Isolation and Characterization of Simian Immunodeficiency Virus from Mandrills in Africa and Its Relationship to Other Human and Simian Immunodeficiency Viruses

HAJIME TSUJIMOTO,1,* ROBERT W. COOPER,2 TOSHIKAI KODAMA,1 MASASHI FUKASAWA,1 TOMOYUKI MIURA,1 YOSHIHIRO OHTA,1 KOH-ICHI ISHIKAWA,1 MASUYO NAKAI,1 ERIC FROST,2 GEORGE E. ROELANTS,2 JEAN ROELANTS,2 AND MASANORI HAYAMI1

Department of Animal Pathology, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108,1 and Osaka Medical College, Takatsuki, Osaka 569,3 Japan, and Centre International de Recherches Médicales de Franceville, B.P. 769, Franceville, Gabon2

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Two isolates of simian retrovirus related to the human immunodeficiency virus (HIV) were obtained from apparently healthy mandrills, Papio (Mandrillus) sphinx, in western equatorial Africa. This virus, designated SIVMND (simian immunodeficiency virus from mandrills), appeared morphologically similar to HIV by electron microscopy, showed Mg2+-dependent reverse transcriptase activity, and induced cytopathic effect in human CD4-positive cells. Western blotting (immunoblotting) analyses revealed that the gag and pol products of SIVMND showed cross-reactivity with those of known HIVs and SIVs. Molecular clones covering full-length viral DNA were obtained from closed circular extrachromosomal DNA of SIVMND-infected cells. By clone-on-clone hybridization with known retroviruses of the HIV and SIV groups, SIVMND showed similar cross-hybridization with HIV-1, HIV-2, SIVAGM (African green monkey-derived SIV), and SIVMAC (rhesus macaque-derived SIV) in the gag and pol regions only at low stringency but not at high stringency, a result indicating that SIVMND is a new member of the HIV-SIV group. The existence of distinct SIVs in different monkey species suggest that recent interspecies transfer of HIV-SIV is unlikely in nature.

The epidemic of acquired immunodeficiency syndrome (AIDS) began in 1981 in the United States and apparently as early as the mid-1970s in Central Africa and has now spread worldwide (6, 31). Human immunodeficiency virus type 1 (HIV-1) belonging to the lentivirus family (13), which was formerly called lymphadenopathy-associated virus (2), human T-lymphotropic virus type III (30), or AIDS-associated retrovirus (24), is known to be an etiological agent of AIDS in humans.

A simian retrovirus antigenically related to HIV was first isolated from a rhesus macaque, Macaca mulatta, with an AIDS-like syndrome at a U.S. primate center (7, 22). The virus was initially designated as rhesus macaque-derived simian T-lymphotropic virus type III but is now called rhesus macaque-derived simian immunodeficiency virus, SIVMAC. Subsequently, a large proportion of African green monkeys (Cercopithecus aethiops) (16, 20, 21, 28) and sooty mangabeys (Cercopithecus atys) (12, 26, 27) were shown to be naturally infected with HIV-related retroviruses but were apparently free of AIDS-like disease. A second human AIDS-related retrovirus, HIV-2, isolated from patients with AIDS in West Africa, was shown to be antigenically closer to SIVMAC than to HIV-1 (5). Nucleotide sequence analyses indicate that HIV-2 is closely related to SIVMAC, with about 75% overall nucleotide sequence homology, but only distantly related to HIV-1, with about 40% overall homology (4, 10, 14).

On the basis of these observations, some workers suggested that monkeys might be a reservoir for an etiological agent of AIDS in humans (20, 21, 27). To understand the origin, natural history, and evolutionary relationships of viruses of the HIV and SIV groups, it will be necessary to obtain isolates from many more species of naturally infected primates and to study their genetic relationship. Herein we report the isolation of a new SIV from wild-caught mandrills, Papio (Mandrillus) sphinx, in Gabon, the cloning of the entire viral genome, and its comparison with other viruses of the HIV and SIV groups.

MATERIALS AND METHODS

Animals. Of 16 wild-caught mandrills in a semi-free-range breeding colony maintained by the Primatology Center of the Centre International de Recherches Médicales de Franceville in Gabon, two adult animals (a breeding male and female) were found to be weakly seropositive for SIV from African green monkey, SIVAGM, by immunofluorescence and Western blotting (immunoblotting) analyses (28). Because these adults had been seropositive since their acquisition by the center as juveniles in 1979 and 1983, respectively, it is virtually certain that they had become infected in the wild. All animals in this colony, including these two SIV-seropositive adults, were healthy and with no symptoms of AIDS-like disease.

