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Detection of Occult Simian Immunodeficiency Virus SIVsmm Infection in Asymptomatic Seronegative Nonhuman Primates and Evidence for Variation in SIV *gag* Sequence between In Vivo- and In Vitro-Propagated Virus

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Polymerase chain reaction techniques were used to identify simian immunodeficiency virus (SIV) SIVsmm *gag* sequences in genomic DNA isolated from peripheral blood mononuclear cells from naturally infected asymptomatic seropositive and seronegative sooty mangabeys (*Cercocebus atys*) and from experimentally infected but asymptomatic rhesus macaques (*Macaca mulatta*). The results indicate that most if not all SIV-seronegative mangabeys from the colony at the Yerkes Primate Center are in fact infected with SIVsmm despite their lack of humoral immune response, confirming previous immunological and virological observations made by our laboratory. Sequence analysis of these particular *gag* fragments from the mangabey revealed an average of 88% nucleotide sequence homology but 97% amino acid identity with the previously published sequence of the SIVsmmH4 clone. The significance of this finding relative to the asymptomatic state of SIV-infected mangabeys and disease-susceptible SIV-infected rhesus macaques is discussed.

Various nonhuman primates have been proven useful for the study of human AIDS (4, 5, 17). Among these models, sooty mangabey (*Cercocebus atys*) monkeys are naturally infected with the simian immunodeficiency lentivirus (SIV) SIVsmm, which is closely related to human immunodeficiency virus type 2 (HIV-2) and HIV-1, although no retrovirus-induced clinical symptoms have so far been observed in these monkeys (10). Inoculation of rhesus macaques (*Macaca mulatta*) or pigtailed macaques (*M. nemestrina*) with SIVsmm leads to an immunodeficiency syndrome characterized by weight loss, diarrhea, opportunistic infections, loss of CD4-bearing T cells, and finally death (17). Approximately 80% of the mangabeys in the colony housed at the Yerkes Regional Primate Research Center (Emory University, Atlanta, Ga.) show SIV-specific serum antibodies by enzyme-linked immunosorbent assay (ELISA) and Western immunoblot analysis. Most seronegative animals are young (under 2 years of age), and seroconversion seems to coincide with sexual maturity, suggesting that sexual transmission is the predominant route of transmission. Three sets of findings made in our laboratory, however, have cast serious doubts about the reliability of serology alone for the detection of SIV infection. (i) It was demonstrated that circulating B cells of these seronegative subjects are capable of secreting SIVsmm-specific antibodies in vitro when polyclonally stimulated with pokeweed mitogen (PWM) (23). (ii) Circulating $CD8^+$ T cells of seronegative mangabeys markedly inhibit the replication of SIVsmm in autologous cells cultured in vitro (19). This property was previously shown to be present in $CD8^+$ cells from HIV-1-infected humans (21) and SIVmac-infected rhesus macaques (15) but not from uninfected humans or rhesus macaques. (iii) Whereas coculture of unfractionated peripheral blood mononuclear cells (PBMCs) from seronegative mangabeys with $CD4^+$ cell lines failed to yield viral replication in vitro, selective depletion of $CD8^+$ T cells before coculture resulted in detectible amounts of SIV-specific reverse transcriptase activity in the cell-free supernatant fluids (19).

To ascertain the status of infection of these seronegative monkeys, we utilized the highly sensitive and specific polymerase chain reaction (PCR) technique with amplimers and probes based on previously published sequences of the SIV *gag* region (3, 8, 9, 11, 12). The results confirmed our previous observations and hypothesis that, indeed, SIV-seronegative mangabeys are immunized and infected; in addition, data derived by PCR suggest that the predominant SIV isolates in naturally infected and asymptomatic mangabeys are certainly genetically distinct from the SIVsmm isolated by in vitro culture.

MATERIALS AND METHODS

Animals. The rhesus macaques and sooty mangabey monkeys used in this study were housed in colonies at the Yerkes Regional Primate Research Center. All animals were maintained according to the guidelines of the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, and the Health and Human Services guidelines for the care and use of laboratory animals.

Media. RPMI 1640 (GIBCO, Grand Island, N.Y.) was used throughout this study supplemented with 50 μg of gentamicin sulfate (Roche, Nutley, N.J.) per ml, 2 mM L-glutamine (GIBCO), and 10% fetal bovine serum (Hyclone, Fort Logan, Utah).

PBMC isolation. PBMCs were separated from fresh heparinized venous blood onto 54% Percoll (Pharmacia, Piscat-

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TABLE 1. Oligonucleotides used for the PCR amplification and hybridization detection of the amplified target fragments

Name	Sequence	Position[a]	T_m (°C)[b]
SIVgagF	AGCTGCCGATTGGGATTTACAACAC	1678–1702	62.9
SIVgagP	TTCTTACACATTTCTGCAGCCCTAATTG	1880–1853	61.5
SIVgagR	ATGGCTCTTTTGGTCCTTGTTTCAC	1931–1907	60.4
2U9G5	GGAATTC TTTAAGCGCTATCAGAAGGCTGCAC	1568–1593	65.2
2U9G3	GGAATT CCTGACAGGCTGTCAGCATTTCTTC	2108–2084	68.6

[a] Base pair counts based on the SIVsmmH4 sequence (12).
[b] Melting temperatures (T_ms) were calculated only for the annealing portion of the primers and probes, as previously described (7).

away, N.J.) gradients. The plasma was analyzed for SIV antibodies by ELISA and Western blot assay.

ELISA. The ELISA was performed by adsorbing SIVsmm (produced as a single lot by ABI, Columbia, Md.) in bicarbonate buffer overnight onto poly-L-lysine-coated 96-well plates. The plates were then blocked with 5% fetal calf serum in phosphate-buffered saline; then 0.1-ml samples of the sera to be tested (diluted 1:50) or 0.1 ml of undiluted supernatant fluids from the PWM cultures were incubated for 2 h at 37°C. After appropriate washing, all wells received 0.1 ml of a 1/1,000 dilution of goat anti-human immunoglobulin conjugated to alkaline phosphatase (Fisher Biotech, Pittsburgh, Pa.). After incubation for 1 h at 4°C, the appropriate substrate (*p*-nitrophenyl phosphate disodium) was added, and the development of color in each well was read by using an ELISA plate reader (Titertek Multiskan, Arlington, Va.) at a wavelength of 405 nm. A reading of 3 standard deviations above background or higher was considered positive.

