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Evidence for Simian Immunodeficiency Virus–Specific IgM and IgG Response in Peripheral Blood Mononuclear Cells of Serum Enzyme-Linked Immunosorbent Assay–Negative Nonhuman Primates

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Summary: *In vitro polyclonal activation of peripheral blood mononuclear cells* (PBMCs) from 70% of the simian immunodeficiency virus (SIV) serum enzyme-linked, immunosorbent assay (ELISA)–negative sooty mangabeys leads to synthesis and release of low but significant and reproducible levels of SIV-reactive antibodies, as determined by ELISA and Western blot analysis. The predominant isotype of SIV-reactive antibodies in the pokeweed mitogen (PWM) supernatant fluids from serum ELISA-negative mangabeys is IgM, whereas the predominant isotype of SIV-reactive antibodies in seropositive mangabeys is IgG. Depletion of $CD8^+$ cells led to a marked increase in the levels of SIV-reactive antibodies detected in supernatant fluids from PWM-induced cultures from the serum ELISA-negative mangabeys. No evidence for such SIV-reactive antibodies has been found, to date, *in similar unfractionated* or $CD8^+$ T-cell-depleted PWM-induced PBMC cultures from uninfected macaques. Supernatant fluids from PWM cultures of PBMCs from a select group of serum ELISA-negative mangabeys, when concentrated five times, were shown to give a Western blot profile against SIV, similar to the profile seen with plasma from seropositive infected macaques and mangabeys. Evidence is presented to show that these serum ELISA-negative mangabeys are most likely latently infected with SIV. This evidence, which was obtained in samples from such ELISA-negative mangabeys, includes the detection of reverse transcriptase activity and the presence of SIV p27 in supernatant fluids of phytohemagglutinin-stimulated PBMCs in vitro. In addition, the data show the presence of $CD8^+$ T cells that regulate SIV-specific Ig synthesis and show the detection of *gag* sequences by the polymerase chain reaction. Thus, the PWM assay described herein may provide a valuable additional tool for detection of lentivirus infection before or in the absence of seroconversion. Key Words: Simian immunodeficiency virus infection—Nonhuman primates—*Polyclonal activation*—Latent infection—Tolerance.

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Conventional methods for determining infection with lentiviruses rely upon the detection of viral-specific antibodies in the appropriate serum specimen. Such methodology, however, is dependent on

the ability of the viral antigens to elicit an immune response and on the ability of the host to mount a specific immune response. In this regard, numerous reports have been published that document the absence of virus-specific antibodies in the presence of lentivirus infections in nonhuman primates and cats (1–3) and a low incidence of human immunodeficiency virus type 1 (HIV-1) and human T-lymphotropic virus type 1 (HTLV-1) infections (4–10). The occurrence of this phenomenon, the precise mechanisms that lead to such a state, and its implications can be best studied in animal models of lentivirus infection in which such a phenomenon has been reported. Our laboratory has documented the presence of occult lentivirus infection in a colony of sooty mangabeys (*Cercocebus atys*), a species from West Africa (1) in which infection is naturally transmitted by routes that have yet to be determined. However, such infection does not appear to induce disease in its natural host (11). The mechanisms by which such a natural host develops protective immunity against lentivirus-induced disease are far from clear, and the elucidation of these mechanisms is clearly of paramount importance since it may provide a rationale for vaccine strategies against human HIV-1 infection. Toward this goal, our laboratory has been studying humoral and cellular responses to the simian immunodeficiency virus (SIV) in naturally infected but clinically asymptomatic sooty mangabeys and in rhesus macaques (*Macaca mulatta*), which (in contrast to the mangabeys), when experimentally injected with an SIV pool isolated from sooty mangabeys (SIVsmm), develop infection that results in disease similar to that seen with HIV-1 infection of humans (12).

Conventional serological studies conducted on the colony of sooty mangabeys at the Yerkes Regional Primate Research Center (YRPRC) by using the commercially available HIV-2 kit showed that >75% of the animals were seropositive and that seroconversion often coincided with the onset of sexual maturity in this colony. Analysis of serum samples from these SIV-seropositive mangabeys confirmed reactivity to most, if not all, major proteins of SIV, as determined by RIPA and Western blot analysis, with results similar to those obtained with serum samples from rhesus or pigtailed macaques experimentally infected with SIVsmm. On the other hand, in vitro culture of peripheral blood mononuclear cells (PBMCs) with pokeweed mitogen (PWM), a polyclonal activation agent, provided evidence of some intriguing differences between the two species. Data are presented that show the secretion of SIV-specific antibodies in culture supernatant fluids of PWM-stimulated PBMCs not only from seropositive mangabeys and macaques experimentally infected with SIVsmm but also from mangabeys that were serum enzyme-linked immunosorbent assay (ELISA) negative by conventional serology. In contrast, such reactivity was never observed in PWM cultures of PBMCs from uninfected rhesus macaques, chimpanzees, and pigtailed macaques that were seronegative by similar tests. The synthesis of SIV-reactive antibodies, in concert with the findings of reverse transcriptase (RT) activity, detection of p27 *gag* proteins, and detection of *gag* sequences by the polymerase chain reaction (PCR) in PBMCs of serum ELISA-negative mangabeys, provides evidence that virus transmission must occur in this naturally infected species long before seroconversion. These data suggest that there is clearly a stage during natural SIV infection when the monkeys are infected but remain serum ELISA negative, for reasons that are not clear at present.

