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Classic AIDS in a Sooty Mangabey after an 18-Year Natural Infection

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Prevailing theory holds that simian immunodeficiency virus (SIV) infections are nonpathogenic in their natural simian hosts and that lifelong infections persist without disease. Numerous studies have reported that SIV-infected sooty mangabeys (SMs; *Cercocebus atys*) remain disease free for up to 24 years despite relatively high levels of viral replication. Here, we report that classic AIDS developed after an 18-year incubation in an SM (E041) with a natural SIVsm infection. Unlike that described in previous reports of SIV-related disease in SMs, the SIVsm infecting E041 was not first passaged through macaques; moreover, SM E041 was simian T-cell leukemia virus antibody negative. SM E041 was euthanized in 2002 after being diagnosed with severe disseminated B-cell lymphoma. The plasma virus load had been approximately the same for 16 years when a 100-fold increase in virus load occurred in years 17 and 18. Additional findings associated with AIDS were $CD4^+$-cell decline, loss of p27 core antibody, and loss of control of SIVsm replication with disseminated giant cell disease. These findings suggest that the time to development of AIDS exceeds the average lifetime of SMs in the wild and that the principal adaptation of SIV to its natural African hosts does not include complete resistance to disease. Instead, AIDS may develop slowly, even in the presence of high virus loads. However, a long-term relatively high virus load, such as that in SM E041, is consistent with AIDS development in less than 18 years in humans and macaques. Therefore, the results also suggest that SMs have a special mechanism for resisting AIDS development.

The simian immunodeficiency virus (SIV) group naturally infects 35 different simian species in sub-Saharan Africa (1, 33). Because cross-species transmission to humans in the 20th century was the root cause of the AIDS pandemic (6, 13, 37), the major threat posed by SIVs is that most have the capacity to grow in vitro in human cells (19) and therefore have the potential for continued spread to humans. SIV-related viruses have clear pathogenic potential in humans and macaques. SIV- or human immunodeficiency virus (HIV)-induced immune suppression results in opportunistic infections and malignancies and is associated with high viral loads, depletion of $CD4^+$ T lymphocytes in various tissues, and loss of the anti-lentiviral Gag and Pol antibodies (21). However, the most intriguing aspect of SIV infections in their natural hosts is that they repeatedly have been reported to be nonpathogenic, lifelong infections that are characterized by relatively high viral loads in peripheral blood and tissues (2–4, 6, 7, 15, 30). Numerous studies have investigated the lack of a relationship between high SIV replication and disease (2–4, 6, 7, 15, 30, 31, 34, 38). If SIVs are nonpathogenic in their natural host, then this represents an exception in that disease is common in mammals infected with lentiviruses (28). The outcome of chronic lentiviral infection is immune suppression or a variety of diseases, including neurological disorders, arthritis, and others (28). It would be logical to expect SIV to cause disease, but the prevailing theory is that the lack of disease in natural SIV infections is due to viral adaptation following a long coevolution (13, 20, 31, 34, 38). However, this theory was generated after relatively short periods of observation. Here, we report a case of full-blown AIDS in a sooty mangabey (SM) after an 18-year natural SIV infection. AIDS in this SM was characterized by clinical, virological, and immunopathological findings that were indistinguishable from end-stage AIDS in macaques, including high viral loads, low $CD4^+$-T-cell counts, SIV giant cell disease, and B-cell lymphoma.

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MATERIALS AND METHODS

Histologic examination. The normal tissues and tumoral masses were fixed in 10% buffered formalin for 48 h, embedded in paraffin, and stained according to the conventional hematoxylin-eosin (HE) technique. The neoplastic samples were histologically examined and classified according to the National Cancer Institute Working Formulation and the Revised European-American Lymphoma Classification.

Immunohistochemistry. Immunohistochemical determinations for T cells, B cells, macrophages, proliferation markers, and SIV antigen were performed on formalin-fixed, paraffin-embedded material by using an avidin-biotin complex enzyme technique. The following primary antibodies were used: CD3, L26, HAM56, Ki-67 (all supplied by DAKO Corporation, Carpinteria, Calif.), and p27 (Mardex Diagnostics, Carlsbad, Calif.). Corresponding negative control sections were stained with an irrelevant-isotype antibody. Lymph nodes from an SIVsm-negative SM and a noninfected macaque were used as negative controls for the SIV staining.