Western blot. Purified SIVsmm was separated on a 10% polyacrylamide minigel (Bio-Rad, Richmond, Calif.) before being electrically transferred to nitrocellulose (Schleicher & Schuell, Keene, N.H.). After blocking with 10% normal goat serum in phosphate-buffered saline, individual strips of the blot were incubated for 2 h with 1/40 dilutions of the sera to be tested, washed, and incubated with biotinylated goat anti-human immunoglobulin G (Fisher) and then avidin peroxidase conjugate (Bio-Rad); the color was developed with 4-chloro-1-naphtol (Bio-Rad).

PWM assay. In vitro stimulation of PBMCs with PWM was performed as previously described (23). In brief, PBMCs (2×10^6/ml) were cultured in 2 ml of medium for 5 to 7 days with a previously determined optimal concentration of PWM (GIBCO). The cultures were incubated at 37°C in a 7% CO_2 humidified atmosphere. The cultures were then centrifuged ($150 \times g$), and the supernatant fluid was harvested and assayed for SIV antibodies with the ELISA and Western blot assay described above.

DNA preparation. PBMCs were washed three time with phosphate-buffered saline and suspended in PCR buffer (50 mM KCl, 10 mM Tris [pH 8.3], 2.5 mM $MgCl_2$, 1 mg of gelatin per ml, 0.45% Nonidet P-40, and 0.45% Tween 20) with 60 μg of proteinase K (Sigma Chemicals, St. Louis, Mo.) per ml at a concentration of 10^7 PBMCs per ml. Lysis was allowed to proceed overnight at 56°C. Unless erythrocyte contamination was visible, the samples were used without further purification for the PCR reaction.

PCR amplification. PCR amplification was performed as described by Ou et al. (18) with slight modifications. Briefly, 25-μl samples of the lysates (corresponding to DNA from 2.5×10^5 PBMCs) were denatured at 95°C for 3 min. Then 75 μl of the PCR cocktail, consisting of PCR buffer, 200 μM deoxynucleotide triphosphates, 1 μM of each amplimer, and 2 U of *Taq* polymerase (Cetus, Emeryville, Calif.), was added. The samples were then subjected to 35 cycles of annealing (55°C), elongation (72°C, 30 to 60 s), and denaturation (94°C, 20 s) with ramping times of 30 s, 1 min, and 1.5 min, respectively, in a thermocycler (Perkin Elmer, Norwalk, Conn.). Amplification products were fractionated by electrophoresis through 1.8% agarose gels and visualized with ethidium bromide staining. The DNA was then denatured, neutralized, and blotted overnight onto nitrocellulose (Schleicher & Schuell) by capillary transfer. The blots were baked for 30 min at 80°C and then prehybridized for 1 h at 42°C in 50 mM Tris (pH 7.5)–0.9 M NaCl–0.1% sodium dodecyl sulfate–6 mM EDTA–0.25% nonfat dry milk. A ^{32}P-labeled oligonucleotide probe was then added to the prehybridization medium and hybridized to the blots overnight at 42°C. The blots were washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate for 15 min at 42°C and autoradiographed with Kodak XAR2 film with one screen for 6 to 12 h at −70°C.

Cloning and sequence analysis. The 554-bp fragments amplified from the seropositive (FWb) and the seronegative (FMh) sooty mangabeys with the 2U9G5 and 2U9G3 amplimers (Table 1) were digested with *Eco*RI (Pharmacia), ligated into the *Eco*RI site of M13mp18 (Pharmacia), and cloned in *Escherichia coli* XL-1 (Stratagene, La Jolla, Calif.). Clones containing the desired insert were identified by plaque hybridization (2), expanded, and sequenced with the Sequenase kit (U.S. Biochemical Corp., Cleveland, Ohio) and dye-labeled M13 primers (Applied Biosystems, Foster City, Calif.). The reading of the sequence was performed by an Applied Biosystems 370A DNA sequencer with a 10% polyacrylamide gel in 8 M urea under a constant voltage of 1,100 V.

DNA analysis. Sequence alignments, homology searches, and secondary structure predictions were performed by using the GCG program (University of Wisconsin, Madison) (6). Figure 1 represents the optimal alignment of SIVsmmH4 (12), SIVmac251 (8), SIVmac142 (3), HIV-2rod (11), and SIVagmTYO (9) corresponding to the *gag* region, nucleotides 1568 and 2108 of SIVsmmH4. This alignment allowed for an optimal choice of primers and probes in relation to their degree of conservation among these related lentiviruses, which are thought to have evolved from a common ancestor (12).

RESULTS

Experimental design. Heparinized blood was obtained from a series of normal (uninfected) and experimentally infected rhesus macaques and a series of sooty mangabeys. Plasma was separated from the cells and analyzed for the presence of SIV-reactive antibodies. One aliquot of Percoll-

TABLE 2. Serologic status of rhesus macaques and sooty mangabeys for SIV tested by ELISA and Western blot (WB) assay

Monkey species[a]	Animal	Serology (ELISA/WB)	PWM[b]	PCR H4[c]	PCR cons[d]
Rh	RUg-1	−	−	−	−
Rh	RBj-1	−	−	−	−
Rh	RIg-1[e]	+	+	+	+
Rh	RRm-1[e]	+	+	+	+
Rh	RJq[e]	+	+	+	+
STM	HSa	+	+	−	+
SM	FIi	−	+	−	+
SM	FMh	−	+	−	+
SM	FRf	−	+	−	+
SM	FSg	−	+	−	+
SM	FZg	−	+	−	+
SM	FBi	+	+	−	+
SM	FGb	+	+	−	+
SM	FOc	+	+	−	+
SM	FWb	+	+	−	+

[a] Monkey species used: Rh, rhesus macaques; STM, stumptail macaques; SM, sooty mangabeys.

[b] Results of the ELISA and Western blot assay for SIVsmm antibodies in cell supernatant fluids from monkey PBMCs that were stimulated for 5 to 7 days in vitro with PWM.

[c] PCR results with the SIVgagF and SIVgagR amplimers hybridized to the SIVgagP probe.

[d] PCR results with the consensus amplimers 2U9G5 and 2U9G3 hybridized to the SIVgagF probes.

[e] Rhesus macaques that had been experimentally inoculated with SIVsmm-9. SIVsmm-9 was a virus isolated from a sooty mangabey after coculture in vitro with human PHA-P blasts.

purified PBMCs was cultured with PWM, and the supernatant fluid was assayed for SIV-reactive antibodies. Another PBMC aliquot was lysed to analyze its DNA by PCR techniques. To date, blood samples of a total of 11 uninfected macaques, 19 experimentally infected macaques, and 37 sooty mangabeys have been screened with the assay procedures listed above. Representative data of one experiment are summarized in Table 2.