MATERIALS AND METHODS

Animals

All monkeys used in this study are housed at the Yerkes Regional Primate Center at Emory University. They are maintained in accordance with the instructions of the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, and the U.S. Public Health Service guidelines, as outlined in the *Guide for the Care and Use of Laboratory Animals*.

PWM Assay

For the polyclonal PWM assay, PBMCs were incubated for 2 h at room temperature (to remove cytophilic immunoglobulins), washed with medium, and then resuspended at 2×10^6 cells/ml. Cultures were performed in triplicates; they contained medium alone (spontaneous immunoglobulin) or a 1:500 final dilution of PWM (GIBCO, Grand Island, NY, U.S.A.) and were incubated for 6 days at 37°C in a 7% CO_2-humidified incubator. The cultures were then centrifuged, and the undiluted supernatant fluid was assayed for total immunoglobulin (Ig) and SIV-specific Ig on the same day. The supernatant fluid and the stored plasma from the same blood samples were assayed in parallel. Samples of the supernatant fluid were also assayed for total protein content by using the Biorad protein assay kit (Biorad, Richmond, CA, U.S.A.).

Media

Medium used throughout this study consisted of RMPI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin,

2 mM L-glutamine (all from GIBCO), and a 10% heat-inactivated (56°C, 30 min) preselected lot of fetal bovine serum (Hyclone Corp., Logan, UT, U.S.A.). In some experiments, this medium was further supplemented with 100 U/ml of r-IL2 (courtesy of Hoffman-LaRoche Labs, Nutley, NJ, U.S.A.).

ELISA

Our laboratory has standardized an ELISA for the detection of antibodies (total, IgM, and IgG SIV-specific) in serum samples from macaques and mangabeys. The SIV used was an isolate from a sooty mangabey (SIVsmm) that was grown in the H9 cell line, was purified on a sucrose gradient, contained 2×10^5 tissue culture median infectious dose ($TCID_{50}$) of virus, and was estimated to contain 10^{11} virus particles/ml (Advanced Biotechnology Inc., Silver Spring, MD, U.S.A.). A single stock of this virus preparation was used throughout this study. Stock suspension, 0.1 ml, was incubated with a 0.1-ml mixture of Triton X-100 for 10 min at room temperature; then this 0.2-ml mixture was added to 49.8 ml of a standard coating buffer. This diluted virus suspension was dispensed in a volume of 0.2 ml to individual wells of a poly-L-lysine precoated 96-well Immulon II microtiter plate, and the plate was incubated overnight at 4°C. Subsequently, the wells were washed twice in phosphate-buffered saline (PBS) and used for the detection of SIV-specific antibodies, according to standard ELISA techniques. The alkaline phosphatase goat anti-human Ig and affinity-purified anti-human IgM and IgG (Southern Biotech, Birmingham, AL, U.S.A.) developing antibodies were prescreened for their reactivity against macaque and mangabey antibodies before use. In several of the assays, the commercially available HIV-2 ELISA kit (Genetic Systems, Seattle, WA, U.S.A.) was used in parallel to compare results obtained by the ELISA established in our laboratory. Results obtained using our laboratory-based ELISA and the HIV-2 kit were essentially similar, except for the developing antibody supplied with the HIV-2 kit, which failed to detect IgM antibodies from mangabeys and therefore was replaced with a developing anti-human IgM antibody that showed similar affinity for IgM of the two species. For purposes of conciseness, data obtained with only our laboratory-established ELISA are presented. Finally, the optimal detection of plasma SIV-specific antibody required incubation for 2 h, whereas optimal detection of SIV-specific antibodies in supernatant fluids from PWM cultures required overnight incubation at room temperature.

Western Blot Assays

Each plasma sample and PWM supernatant fluid were also tested by Western blot assays. The antigen used was the same pool of SIVsmm. Standard techniques were applied, and incubation times and dilutions were optimized for plasma and for PWM culture supernatant fluids. Individual strips were blocked in PBS with 20% horse serum plus 20% calf serum overnight at 4°C, rinsed with PBS, and incubated for 2 h at room temperature with a 1:50 dilution of the plasma sample or overnight in a 37°C-humidified incubator with undiluted (unless otherwise indicated), freshly obtained (same day of harvest) supernatant fluid from the PWM cultures to be tested. The strips were rinsed with PBS, incubated with 2% powdered milk, rinsed again with PBS, and then incubated with HRPO-conjugated goat anti-human Ig reagent (diluted 1:250) for 1 h at room temperature, followed by rinsing and the addition of the carbazol substrate.

