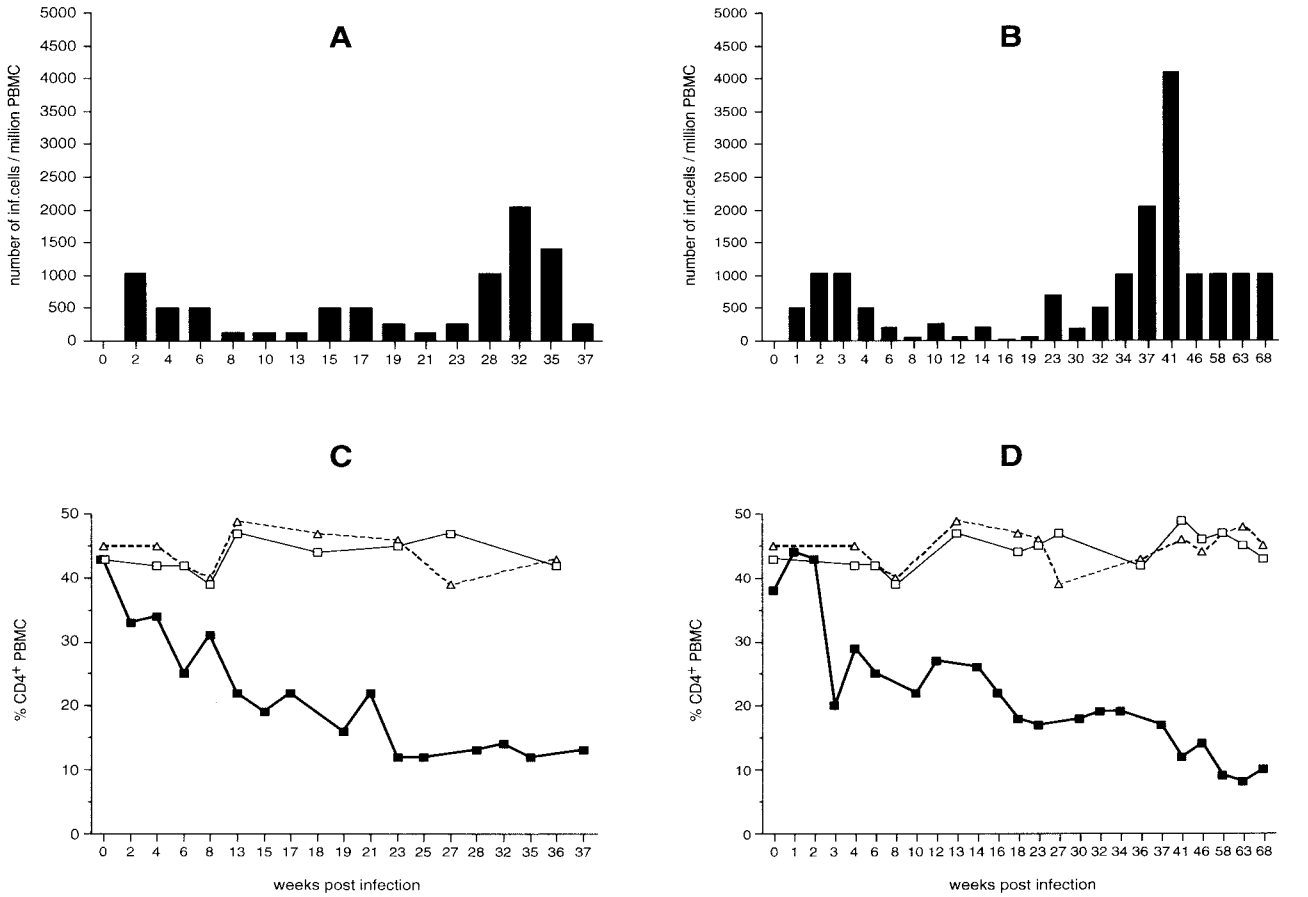

FIG. 3. Virus load (A and B) and changes in $CD4^+$ lymphocyte populations (C and D) of rhesus monkeys WT (A and C) and L52 (B and D) after infection with molecularly cloned SIV_{F359}. A progressive decline of $CD4^+$ T lymphocytes in infected rhesus monkeys (WT and L52) (■) compared to control monkeys 8637 (□) and 8711 (△) is seen.

variant from the original SIV_{B670} infection and represented the highest genome stability during the passage experiment (Fig. 5). Clones obtained during subsequent passages represented viral variants that were more closely related to SIV_{B670}-CL12 than to any other B670 clone (Fig. 5). This indicated that SIV_{B670}-CL12 possessed the optimal fitness for replication in both B670 and the animals to which it was transmitted. Indeed, based on our observations of high persistent virus loads in the most rapidly progressing animals (27), certain variants may have acquired mutations making them relatively resistant to neutralization (32). V1 sequences derived from passage samples were compared to SIV_{B670}-CL12, which had shown the highest homology with evolving variants during the passage study. Specifically, during the subsequent passages the number of different V1 clones decreased from four (out of four) to two (out of four) (Fig. 5). These findings demonstrated that during in vivo passage there was a selection for the most fit variants (32). The molecular clone SIV_{F359} represented the dominant variant and had retained the identical V1 sequences observed in P4.1, P4.2, and P4.3 envelope clones (Fig. 6).