Detection of SIV-reactive antibodies. Uninfected macaques do not have detectable SIV antibodies in their sera and are negative by the PWM assay (and PCR assays) (Table 2). All three experimentally infected rhesus macaques, a naturally infected stumptail macaque, and four of the sooty mangabeys tested in this experiment had SIV-reactive antibodies and were positive by the PWM assay. Consistent with our previous findings (23), although plasma from four sooty mangabeys showed no detectable SIV-reactive antibodies, supernatant fluids from PWM culture of PBMCs of these four animals had readily detectable SIV-reactive antibodies as determined by ELISA and confirmed (detection of at least three bands of major SIV proteins) by the Western blot assay (23).

PCR amplification of SIV *gag* fragments. Since some sooty

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           1568                                           1617
 SIVsmmH4  TTTCAAGCGC TATCAGAAGG CTGCACTCCC TATGATATTA ATCAAATGCT
SIVmac251  .....G..A. .G........ ......C... .....C.... ....G...T.
SIVmac142  .....G..A. .G........ ....CTC... .....C.... ....G...T.
 HIV-2rod  .....G..A. .C........ ......G... ........C. .C........
SIVagmTYO  ........C. .......... ......A... .....C..T. ....G.....

Consensus  TTTCAgGCaC T*TCAGAAGG CTGCac*CCC TATGAcATtA AtCAgATGcT

           1618                                           1667
 SIVsmmH4  AAATTGTGTA GGAGAACATC AGGCAGCCAT GCAAATTATT AGAGAGATTA
SIVmac251  .........G .....C.... .A..G..T.. ...G.....C .....T....
SIVmac142  .........G .....C.... .A..G..T.. ...G..C..C .....T....
 HIV-2rod  T........G ..C..C.... .A........ ...G..A..C ..G.......
SIVagmTYO  T...GTGC.. .....T.... .A.GG..AT. A.....AG.G .A......C.

Consensus  aAATtgtgTg GGaGAcCATC AaGcgGC*aT gCAgAT*aTc AgaGAgATtA

           1668                                           1714
 SIVsmmH4  TAAATGAAGA AGCTGCCGAT TGGGATTTAC AACACCCGCA AC   CAGGT
SIVmac251  .......G.. G.....A... .....C..G. .G.....A.. ..   ...C.
SIVmac142  .A.....G.. G.....A... .....C..G. .G.....A.. ..   A..C.
 HIV-2rod  .C.....G.. ...A..A..A ......G.G. ....T..AAT .........C
SIVagmTYO  .T........ ...A...C.G ......G..A C......A.T ..CCG....A

Consensus  TaAATGAgGA aGCtGCagAt TGGGAttTgc aaCAcCCaca AC...cAGgt

           1715                                           1764
 SIVsmmH4  CCACTACCAG CAGGGCAACT TAGAGAGCCA AGAGGATCAG ACATTGCAGG
SIVmac251  ....A..    A...A..G.. T..G.....G TC........ .T........
SIVmac142  ....A..    A...A..G.. T..G.....G TC........ .T........
 HIV-2rod  ..CT...... .G.....G.. .........A ..G.....T. ....A.....
SIVagmTYO  ..C....... ....A..G.. C..G..C..T C.C..C.... .T..A.....

Consensus  CCactACcag caGGaCAgCT tAGgGAgCC* *gaGGaTCaG AtATtGCAGG

           1765                                           1814
 SIVsmmH4  AACTACTAGT ACAGTAGATG AACAAATCCA ATGGATGTAC AGGCAACAAA
SIVmac251  ...A...... T......... .......... G......... ..A.....G.
SIVmac142  ...A...... ........A. .......... G......... ..A.....G.
 HIV-2rod  G..A..A..C ........A. ....G..... G.......TT ....C.....
SIVagmTYO  G..C..C..C T.....C.A. ....GT.AG. ......C..T .CTGCTA.CC

Consensus  aACaACtAGt aCAGTAgAaG AACAaaTccA gTGGATgTac Ag*caacA*a

           1815                                           1864
 SIVsmmH4  ACCCCATACC AGTAGGCAAC ATTTATAGAA GGTGGATCCA ATTAGGGCTG
SIVmac251  .......... .......... .....C..G. .A........ .C.G...T..
SIVmac142  .......... .......... .....C..G. .A........ .C.G...T..
 HIV-2rod  .T..TG.... ......A... ..C....... .A........ GA....AT..
SIVagmTYO  C..GGG..GA T.....TGC. ..C..CC.G. .A.....TAT TC....A..T

Consensus  acCccaTAcc aGTAGGcaaC ATtTAcaGgA GaTGGATcca acTaGGgtTg

           1865                                           1914
 SIVsmmH4  CAGAAATGTG TAAGAATGTA TAACCCAACA AACATTTTAG ATGTGAAACA
SIVmac251  ..A....... .C........ .......... ......C... ....A.....
SIVmac142  ..A....... .C........ .......... ......C... ....A.....
 HIV-2rod  .....G.... .C..G..... C.....G..C .....CC... .CA.A.....
SIVagmTYO  ..A..G.... .C.A...... C......GT. TCAG.CC... .CA.T.GG..

Consensus  CAaAAaTGTG TcAgaATGTA tAACCCaaca aacaTtcTAG AtgTaAaaCA

           1915                                           1964
 SIVsmmH4  AGGACCAAAA GAGCCATTTC AAAGCTACGT AGATAGATTC TACAAAAGTC
SIVmac251  ...G...... .......... .G.....T.. ...C..G... ........CT
SIVmac142  ...G...... .......... .G.....T.. ...C..G... .........T
 HIV-2rod  G........G .....G..C. .......T.. .......... ........CT
SIVagmTYO  G.....T... .....C..CA .GGAT..T.. G..C.....T .....GGCAA

Consensus  aGGaCCaAAa GAGCCaTTtc AgagcTAtGT aGAcAGaTTc TACAAaag*t

           1965                                           2014
 SIVsmmH4  TAAGAGCAGA GCAAACAGAT CCCGCAGTAA AGAATTGGAT GACCCAAACA
SIVmac251  .......... A......... G.A....... .......... ...T......
SIVmac142  .......... A......... ..A....... .......... ...T......
 HIV-2rod  .G..G..... A......... ..A.....G. .......... ..........
SIVagmTYO  .T........ A...G.CTCA GGG.A...G. .AC.A..... ...AG..T..

Consensus  TaAGaGCAGA aCAAaCagat ccaGcAGTaA AgaAtTGGAT GAC.cAAaCA

           2015                                           2064
 SIVsmmH4  CTGCTGATTC AAAATGCTAA CCCAGATTGT AAACTAGTGC TCAAGGGTCT
SIVmac251  .......... .......... .........C ..G....... .G.....G..
SIVmac142  .......... .......... .........C ..G....... .G.....G..
 HIV-2rod  .....AG.A. .......C.. ......C..T ...T...... .A..A..A..
SIVagmTYO  T.A..C.... .......... T......... ..GG.CA.C. .G.....C..