Virus isolation. Peripheral-blood mononuclear cells from the two seropositive mandrills (2 × 106 cells per ml) were stimulated with 25 μg of concanavalin A per ml for 24 h and cultured in RPMI 1640 growth medium supplemented with 20% heat-inactivated fetal calf serum, antibiotics, and 10% crude interleukin-2. After cultivation for 3 to 7 days, these mandrill cells were cocultivated with Mol-4 clone 8 cells (23), which are highly susceptible to HIV-1 infection and its cytopathogenicity. After cocultivation, the medium without interleukin-2 was replaced every 3 days, and the cultures were examined for the expression of cytopathic effects (CPE). The expression of viral proteins in cultured cells was detected by immunofluorescence assay as described previ-
ously (28). The release of virus particles was examined by reverse transcriptase (RT) assay (28) and electron microscopy. When the production of SIV in the Molt-4 clone 8 cells was confirmed, the supernatant was inoculated into uncloned Molt-4 cells to obtain a persistently virus-infected cell line showing no CPE.

**Western blotting.** For both mandrill isolates, virions were concentrated and purified from the culture supernatant of the persistently virus-infected Molt-4 cells by sucrose gradient ultracentrifugation as described previously (28). HIV-1 and SIV<sub>AGM</sub> virions were also prepared from H9 cells infected with human T-lymphotropic virus type III<sub>r</sub> (30) and Molt-4 cells infected with SIV<sub>AGM</sub>[TYO-1] (previously called SIV (AGM-1)) (28), respectively, by the same procedures. These virion suspensions were treated with 2% sodium dodecyl sulfate and 5% 2-mercaptoethanol, boiled briefly, and subjected to electrophoresis on 12% polyacrylamide slab gels. After electrophoresis, the size-fractionated proteins were transferred to Durapore filters (Millipore Corp., Bedford, Mass.) by electroblotting. The filters were incubated with 5% fetal calf serum to block nonspecific reactions and then cut into strips. These strips were then washed, incubated with biotin-labeled sheep anti-human immunoglobulins at 37°C for 1 h and then with biotinylated peroxidase-streptavidin complex (Amersham, Tokyo, Japan) at room temperature for 30 min. The strips were then washed, and peroxidase activity was detected by the addition of a substrate solution containing 0.5 mg of diaminobenzidine per ml supplemented with 0.01% hydrogen peroxide.

**DNA clones of reference virus strains.** A 32P-labeled pSAH12 probe was used to detect the genome of immunodeficiency virus from mandrills (SIV<sub>MND</sub>) for molecular cloning. This probe is a full-length clone of SIV<sub>AGM</sub> isolated in our laboratory (11). Molecular clones of HIV-1, HIV-2, SIV<sub>MAC</sub>, and SIV<sub>AGM</sub> were used for comparison with SIV<sub>MND</sub>. A nearly full-length DNA clone of HIV-1 containing a 9-kilobase (kb) S<sub>ac</sub>1 fragment (pBT-1) (1) was kindly provided by Luc Montagnier. The HIV-2 clone used was pGH-1, which covers the entire genome of HIV-2(GH-1) and was isolated from a patient with AIDS in Ghana (K. Ishikawa, H. Tsujimoto, M. Nakai, J. A. A. Mingle, M. Osei-Kwasi, S. E. Aggrey, V. B. A. Netty, S. N. Afokwa, M. Fukasawa, T. Kodama, M. Kawamura, and M. Hayami, manuscript submitted for publication); its approximate nucleotide sequence homology with the Institute Pasteur isolate, HIV-2<sub>ROD</sub> (14), is 90% in the gag and pol regions and 80% in the env region. The SIV<sub>MAC</sub> (simian T-lymphotropic virus type III) probes used were pK2-10BamA (gag-pol) (18) and pK2-OLTR (3' open reading frame-long terminal repeat [LTR]), which were kindly provided by James I. Mullins.