To determine the phenotype of the SIV-infected cells, a double-immunofluorescence staining technique was used. Briefly, a 1-h incubation with a macrophage (HAM56) and an anti-SIV primary antibody (p27) was performed, followed by incubation with secondary antibodies conjugated with Alexa fluorochromes 488 and 568. The nuclei were stained with TO-PRO-3 (Molecular Probes, Eugene, Ore.), and sections were mounted with an antiquenching solution (Sigma, St. Louis, Mo.). Slides were examined using a true confocal laser scanning microscope (Leica, Wetzlar, Germany) equipped with three lasers that

span from the visible to the far-red side of the spectrum. Differential interference contrast was used for the observation of nonstained specimen during fluorescent confocal image collection.

In situ hybridization. Paraffin sections from the lymphoma tissue were examined by in situ hybridization for the detection of Epstein-Barr virus (EBV) and SIV. For in situ hybridization of EBV, a fluorescein-conjugated peptide nucleic acid probe complementary to the two nuclear EBV-expressed RNAs was used (DAKO Corporation, Carpinteria, Calif.). A DAKO Universal peptide nucleic acid in situ hybridization detection kit was used thereafter for detection of the fluorescent probe. The in situ hybridization of SIV was done as previously described (42), with a digoxigenin-conjugated riboprobe. For the negative controls, sections processed without conjugated probes for EBV, or the sense riboprobe for SIV, and sections from an SIV-seronegative SM were used. In addition, an EBV-positive lymphoma and tissues from an SIV-infected macaque were used as positive controls.

Antibody test by Western blotting. Anti-SIVsm antibody was tested by using commercially available SIV Western blot kits (Zeptometrix, Buffalo, N.Y.) according to the manufacture's instructions.

Quantification of plasma viral load. Blood was collected with EDTA as the anticoagulant. Plasma or serum was frozen at −80°C and analyzed by branched DNA (bDNA) as described previously (23) at Bayer Reference Testing Laboratory (Emeryville, Calif.). The limit of detection was 125 copies/ml.

Determination of $CD4^+$- and $CD8^+$-T-lymphocyte subsets. The numbers of CD4 and CD8 cells in peripheral blood were determined by the true-count method as previously described (23). For tumor tissue samples, a cellular suspension was obtained by mincing and pressing the sample through a nylon mesh screen. The cells were then washed twice in complete RPMI 1640 and adjusted to 10^7 cells/ml for flow cytometry staining. The cells were stained with 20 μl of anti-human CD3–fluorescein isothiocyanate (clone SP34) (Becton Dickinson immunocytometry system) and anti-CD20 antibodies in the dark for 30 min at 4°C, washed once in phosphate-buffered saline, and fixed overnight in 2% paraformaldehyde. To determine the numbers of tumoral proliferating cells, intracellular stainings for Ki-67 were performed after the permeabilization of the samples with saponin. The cells were acquired with a FACSCalibur flow cytometer (Becton Dickinson) and analyzed with Cell Quest software (Becton Dickinson). At least 50,000 events per sample were acquired.

Reverse transcriptase PCR and phylogenetic analysis. Total RNA was extracted from plasma samples collected from SM E041 in 1986, 2000, and 2002 by using a QIAamp viral RNA mini-kit (QIAGEN, Valencia, Calif.). Reverse transcriptase PCR was performed. The *env* primers and PCR thermocycling conditions are described elsewhere (23). PCR products were purified (QIAGEN) and directly sequenced in an ABI automated DNA sequencer. The *env* sequences were aligned with SIVsm and SIVmac reference sequences from the Los Alamos National Laboratory HIV sequence database (http://hiv-web.lanl.gov) by using the CLUSTAL_X program (40). The alignment was manually adjusted, and poorly aligned regions were excluded. Gap-containing sites were removed prior to analysis. A phylogenetic tree was constructed by using the neighbor-joining method (36) in the Phylip package, and reproducibility of the branching orders was estimated by 1,000 bootstraps.