Depletion of $CD8^+$ T Cells

Ficoll-hypaque density gradient purified and washed PBMCs were unfractionated or depleted of $CD8^+$ T cells before culture in select assays. For depletion of $CD8^+$ T cells, the PBMCs were incubated for 30 min on ice with 2–5 μg of monoclonal anti-CD8 antibody (Leu-2A; Becton-Dickinson, Mountain View, CA, U.S.A.) per 10^7 cells in 1 ml of medium. The cell suspension was washed twice in medium and then incubated for 40 min on ice with anti-mouse Ig-coated magnetic beads (Dynabead Corp., Long Island, NY, U.S.A.) at a concentration of 10–50 beads/cell. The bead-conjugated cells were removed by placing them in a magnetic field for 2 min. The nonconjugated cells (CD8 depleted) in the supernatant fluid were removed by aspiration, centrifuged at 150 *g*, resuspended in fresh medium, and counted. The beads that were conjugated with cells were allowed to incubate overnight at 37°C, and the dissociated cells were gently removed and used as a source of enriched $CD8^+$ T cells in select experiments. Samples of the cell suspension were subjected to flow analysis to determine the degree of purity. These experiments found <1% $CD8^+$ cells in the depleted population and >90% $CD8^+$ cells in the enriched population. For studies of RT activity, the cell cultures were adjusted to contain equal numbers of $CD4^+$ cells in the unfractionated and CD8-depleted cultures to allow for meaningful comparison of RT activity.

Reverse Transcriptase Activity

Unfractionated and $CD8^+$ T-cell-depleted PBMCs were cultured at 2×10^6/ml and contained an equal number of total $CD4^+$ T cells (6×10^5/culture) in a total volume of 2 ml of medium containing 100 U/ml of recombinant interleukin-2 (rIL-2) and 0.1% phytohemagglutinin-P (PHA-P) (Difco, Detroit, MI, U.S.A.) in 24-well plates. The cultures were incubated in a 37°C incubator in an atmosphere of 7% CO_2 humidified air, and supernatant fluid was removed at days 7, 10, 14, 18, 21, 28, and 35. Samples were assayed undiluted for RT activity as previously described (13) and for levels of p27 by the p27 SIV capture assay (Coulter, Hialeah, FL, U.S.A.). Peak RT activity was observed in the supernatant fluids from the PBMCs of seropositive mangabeys and macaques between days 10 and 14 and between days 18 and 28 in cultures from the serum ELISA-negative mangabeys. Peak p27 levels correspond to peak RT levels in 30 of the 32 samples. The two exceptions were in cultures from one seropositive macaque and one serum ELISA-negative mangabey, with the peak RT activity occurring on day 14 and the peak p27 level on day 18 for the macaque, and peak RT and p27 levels occurring on day 21 and day 28, respectively, for the serum ELISA-negative mangabey. Only the highest RT and p27 levels are reported here, and for brevity the means of mean values have been derived from these results. For purposes of comparison, mean optical density values of 0.84 and 1.67 were obtained using 0.392 and 0.784 ng/ml of the recombinant p27 supplied with the kit (Coulter, Hialeah, FL, U.S.A.).

Polymerase Chain Reaction Analysis

PBMCs were washed three times with PBS and resuspended in PCR buffer (50 mM KCl, 10 mM Tris, 2.5 mM $MgCl_2$, 1 mg/ml

TABLE 1. *Isotype-specific SIV-reactive antibodies in PWM-induced PBMC cultures from seropositive and seronegative rhesus macaques and mangabeys*[a]

Supernatant fluid source	ELISA O.D.$_{405\ nm}$ (mean ± SD)		
	Ig (total)	IgM	IgG
Seropositive macaques (n = 10)	1.879 ± 0.258	0.129 ± 0.061	2.108 ± 0.231
Seropositive mangabeys (n = 10)	2.214 ± 0.417	0.459 ± 0.214	1.996 ± 0.314
Seronegative macaques (n = 20)	0.072 ± 0.029	0.066 ± 0.014	0.074 ± 0.031
Seronegative mangabeys (n = 17)	0.627 ± 0.398	0.837 ± 0.159	0.226 ± 0.117

[a] Samples of freshly harvested undiluted supernatant fluid from individual PWM cultures of PBMCs from the monkeys were assayed by using a goat anti-human Ig (for total Ig) and a goat anti-human IgM and IgG (previously screened for cross-reactivity against macaque and mangabey IgM and IgG, respectively). Background O.D. readings were <0.07 for all three developing reagents. The data represent the mean O.D.$_{405\ nm}$ ± SD observed in the sample numbers assayed.

type of reactive antibodies from the seropositive macaques and mangabeys was IgG, whereas the predominant isotype seen in supernatant fluids from the serum ELISA-negative mangabeys was IgM. It should be noted, nonetheless, that there is significant IgG reactivity seen in the supernatant fluids from the serum ELISA-negative mangabeys. In addition, the failure to detect antibodies in the test fluids from the seronegative macaques did not stem from the inability of the developing antibodies to react with IgM and IgG from this species.

Western Blot Analysis of Plasma and Corresponding PWM-induced Supernatant Fluids

To further confirm the specificity of the ELISA results, samples of the plasma diluted 1:50 and corresponding undiluted supernatant fluid from the PWM-induced PBMC cultures that were used for ELISA were also analyzed for reactivity against SIVsmm by using the Western blot assay. Plasma and undiluted supernatant fluids from each of the seropositive macaques and mangabeys gave multiple bands against most, if not all, SIV proteins (data not shown). Plasma and undiluted supernatant fluid from each of the seronegative uninfected macaques failed to show any detectable band. However, of interest was the observation that whereas plasma from all 22 serum ELISA-negative mangabeys, even when undiluted, failed to show detectable bands, supernatant fluid from PWM cultures of PBMCs from the same 17 of 22 serum ELISA-negative mangabeys that showed positive ELISA results (Fig. 1) gave at least two bands (gp130 and p27). Twelve of these 17 samples showed a third band (p55), and five of these 17 showed four major bands (gp130, p66, p55, and p27) and a minor gp30 band.

