Immune Activation and Viral Burden in Acute Disease Induced by Simian Immunodeficiency Virus SIVsmmPBj14: Correlation between In Vitro and In Vivo Events

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The simian immunodeficiency virus SIVsmmPBj14 (SIV-PBj14) is an atypical lentivirus that causes acute disease and death in pig-tailed macaques and in vitro replicates efficiently in resting macaque lymphocytes and activates and induces proliferation of lymphocytes. The present study was conducted to test the hypothesis that production of large quantities of SIV-PBj14 induces widespread immune activation and elaboration of cytokines which lead directly to the death of infected pig-tailed macaques. Following intravenous inoculation of pig-tailed macaques with SIV-PBj14, acute disease developed and was characterized by high levels of plasma viremia, p27^{gag} antigenemia, tumor necrosis factor alpha, and interleukin-6 (IL-6). All animals died within 10 days of infection, at which time some animals had as many as 100% CD4⁺ cells in the periphery and lymphoid tissues infected. During the last few days before death, titers of infectious virus in blood increased as much as 10⁵-fold. By using dual-label immunofluorescence assays for detection of cell surface activation markers, both CD4⁺ and CD8⁺ lymphocytes were shown to express the IL-2 and transferrin receptors following either in vivo or in vitro infection with SIV-PBj14. Furthermore, in vitro infection of quiescent macaque lymphocytes by SIV-PBj14 was accompanied by proliferation of both CD4⁺ and CD8⁺ lymphocyte subsets, as measured by incorporation of [³H]thymidine. Increases in numbers of activated lymphocytes and levels of proinflammatory cytokines in plasma coincided with increased amounts of detectable virus in vivo. Clinical signs of disease and pathological findings were most consistent with death from a shock-like syndrome, in which acute-phase inflammatory cytokines are known to play a major role. Tumor necrosis factor alpha, IL-2, and IL-6 were detected in some cultures infected with SIV-PBj14, but this finding was not consistent. When cytokines were detected, their concentrations were essentially no different from those found in control cultures infected with SIVsmm9, a prototypic strain from which SIV-PBj14 was derived. The in vivo results suggest a synergistic cycle of activation of lymphocytes and monocytes, elaboration of cytokines, and virus production that accelerates uncontrolled and culminates in death. The observed correlations between in vivo and in vitro activation events following SIV-PBj14 infection validate the use of in vitro studies to clarify lentivirus-lymphocyte interactions that may contribute to the virulence of SIV-PBj14.

Infection of macaques with most simian immunodeficiency viruses (SIV) results in an AIDS-like disease similar to that seen with humans infected with human immunodeficiency viruses (HIV) (10, 14, 34, 36, 45, 47). An exception, however, is SIVsmmPBj14 (SIV-PBj14), which was isolated from a pig-tailed macaque at the time of its death, 14 months after infection with SIVsmm9, a prototypic SIV originally obtained from a naturally infected sooty mangabey monkey (*Cercopithecus atys*) (18). SIV-PBj14 is an atypical lentivirus for several reasons: (i) it induces acute disease and death within 6 to 10 days, not only in pig-tailed (*Macaca nemestrina*), cynomolgus (*Macaca fascicularis*), and some rhesus (*Macaca mulatta*) macaques but also in its natural host, sooty mangabeys (19, 35, 50, 51); (ii) unlike other primate lentiviruses that require lymphocyte activation for replication, SIV-PBj14 replicates efficiently in resting pig-tailed macaque peripheral blood mononuclear cells (PBMC) (17, 41); and (iii) it activates and induces proliferation of resting lymphocytes (17, 20, 41). These latter two properties were identified in in vitro experiments, but they are consistent with in vivo pathogenesis, which is characterized by extensive lymphoid hyperplasia and is espe-

cially prominent in the spleen, mesenteric lymph nodes, and gut-associated lymphoid tissues (17, 19, 20, 41). Biologically and molecularly cloned viruses that exhibit these same general properties, albeit with some variation in pathogenesis, have been derived from SIV-PBj14 (12, 41). In particular, a molecular clone, SIV-PBj14-mcl1.9, reproducibly induces the same acute disease syndrome, including profuse diarrhea, in pig-tailed macaques; however, most animals survive, and a prolonged, clinically asymptomatic infection ensues (28).