RESULTS

Clinical and pathological findings of AIDS in an SM. SM E041 was 21 years old when it was euthanized with signs of chronic illness. At necropsy, multiple lymphomas were found throughout the abdominal and thoracic cavities. The largest mass was in the mesenteric lymph node, but several discrete masses were observed throughout the thoracic lymph duct along the aorta. Histologically, the tumor masses consisted of sheets of densely packed round cells that completely effaced tissue architecture (Fig. 1A). The neoplastic cells were moderate to large lymphoid cells with moderate pleomorphism. Mitotic cells with areas of hemorrhage and necrosis were frequently observed, indicating a high cell turnover. A particular morphological feature was the presence of a relatively high number of multinucleated giant cells, usually around and within the tumors. The giant cells were also found in normal areas of the lymph nodes, in the brain, in the lung (Fig. 1D), in the tongue papillae, and in the palatine tonsils. These cells were SIVmac p27 antigen positive and are common in SIVmac infection in macaques; their presence supports a diagnosis of SIVsm-associated lymphoma in SM E041. Flow cytometry and immunohistochemistry (Fig. 1B) confirmed that the neoplastic cells were CD3 negative and CD20 positive (B cells). Most were also Ki-67 positive, indicating a high cell turnover (data not shown). Based on these results, a diagnosis of immunoblastic B-cell lymphoma with plasmacytoid differentiation was made. In situ hybridization for EBV was negative for all tissues (data not shown).

Natural history of SIVsm infection in the SM colony at TNPRC. SM E041 was a male born in January 1981 at the Yerkes National Primate Research Center (YNPRC) and transferred to the Tulane National Primate Research Center (TNPRC) in July 1983.

According to TNPRC records, the TNPRC housed 86 SMs, 74 of which originated from the YNPRC mangabey colony in the 1980s. They were used for experimental leprosy infection (17, 18) because of their unique susceptibility to *Mycobacterium leprae* (16, 25, 46). A high prevalence of SIV infection has been reported for the YNPRC colony (10), and 90% of SMs were SIVsm infected when they were moved to TNPRC (29).

SM A015 was naturally infected with *M. leprae* and was the first donor of *M. leprae*-infected lepromatous tissue for subsequent experiments. SM A022 was inoculated with lepromatous tissue from SM A015 in 1980. In 1984, at the age of 3.2 years, E041 was intravenously inoculated with a tissue extract of an *M. leprae* lesion from A022. In summary, A015 donated tissue to A022, and then A022 donated tissue to E041. No signs of leprosy were seen in E041 during the 18 years of observation. There was also no histological evidence of leprosy at necropsy (data not shown).

Dynamics of anti-SIVsm by Western blot analysis. To determine when SIVsm infection began in the colony and whether SIVsm infection was inadvertently caused by intravenous *M. leprae* inoculation, a retrospective analysis of the SIVsm infection status from SMs in the leprosy study was performed. Serial serum or plasma specimens obtained before and after *M. leprae* inoculation were analyzed by Western blotting for anti-SIVsm antibody. A015 was anti-SIV antibody negative and anti-simian T-cell leukemia virus (STLV) positive, findings consistent with a previous report (29). A022 was SIV antibody positive prior to *M. leprae* inoculation (data not shown).

The dynamics of SIVsm-specific antibody in serial samples collected from SM E041 between 1984 and 2002 are shown in Fig. 2. In samples taken in February 1984, before the inoculation of E041 with lepromatous A022 tissue, Western blot assays for SIV revealed that E041 was positive for SIV antibody (Fig. 2, lanes 84a and 84b). These results were confirmed by the simian retrovirus laboratory at the California National Primate Research Center. E041 was also negative for simian retrovirus type D and STLV antibody (data not shown). Antibody to SIV p27 remained detectable until 1995 (Fig. 2, lane 95). Between 1995 and 2000, p27 antibody decreased (Fig. 2, lanes 95 and 00). Samples tested positive for p27 antigenemia in 2000 (125 pg/ml), and the antigenemia increased to 8,763 pg/ml in 2002. p27 antigenemia had been undetectable in all previous samples. In HIV-infected persons, p24 antigenemia tests are positive only during primary infection, and p24 antigenemia then becomes undetectable. The reappearance of p24