Phylogenetic analysis of SIV_{F359}. Phylogenetic analysis of *env* sequences using the neighbor-joining and general time reversible (GTR) maximum-likelihood method (17) confirmed that SIV_{F359} clustered together with SIV_{sm} clones such as SIV_{sm9} and $SIV_{smmPBj4.41}$ and was not closely related to SIV_{mac} clones such as SIV_{mac251}, SIV_{mac239}, and $SIV_{mac1A11}$. As could be expected from the origin of SIV_{F359}, this clone was most closely related to but distinct from SIV_{B670} (only the envelope sequence is available) (Fig. 7A). SIV_{B670} had been isolated from a rhesus macaque infected with material containing SIV from a sooty mangabey housed at the Tulane Primate Center (49). These two viruses branch separately from other characterized SIV_{sm} viruses, which had also been derived from sooty mangabeys but originated from the Yerkes primate colony (18). Interestingly, these viruses formed a unique cluster of strains which branched separately from those macaque-adapted isolates derived from the New England (mac251 and mac239) and California (mac1A11) Regional Primate Centers (38). Neighbor-joining phylogenetic analysis performed on the entire sequence showed similar relationships within the SIV cluster (Fig. 7B). The predicted amino acid sequences of SIV_{F359} proteins were more similar to those of SIV_{smH4} (87%) and $SIV_{smmPBj4.41}$ (88%) than to those of the SIV_{mac} cluster (83%) (Table 2). The greatest similarity with other SIVs was present in the *gag* (average, 92%), *pol* (average, 93%), and *vpx* (average, 92%) genes, whereas the greatest divergence was seen in the *nef* (average similarity, 75%) and *tat* (average similarity, 72%) genes (Table 2).

Virulence of the SIV_{F359} molecular clone. To determine if the SIV_{F359} molecular clone had retained the same pathogenic

TABLE 1. Comparison of coreceptor use and cell tropism of the well-defined T-cell-tropic and macrophage-tropic SIV_{mac} strains compared to SIV_{F359}[a]

Property	Molecular clone			Parental strain SIV_{8980}
	SIV_{mac239}	SIV_{mac316}	SIV_{F359}	
Macrophage tropism				
Infection	−	+	+	+
Productive replication	−	+	−	−
Coreceptor use				
CCR1	−	−	−	−
CCR2B	−	−	−	−
CCR3	−	+	−	−
CCR4	−	−	−	−
CXCR4	−	−	−	−
CCR5	+	+	+	+
BOB/GPR15	+	+	−	−
Bonzo/STPR	+	+	−	−

[a] Infection by SIV_{mac239}, SIV_{mac316}, and SIV_{F359} was in HOS-CD4$^+$, GHOST, and U87 cell lines stably expressing the human coreceptors listed (partial list).

potential as the parental SIV_{8980} isolate, eight additional animals were infected with the same dose of the SIV_{F359} virus stock. As illustrated in Fig. 8, animals infected with the SIV_{F359} stock had survival curves very similar to those of animals infected with the SIV_{8980} stock from which it was derived, and SIV_{F359} was much more pathogenic than the original prepassage SIV_{B670} isolate. The molecular clone was slightly less pathogenic, as could be seen by the slight shift of the survival curve to the right. This, however, could be a consequence of the genetically homogeneous nature of the SIV_{F359} inoculum compared to the more heterogeneous SIV_{8980} inoculum, which represented a highly virulent quasispecies. Indeed, a heterogeneous quasispecies is more likely to escape from the immune responses of an outbred host.

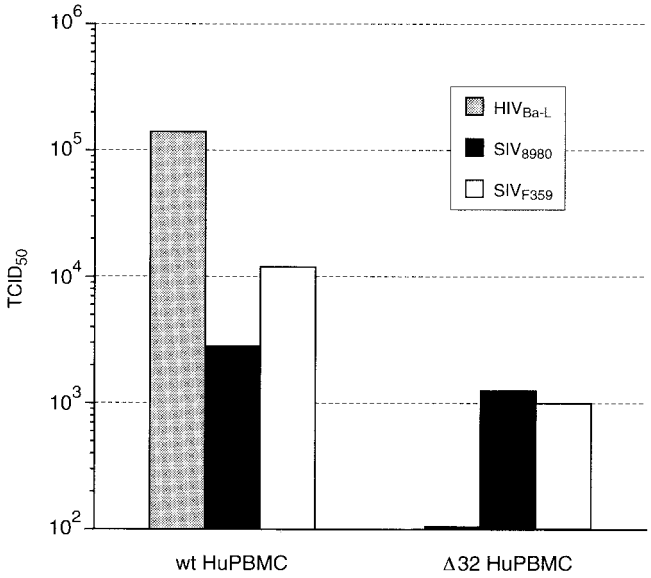

FIG. 4. Abilities of HIV-1_{Ba-L}, SIV_{F359}, and SIV_{8980} to grow on normal human PBMC (wt HuPBMC) versus PBMC homozygous for the Δ32 deletion in the CCR5 receptor gene (Δ32 HuPBMC). The CCR5-restricted HIV_{Ba-L} isolate could replicate only in wild-type human PBMC, while SIV_{F359} and SIV_{8980} were found to replicate in both Δ32 PBMC and normal human PBMC.