The assignments of specificity for some of these bands were based on the reactivity of monoclonal antibodies against *gag* (AIDS Reference and Reagents Depository, Rockville, MD, U.S.A.) and a monoclonal anti-HIV-1 gp41 antibody that cross-reacts with SIV *env* transmembrane (TM) protein at the 30-kD level. This reactivity at the 30-kD level for SIV *env* agrees with previous data that indicate that the TM of SIV cultured in the H9 cell line in vitro is truncated (15). It was reasoned that concentration of the supernatant fluid before Western blot analysis might facilitate the detection of low levels of other SIV-specific antibodies.

To address this issue, 20-ml cultures of PBMCs (2×10^6/ml) from three of the five serum ELISA-negative mangabeys whose PWM-induced PBMC supernatant fluid gave four to five bands and, for comparison, similar cultures of PBMCs from three uninfected seronegative macaques, three seropositive mangabeys, and three seropositive macaques were stimulated with PWM for 6 days, and the supernatant fluids were collected. Whereas PWM-induced supernatant fluid from the seropositive macaques and mangabeys was analyzed undiluted, the supernatant fluid from the serum ELISA-negative mangabeys and macaques was concentrated five times, using Centricon ultrafiltration devices, and subjected to Western blot analysis. In addition, Western blot analysis was performed in parallel with plasma from the same blood sample that was used for PWM cultures, which was diluted 1:50 from the seropositive animals and 1:1 from the serum ELISA-negative animals.

Figure 2 shows the results obtained with plasma (top panel) and the corresponding supernatant fluids (bottom panel). As seen, multiple SIV-reactive antibodies were visualized with plasma samples from both the seropositive rhesus macaques and mangabeys (top panel), with no discernible bands seen with plasma from the serum ELISA-negative macaques and mangabeys. Undiluted supernatant fluids from the seropositive rhesus macaques and mangabeys also showed multiple SIV-reactive

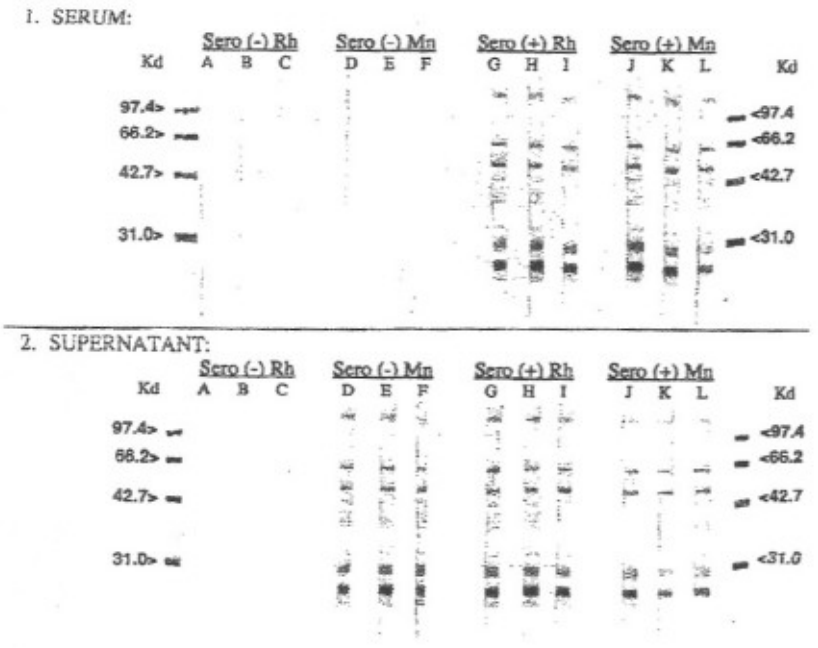

FIG. 2. Plasma and corresponding PWM-induced supernatant fluids from three seronegative uninfected rhesus macaques [sero (−) *Rh*], a select group of three serum ELISA-negative mangabeys [sero (−) Mn], three seropositive experimentally infected rhesus macaques [sero (+) Rh], and three seropositive naturally infected mangabeys [sero (+) *Mn*] were analyzed by Western blot against the SIVsmm preparation. The plasma samples from the serum ELISA-negative and the seropositive animals were diluted 1:2 and 1:50, respectively. The supernatant fluids from the seropositive monkeys were used undiluted, whereas the supernatant fluids from the serum ELISA-negative monkeys were concentrated fivefold before assay.

bands (bottom panel). The fivefold concentration of supernatant fluids from seronegative macaques showed no detectable bands. In marked contrast, the fivefold concentration of supernatant fluids from the PWM-induced PBMC cultures of the serum ELISA-negative mangabeys showed multiple SIV-reactive bands that were indistinguishable in profile from those seen with plasma or supernatant fluids from the seropositive animals. It should be noted that this complete profile is seen in only five of the 22 (22.7%) serum ELISA-negative mangabeys, with 12 additional samples of the 22 (54.5%) giving only two bands, corresponding to *env* and *gag* proteins, even after fivefold concentration of their respective supernatant fluids.