Consensus  cTgCTgaTtC AAAATGCtAA cCCAGAtTGt AAgcTagTgC TgAAgGG*CT

           2065                                 2108
 SIVsmmH4  GGGCATGAAT CCCACTTTAG AAGAAATGCT GACAGCCTGT CAGG
SIVmac251  ...TG..... .....CC... .......... ...G..T... ..A.
SIVmac142  ...T.C.... .....CC... .......... ...G...... ..A.
 HIV-2rod  A..G.....C ..T..C.... ....G..... ...C...... ....
SIVagmTYO  A..A...C.C .....CC.T. ........T. A..G..T... ....

Consensus  gGG*atGaAt CCcACccTaG AAGAaATGcT gACgGCcTGT CAgG
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FIG. 1. Alignment of various related SIVs and HIV-2 for the *gag* fragment, nucleotides 1568 and 2108 of SIVsmmH4. Also shown are bp 1560 to 2097 of SIVmac251 (8), bp 1070 to 1607 of SIVmac142 (3), bp 1065 to 1605 of HIV-2rod (11), and bp 969 to 1512 of SIVagmTYO (8). Bases identical to those of the SIVsmmH4 sequence areindicated (.). The lower line represent the consensus sequence derived from the alignment: Uppercase letters represent a perfect match, and lowercase letters represent one or two mismatching bases; *, more than two mismatching bases per position.

FIG. 2. PCR amplification with SIVgagF and SIVgagR as primers and detection of the amplified fragments by hybridization with SIVgagP. Lanes: A and B, results obtained with a plasmid construct containing the SST-I insert of SIVsmm (courtesy of R. C. Desrosiers) amplified at 10^{-6} and 10^{-4} dilutions, respectively; C and D, results of two rhesus macaques experimentally infected with SIVsmm-9; E and F, results with two uninfected rhesus macaques.

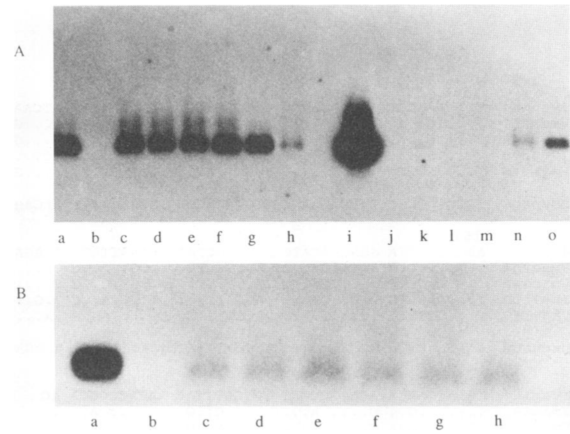

FIG. 3. Detection of SIV sequences in DNA samples from seronegative and seropositive sooty mangabeys and SIV-infected macaques by PCR amplification and hybridization with broadly reactive oligonucleotides. (A) PCR amplification and Southern blot hybridizations were performed with 2U9G5 and 2U9G3 as primers and SIVgagF as a detection probe, respectively. (B) Amplification products b through h were reamplified with the SIVgagF and SIVgagR primers and hybridized to SIVgagP. Lanes: a, positive controls, DNA samples from the seropositive mangabey FWb (A) and from the SIVsmm-infected rhesus macaque RJq (B); b, primers only; c, g, and k, DNA samples from PBMCs of seropositive mangabeys; d, e, f, and h, DNA samples from PBMCs of seronegative mangabeys; i, DNA sample from in vitro cocultured PBMCs of a seropositive stumptailed macaque with human $CD4^+$ cells; l and m, DNA samples from PBMCs of uninfected rhesus macaques; n and o, DNA from PBMCs of rhesus macaques experimentally infected with SIVsmm-9.

mangabeys remain seronegative for prolonged periods of time (up to 12 years), it became necessary to confirm their status of infection by a highly specific and sensitive detection method for SIV. We deliberately chose to amplify a fragment in the *gag* region to combine specificity with a high degree of conservation (12). Based on the published sequence of SIVsmmH4 (12), we first chose a set of primers (SIVgagF and SIVgagR) and a probe (SIVgagP) specific for SIVsmm (Table 1 and Fig. 1) as opposed to the closely related SIVmac251 (8). This primer pair clearly amplified the expected 254-bp fragment from the DNA of PBMCs obtained from experimentally infected rhesus macaques. This amplified product hybridized with the SIVgagP probe (Fig. 2), whereas DNA samples from uninfected macaques remained negative. Of interest was our observation that DNA samples from PBMCs of 36 seronegative and seropositive mangabeys tested to date failed to provide positive results (Table 2; data not shown). Consequently we designed a new set of primers (Table 1) aimed at the most conserved regions of the *gag* gene by using the alignment presented in Fig. 1. Table 2 and Fig. 3A illustrate the results obtained by PCR amplification with the 2U9G5 and 2U9G3 amplimers and hybridization with SIVgagF. DNA samples from four of four seropositive mangabeys and four of four seronegative mangabeys showed a PCR-amplified product whose identity was confirmed by hybridization. DNA samples from PBMCs of uninfected rhesus macaques (Fig. 3A) and STLV-1-infected rhesus cells (data not shown) remained negative upon repeated testing with the primer pair and probe. DNA prepared from $CD4^+$ cell lines infected in vitro with SIVsmm (Fig. 3A, lane l) or SIV isolated from a stumptailed macaque from the Yerkes colony (lane h) naturally infected with as yet an unknown SIV strain, gave strong positive signals as compared with those of DNA prepared from PBMCs of experimentally infected rhesus macaques, which generally show a comparatively weaker amplification reaction. Dilution of mangabey PBMCs or PBMCs from experimentally infected rhesus macaques with PBMCs of uninfected macaques before DNA extraction resulted in loss of the signal at a 10^{-1} dilution as a general limit of sensitivity for that assay (data not shown).

Nested PCR. Since the fragment amplified with the primers 2U9G5 and 2U9G3 includes the fragment amplified with the SIVgagF and SIVgagR amplimers, we reamplified the product of the consensus amplification with the internal SIVsmmH4-specific primer pair by using a nested PCR technique. A strong band could be detected with ethidium bromide staining at the expected molecular weight for both mangabeys and infected rhesus DNA (data not shown). Only the the DNA from PBMCs of rhesus macaques, however, bound the SIVsmm-specific probe SIVgagP to a significant level (Fig. 3B). DNA samples from PBMCs of mangabeys, although clearly positive after the consensus amplification when hybridized with a consensus probe (Fig. 3A), bound the SIVgagP only weakly (Fig. 3B).