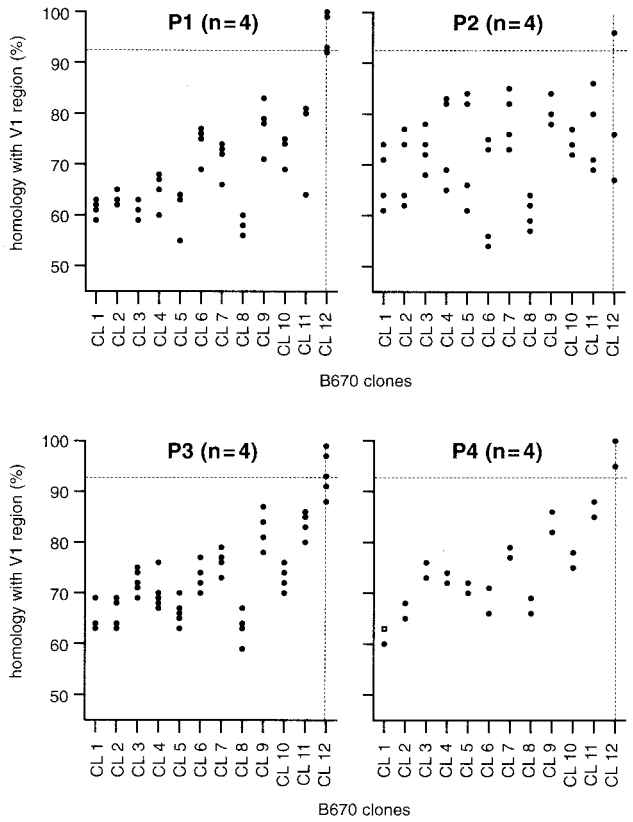

FIG. 5. Comparison of V1 sequences of envelope clones that emerged during the in vivo passages (P1 to P4) to obtain SIV_{8980}, from which the molecular clone SIV_{F359} was derived, and V1 sequences of the viral variants (CL1 to CL12) which were present in the original SIV_{B670} inoculum (62, 64).

DISCUSSION

We have utilized a novel cloning strategy to derive an infectious pathogenic molecular clone of a primate lentivirus without cell culture passage directly from serum. This molecular clone from serum (SIV_{F359}) has unique properties. It is highly cytopathic in vitro and causes marked syncytia in lymphoid tissues in vivo. It readily infects activated but not resting rhesus lymphocytes. Infection of MDM cultures was demonstrated; however, in contrast to the case for SIV_{mac316}, no virus production in the supernatant was detectable. Coreceptor use of this clone was restricted to CCR5 only, in contrast to that of other well-characterized molecular clones (SIV_{mac239} [T-cell tropic] and SIV_{mac316} [macrophage tropic]) studied for comparison. Surprisingly, although this virus did not use any of the 13 known primate lentivirus coreceptors, it could replicate on human cells defective for CCR5.

The use of molecular clones derived from extracellular virions may provide additional insight into the evolution of the lentiviral pathogen. For instance, this cloning strategy may aid the analysis of the specific biological properties of viral variants in plasma or serum which have escaped host immune responses at a specific point in time. Similarly, the study of virus populations which arise and become predominant in various extracellular compartments is feasible using this methodology. Studying the evolution of viruses using this strategy has a

```
P1 (n=4)
B670 CL12 WGLTGNAATTTTTTTTASTTTP.KGRADVVNETSSCVKNNNCTGLEQEP
P1.1      ..................A...S.........................
P1.2      ........................EE........P.............
P1.3      .........I.....................................
P1.4      ............I..................................

P2 (n=4)
B670 CL12 WGLTGNAA.TTTTTTTTASTTTPKGRADVVNETSSCVKNNNCTGLEQEP
P2.1      ......VEA.............S.EE.......................
P2.2      ......VEA........-----..........................
P2.3      ..................-..............................
P2.4      ............I..................................

P3 (n=4)
B670 CL12 WGLTGNAATTTTTTTTASTTTPKGRADVVNETSSCVKNNNCTGLEQEP
P3.1      ............I...-.....EE........................
P3.2      ..................-..............................
P3.3      ................--........N......................
P3.4      ............I...........N......................

P4 (n=4)
B670 CL12 WGLTGNAATTTTTTTTASTTTPKGRADVVNETSSCVKNNNCTGLEQEP
P4.1      ..................-..............................
P4.2      ..................-..............................
P4.3      ..................-..............................
P4.4      ........................N.......................
```

FIG. 6. Amino acid sequence alignments of the V1 envelope region of the predominate clone 12 (CL12) in the prepassage SIV_{B670} inoculum. This sequence persisted through the passage as the diversity of the inoculum declined (Fig. 4). The CL12 V1 sequences are compared with the sequences of clones derived from the four different passages (P1 to P4). Clone P4.3 represents the same V1 sequence found in the molecular clone, SIV_{F359}, and which was originally found in CL12 within the quasispecies found in the prepassage SIV_{B670} inoculum.

number of important advantages over conventional techniques that involve cloning proviruses from infected lymphocytes.

The SIV_{F359} molecular clone was derived from a rhesus macaque which had developed AIDS following the fourth in vivo passage of virus derived from SIV_{B670}. The virus isolate SIV_{B670} originated from one of the first reports of simian AIDS in macaques and since has been used in a wide variety of studies (1, 4, 49). Phylogenetic analyses using available *env* genes or entire SIV genomes revealed that SIV_{B670} and SIV_{F359} branch off between two separate clusters of SIV_{sm} clones, represented by SIV_{smm9} and $SIV_{smmPBj4.41}$, derived from the Yerkes Primate Colony, and SIV_{mac} clones, such as SIV_{mac251} and SIV_{mac239}, derived from the New England Primate Colony (Fig. 7). As could be expected from the origin of SIV_{F359}, this clone was related to but distinct from SIV_{B670}. Originally SIV_{B670} had been isolated from a rhesus macaque (B670) infected with material containing SIV originally derived from a sooty mangabey from the Tulane Primate Center (49). Using the entire sequence for phylogenetic analysis, SIV_{F359} was found to branch separately between other characterized SIV isolates derived from sooty mangabeys or macaques (Fig. 7). The characteristics of the envelope evolution of SIV_{B670} during serial passage to animal 8980, from which SIV_{F359} was derived, have recently been described (64).