**Molecular cloning.** Extrachromosomal DNA was stably isolated essentially by the Hirt method (19) from a 24-h culture of Molt-4 clone 8 cells inoculated with 10<sup>8</sup> to 10<sup>5</sup> 50% tissue culture infective dose per ml of SIV<sub>MND</sub>[GB-1] and showing prominent CPE such as syncytium formation and ballooning. The DNA sample was treated with proteinase K (50 µg/ml) at 37°C for 2 h, extracted with phenol and chloroform-isoamyl alcohol (24:1), precipitated with ethanol, and then treated with RNase A (50 µg/ml). Closed circular DNA of SIV<sub>MND</sub>, which was shown to have a single XhoI site in the circular genome, was linearized by digestion with XhoI. The linearized viral DNA was fractionated by 0.8% agarose gel electrophoresis, and the DNA fraction of 8 to 10 kb was electroeluted from the gel and extracted with phenol and chloroform-isoamyl alcohol. The XhoI-digested DNA fraction containing linearized SIV<sub>MND</sub> DNA was ligated with XhoI-digested lambda-ong-C vector arms (Stratagene, San Diego, Calif.) and packaged in vitro with Gigapack Gold (Stratagene), and the recombinant phages were plated and screened in situ with a 32P-labeled full-length SIV<sub>AGM</sub> probe after transfer to nylon membrane filters. Several signal-positive plaques were purified and expanded, and the insert DNA in phage vector was cut out by XhoI digestion and recloned into a pUC119 plasmid vector (33).

**Southern blot hybridization.** Undigested and restriction endonuclease-digested DNA samples were subjected to electrophoresis in 0.8% agarose gel and transferred to nylon membrane filters. The filters were then irradiated with UV light to cross-link them to the DNAs. Hybridization at low stringency was carried out at 37°C in a mixture of 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 25% formamide, 5× Denhardt solution, 0.1% sodium dodecyl sulfate, 10 mM EDTA, 20 µg of denatured salmon sperm DNA per ml, and a 32P-labeled probe (2 × 10<sup>6</sup> to 5 × 10<sup>6</sup> cpm/ml). After hybridization, the filters were washed three times at 50°C with a solution containing 2× SSC, 0.1% sodium dodecyl sulfate, and 1 mM EDTA. This low-stringency condition corresponds to T<sub>m</sub> = 40°C, a value calculated by using the standard formula and taking the GC content of HIV-1 as 42% (34). A hybridization solution containing 30% formamide at 37°C and a washing solution containing 0.5× SSC at 50°C were used for a medium-stringency condition (T<sub>m</sub> = 30°C), and 40% formamide hybridization solution at 37°C and a washing solution containing 0.2× SSC at 55°C were used for a high-stringency condition (T<sub>m</sub> = 23°C). These filters were then exposed to X-ray films for 12 to 36 h at −70°C.

**RESULTS**

**Virus isolation.** Peripheral-blood mononuclear cells from the two seropositive mandrills were cultured for 3 to 7 days and then cocultivated with Molt-4 clone 8 cells. After cocultivation for 1 to 2 weeks, characteristic giant-cell formation like that in HIV-infected Molt-4 clone 8 cells was observed in both cultures from the mandrills. On immunofluorescence assay, these giant cells and some normalized cells gave strong positive reactions with sera from these two seropositive mandrills but only weakly positive reactions with anti-HIV-1-positive human sera and anti-SIV<sub>AGM</sub>-positive African green monkey sera.

The culture supernatants of these Molt-4 clone 8 cells showing marked CPE contained high Mg<sup>2+</sup>-dependent RT activity. The RT activity reached a maximum of more than 1 × 10<sup>6</sup> cpm/ml, and the RT activity with Mg<sup>2+</sup> was more than 10 times that with Mn<sup>2+</sup>. Electron microscopic examination of the cells showing CPE revealed mature virus particles of 100- to 120-nm diameter in the extracellular space (Fig. 1). The mature particles consisted of a condensed core that appeared tubular-prismatic or centrosymmetrical, depending on the plane of section, and an envelope with relatively prominent knobs. Particles budding from the cell membrane in which the electron-dense shell was closely apposed to the envelope were also seen. These two SIV isolates were designated SIV<sub>MND</sub>[GB-1] and SIV<sub>MND</sub>[GB-2].

As CPE in the Molt-4 clone 8 cells became prominent, cell proliferation became markedly impaired. Therefore, the supernatant of the infected cells was inoculated into uncloned Molt-4 cells to obtain persistently infected cell lines. At 1 or 2 weeks after inoculation, the Molt-4 cells showed...
CPE which was not so marked as that for clone 8 cells. After 4 to 5 weeks, the CPE disappeared, and normal cell proliferation resumed, but almost all the cells from the two Molt-4 cell lines expressed viral antigens that were detected with seropositive mandrill sera by immunofluorescence. Furthermore, high RT activity and abundant virus particles were detected in these cell cultures.