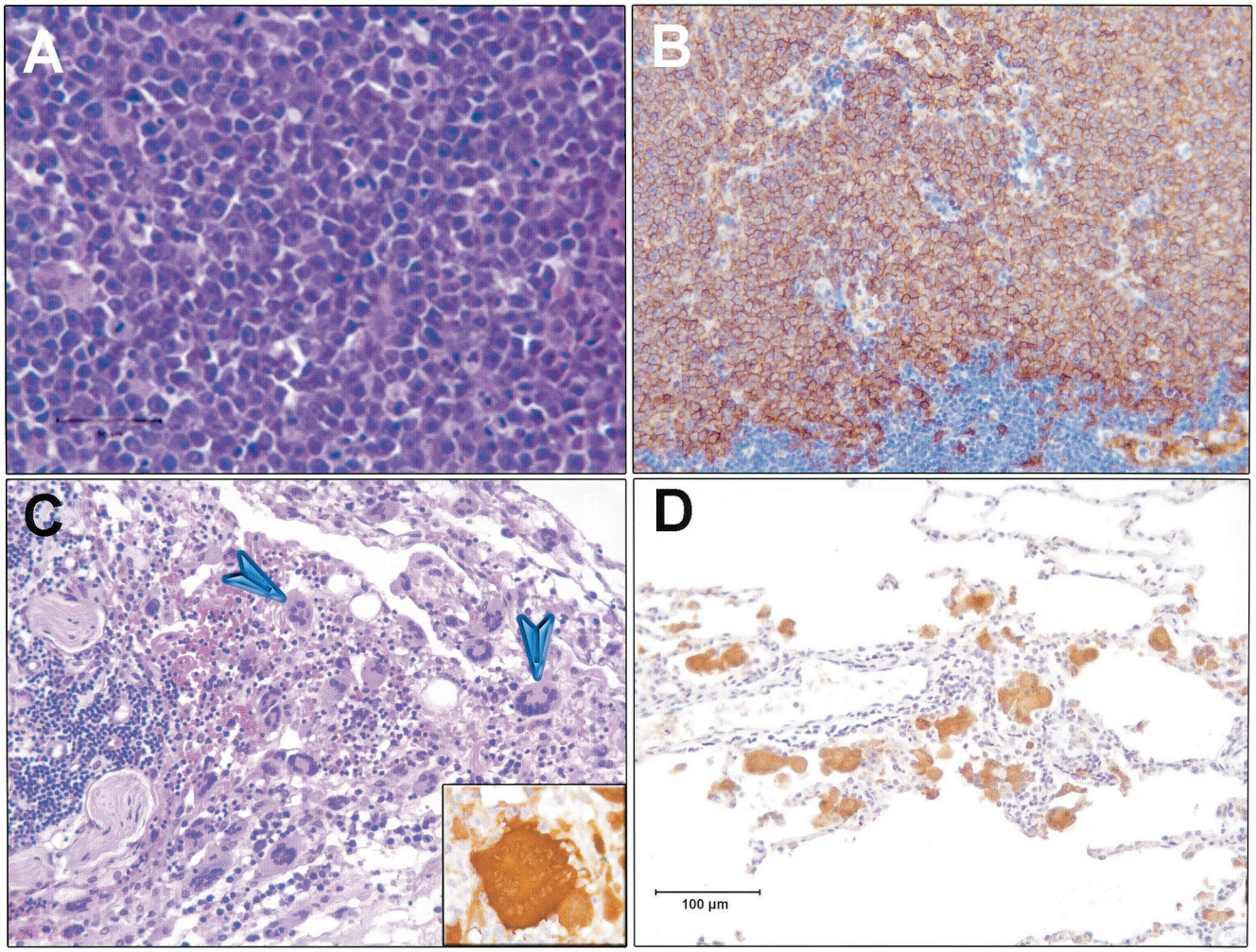

FIG. 1. (A) HE-stained sections of lymphoma tissue. The tumor consisted of sheets of densely packed round cells that completely effaced the architecture of the mesenteric lymph nodes. Neoplastic cells were moderate- to large-sized lymphoid cells with moderate pleomorphism. (B) Immunophenotyping of neoplastic cells. The majority of large, atypical cells were $CD20^+$ (B cells) as demonstrated by immunohistochemistry (stained brown). (C) HE stain of giant cells (arrows) in the connective tissue surrounding lung hilus. Inset, a giant cell that tested positive for SIV by immunohistochemistry (stained brown). (D) Lung showing multiple $SIVp27^+$ giant cells (stained brown). Giant cell disease is also characteristic of AIDS seen in macaques infected with SIVsm. Immunohistochemistry was performed as described previously (44, 45).

antigenemia is considered a surrogate marker for AIDS (21). These findings are highly consistent with a diagnosis of AIDS and also show that E041 was SIVsm infected before the leprosy experiments began. E041 was infected while in the Yerkes or Tulane colony. The natural spread of SIV between group-housed mangabeys is common (10, 29) at both the Tulane and Yerkes primate centers.

Genetic and biological characteristics of SIVsmE041. The virus from SM E041 was designated SIVsmE041. A region of *env* (436 bp) was sequenced from blood samples collected in 1986, 2000, and 2002 (SIVsmE041-86, SIVsmE04-00, and SIVsmE041-02, respectively). Phylogenetic analysis showed SIVsmE041-86 and SIVsmE041-00 clustered together (Fig. 3). SIVsmE041-02 was more distant from SIVsmE041-86 and SIVsmE041-00. Seven nucleotide mutations were observed in SIVsmE041-02, and four were observed in