Comparison of the biological characteristics of the clone with those of the original SIV_{8980} isolate should demonstrate that we cloned the dominant virulent variant. For instance, coreceptor usage (55, 57) and the ability to replicate in resting versus activated $CD4^+$ T cells (15) and macrophages (58, 67) are characteristics considered to influence pathogenicity. In this regard, no differences were observed between the cloned virus and the virus isolate in that both recognized only the CCR5 coreceptor and neither replicated in resting PBMC or macrophages. The subsequent in vivo passages of SIV_{B670} resulting in SIV_{8980} were carefully monitored. The original SIV_{B670} inoculum was also used in studies performed by Trichel et al. (62) and Amedee et al. (1). They had determined the number of different genotypes in the SIV_{B670} inoculum based on sequence divergence in the first hypervariable region (V1) (5, 54). Twelve different genotypes could be recognized in

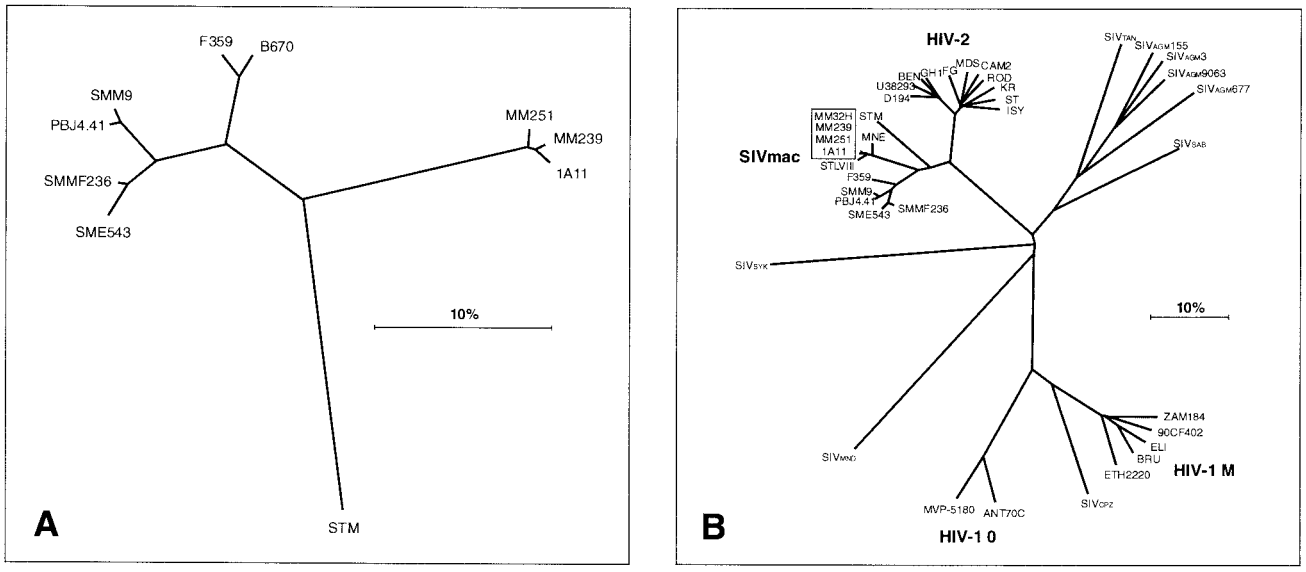

FIG. 7. (A) Phylogenetic tree based on the SIV_{F359} sequence compared with envelope sequences from the parental SIV_{B670} and compared to less related SIV_{mac251}, SIV_{sm}, and SIV_{stm} sequences. The tree was constructed using a GTR maximum-likelihood analysis. (B) Phylogenetic analysis based on full-length sequences, comparing the SIV_{F359} molecular clone to other SIV, HIV-1, and HIV-2 clones. Phylogenetic trees were constructed by the neighbor-joining method using the PHYLIP program (version 3.5c).

TABLE 2. Predicted amino acid similarity of SIV_{F359} to other SIV molecular clones

SIV_{F359} protein	% Similarity								
	SIV_{B670}	SIV_{SmH4}	$SIV_{smmPBj4.41}$	SIV_{mac239}	$SIV_{mac1A11}$	SIV_{mac}	SIV_{Stm}	SIV_{Mne}	Mean
Gag		96	95	90	91	92	88	93	92
Pol		97	95	92	91	92	91	93	93
Vif		93	89	84	84	85	78	84	85
Vpx		94	92	94	94	93	88	88	92
Vpr		81	96	87	84	86	88	89	87
Tat		75	80	65	80	75	69	62	72
Rev		78	81	77	76	75		80	78
Env	95	88	86	83	80	78	83	83	84
Nef		81	75	78	74	66	77	75	75
Mean		87	88	83	84	82	83	83	

this isolate (SIV_{B670}-CL1 to -CL12). The envelope sequence of SIV_{B670}-CL12 was one of the sequence variants present in the original SIV_{B670} stock. During our passage experiment, the sequence variant SIV_{B670}-CL12 had acquired an optimal fitness, represented by its prevalence in the early SIV_{B670} isolate and in its maintenance during the passages. Indeed, the SIV_{F359} molecular clone had the highest homology (96%) with SIV_{B670}-CL12 and therefore is representative of the dominant virus variant of SIV_{B670}.