Analysis of virion proteins. The purified virions of two SIV<sub>MND</sub> isolates collected from the culture supernatant of persistently infected Molt-4 cells were analyzed by Western blotting for identification of the SIV<sub>MND</sub> proteins and their cross-reactivity with HIV-1 and SIV<sub>AGM</sub> (Fig. 2). When the virion proteins of SIV<sub>MND</sub> were incubated with the serum of a patient with AIDS containing anti-HIV-1 antibody, four major bands at positions of approximately 66, 55, 31, and 26 kilodaltons, corresponding to the pol and gag gene products of HIV-1 (8, 25), were specifically identified. Anti-SIV<sub>AGM</sub>-positive African green monkey serum reacted with the protein of a smeared band at approximately 30 kilodaltons, corresponding to the gp32 transmembrane protein of SIV<sub>AGM</sub> (28), and with the gag and pol gene products of SIV<sub>MND</sub>. The serum of the mandrill from which SIV<sub>MND</sub>[GB-1] was isolated also recognized a 120 kilodalton-protein of SIV<sub>MND</sub>, which should correspond to the surface protein of the envelope. The mandrill serum reacted mainly with env gene products and scarcely reacted with the gag and pol gene products of even SIV<sub>MND</sub>. These Western blotting analyses indicated that the gag and pol gene products of SIV<sub>MND</sub> showed distinct cross-reactivity with those of HIV-1 and SIV<sub>AGM</sub> but that the env gene products of SIV<sub>MND</sub> partially cross-reacted only with those of SIV<sub>AGM</sub>. Furthermore, the molecular weights of a gag protein (p26) and transmembrane protein (gp30) in SIV<sub>MND</sub> were slightly different from their corresponding proteins in HIV-1 (9) and SIV<sub>AGM</sub> (28).

Molecular cloning. Immunologic cross-reactivity between SIV<sub>MND</sub> and SIV<sub>AGM</sub> indicated the possible value of cloned SIV<sub>AGM</sub> DNA as a probe for detecting the SIV<sub>MND</sub> genome. Hirt supernatant DNA prepared from Molt-4 clone 8 cells showing marked CPE after infection with SIV<sub>MND</sub> was subjected to Southern blot hybridization with an SIV<sub>AGM</sub> full-length probe, pSAH12.

With these DNAs, faint specific hybridizing bands were detected only at low stringency (T<sub>m</sub> - 40°C) after a long exposure (Fig. 3A) and not at medium (T<sub>m</sub> - 30°C) or high stringency. The undigested Hirt supernatant gave a band of approximately 9.4 kb and two very faint bands of about 5 kb. Probably, the former band corresponded to linear unintegrated viral DNA, whereas the latter bands originated from the closed circular forms with one and two LTRs (29). After XbaI digestion, the two bands of closed circular DNA were shifted to a position of about 9.4 kb, and the linear viral DNA was cut into two fragments of 5.6 and 3.8 kb, a result indicating that XbaI has a single cutting site in the SIV<sub>MND</sub> genome. The XbaI-digested Hirt supernatant DNA of SIV<sub>MND</sub>-infected cells was ligated to XbaI-digested lambda-
BamHI, PstI, from plasmid SIV and were groups full-length DNAs between SIVMND DNA fragmentation detected under were bands when they in size cell lines, with genome The bands detected with and SIVMND[GB-2] clones, eight these SIVMAC, of SIVMAC(pK2-lOBamA), that the ongoing-C vector arms and packaged. Of about $5 \times 10^4$ plaques, 10 clones showed a positive signal on in situ screening with the SIVAGM probe under conditions of low stringency. Of these clones, eight had an insert of 8.8 kb and two had an insert of 9.5 kb. Restriction endonuclease mapping indicated that the clone with the longer insert (pSMH103) had two additional adjoining PstI and XhoI sites. Thus the long and short inserts may correspond to the full-length SIVMND genome with two and one LTRs, respectively.