This finding has been further confirmed by assaying the consensus amplification of DNA samples of two seropositive and one seronegative mangabeys and one experimentally infected rhesus macaque with three different probes covering three different areas of the amplified fragment (Fig. 4). Whereas the SIVgagF and SIVgagR probes (conserved sequences) readily hybridized to the fragment amplified from both rhesus macaque and mangabey DNA samples, the SIVsmmH4-specific SIVgagP probe only hybridized to the fragment amplified from the rhesus macaque DNA sample. Amplified DNA samples from sooty mangabeys, whether seropositive or not, consistently failed to hybridize to the SIVgagP probe.

As alluded to above, these are representative data; analyses of additional samples from each set of monkeys have so far given identical results. These data demonstrate two basic results. First, seronegative mangabeys are in fact infected

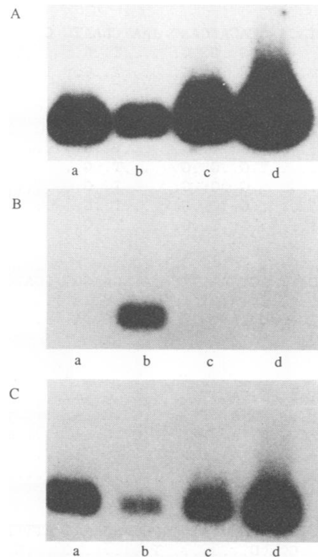

FIG. 4. Hybridization of a PCR-amplified SIVsmm *gag* fragment to various oligonucleotide probes. DNA samples from the PBMCs of two SIV-seropositive sooty mangabeys (lanes a and d), one seronegative mangabey (lane c), and one experimentally infected rhesus macaque (lane b) were amplified as described in the legend to Fig. 1 with the 2U9G5 and 2U9G3 amplimers and hybridized with the radiolabeled oligonucleotide probes SIVgagF (A), SIVgagP (B), and SIVgagR (C).

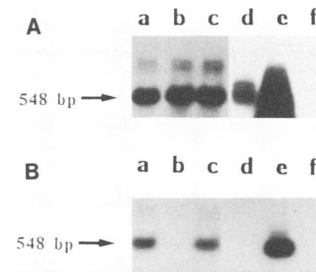

FIG. 5. Hybridization of the consensus SIVgagF probe (A) and the SIVsmmH4-specific SIVgagP probe (B) to 2U9G5 and 2U9G3 amplification products from DNA samples of mangabey PBMCs before and after in vitro culture. Lanes: a, positive control DNA sample from an experimentally infected rhesus macaque; b and d, DNA samples from fresh mangabey PBMCs; c and e, DNA samples from PHA-P-stimulated mangabey PBMCs cultured in vitro for 5 days; f, negative control DNA sample from an uninfected rhesus macaque.

with SIVsmm. Second, although the highly specific SIVsmm primer pair and probe were able to amplify and detect SIV sequences present in rhesus macaques experimentally infected with an SIV isolated by in vitro cocultivation techniques from PBMCs of sooty mangabeys, the same oligonucleotide set failed to detect sequences of SIV present in sooty mangabeys. It was reasoned that this was most likely due to differences in this specific *gag* region of the SIV present in sooty mangabeys in vivo and that cocultivation of mangabey PBMCs with human PHA-P blasts (which was the source of the SIV used to infect the rhesus macaques) resulted in the selection of a variant.

To verify this hypothesis, we amplified DNA extracted from mangabey PBMCs before and after in vitro culture in media containing PHA-P. The results of DNA samples from stimulated and control PBMCs from two mangabeys are shown in Fig. 5. After 5 days of culture in vitro, the PCR-amplified product hybridized to the SIVsmmH4-specific probe, whereas the product amplified from DNA of fresh PBMCs did not. This effect, if not systematic for every mangabey PBMC culture in such a short period of time, was consistently observed with DNA samples from mangabey PBMCs cultured or cocultured with human PHA-P blasts in vitro after various periods of time.

Cloning and sequence analysis of the amplified SIVsmm *gag* fragment. In view of the different clinical courses of SIV infection between macaques and mangabeys, sequence differences in the predominant SIVsmm isolate for each monkey species might account for the natural resistance of mangabeys to the SIV pathogenic effects.

We therefore analyzed the sequence of the fragment amplified from DNA of a seropositive mangabey FWb and of a seronegative mangabey FMh with the 2U9G5 and 2U9G3 amplimers, in the hope of determining amino acid differences that may account for differential humoral or cellular reactivity between seropositive and seronegative but infected mangabeys and also between mangabeys and macaques (1, 20). Sequence analyses (Fig. 6) show a sequence homology of over 97% between the *gag* fragments amplified from the SIVsmm present in mangabeys and only 88% sequence homology with the same fragment in the SIVsmmH4. The latter, however, is comparable to the overall homology observed for the whole *gag* region for various SIV and HIV-2 isolates (12). The binding site for SIVgagP shows a 9- to 10-base change for a total nucleotide length of 28 bases, which supports the observed lack of hybridization of this probe with DNA samples from PBMCs of the mangabeys. At the amino acid level, however, only 3% or less of the residues vary. For the *gag* residues 224 (all clones), 227 (SIVsmmfa1), 271 (SIVsmmfa2), 302 (FMh1), and 342 (FMh10), a notable decrease in hydrophilicity and antigenicity profiles (as predicted by the Kyte-Doolittle and the Jameson-Wolf algorithms, respectively) was found as compared with the SIVsmmH4 *gag* amino acid sequence. A premature stop codon was found at amino acid position 261 for the clone FMh4.

DISCUSSION

PCR as a detection tool for occult SIV infection. The broad reactivity of the consensus primer pair and probe underscores the value of PCR as a highly sensitive diagnostic tool for screening nonhuman primates before their introduction to a colony or their involvement in studies with infectious agents.