SIVsmE041-00 relative to the sequence for SIVsmE041-86. These mutations may have accumulated because of the relatively high level of replication during the last 2 years of the infection. Attempts to amplify SIV RNA from the 1984 samples were negative. However, the 1984 samples were positive for bDNA (see below).

Sequence comparisons between SIVsmA022 and SIVsmE041 showed that the two viruses, although belonging to the same SIVsm lineage, did not cluster closely within the group. This is a second line of evidence that E041 was not infected from lepromatous tissue from A022. This finding is important because it shows that the pathogenic virus SIVsmE041 was not a serially passaged virus but instead was naturally acquired from the SM colony. SIVsm group 1 contains SIV-PBj, SIV-B670, and SIVsmH4 (Fig. 3). It should be noted that SIVsm strains in this group were pathogenic in Asian macaques; for instance, SIV-PBj was pathogenic in pig-tailed macaques (*Macaca nemestrina*) and cynomolgus monkeys (*Macaca fascicularis*) (11, 22).

Since approximately 40 to 50% of the cases of HIV infection that progress to AIDS are associated with a switch in virus tropism from R5 to X4 viruses, the tropism of the SIVsmE041-

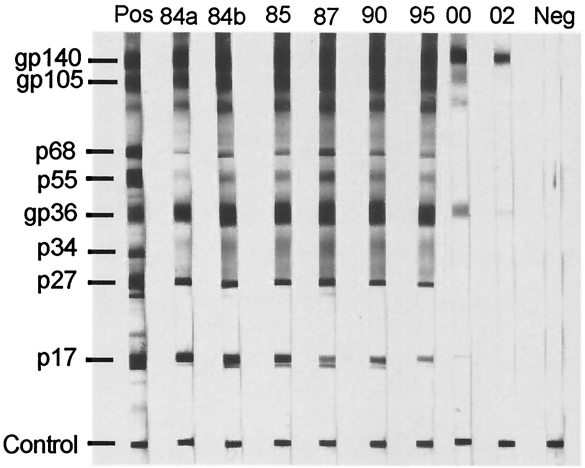

FIG. 2. Western blot analysis of plasma from E041 over an 18-year period of SIV infection. p27 antibody was detected from 1984 (lanes 84a and 84b) through 1995 (lanes 85, 87, 90, and 95). p27 antibody was undetectable in 2000 (lane 00) and on the day of euthanasia in 2002 (lane 02). gp140 antibody was the only SIV antibody detected in 2002 (lane 02). Specimens 84a and 84b were taken prior to *M. leprae* inoculation. SIV antibody-positive (lane Pos) and -negative (lane Neg) controls are shown.