The in vivo experiments in rhesus macaques demonstrated similar clinical and pathological characteristics of SIV_{F359} and SIV_{8980}. Several $SIV_{sm/mac}$ clones have been reported to cause different patterns of disease, including attenuated virulence, compared to the virus isolates from which they were derived. With specific regard to the first SIV molecular clones which were derived, viral adaptation and attenuation often occurred as a result of in vitro propagation. This attenuation phenomenon may also be due to a failure to clone the dominant virus variant, possibly as a consequence of using proviral DNA as biological template or due to the use of biological material that contained a composition of early (macrophage-tropic, slow-replicating, non-syncytium-inducing) and late (T-cell-tropic, fast-replicating, syncytium-inducing) variants. It may also reflect a certain synergistic effect of a quasispecies that does not exist in the case of a single molecularly cloned virus. A study demonstrating a regulatory effect on HIV replication mediated by defective proviruses has provided evidence in that direction. Convincing data have started to accumulate which explain the progression to AIDS in terms of the biological properties of virus variants which emerge during the course of infection. However, results from other studies suggest a more complex cascade of events in which variants in combination with host-specific factors are involved in AIDS pathogenesis.

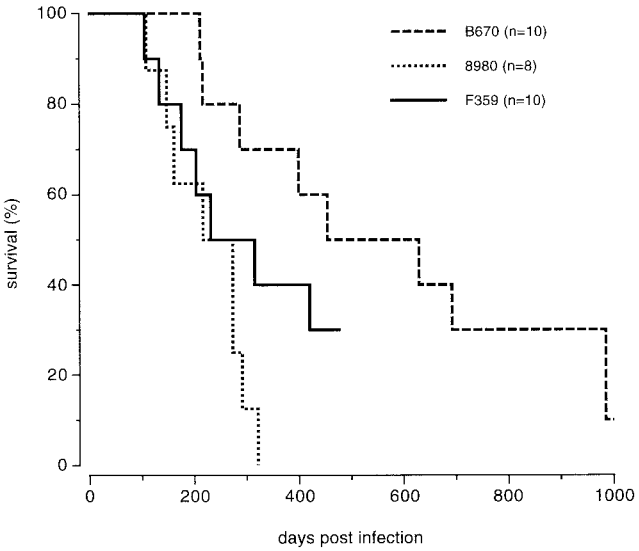

FIG. 8. Comparison of survival of groups of animals infected with the original prepassage SIV_{B670} isolate, the postpassage SIV_{8980} isolate, and the subsequently derived SIV_{F359} molecular clone as illustrated by a Kaplan-Meier plot.

In summary, we have utilized a new cloning strategy to generate infectious molecular clones of lentiviruses from extracellular viral RNA in body fluids. This has facilitated the isolation of a unique pathogenic molecular clone designated SIV_{F359}. Characterization of SIV_{F359} in vivo and in vitro revealed that it was highly pathogenic, causing marked syncytial giant cell formation in situ in lymph nodes and CPE in rhesus $CD4^+$ T cells in vitro. It was found to have limited and novel coreceptor usage (CCR5 and a yet-undefined coreceptor) and could infect but did not result in detectable virus production in rhesus MDM. Upon the addition of autologous lymphocytes to these MDM, infection was transmitted and resulted in high concentrations of virus production. This SIV_{F359} molecular clone proved to be able to cause a rigorous infection and AIDS in rhesus macaques. It was genetically distinct from other molecular clones, mapping between two clusters of previously characterized groups of SIV clones (the SIV_{sm} and SIV_{mac} molecular clones, respectively). The SIV_{F359} molecular clone provides further insight into the nature of end-stage variants and the characteristics associated with accelerated disease progression.

REFERENCES

1. **Amedee, A. M., N. Lacour, J. L. Gierman, L. N. Martin, J. E. Clements, R. Bohm, Jr., R. M. Harrison, and M. Murphey-Corb.** 1995. Genotypic selection of simian immunodeficiency virus in macaque infants infected transplacentally. J. Virol. **69:**7982–7990.
2. **Balzarini, J., L. Naesens, J. Slachmuylders, H. Niphuis, I. Rosenberg, A. Holy, H. Schellekens, and E. De-Clercq.** 1991. 9-(2-Phosphonylmethoxyethyl)adenine (PMEA) effectively inhibits retrovirus replication in vitro and simian immunodeficiency virus infection in rhesus monkeys. AIDS **5:**21–28.
3. **Barbareschi, M., S. Girlando, F. M. Mauri, S. Forti, C. Eccher, F. A. Mauri, R. Togni, P. Dalla Palma, and C. Doglioni.** 1994. Quantitative growth fraction evaluation with MIB1 and Ki67 antibodies in breast carcinomas. Am. J. Clin. Pathol. **102:**171–175.