As shown in humans, the absence of serum antibodies to HIV-1 does not warrant the absence of viral infection, particularly in individuals at high risk (14, 16, 22, 24). A colony of nonhuman primates in a primate center has certainly to be considered at high risk for SIV infection, due to the close contact between animals inside a colony or even between various colonies. This is highlighted by the discovery of new lentivirus infections in several additional nonhuman primate species (unpublished data), often in the absence of clinical symptoms and eventually in the absence of humoral response. Such an example is illustrated by the result shown in Fig. 3A (lane h), which demonstrates that DNA samples prepared from coculture of PBMCs of a stumptailed macaque (*M. arctoides*) with a human $CD4^+$ cell

```
            1568                                        1617
  SIVsmmh4  TTTCAAGCGC TATCAGAAGG CTGCACTCCC TATGATATTA ATCAAATGCT
 SIVsmmFa1  .......... .......... .......... .....C..C. ..........
 SIVsmmFa2  .......... .......... .......... .....C..C. ..........
      FWb1
      FWb2

      FMh1                            ....... .....C..C. ..........
      FMh3   ......... .......... .......... .....C..C. ..........
      FMh4          .. .......... .......... .....C..C. ..........
      FMh5  .......... .......... .......... .....C..C. ..........
     FMh10

            1618                                        1667
  SIVsmmh4  AAATTGTGTA GGAGAACATC AGGCAGCCAT GCAAATTATT AGAGAGATTA
 SIVsmmFa1  .......... .......... .......... .......... ..........
 SIVsmmFa2  .......... .......... .......... .......... ..........
      FWb1
      FWb2

      FMh1  .......... .......... .......... .......... .....A....
      FMh3  .......... .......... .......... .......... .....A....
      FMh4  .......... .......... .......... .......... .....A....
      FMh5  .......... .......... .......... .......... .....A....
     FMh10

            1668                                        1717
  SIVsmmh4  TAAATGAAGA AGCTGCCGAT TGGGATTTAC AACACCCGCA ACCAGGTCCA
 SIVsmmFa1  .......... ......T... ......C... .......... G.........
 SIVsmmFa2  .....A.... ......T... .......... .......... G.........
      FWb1
      FWb2

      FMh1  .......... ......T... ......C... .......... G.........
      FMh3  .......... ......T... ......C... .......... G.........
      FMh4  .......... ......T... ......C... .......... G.........
      FMh5  .......... ......T... ......C... .......... G.........
     FMh10

            1718                                        1767
  SIVsmmh4  CTACCAGCAG GGCAACTTAG AGAGCCAAGA GGATCAGACA TTGCAGGAAC
 SIVsmmFa1  A......... TA.....C.. .......... ........T. ..........
 SIVsmmFa2  A......... .A.....C.. .......... ........T. ..........
      FWb1
      FWb2

      FMh1  A......... .A.....C.. .......... ........T. ..........
      FMh3  A......... .A.....C.. .......... ........T. ..........
      FMh4  A......... .A.....C.. .......... ........T. ..........
      FMh5  A......... .A.....C.. .......... ........T. ..........
     FMh10                                   ......T. ..........

            1768                                        1817
  SIVsmmh4  TACTAGTACA GTAGATGAAC AAATCCAATG GATGTACAGG CAACAAAACC
 SIVsmmFa1  C......... ..G..C..G. ....T..G.. ......T... ........T.
 SIVsmmFa2  C......... ..G..C..G. ....T..G.. ......T... ........T.
      FWb1
      FWb2

      FMh1  C......... ..G..C..G. ....T..G.. ......T... ........T.
      FMh3  C......... ..G..C..G. ....T..G.. ......T... ........T.
      FMh4  C......... ..G..C..G. ....T..G.. ......T... ........T.
      FMh5  C......... ..G..C..G. ....T..G.. ......T... ........T.
     FMh10  C......... ..G..C..G. ....T..G.. ......T... ........T.

            1818                                        1867
  SIVsmmh4  CCATACCAGT AGGCAACATT TATAGAAGGT GGATCCAATT AGGGCTGCAG
 SIVsmmFa1  .TG....... G..A..T... ..C..G..A. ........C. T..AT.A..A
 SIVsmmFa2  .TG....... C..A...... ..C..G..A. ........C. T.CAT.A..A
      FWb1
      FWb2

      FMh1  .TG....... G..A..T... ..C..G..A. ........C. T..AT.A..A
      FMh3  .TG....... G..A..T... ..C..G..A. ........C. T..AT.A..A
      FMh4  .TG....C.. GT.A..T... ..C..G..A. ........C. T..AT.A..A
      FMh5  .TG....... G..A..T... ..C..G..A. ........C. T..AT.A..A
     FMh10  .TG....... G..A..T... ..C..G..A. ........C. T..AT.A..A

            1868                                        1917
  SIVsmmh4  AAATGTGTAA GAATGTATAA CCCAACAAAC ATTTTAGATG TGAAACAAGG
 SIVsmmFa1  ..G.....C. .G........ T......... ...C.G..C. .A.....G..
 SIVsmmFa2  ..G.....C. .G.....C.. T......... ...C.
      FWb1                                                .G..
      FWb2                                                  ..

      FMh1  ..G.....C. .G........ T......... ...C.G..C. .A.....G..
      FMh3  ..G.....C. .G........ T......... ...C.G..C. .A.....G..
      FMh4  ..G.....C. .G........ T.
      FMh5  ..G.....C. .G........ T......... ...C.G..C. .A.....G..
     FMh10  ..G..C..C. .G........ T......... ...C.G..C. .A.....G..

            1918                                        1967
  SIVsmmh4  ACCAAAAGAG CCATTTCAAA GCTACGTAGA TAGATTCTAC AAAAGTCTAA
 SIVsmmFa1  G..G...... .....C..G. ....T..... C..G...... .....CT...
 SIVsmmFa2
      FWb1  G..G...... .....C..G. ....T..... C..G...... .....CT...
      FWb2  G..G...... .....C..G. ....T..... C..G...... .....CT...

      FMh1  G..G...... ....AC..G. ....T..... C..G..A.
      FMh3  G..G...... .....C..G. ....T..... C..G...... C....CT...
      FMh4
      FMh5  G..G...... .....C..G. ....T..... C..G...... .....CT...
     FMh10  G..G...... .....C..G. ....T..... C..G...... .....CT...
```

FIG. 6. Nucleotide sequence of the SIVsmm gag fragment amplified by the 2U9G5 and 2U9G3 primers from the DNA of two sooty mangabeys. Base numbering refers to the SIVsmmH4 sequence (13) represented on the first row. Bases identical to those of SIVsmmH4 are indicated (.). SIVsmmFA1, SIVsmmFA2, FWb1, and FWb2 represent four clones of the in vivo sequence of SIVsmm isolated from the seropositive sooty mangabey monkey FWb. FMh1-10 represent five clones amplified and isolated from the seronegative sooty mangabey FMh.

line gave positive PCR reactions despite the unknown origin of the SIV in this monkey species (unpublished data).