02 virus was tested on various ghost cells expressing different SIV coreceptors (5). The major coreceptor remained R5 (data not shown).

Virological and immunological findings of SIVsm-induced AIDS. The dynamics of the HIV or SIV viral load is the best predictor of evolution toward AIDS in pathogenic models (27). We investigated the SIVsmE041 viral load over an 18-year period by bDNA assay. E041 was infected with SIVsm from February 1984 to the day of euthanasia in October 2002. No samples taken prior to 1984 were available. The SIV plasma virus load in 1984 was almost 80,000 copies/ml and had already reached an apparent set point before E041 was inoculated with lepromatous tissue (Fig. 4). The plasma viral load remained approximately constant (about 120,000 copies/ml) for 11 years, until 1995. These values are in the same range as those described for most SIVsm-infected SMs and also for SIVagm-infected African green monkeys or SIVmnd-1-infected mandrills during the chronic phase of SIV infection (3, 15, 31). Between 1996 and 2002, the plasma virus load increased 100-fold to 10^7 copies/ml on the day of euthanasia, when E041 was 21 years of age. Similar increases occur in AIDS patients, and the results for E041 also paralleled the serological markers of disease progression.

Based on immunophenotyping of T lymphocytes and the percentages of CD3 cells, the levels of CD4 cells in the blood were 21.7% (1,475 cells/ml) in 1984, 20% (1,340 cells/ml) in 1985, and 25% (2,836 cells/ml) in 1986. Sixteen years later, in 2000, CD4 cells were at 25% (718 cells/ml). In contrast, by October 2002, $CD4^+$ cells were significantly depleted in peripheral blood, with a value of 78 cells/ml or 13.8%. The percentage of $CD4^+$ cells in the small intestine was 36%, with $CD4^+$ $CCR5^+$ cells at 0.3%. This percentage of $CD4^+$ $CCR5^+$ cells in the gut is consistent with the finding of AIDS in macaques (43). However, normal values for intestinal $CD4^+$ $CCR5^+$ cells in the guts of uninfected SMs have not been established. Due to the endangered species status of SMs at the TNPRC, normal values could not be obtained from the few remaining SIV-negative animals in the colony.

DISCUSSION

This case provides compelling evidence that SIV infection in a naturally infected African monkey can lead to AIDS. Other cases of immune suppression have been reported in African nonhuman primates as a consequence of natural SIV infection. YNPRC documented that three natural SIVsm and STLV-1 infections in SMs progressed to AIDS-like diseases, but the possibility of a role for STLV-1 in these cases could not be ruled out (26). SIV-PBj, a virus passaged in macaques, induces acute disease and death not only in pigtailed and cynomolgus