Role of CD8$^+$ T Cells in PWM-induced Ig Biosynthesis In Vitro

Several laboratories have documented the presence of antigen-specific and antigen-nonspecific regulatory CD8$^+$ T cells in a variety of immunological assays (16,17). The finding by Reinherz and Schlossman (18) that CD8$^+$ T cells can regulate PWM-induced Ig biosynthesis in vitro prompted us to determine whether the lack of SIV-reactive serum antibody in the serum ELISA-negative mangabeys that appear to have a sufficient number of "SIV primed" peripheral B cells may be subjected to functional regulation by CD8$^+$ T cells.

Unfractionated and CD8-depleted PBMCs from uninfected rhesus macaques (n = 6), experimentally infected rhesus macaques (n = 10), serum ELISA-negative mangabeys (n = 12), and seropositive mangabeys (n = 10) were cultured for 6 days in medium containing PWM. The supernatant fluid was collected and analyzed freshly for the levels of total Ig by RID and for SIV-specific Ig by ELISA. As seen in Table 2, the total Ig secreted by unfractionated cells from all samples was essentially similar. Depletion of CD8$^+$ T cells before culture markedly enhanced the total level of Ig secreted (approximately fourfold). The relative amount of SIV-specific antibody that was secreted by unfractionated PBMCs from seropositive macaques and mangabeys was again essentially similar. Depletion of CD8$^+$ cells from these seropositive mangabeys and macaques resulted in significant enhancement of SIV-reactive antibodies, which could be appreciated only when the fluid was diluted 1:10 before use. Similarly to our previous findings, significant levels of SIV-reactive antibodies were secreted by unfractionated cells from the 12 serum ELISA-negative mangabeys. These levels markedly increased when CD8$^+$ cells were removed before culture. It is important to note that neither unfraction-

TABLE 2. *Effect of depletion of CD8⁺ T cells on the quantity of total and SIV-specific Ig biosynthesis by PWM* in vitro[a]

Sample source (n)	SIV serology + dilution of S.F.[b]	Total Ig (μg/ml)[c] Mean ± SD		SIV-specific Ig[d] $O.D._{405\ nm}$ (mean ± SD)	
		Unfractionated	$CD8^-$	Unfractionated	$CD8^-$
Macaques (6)	−, undiluted	1.94 ± 0.18	8.76 ± 0.47	0.0069 ± 0.007	0.075 ± 0.012
Macaques (6)	−, 1/10	ND	ND	0.053 ± 0.018	0.063 ± 0.025
Macaques (10)	+, undiluted	2.24 ± 0.24	9.28 ± 0.80	2.356 ± 0.314	2.579 ± 0.294
Macaques (10)	+, 1/10	ND	ND	0.354 ± 0.036	1.396 ± 0.215
Mangabeys (12)	−, undiluted	2.18 ± 0.14	9.02 ± 1.22	0.564 ± 0.088	1.467 ± 0.235
Mangabeys (12)	−, 1/10	ND	ND	0.089 ± 0.041	0.176 ± 0.081
Mangabeys (10)	+, undiluted	1.98 ± 0.18	9.12 ± 1.24	2.578 ± 0.432	2.746 ± 0.319
Mangabeys (10)	+, 1/10	ND	ND	0.415 ± 0.095	1.634 ± 0.167

ND, not done.

[a] Unfractionated or CD8-depleted PBMCs were cultured for 6 days in vitro with PWM, and the supernatant fluids were assayed for total Ig and SIV-specific Ig by ELISA as described in Materials and Methods.

[b] Plasma from each of the blood samples was diluted 1/400 and assayed for SIV-specific antibody by using our standard SIV ELISA. The mean $O.D._{405\ nm}$ for the six seronegative macaques was <0.100, and the mean $O.D._{405\ nm}$ for the 10 seropositive macaques and mangabeys was >2.2. The samples denoted as seronegative mangabeys gave a value of 0.117 ± 0.038.

[c] Total relative Ig was determined by using a quantitative radial immunodiffusion kit.

[d] SIV-specific Ig was determined by using undiluted and 1/10 diluted supernatant fluids.

ated nor CD8-depleted PBMCs from the uninfected rhesus macaques secreted significant levels of SIV-reactive antibodies, whereas both the unfractionated and CD8-depleted PBMCs secreted total Ig levels similar to total Ig levels secreted by the infected macaques and mangabeys, which shows that the failure to detect SIV-reactive antibodies in cultures from the uninfected macaques was not due to the failure of these cells to secrete Ig.

Are Serum ELISA-negative Mangabeys Latently Infected with SIVsmm?

It was reasoned that the presence of B cells that can be induced to secrete SIV-reactive antibodies by PBMCs from many serum ELISA-negative mangabeys most likely stemmed from (a) latent SIV infection, which during the initial infection induced a sufficient number of memory B cells; (b) prior exposure, during fetal life or during birth, to SIV or SIV peptides (due to maternal SIV infection prevalent in this colony) or other cross-reactive peptides; (c) latent infection with other endogenous retroviruses that shared peptide sequences with SIV. To distinguish among these possibilities, PBMCs from serum ELISA-negative mangabeys (n = 12) and, for purposes of control, PBMCs from seropositive mangabeys (n = 6) and seropositive and seronegative macaques (n = 6 and n = 8, respectively) were cultured in vitro in medium containing 0.1% PHA-P and 100 U/ml of recombinant human IL-2. The PBMCs were cultured unfractionated or after depletion of $CD8^+$ cells that were previously shown to regulate virus replication in both human and nonhuman primates (19–22). Supernatant fluids from each of these cultures were assayed for RT activity and p27 levels.