In addition, DNA samples from PBMC of a vast majority of neonatal (<6 months old) seronegative mangabeys were recently tested and were found positive with these oligonucleotides (unpublished data). Two major alternatives might explain these findings: either SIV is endogenous in mangabey monkeys at the Yerkes Primate Center, or the primary route of virus transmission is vertical rather than through sexual contact as previously shown with serological data. In the event of the first hypothesis, the genomic DNA of all mangabey cells should encompass at least one copy of the SIV genome. PCR amplifications of DNA from one Epstein-Barr virus-transformed and cloned (seropositive) mangabey B-cell line and from a herpesvirus-immortalized mangabey $CD8^+$ cell line were repetitively negative (data not shown). In addition, the sensitivity of the PCR assay with the 2U9G5 and 2U9G3 primer pair was analyzed. Cell lines infected in vitro with SIV or HIV-2 and PBMC from sooty mangabeys were subjected to limiting dilution analysis with uninfected H9 cells to provide equal number of cells for each sample before the extraction of DNA. In addition, various concentrations of purified plasmid DNA containing the *Sst*I insert of SIVsmm (8.3-kb insert) was utilized. As little as 2×10^{-3} pg of the plasmid DNA gave a positive signal. Two to three cells from the SIV- or HIV-2-infected cell lines, when added to 2.5×10^5 uninfected H9 cells, resulted in DNA that provided a positive signal (data not shown), whereas at least as many as 2.5×10^4 PBMCs from naturally infected (seropositive) mangabeys were required to yield such a positive signal.

These results strongly argue against the possibility of SIV being endogenous in mangabey monkeys and therefore suggest that the primary mode of transmission of SIV in the naturally infected sooty mangabey colony is vertical rather than sexual. These findings dictate a reevaluation of the data previously obtained with serological techniques.

The detection of SIV sequences in the PBMCs of these seronegative mangabeys not only confirms our previous findings with the PWM assay (23) but in addition supports the view that the secretion of SIV-reactive antibodies by polyclonal activation in vitro was not secondary to previous

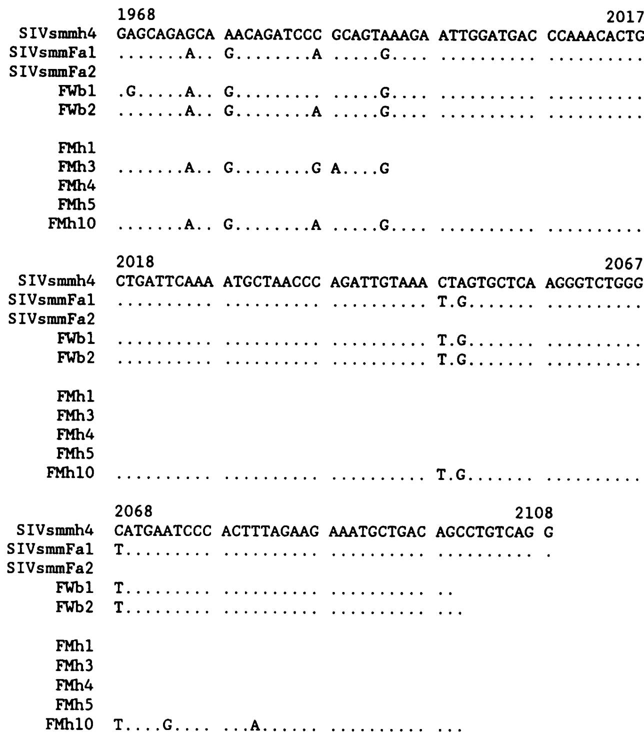

FIG. 6—*Continued*

immunization or the presence of cross-reactive antibodies but was indeed secondary to the presence of infectious virus.

Significance of seronegative, PCR-positive status. One major issue concerning these SIV- and HIV-1-seronegative asymptomatic but infected individuals (14, 15, 22, 23) is to determine whether the harbored virus and/or provirus can be transmitted to naive recipients; if so, will the recipient seroconvert and gradually develop clinical symptoms and disease? For obvious reasons, such an approach cannot be assayed in humans. Experiments were performed in which unfractionated blood from seronegative but PCR-positive sooty mangabey monkeys was transfused to two naive pigtail macaques. In addition, PBMCs from two SIV-seronegative, PCR-positive mangabeys were depleted of $CD8^+$ cells and then cocultured with human PHA-P blasts, and samples of the putative in vitro replicating virus (both cells and supernatant fluids) from each animal were injected into two naive pigtailed macaques. Indeed, such transfusion appears to result in transmission of infection (unpublished data).

Sequence differences between SIVsmm in mangabeys and in macaques. One striking, if not completely unexpected, result of this study is the differences detected in the SIV sequences in the DNA from PBMCs of mangabeys and rhesus macaques experimentally infected with SIVsmm-9. This is particularly true for the results obtained with probe SIVgagP (Fig. 4). We have further found that mangabey PBMCs cultured and/or cocultured with $CD4^+$ cell lines did react with this particular probe (Fig. 5) after various periods of time in culture. This suggests that the in vitro culture conditions strongly select for SIV isolates that are present at almost undetectable levels in the PBMCs of the mangabeys. The short period of time in in vitro culture argues against the possibility that the difference in the *gag* sequence is secondary to random mutations. This is further supported by the data obtained with the nested PCR, in which a modest reaction with SIVgagP was observed after a second round of amplification (Fig. 3B). It is important to note that we were unable to isolate a clone bearing the SIVsmmH4-like sequence from the mangabey PBMCs for the sequencing studies (Fig. 6). Similar results have been reported for the *env* region of SIVsmm upon in vitro passage in human $CD4^+$ cell lines (13).

In rhesus macaques, however, this in vitro-selected isolate remains predominant (Fig. 2), which leads to the following question: is this due to the passage of SIVsmm in in vitro cell culture before inoculation in the animal, or does the macaque immune system exert a selective pressure on SIVsmm similar to the one observed in vitro? The results of the above-mentioned direct blood transfusion from seronegative but PCR positive mangabeys to macaques supports the concept that the in vitro culture system appears to select for a variant present in a very low frequency in genomic DNA of sooty mangabey PBMCs.

Depending on a possible clinical correlation with the emergence of certain SIVsmm isolates, we hope to address the disease resistance of sooty mangabeys compared with the disease-susceptible state of macaques. It is our objective to synthesize peptides based on the variable residues determined by sequencing and then to test them for reactivity against serum antibodies and to identify T-cell epitopes (1, 20). Further detailed sequence analysis of the complete *gag* and *env* genes of the naturally occuring SIVsmm from both seronegative and seropositive sooty mangabeys is currently in progress. Results from such studies may shed light on the impact of viral sequence variation on subsequent immune responses and the consequences of such immune responses in the development of disease resistance in mangabeys and susceptibility in macaques.