4. **Baskin, G. B., L. N. Martin, M. Murphey-Corb, F. S. Hu, D. Kuebler, and B. Davison.** 1995. Distribution of SIV in lymph nodes of serially sacrificed rhesus monkeys. AIDS Res. Hum. Retroviruses **11:**273–285.
5. **Burns, D. P., and R. C. Desrosiers.** 1991. Selection of genetic variants of simian immunodeficiency virus in persistently infected rhesus monkeys. J. Virol. **65:**1843–1854.
6. **Carlson, J. R., T. P. McGraw, E. Keddie, J. L. Yee, A. Rosenthal, A. J. Langlois, R. Dickover, R. Donovan, P. A. Luciw, and M. B. Jennings.** 1990. Vaccine protection of rhesus macaques against simian immunodeficiency virus infection. AIDS Res. Hum. Retroviruses **6:**1239–1246.
7. **Chakrabarti, L., M. Emerman, P. Tiollais, and P. Sonigo.** 1989. The cytoplasmic domain of simian immunodeficiency virus transmembrane protein modulates infectivity. J. Virol. **63:**4395–4403.
8. **Chen, Z., P. Zhou, D. D. Ho, N. R. Landau, and P. A. Marx.** 1997. Genetically divergent strains of simian immunodeficiency virus use CCR5 as a coreceptor for entry. J. Virol. **71:**2705–2714.
9. **Daniel, M. D., and R. C. Desrosiers.** 1989. Use of simian immunodeficiency virus for evaluation of AIDS vaccine strategies. AIDS **3:**S131–S133.
10. **Daniel, M. D., P. K. Sehgal, T. Kodama, M. S. Wyand, D. J. Ringler, N. W. King, D. K. Schmidt, C. D. Troup, and R. C. Desrosiers.** 1990. Use of simian immunodeficiency virus for vaccine research. J. Med. Primatol. **19:**395–399.
11. **Deng, H., R. Liu, W. Ellmeier, S. Choe, D. Unutmaz, M. Burkhart, P. Di Marzio, S. Marmon, R. E. Sutton, C. M. Hill, C. B. Davis, S. C. Peiper, T. J. Schall, D. R. Littman, and N. R. Landau.** 1996. Identification of a major co-receptor for primary isolates of HIV-1. Nature **381:**661–666.
12. **Deng, H. K., D. Unutmaz, V. N. KewalRamani, and D. R. Littman.** 1997. Expression cloning of new receptors used by simian and human immunodeficiency viruses. Nature **388:**296–300.
13. **Desrosiers, R. C., and D. J. Ringler.** 1989. Use of simian immunodeficiency viruses for AIDS research. Intervirology **30:**301–312.
14. **Dewhurst, S., J. E. Embretson, D. C. Anderson, J. I. Mullins, and P. N. Fultz.** 1990. Sequence analysis and acute pathogenicity of molecularly cloned SIVSMM-PBj14. Nature **345:**636–640.
15. **Du, Z., S. M. Lang, V. G. Sasseville, A. A. Lackner, P. O. Ilyinskii, M. D. Daniel, J. U. Jung, and R. C. Desrosiers.** 1995. Identification of a *nef* allele that causes lymphocyte activation and acute disease in macaque monkeys. Cell **82:**665–674.
16. **Edinger, A. L., A. Amedee, K. Miller, B. J. Doranz, M. Endres, M. Sharron, M. Samson, Z. H. Lu, J. E. Clements, M. Murphey-Corb, S. C. Peiper, M. Parmentier, C. C. Broder, and R. W. Doms.** 1997. Differential utilization of CCR5 by macrophage and T cell tropic simian immunodeficiency virus strains. Proc. Natl. Acad. Sci. USA **94:**4005–4010.
17. **Felsenstein, J.** 1989. PHYLIP—Phylogeny Inference Package (version 3.2). Cladistics **5:**164–166.
18. **Fultz, P. N., D. C. Anderson, H. M. McClure, S. Dewhurst, and J. I. Mullins.** 1990. SIVsmm infection of macaque and mangabey monkeys: correlation between in vivo and in vitro properties of different isolates. Dev. Biol. Stand. **72:**253–258.
19. **Gardner, M. B., and P. A. Luciw.** 1989. Animal models of AIDS. FASEB J. **3:**2593–2606.
20. **Goodenow, M., T. Huet, W. Saurin, S. Kwok, J. Sninsky, and S. Wain-Hobson.** 1989. HIV-1 isolates are rapidly evolving quasispecies: evidence for viral mixtures and preferred nucleotide substitutions. J Acquir. Immune Defic. Syndr. **2:**344–352.
21. **Heeney, J. L.** 1996. Primate models for AIDS vaccine development. AIDS **10:**S115–S122.
22. **Hill, C. M., H. Deng, D. Unutmaz, V. N. Kewalramani, L. Bastiani, M. K. Gorny, S. Zolla-Pazner, and D. R. Littman.** 1997. Envelope glycoproteins from human immunodeficiency virus types 1 and 2 and simian immunodeficiency virus can use human CCR5 as a coreceptor for viral entry and make direct CD4-dependent interactions with this chemokine receptor. J. Virol. **71:**6296–6304.
23. **Hirsch, V., D. Adger-Johnson, B. Campbell, S. Goldstein, C. Brown, W. R. Elkins, and D. C. Montefiori.** 1997. A molecularly cloned, pathogenic, neutralization-resistant simian immunodeficiency virus, SIVsmE543-3. J. Virol. **71:**1608–1620.
24. **Hirsch, V. M., P. Edmondson, M. Murphey-Corb, B. Arbeille, P. R. Johnson, and J. I. Mullins.** 1989. SIV adaptation to human cells. Nature **341:**573–574.
25. **Holterman, L., R. Dubbes, J. Mullins, J. Haaijman, and J. Heeney.** 2000. A strategy for cloning infectious molecular clones of retroviruses from serum or plasma. J. Virol. Methods **84:**37–48.
26. **Holterman, L., J. I. Mullins, J. J. Haayman, and J. L. Heeney.** 1996. Direct amplification and cloning of up to 5-kb lentivirus genomes from serum. BioTechniques **21:**312–319.
27. **Holterman, L., H. Niphuis, W. Koornstra, R. Dubbes, P. ten Haaft, and J. L. Heeney.** 2000. The rate of progression to AIDS is independent of virus dose in simian immunodeficiency virus-infected macaques. J. Gen. Virol. **81:**1719–1726.
28. **Holterman, L., H. Niphuis, P. J. ten Haaft, J. Goudsmit, G. Baskin, and J. L. Heeney.** 1999. Specific passage of simian immunodeficiency virus from end-stage disease results in accelerated progression to AIDS in rhesus macaques. J. Gen. Virol. **80:**3089–3097.
29. **Johnson, P. R., and V. M. Hirsch.** 1992. SIV infection of macaques as a model for AIDS pathogenesis. Int. Rev. Immunol. **8:**55–63.