As seen in Fig. 3A and C, unfractionated cells from the seropositive mangabeys and macaques showed high RT activity and p27 levels, as expected. These values reflect peak RT and p27 levels, which occurred between day 10 and day 14. $CD8^+$ T-cell depletion of PBMCs from these seropositive animals, before culture, clearly enhanced both RT activity and p27 levels (see Fig. 3A and C). Supernatant fluid from uninfected and seronegative macaques showed background RT and p27 levels that did not change by $CD8^+$ T-cell depletion (see Fig. 3B and C). In contrast to seronegative macaques, cultures from the serum ELISA-negative mangabeys showed significant RT and p27 levels, which were markedly enhanced by depletion of $CD8^+$ T cells (see Fig. 3B and C). Peak RT activity in these cultures occurred on days 18–28. However, repeated attempts to culture virus from such supernatant fluid by transferring them to fresh uninfected PHA-P blasts from human or rhesus macaques failed to lead to virus isolation. The reasons for this failure are not clear.

To further verify the results of the RT and p27 capture assay, the PCR technique was used with a set of *gag*-specific primer pairs and probes as previously described (1). DNA samples from six serum ELISA-negative mangabeys (confirmed by ELISA and Western blot) and, for purposes of control, DNA samples from a seropositive mangabey and an

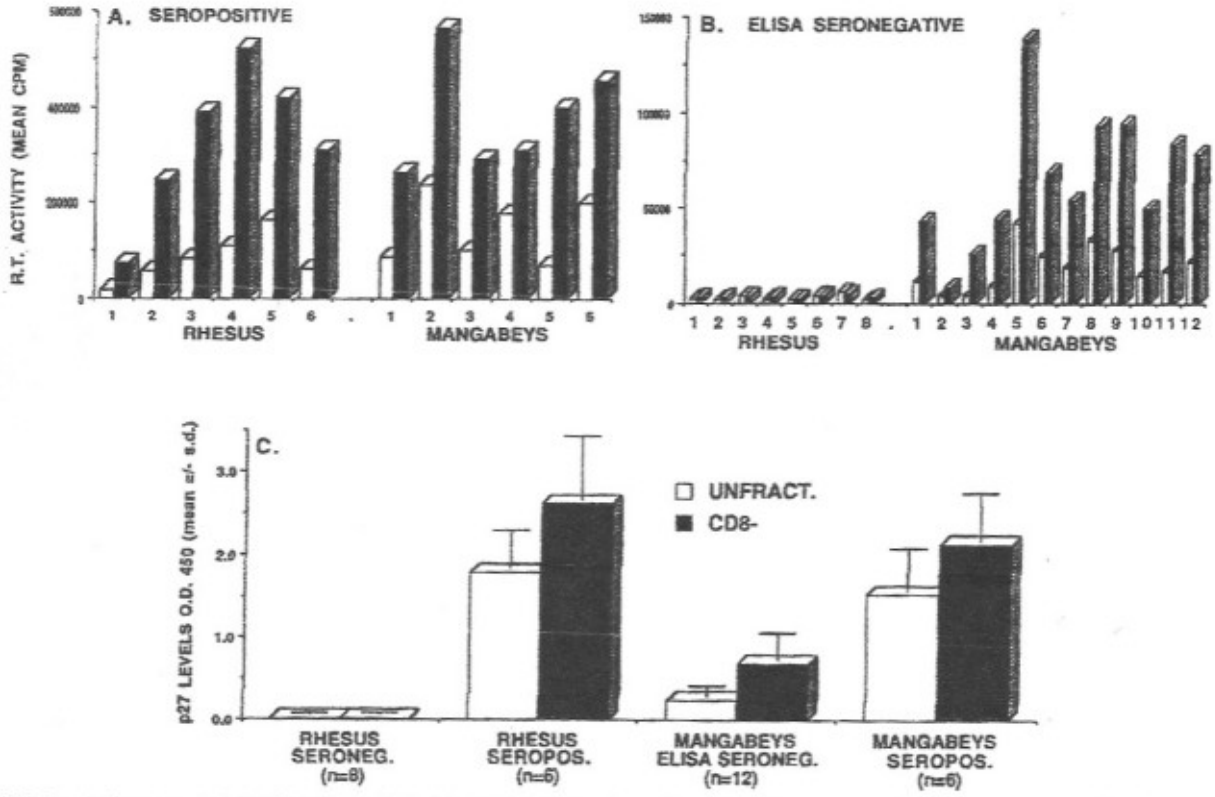

FIG. 3. Unfractionated (□) $CD8^+$ T-cell-depleted (■) PBMCs from six seropositive rhesus macaques and mangabeys, eight seronegative rhesus macaques, and 12 serum *ELISA-negative mangabeys were cultured in vitro in medium* containing PHA-P (0.1%) and r-IL2 (100 U/ml). On days 7, 10, 14, 18, 21, 28, and 35, supernatant fluids from individual cultures were monitored for RT activity and levels of p27. Results shown represent only peak levels of RT and p27. **A,B:** Results of RT activity of cultures from seropositive and serum ELISA-negative animals, respectively. **C:** Mean peak p27 levels observed in cultures from the serum ELISA-negative and seropositive animals.