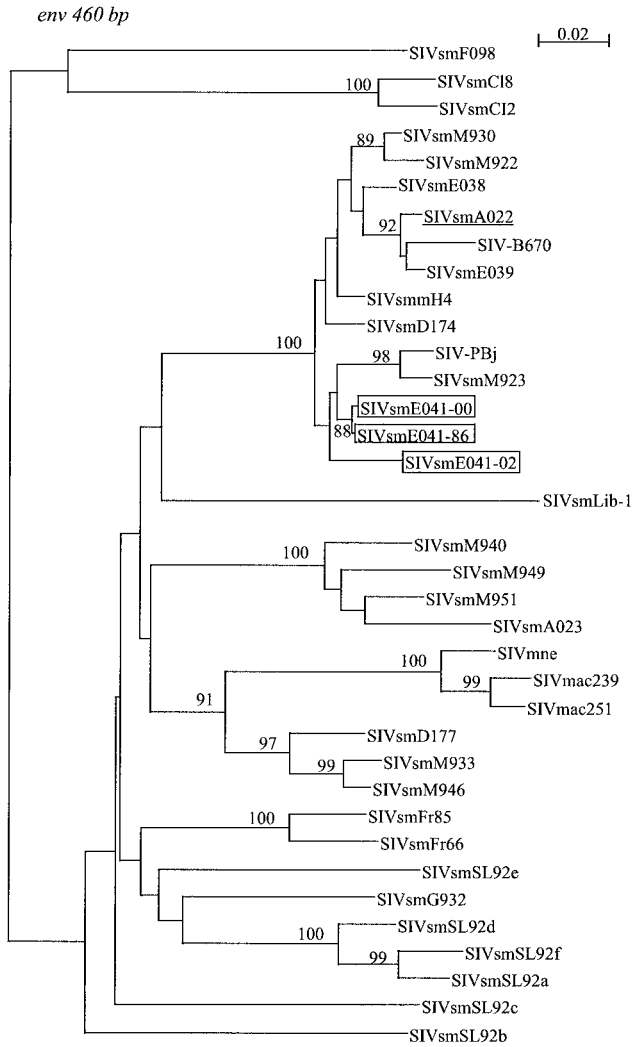

FIG. 3. Phylogenetic analysis of 460-bp *env* sequences of SIVsmE041 in 1986, 2000, and 2002 (boxed). These sequences were compared with known SIVsm reference sequences from the GenBank database. Phylogenetic trees were estimated by the neighbor-joining method (36). The reliability was estimated from 1,000 bootstrap replicates; bootstrap values of less than 70% are not shown on the branches. SIVsm A022 (underlined) showed a different branching order from that of SIVsm E041 (boxed).

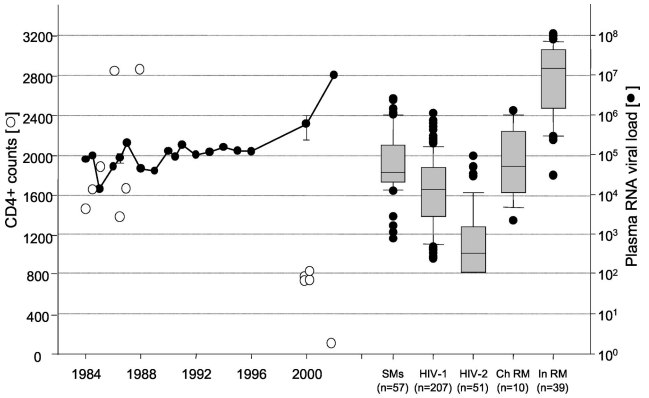

FIG. 4. Plasma RNA viral load in SM E041 over an 18-year period. The plasma viral load was about 80,000 to 110,000 copies/ml over an 11-year period, from 1984 to 1995. The virus load with AIDS increased 100-fold between 1996 and 2002, the time of euthanasia. CD4 cell numbers were between 1,500 and 2,700 cells/μl from 1984 through 1986. No CD4 cell data were available between 1985 and 2000. By 2000, the CD4 cell count had declined to 765, and it dropped to 50 cells/μl on the day of euthanasia. The mean plasma viral loads of SIVsm-infected SMs, HIV-1- or HIV-2-infected persons, and experimentally SIVmac-infected Chinese (Ch RM) and Indian (In RM) rhesus monkeys (24) are also shown for comparison.

macaques but also in SMs (11, 22). However, although SIV-PBj had been derived from naturally infected SMs, it was passaged in a pigtailed macaque. Moreover, SIV-PBj contains mutations not found in naturally infected SMs (8). The parent virus of SIV-PBj, SIVsm9, which was isolated from a naturally infected SM, induces a slowly progressing AIDS-like disease in rhesus monkeys (*Macaca mulatta*) and pig-tailed macaques (*Macaca nemestrina*) (9, 10, 12) but has not been shown to be pathogenic in mangabeys.