30. **Johnson, P. R., D. C. Montefiori, S. Goldstein, T. E. Hamm, J. Zhou, S. Kitov, N. L. Haigwood, L. Misher, W. T. London, and J. L. Gerin.** 1992. Inactivated whole-virus vaccine derived from a proviral DNA clone of simian immunodeficiency virus induces high levels of neutralizing antibodies and confers protection against heterologous challenge. Proc. Natl. Acad. Sci. USA **89:**2175–2179.
31. **Kestler, H., T. Kodama, D. Ringler, M. Marthas, N. Pedersen, A. Lackner, D. Regier, P. Sehgal, M. Daniel, and N. King.** 1990. Induction of AIDS in rhesus monkeys by molecularly cloned simian immunodeficiency virus. Science **248:**1109–1112.
32. **Kimata, J. T., L. Kuller, D. B. Anderson, P. Dailey, and J. Overbaugh.** 1999. Emerging cytopathic and antigenic simian immunodeficiency virus variants influence AIDS progression. Nat. Med. **5:**535–541.
33. **Kirchhoff, F., S. Pohlmann, M. Hamacher, R. E. Means, T. Kraus, K. Uberla, and P. Di Marzio.** 1997. Simian immunodeficiency virus variants with differential T-cell and macrophage tropism use CCR5 and an unidentified cofactor expressed in CEMx174 cells for efficient entry. J. Virol. **71:**6509–6516.
34. **Kodama, T., D. P. Burns, H. W. de Kestler, M. D. Daniel, and R. C. Desrosiers.** 1990. Molecular changes associated with replication of simian immunodeficiency virus in human cells. J. Med. Primatol. **19:**431–437.
35. **Kodama, T., D. P. Wooley, Y. M. Naidu, H. de Kestler, M. D. Daniel, Y. Li, and R. C. Desrosiers.** 1989. Significance of premature stop codons in *env* of simian immunodeficiency virus. J. Virol. **63:**4709–4714.
36. **Kornfeld, H., N. Riedel, G. A. Viglianti, V. Hirsch, and J. I. Mullins.** 1987. Cloning of HTLV-4 and its relation to simian and human immunodeficiency viruses. Nature **326:**610–613.
37. **Kozal, M. J., R. W. Shafer, M. A. Winters, D. A. Katzenstein, and T. C. Merigan.** 1993. A mutation in human immunodeficiency virus reverse transcriptase and decline in CD4 lymphocyte numbers in long-term zidovudine recipients. J. Infect. Dis. **167:**526–532.
38. **Lackner, A. A., P. F. Moore, P. A. Marx, R. J. Munn, M. B. Gardner, and L. J. Lowenstine.** 1990. Immunohistochemical localization of type D retrovirus serotype 1 in the digestive tract of rhesus monkeys with simian AIDS. J. Med. Primatol. **19:**339–349.
39. **Landau, N. R., and D. R. Littman.** 1992. Packaging system for rapid production of murine leukemia virus vectors with variable tropism. J. Virol. **66:**5110–5113.
40. **Landberg, G., E. M. Tan, and G. Roos.** 1990. Flow cytometric multiparameter analysis of proliferating cell nuclear antigen/cyclin and Ki-67 antigen: a new view of the cell cycle. Exp. Cell. Res. **187:**111–118.
41. **Letvin, N. L., M. D. Daniel, P. K. Sehgal, R. C. Desrosiers, R. D. Hunt, L. M. Waldron, J. J. MacKey, D. K. Schmidt, L. V. Chalifoux, and N. W. King.** 1985. Induction of AIDS-like disease in macaque monkeys with T-cell tropic retrovirus STLV-III. Science **230:**71–73.
42. **Li, Y., H. Hui, C. J. Burgess, R. W. Price, P. M. Sharp, B. H. Hahn, and G. M. Shaw.** 1992. Complete nucleotide sequence, genome organization, and biological properties of human immunodeficiency virus type 1 in vivo: evidence for limited defectiveness and complementation. J. Virol. **66:**6587–6600.
43. **Li, Y., J. C. Kappes, J. A. Conway, R. W. Price, G. M. Shaw, and B. H. Hahn.** 1991. Molecular characterization of human immunodeficiency virus type 1 cloned directly from uncultured human brain tissue: identification of replication of replication-competent and -defective viral genomes. J. Virol. **65:**3978–3985.
44. **Liu, R., W. A. Paxton, S. Choe, D. Ceradini, S. R. Martin, R. Horuk, M. E. MacDonald, H. Stuhlmann, R. A. Koup, and N. R. Landau.** 1996. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. Cell **86:**367–377.
45. **Marthas, M. L., R. A. Ramos, B. L. Lohman, K. K. Van-Rompay, R. E. Unger, C. J. Miller, B. Banapour, N. C. Pedersen, and P. A. Luciw.** 1993. Viral determinants of simian immunodeficiency virus (SIV) virulence in rhesus macaques assessed by using attenuated and pathogenic molecular clones of SIVmac. J. Virol. **67:**6047–6055.
46. **McGowan, I., A. S. Hawkins, and I. V. Weller.** 1993. The natural history of cryptosporidial diarrhoea in HIV-infected patients. AIDS **7:**349–354.
47. **Meyerhans, A., R. Cheynier, J. Albert, M. Seth, S. Kwok, J. Sninsky, L. Morfeldt-Manson, B. Asjo, and S. Wain-Hobson.** 1989. Temporal fluctuations in HIV quasispecies in vivo are not reflected by sequential HIV isolations. Cell **58:**901–910.
48. **Mori, K., D. J. Ringler, T. Kodama, and R. C. Desrosiers.** 1992. Complex determinants of macrophage tropism in *env* of simian immunodeficiency virus. J. Virol. **66:**2067–2075.
49. **Murphey-Corb, M., L. N. Martin, S. R. Rangan, G. B. Baskin, B. J. Gormus, R. H. Wolf, W. A. Andes, M. West, and R. C. Montelaro.** 1986. Isolation of an HTLV-III-related retrovirus from macaques with simian AIDS and its possible origin in asymptomatic mangabeys. Nature **321:**435–437.
50. **Naidu, Y. M., H. W. Kestler, Y. Li, C. V. Butler, D. P. Silva, D. K. Schmidt, C. D. Troup, P. K. Sehgal, P. Sonigo, M. D. Daniel, and R. C. Desrosiers.** 1988. Characterization of infectious molecular clones of simian immunodeficiency virus (SIV mac) and human immunodeficiency virus type 2: persistent infection of rhesus monkeys with molecularly cloned SIV mac. J. Virol. **62:**4691–4696.