uninfected seronegative rhesus macaque were subjected to PCR analysis. As seen in Fig. 4, DNA from the seropositive mangabey gave a distinct signal (lane a), and no band was seen with the sample from the seronegative macaque (lane i). In contrast,

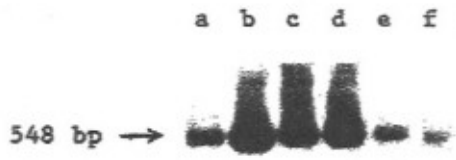

FIG. 4. PCR analysis of DNA samples from a seropositive experimentally infected rhesus macaque (lane a), six serum ELISA-negative mangabeys (lanes b, c, d, e, f, and g), and an uninfected seronegative rhesus macaque (lane i). Lane h represents the negative control that contained the cocktail used for the assay. The PCR used a set of *gag*-specific primer pairs and a *gag*-specific probe (sequence 1678–1702), as described in Materials and Methods. The PCR-amplified products were electrophoresed on an agarose gel, and Southern blots were performed by using standard techniques.

DNA samples from three serum ELISA-negative mangabeys (lanes b, c, and d) gave strong signals, and samples from two of the other serum ELISA-negative mangabeys gave weaker signals (lanes e and f). The signal from the sixth serum ELISA-negative mangabey could be visualized by the naked eye on the x-ray film but could not be adequately photographed (lane g). The serum ELISA-negative mangabey represented by lane b died a year later (still seronegative at death), and the cause of death remains unknown. The serum ELISA-negative mangabeys represented by lanes c, d, e, and f each seroconverted at 8, 7, 7, and 6 months, respectively, following the tests described herein, whereas the mangabey represented by lane g remains serum ELISA negative to date (>2 years since the study began). The *gag* region amplified from the serum ELISA-negative mangabey (see lane b) has been sequenced and shown to be 88%

homologous to the sequences published for SIVsmmH4 (1).

DISCUSSION

The data from these studies provide reasonable evidence for the presence of many sooty mangabeys in our colony at the YRPRC that are serum ELISA negative but nonetheless have PBMCs that can be induced in vitro to secrete both IgM and IgG antibodies that react with SIV. There were 17 samples that clearly gave positive results. However, there were five that were nonreactive. This finding did not stem from sampling error, since these tests were repeated three times on samples from the same monkey within a 3-month period, and similar results were obtained each time. It was also not due to the age of these five animals, since they were of the same age or older than some of the 17 that gave positive results. In addition, they were from the same breeding compounds. The predominant SIV-reactive antibody in the supernatant fluids of these 17 mangabeys was determined to be IgM. IgM antibodies have been shown by several laboratories to have polyspecific reactivity. Although these results could be secondary to such polyreactivity, it is important to note certain unique features of these findings.

First of all, such reactivity was never observed in supernatant fluids from PBMC cultures of ≥20 uninfected rhesus macaques, suggesting an element of species specificity for such reactivity. Second, absorption of samples of reactive supernatant fluids from serum ELISA-negative mangabeys with the uninfected H9 cell line (used for preparation of the SIV) failed to diminish the SIV antibody reactivity (data not shown). Third, it is important to note that not only significant IgM antibodies but also IgG antibodies reactive with SIV were detected in such supernatant fluids (see Table 1). Fourth, the supernatant fluids contain IgG antibodies that clearly react with SIV proteins by Western blots, showing bands of 130 and 27 kD in all 17 samples; bands of 130, 55, and 27 kD in 12 of 17 samples; and bands of 130, 66, 55, and 27 kD in five of 17 samples. Finally, when these five samples were concentrated five times, their Western blot profiles were indistinguishable from the profiles seen with supernatant fluids or plasma samples from the seropositive macaques and mangabeys (see Fig. 2). No unique age, sex, or prior clinical history could be associated with these five mangabeys. Thus, it is unlikely that SIV antibody reactivity in such supernatant fluids is entirely due to the presence of nonspecific antibodies.

There are several possible explanations for the findings of this study. First, one can argue that the serum ELISA-negative mangabeys are indeed uninfected since, to date, it has been impossible to culture replication-competent SIV from these animals by a variety of techniques, such as cocultures with $CD4^+$ cell lines and normal human or nonhuman primate PHA blasts. It is thus possible that this species, over a period of time, has evolved a repertoire of B cells that are primed against a variety of ubiquitous antigens that cross-react with determinants of SIV. This would explain the reactivity by Western blots to *env* and *gag* sequences by supernatant fluids from PWM cultures from the serum ELISA-negative mangabeys. Second, it can also be argued that the few serum ELISA-negative mangabeys whose PWM-induced PBMC-cultured supernatant fluids, when concentrated, exhibited a profile similar to the profile exhibited by serum samples and PWM supernatant fluid from the seropositive animals represented mangabeys that were in the window period between infection and seroconversion. As stated herein, these animals were maintained in a colony with free interaction among various monkeys (serum positive and serum negative). Longitudinal studies of these mangabeys and a large number of other mangabeys have shown that window periods are highly variable. Of the five mangabeys whose PBMCs secreted multiple SIV-reactive antibodies (described previously), two seroconverted within 6 months, two seroconverted 1½–2 years from the time this study began, and one remains serum ELISA negative. The four seroconverters were also virus culture–positive at the time of seroconversion. It is of interest to note that the number of B cells that secrete SIV-specific antibodies, as determined by the ELISPOT assay using PWM-stimulated PBMCs, was determined to be between 100 and 330/10^6 input cells from SIV-seropositive mangabeys and macaques. Although no spots were noted in similar cultures from uninfected rhesus macaques, PBMCs from serum ELISA-negative mangabeys gave values between 30 and 65 spots/10^6 input cells.