Lymphoproliferative disease, CD4 depletion, and opportunistic infection were found in an African green monkey that had been naturally infected with both SIVagm and STLV-1 more than 10 years earlier (41). This case suggested that AIDS occurred as a result of SIVagm-induced immune suppression in the natural host. A second case of immune suppression in a naturally SIVmnd-2-infected mandrill (*Mandrillus sphinx*) has been reported (39). A female mandrill born in Sarasota, Fla., in 1971 and housed in the San Diego Wild Animal Park since 1984 was tested and found to be SIVmnd-2 infected (39). This mandrill died at 18 years of age from persistent diarrhea and weight loss, unresponsive invasive *Balantidium coli* infection, and disseminated atypical mycobacteriosis. Although viral load analyses were not done, the clinical signs, presence of opportunistic infections, and $CD4^+$-T-cell depletion were consistent with the classical definition of AIDS. Finally, a female mandrill in Gabon in a semifree-ranging colony since 1983 was diagnosed as SIVmnd-1 infected in 1988 (32). This animal had been infected in the wild before 1985, and it died in 2000 at the approximate age of 20 years. Sequential plasma samples collected since 1985 showed a significant increase in viral load (>0.5 log) that paralleled a significant decrease in both anti-gp36 and anti-V3 antibody titers. Weight loss, as well as $CD4^+$-T-cell depletion from 19% in 1995 to 6.6% in 2000, was also recorded. The major weakness of this study was that no necropsy was done, because the dead mandrill was not found in the large forested outdoor enclosure that housed the mandrill colony. Altogether, the data show that lentivirus-induced immune suppression can occur but may require long incubation periods. In all cases thus far, including that of SM E041, the time to disease was at least 10 years and possibly more than 18 years.

The long incubation period to development of AIDS, in spite of a high virus load, may represent the major mechanism of adaptation of SIVsm to its natural host. The average age of SMs euthanized at the TNPRC for all reasons was 14 years. At 21 years, E041 was the third-oldest SM to be euthanized at the TNPRC in 20 years of record keeping. When the highest and lowest values were dropped, E041 had an average plasma virus load of 95,818 ± 40,540 copies/ml (mean ± standard deviation) for 11.6 years. This set point for E041 parallels the plasma virus load seen in SIVmac infections in rhesus monkeys of Chinese origin (Fig. 4). However, AIDS developed in 2 to 4 years in six SIV-infected Chinese-origin macaques with similar or lower plasma virus loads (24). HIV-1 set points are generally lower than those of E041 (Fig. 4), with an average value of 11,508 copies/ml in 177 patients (14). However, AIDS develops in most humans in 10 to 12 years in untreated cases (27). E041, therefore, had a longer course of disease than would be expected based on its relatively high virus load compared to macaque and human plasma virus loads (Fig. 4). The data suggest, therefore, that SMs have a special mechanism for resisting AIDS development.

In the captive colony of SMs at the TNPRC, plasma virus loads range from <200 to 1,900,000 copies/ml (23). A retrospective analysis of the results of 30 necropsies of SIV-infected SMs aged 7 to 23 years revealed no evidence of AIDS. However, scattered giant cells were seen in several individuals (data not shown). The evolutionary adaptation appears to be that the incubation period exceeds the normal life span of most SMs in captivity and almost certainly exceeds that of SMs in the wild. For mandrills, this hypothesis was proposed by Pandrea et al. (32). The present study adds an additional species and data to support the hypothesis. Complete resistance to SIV AIDS is apparently not the primary adaptation, as has been previously believed.

An understanding of this adaptation mechanism will be a useful model for the prevention of the progression to AIDS in humans. The same adaptation mechanism is desirable with highly active antiretroviral therapy, whose goal is to transform HIV infection into a chronic, long-lasting infection without AIDS. Recent data on effective AIDS vaccines in nonhuman primates showed that the same objective may be attained with an effective vaccine (35). This report provides evidence that studying the SM model may lead to improved vaccine and therapeutic strategies to achieve long-term nonprogression. The study of lentiviral infections in African primates will provide valuable insights not only for meeting the major objective of suppressing HIV infections, but also for maintaining a disease-free state in HIV-infected individuals.

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