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REFERENCES

1. **Ansari, A. A., J. D. Powell, P. E. Jensen, T. Yehuda-Cohen, H. M. McClure, D. C. Anderson, P. N. Fultz, and K. W. Sell.** 1990. Requirements for SIVsmm antigen specific in vitro proliferation of peripheral blood mononuclear cells from experimentally infected rhesus macaques and naturally infected sooty mangabeys. AIDS **4**:399–407.
2. **Benton, W. D., and R. W. Davis.** 1977. Screening gt recombinant clones by hybridization to single plaques *in situ*. Science **196**: 180–182.
3. **Chakrabarti, L., M. Guyader, M. Alizon, M. D. Daniel, R. C. Desrosiers, P. Tiollais, and P. Sonigo.** 1987. Sequence of simian immunodeficiency virus from macaque and its relationship to other human and simian retroviruses. Nature (London) **328**:543–547.
4. **Daniel, M. D., N. L. Letvin, N. W. King, M. Kannagi, P. K. Sehgal, R. D. Hunt, P. J. Kanki, M. Essex, and R. C. Desrosiers.** 1985. Isolation of T-cell tropic HTLV-III-like retrovirus from macaques. Science **228**:1201–1204.
5. **Desrosiers, R. C.** 1988. Simian immunodeficiency viruses. Annu. Rev. Microbiol. **42**:607–625.
6. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic

Acids Res. **12**:387–395.

7. **Freier, S. M., R. Kierzek, J. A. Jaeger, N. Sugimoto, M. H. Caruthers, T. Neilson, and D. H. Turner.** 1986. Improved free-energy parameters for predictions of RNA duplex stability. Proc. Natl. Acad. Sci. USA **83**:9373–9377.
8. **Franchini, G., C. Gurgo, H. G. Guo, R. C. Gallo, E. Collati, K. A. Fargnoli, L. F. Hall, F. Wong-Staal and M. S. Reitz, Jr.** 1987. Sequence of simian immunodeficiency virus and its relationship to the human immunodeficiency viruses. Nature (London) **328**:539–543.
9. **Fukasawa, M., T. Miura, A. Hasegawa, S. Morikawa, H. Tsujimoto, K. Miki, T. Kitamura and M. Hayami.** 1988. Sequence of simian immunodeficiency virus from African green monkey, a new member of HIV/SIV group. Nature (London) **333**:457–461.
10. **Fultz, P. N., H. M. McClure, D. C. Anderson, A. S. Swenson, R. Anand, and A. Srinivasan.** 1986. Isolation of a T-lymphotropic retrovirus from naturally infected sooty mangabey monkeys (*Cercocebus atys*). Proc. Natl. Acad. Sci. USA **83**:5286–5290.
11. **Guyader, M., M. Emerman, P. Sonigo, F. Clavel, L. Montagnier, and M. Alizon.** 1986. Molecular cloning and polymorphism of the human immunodeficiency virus type 2. Nature (London) **324**:691–695.
12. **Hirsch, V. M., R. A. Olmsted, M. Murphey-Corb, R. H. Purcell, and P. R. Johnson.** 1989. An African primate lentivirus (SIVsm) closely related to HIV-2. Nature (London) **339**:389–391.
13. **Hirsch, V. M., P. Edmonson, M. Murphey-Corb, B. Arbeille, P. R. Johnson, and J. I. Mullins.** 1989. SIV adaptation to human cells. Nature (London) **341**:573–574.
14. **Imagawa, D. T., M. H. Lee, S. M. Wolinsky, K. Sano, F. Morales, S. Kwok, J. J. Sninsky, P. G. Nishanian, J. Giorgi, J. L. Fahey, J. Dudley, B. R. Visscher, and R. Detels.** 1989. Human immunodeficiency virus type 1 infection in homosexual men who remain seronegative for prolonged periods of time. N. Eng. J. Med. **320**:1458–1462.
15. **Kannagi, M., L. U. Chalifoux, C. I. Lord, and N. L. Letvin.** 1988. Suppression of simian immunodeficiency virus replication in vitro by $CD8^+$ lymphocytes. J. Immunol. **140**:2237–2242.
16. **Loche, M., and B. Mach.** 1988. Identification of HIV-infected seronegative individuals by a direct diagnostic test based on hybridization to amplified viral DNA. Lancet **ii**:418–421.
17. **McClure, H. M., D. C. Anderson, P. N. Fultz, A. A. Ansari, E. Lockwood, and A. Brodie.** 1989. Spectrum of disease in macaques monkeys chronically infected with SIV/SMM. Vet. Immunol. Immunopathol. **21**:13–24.
18. **Ou, C. Y., S. Kwok, S. W. Mitchell, D. H. Mack, J. J. Sninsky, J. W. Krebs, P. Feorino, D. Warfield, and G. Schochetman.** 1988. DNA amplification for direct detection of HIV-1 in DNA of peripheral blood mononuclear cells. Science **237**:295–297.
19. **Powell, J. D., T. Yehuda-Cohen, F. Villinger, H. M. McClure, K. W. Sell, and A. A. Ansari.** 1990. Inhibition of SIVsmm replication in vitro by $CD8^+$ cells from SIV/SMM infected seropositive clinically asymptomatic sooty mangabeys. J. Med. Primatol. **19**:239–249.
20. **Powell, J. D., F. Villinger, T. Yehuda-Cohen, M. Vuchetich, H. M. McClure, K. W. Sell, and A. A. Ansari.** 1990. Identification of SIVsmm viral proteins that induce T cell response in experimentally infected rhesus macaques and naturally infected sooty mangabeys by the cellular Western blot assay. J. Med. Primatol. **19**:227–238.
21. **Walker, C. M., D. J. Moody, D. P. Stites, and J. A. Levy.** 1986. $CD8^+$ lymphocytes can control HIV infection *in vitro* by suppressing virus replication. Science **234**:1563–1566.
22. **Wolinsky, S. M., C. R. Rinaldo, S. Kwok, J. J. Sninsky, P. Gupta, D. Imagawa, H. Farzadegan, L. P. Jacobson, K. S. Grovit, M. H. Lee, J. S. Chmiel, H. Ginzburg, R. A. Kaslow, and J. P. Phair.** 1989. Human immunodeficiency virus type 1 (HIV-1) infection. A median of 18 months before a diagnostic Western blot. Ann. Intern. Med. **111**:961–972.
23. **Yehuda-Cohen, T., J. D. Powell, F. Villinger, H. M. McClure, and A. A. Ansari.** Submitted for publication.
24. **Jehuda-Cohen, T., B. A. Slade, J. D. Powell, F. Villinger, B. De, T. M. Folks, H. M. McClure, K. W. Sell, and A. A. Ansari.** 1990. Polyclonal B cell activation reveals anti HIV-1 antibodies in HIV-1 seronegative individuals. Proc. Natl. Acad. Sci. USA **87**:3972–3976.