51. **Niedrig, M., J. P. Rabanus, J. L'Age Stehr, H. R. Gelderblom, and G. Pauli.** 1988. Monoclonal antibodies directed against human immunodeficiency virus (HIV) gag proteins with specificity for conserved epitopes in HIV-1, HIV-2 and simian immunodeficiency virus. J. Gen. Virol. **69:**2109–2114.
52. **Novembre, F. J., P. R. Johnson, M. G. Lewis, D. C. Anderson, S. Klumpp, H. M. McClure, and V. M. Hirsch.** 1993. Multiple viral determinants contribute to pathogenicity of the acutely lethal simian immunodeficiency virus SIVsmmPBj variant. J. Virol. **67:**2466–2474.
53. **Novembre, F. J., M. M. Saucier, V. M. Hirsch, P. R. Johnson, and H. M. McClure.** 1994. Viral genetic determinants in SIVsmmPBj pathogenesis. J. Med. Primatol. **23:**136–145.
54. **Overbaugh, J., L. M. Rudensey, M. D. Papenhausen, R. E. Benveniste, and W. R. Morton.** 1991. Variation in simian immunodeficiency virus *env* is confined to V1 and V4 during progression to simian AIDS. J. Virol. **65:**7025–7031.
55. **Paxton, W. A., and S. Kang.** 1998. Chemokine receptor allelic polymorphisms: relationships to HIV resistance and disease progression. Semin. Immunol. **10:**187–194.
56. **Robinson, H. L., S. Lu, F. Mustafa, E. Johnson, J. C. Santoro, J. Arthos, J. Winsink, J. I. Mullins, D. Montefiori, and Y. Yasutomi.** 1995. Simian immunodeficiency virus DNA vaccine trial in macaques. Ann. N.Y. Acad. Sci. **772:**209–211.
57. **Ross, T. M., P. D. Bieniasz, and B. R. Cullen.** 1999. Role of chemokine receptors in HIV-1 infection and pathogenesis. Adv. Virus Res. **52:**233–267.
58. **Simon, M. A., L. V. Chalifoux, and D. J. Ringler.** 1992. Pathologic features of SIV-induced disease and the association of macrophage infection with disease evolution. AIDS Res. Hum. Retroviruses **8:**327–337.
59. **Smith, S. W., R. Overbeek, C. R. Woese, W. Gilbert, and P. M. Gillevet.** 1994. The genetic data environment an expandable GUI for multiple sequence analysis. Comput. Appl. Biosci. **10:**671–675.
60. **Stott, E. J., W. L. Chan, K. H. Mills, M. Page, F. Taffs, M. Cranage, P. Greenaway, and P. Kitchin.** 1990. Preliminary report: protection of cynomolgus macaques against simian immunodeficiency virus by fixed infected-cell vaccine. Lancet **336:**1538–1541.
61. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
62. **Trichel, A. M., E. D. Roberts, L. A. Wilson, L. N. Martin, R. M. Ruprecht, and M. Murphey-Corb.** 1997. SIV/DeltaB670 transmission across oral, colonic, and vaginal mucosae in the macaque. J. Med. Primatol. **26:**3–10.
63. **Uberla, K., C. Stahl-Hennig, D. Bottiger, K. Matz-Rensing, F. J. Kaup, J. Li, W. A. Haseltine, B. Fleckenstein, G. Hunsmann, B. Oberg, et al.** 1995. Animal model for the therapy of acquired immunodeficiency syndrome with reverse transcriptase inhibitors. Proc. Natl. Acad. Sci. USA **92:**8210–8214.
64. **Valli, P. J., V. V. Lukashov, J. L. Heeney, and J. Goudsmit.** 1998. Shortening of the symptom-free period in rhesus macaques is associated with decreasing nonsynonymous variation in the *env* variable regions of simian immunodeficiency virus SIVsm during passage. J. Virol. **72:**7494–7500.
65. **Vartanian, J. P., A. Meyerhans, B. Asjo, and S. Wain-Hobson.** 1991. Selection, recombination, and G→A hypermutation of human immunodeficiency virus type 1 genomes. J Virol. **65:**1779–1788.
66. **Wei, X., S. K. Ghosh, M. E. Taylor, V. A. Johnson, E. A. Emini, P. Deutsch, J. D. Lifson, S. Bonhoeffer, M. A. Nowak, and B. H. Hahn.** 1995. Viral dynamics in human immunodeficiency virus type 1 infection. Nature **373:**117–122.
67. **Wodarz, D., A. L. Lloyd, V. A. Jansen, and M. A. Nowak.** 1999. Dynamics of macrophage and T cell infection by HIV. J. Theor. Biol. **196:**101–113.