The finding of SIV-specific B cells in the PBMCs of serum ELISA-negative mangabeys therefore leads one to question whether these mangabeys have an occult SIV infection or are merely exposed to SIV or SIV cross-reactive proteins in the form of

dead or inactivated virus or related infectious agents. In this regard, it is important to note the recent findings from the laboratories of Fauci (23) and Haase (24). The findings of their studies indicate that during the asymptomatic stage of lentivirus infection, there are high relative levels of replication-competent virus in the lymph nodes even though there may be very few infected cells in the peripheral circulation. Thus, in the case of the serum ELISA-negative mangabeys, it is possible that the primed B cells reflect peripheralization of B cells sensitized in lymph nodes. In efforts to address this question, our laboratory employed PCR techniques that we have previously shown to detect as few as two SIV *gag* copies in DNA samples from 2.5×10^5 cells (1) to confirm the infection status of these animals. Although this assay consistently detects SIV *gag* sequences from PBMC DNA samples of experimentally infected macaques, amplification from PBMC DNA of naturally infected mangabeys (particularly serum ELISA-negative animals) sampled over extended periods of time has proved less consistent longitudinally.

Given the sensitivity and broad cross-reactivity of the *gag* primers used, these variable results most likely reflect a low provirus copy number among peripheral blood cells. Infected cells may in fact be present in lymph nodes or spleen, as shown recently by two different laboratories (23,24). Sequence analysis of the *gag* fragment from the serum ELISA-negative mangabey (Fig. 4, lane b), amplified from a sample collected 2 years before death, rules out the possibility of cross-reactivity with non-SIV retroviruses, since the sequence homology to SIVsmmH4 was 88% (1). It can be argued that the serum ELISA-negative mangabeys are infected with replication-incompetent SIV, but two observations made by our laboratory appear to refute this hypothesis. (a) There were three seropositive/virus-positive offspring of serum ELISA-negative mothers [samples from these babies were derived during a time period when the babies had no social contact with other monkeys in the breeding colony (25)]. (b) Data from preliminary experiments show that in vivo hyperactivation of the immune system of serum ELISA-negative mangabeys by a polyclonal activation agent led not only to frank seroconversion but also to the ready isolation of virus (confirmed also by E. M.) from the PBMCs of these animals (A. A. Ansari et al., unpublished observations) even though these animals were housed in individual cages 3 months before activation and for the entire duration of the experiment. It is of interest to note, however, that we failed to obtain positive amplification using a number of *env* and *pol* primers based on the published sequence of SIV in samples from serum ELISA-negative mangabeys that gave clear positive signals with our *gag* primers.

Thus, it is our working hypothesis that in addition to later (re)infection by social contact, a large number of sooty mangabeys in our colony are most likely (a) exposed to SIV proteins, peptides, and live virus during fetal life the replication of which is highly regulated or (b) exposed to virus during or shortly after birth, resulting in the development of partial tolerance and/or split tolerance that leads to regulation of virus replication with concomitant reversible antigen-specific B-cell anergy.

It is important to keep in mind that latent retroviral infection in the absence of detectable antibodies has also been noted in models of feline immunodeficiency virus, bovine immunodeficiency virus, caprine arthritis and encephalitis virus, and visna virus infections of cattle, goats, and sheep (Dr. Mary Saltarelli, Frederick Cancer Center, Frederick, MD, U.S.A., personal communication). Although this assay provides a sensitive complement to PCR detection of early lentivirus or other viral infections, additional studies are required to fully understand this complex host–lentivirus relationship.

Finally, the relevance of the findings reported in this communication to human HIV-1 infection deserves comment. It should be kept in mind that the study of a closed breeding colony of sooty mangabeys that are naturally infected with SIV, in our opinion, represents studies of socially interactive groups of humans who are at very high risk of HIV-1 infection, e.g., intravenous drug abusers. Consequently, we predict similar findings in such groups. In this regard, we have previously found such a phenomenon in blood samples from a population at very high risk of HIV-1 infection (4), a population that could not be prospectively followed owing to the anonymity of the donors. However, subsequent longitudinal studies on a group of 215 individuals being treated for alcohol and drug abuse showed a prevalence rate of 5.6% HIV-1 seropositivity. There were five additional individuals who were serum ELISA negative but positive by PCR and the PWM test. One donor seroconverted within 2 months and subsequently died of AIDS. A second donor seroconverted 2 years later and is still seropositive but asymptomatic. A third donor is still

ELISA seronegative but PCR positive (for both *env* and *gag*) and PWM positive. Two others were lost to follow-up owing to lack of donor cooperation (unpublished data). These data suggest that the incidence of such infected but serum ELISA-negative individuals is, in fact, quite low in this suburban referral drug and alcohol intake treatment center. The test does detect infection during the window period between infection and seroconversion and may even identify some few individuals who are infected but who may never seroconvert. Although our test procedures detect lentiviral humoral immunity before seroconversion, it is important to note similar findings of T-cell immunity in seronegative individuals by the laboratory of Shearer (6).

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