**Detection of viral genome in infected cells.** Hirt supernatant DNA from Molt-4 clone 8 cells infected with SIVMND[GB-1], which was used as a DNA source for molecular cloning, was examined by Southern blot hybridization with the $^{32}$P-labeled cloned SIVMND insert, pSMH103 (Fig. 3B). The undigested DNA gave strong bands of approximately 9.4 kb and two faint bands of about 5 kb, and the digests with several restriction endonucleases also gave distinct bands at high stringency ($T_m - 23^\circ C$), which coincided with the faint bands detected with the SIVAGM probe at low stringency. The SIVMND genome in the chromosomal DNA of the two Molt-4 cell lines persistently infected with SIVMND[GB-1] and SIVMND[GB-2] was then examined. For both of these cell lines, bands that hybridized distinctly and corresponded in size to internal fragments of the cloned SIVMND genome were detected under conditions of high stringency. The DNA fragment patterns of these two cell lines with BamHI, PstI, HindIII and XbaI were identical. These SIVMND-infected cell DNAs did not give any detectable bands when they were probed with cloned HIV-1, HIV-2, SIVMAC, or SIVAGM under the same conditions.

**Homology between SIVMND and other viruses of the HIV and SIV groups.** The approximate sequence homologies between SIVMND and known viruses of the HIV and SIV groups were then examined. First, full-length or nearly full-length DNAs of HIV-1 (pBT-1), HIV-2 (pGH-1), SIVMAC (pK2-10BamA), and SIVAGM (pSAH12) excised from plasmid clones were blot hybridized to a $^{32}$P-labeled...

**FIG. 2.** Identification of SIVMND proteins and their cross-reactivity with those of HIV-1 and SIVAGM by Western blot analysis. Purified virions of SIVMND[GB-1] (lanes 1), SIVMND[GB-2] (lanes 2), SIVAGM (lanes 3), and HIV-1 (lanes 4) were examined by Western blot analysis with sera from a patient with AIDS and seropositive for HIV-1 (A), an African green monkey seropositive for SIVAGM (B), and the mandrill from which SIVMND[GB-1] was isolated (C).

**FIG. 3.** Detection of unintegrated viral DNA in SIVMND-infected cell line. (A) Hirt supernatant DNA prepared from approximately $1 \times 10^7$ Molt-4 clone 8 cells infected with SIVMND[GB-1] (lane 1) and its digest with XbaI (lane 2) were subjected to Southern blotting analysis with a $^{32}$P-labeled full-length clone of SIVAGM, pSAH12, under conditions of low stringency ($T_m - 40^\circ C$). (B) The same Hirt supernatant DNA preparation was blot analyzed by using a $^{32}$P-labeled SIVMND full-length clone, pSMH103, under conditions of high stringency ($T_m - 23^\circ C$). Results for undigested Hirt supernatant (lane 1) and its digests with XbaI (lane 2), BamHI (lane 3), and EcoRI (lane 4) are shown.
SIV\textsubscript{MND} full-length probe (pSMH103) under various degrees of stringency (Fig. 4A). Under low stringency (\(T_m = 40^\circ\text{C}\)), distinct cross-hybridization was observed between SIV\textsubscript{MND} and all of the viruses HIV-1, HIV-2, SIV\textsubscript{MAC}, and SIV\textsubscript{AGM}. However, under medium- \(T_m = 30^\circ\text{C}\) and high-stringency \(T_m = 23^\circ\text{C}\) conditions, cross-hybridization was very faint or insignificant, a result indicating that the approximate sequence homology between SIV\textsubscript{MND} and the other four HIV or SIV clones was only 50 to 70\% even in the regions showing cross-hybridization. These results provided no evidence that SIV\textsubscript{MND} cross-hybridized especially strongly with any one of these HIVs or SIVs. On the basis of these data, the SIV\textsubscript{MND} isolated and molecularly cloned in this study can be regarded as a new virus belonging to the HIV-SIV group.

To localize the regions in the SIV\textsubscript{MND} genome that cross-hybridized with HIV-1, DNA fragments of SIV\textsubscript{MND} (pSMH103) digested with appropriate restriction enzymes were blot hybridized to \(^32\)P-labeled subgenomic DNAs of HIV-1 under conditions of low stringency (Fig. 4B). Four kinds of subgenomic DNA probes of HIV-1 were prepared by HincII digestion of pBT-1. Distinct bands were detected when the SIV\textsubscript{MND} DNAs were probed with labeled subgenomic probes of pBT-1 that corresponded to the gag and pol regions. In the case of SIV\textsubscript{MND} DNA digested with BamHI and XhoI, a 2.5-kb fragment hybridized with the HIV-1 gag-pol probe, and 3.7- and 1.0-kb fragments hybridized with the pol-sor probe (Fig. 4B). This cross-hybridization data indicated that the gag and pol regions in the SIV\textsubscript{MND} genome had approximately 50 to 70\% homology with those of HIV-1, whereas the homologies in other regions such as env and LTR were lower. From this experiment, putative gag and pol genes could be localized in the cloned SIV\textsubscript{MND} genome by their homology with the corresponding subgenomic fragments of HIV-1. Furthermore, clone-on-clone hybridization experiments of SIV\textsubscript{MND} DNA with HIV-2, SIV\textsubscript{MAC}, and SIV\textsubscript{AGM} probes indicated that SIV\textsubscript{MND} also has approximately 50 to 70\% sequence homology with all of these viruses in the gag and pol regions (data not shown), just as demonstrated with HIV-1. Thus SIV\textsubscript{MND} was shown to be approximately equidistantly related to HIV-1, HIV-2, SIV\textsubscript{MAC}, and SIV\textsubscript{AGM}.
DISCUSSION

The present study provided convincing evidence that mandrills are naturally infected with a retrovirus that is genetically related to HIVs and SIVs but clearly distinct from all other viruses of the HIV and SIV groups that were examined.

Because of antigenic similarities or common African genesis, it has been suggested that HIV may have originated from SIV of African monkeys (20, 21, 27). However, this study and our previous studies of SIV (11, 28) do not support this notion. We have shown by using genomic comparisons that SIVs isolated from mandrills, African green monkeys, and rhesus macaques are all quite distinct. Furthermore, some SIVs are distantly related to both HIV-1 and HIV-2, except for SIVs from Old World monkeys, which showed close relatedness to HIV-2. Although the interspecies transfer of HIVs and SIVs in artificial situations cannot be denied, infections by these viruses seem to be limited to their natural hosts.

In this study, the SIV-MND strains were isolated from mandrills inhabiting western parts of equatorial Africa such as Gabon, the Cameroons, and the Congo (35). On the other hand, SIVAGM isolates were obtained from African green monkeys originally captured in parts of eastern Africa such as Ethiopia and Kenya (28). Seropositive African green monkeys have been reported before from this region (20, 21). These monkeys appear to have been naturally infected with SIVs from olden times, before the AIDS epidemic in humans (17). Therefore, it is conceivable that these SIVs, which exhibit little or no pathogenicity in their natural hosts, have been well conserved in certain species of African nonhuman primates in some areas of Africa.

On the other hand, the origin of SIVMAC (7) is not clear. Numerous serum samples from Asian macaques all gave a negative reaction for HIV or SIV antibodies (16, 26, 28), whereas those from a few colony-born animals gave a positive reaction (3, 7, 22). Considering the genetic divergence between members of the HIV and SIV groups, the relation between SIVMAC and HIV-2 seems exceptionally very close. Further genetic analyses of SIVs from African nonhuman primates will ultimately disclose the natural source of SIVMAC.

The two mandrills from which SIV-MND was isolated were apparently healthy and have not exhibited any signs of immunodeficiency. Moreover, African green monkeys (21, 28) and sooty mangabeys (12, 26, 27) infected with SIV appear free of AIDS-like disease. On the contrary, the rhesus macaque (7, 22, 27) and the pig-tailed macaque (3), which probably are not natural hosts of these viruses, show severe diseases, including AIDS-like disease, when infected with SIV. These differences could be due to different host responses after infection with SIVs, genetic differences among the viruses, or combinations of both factors. SIVs from naturally-infected healthy African nonhuman primates also induce severe CPE in human CD4-positive cells in culture, and high levels of unintegrated viral DNA can be detected in these cells, as with pathogenic human AIDS virus (32) and other lentiviruses (15). It is important to compare the pathogenicities of SIVs in different monkey species.

On the basis of serological observations, some of the many additional African nonhuman primates in which natural SIV infections may well exist include the DeBrazza monkey (28), Sykes monkey (28), talpoin monkey (26), guereza colobus monkey (26), baboons (28), and, possibly, chimpanzee, etc. To better understand the natural history and evolutionary relationships of the viruses of the HIV and SIV groups, further efforts will be required to isolate and characterize new SIVs from as many sources as possible.

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LITERATURE CITED