The natural history of SIV-PBj14-mcl1.9 infection of pig-tailed macaques mimics that of HIV type 1 (HIV-1) infection in that the initial phase is characterized by (i) high virus loads that drop precipitously as humoral immune responses become detectable and (ii) clinical signs of disease, such as lymphopenia, diarrhea, rash, and lymphadenopathy, that also resolve as the amount of virus in the peripheral blood decreases (6, 7, 9, 12, 14, 24, 28). As long-term asymptomatic HIV infections progress to overt disease, not only does viral load in the periphery increase but also there is evidence for abnormal immune activation that becomes more pronounced in parallel with the development of immunosuppression. Indicators of immune activation detected in HIV-infected persons include elevated levels in plasma and cerebrospinal fluid of immunoglobulins (1, 33), CD25 (interleukin-2 receptor alpha chain [IL-2R α]) (21, 26), and the multifunctional inflammatory

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cytokines IL-6 (3, 4, 21, 26) and tumor necrosis factor alpha (TNF- α) (32, 39), among other proteins. IL-6 and TNF- α are of particular interest since both cytokines are known to up-regulate expression of HIV-1 in primary monocyte-derived macrophages (38, 47, 59) and are produced in vitro during interaction between activated CD4⁺ lymphocytes and SIV-infected macrophages (37).

Previously we showed that in vitro infection of resting or quiescent pig-tailed macaque PBMC with SIV-PBj14 resulted in replication and production of high levels of virus, followed by increases both in total numbers of cells and in percentages of cells expressing CD25, a cell surface antigen expressed on activated lymphocytes (61). Furthermore, we showed that both CD4⁺- and CD8⁺-enriched cell populations incorporated [³H]thymidine specifically in response to heat-inactivated SIV-PBj14 (17). However, whether both lymphocyte subsets were activated and induced to proliferate during active replication of SIV-PBj14 in vitro and in vivo was not determined. Activation of both subsets was suggested not only by the in vitro experiments described above but also by in vivo observations that CD4⁺ and CD8⁺ lymphoblasts in approximately equal numbers infiltrated the laminae propriae of gastrointestinal tracts of animals dying of acute SIV-PBj14 infection (20).

Because the acute lethal disease induced by SIV-PBj14 appears to reproduce certain aspects of primary HIV infection, but in a grossly accelerated manner, infection of pig-tailed macaques by this virus may provide insight into HIV acute disease (40). This model also has the advantage that results can be obtained more rapidly than when conventional primate lentiviruses are studied. The present study was undertaken to test the hypothesis that in vivo production of large quantities of SIV-PBj14 induces widespread immune activation associated with elaboration of TNF- α , IL-6, and possibly other acute-phase reactants, culminating in death. In addition, to facilitate identification of mechanisms directly responsible for acute disease, it was important to determine whether in vitro infection of macaque lymphocytes by SIV-PBj14 is predictive of events in vivo. Through combined analyses of in vivo and in vitro events, the SIV-PBj14-macaque model should provide insight into not only lentivirus-PBMC interactions, in general, but also the role of overproduction of cytokines, such as IL-6 and TNF- α , in the pathogenesis of primate lentiviruses.

MATERIALS AND METHODS

Viruses and animals. All virus stocks were generated by one or two infectious passages of the original virus isolates on phytohemagglutinin (PHA; Difco Laboratories, Inc., Detroit, Mich.) P-stimulated normal human PBMC. A biologic clone of SIV-PBj14, SIV-PBj14-bcl3, was generated after two sequential limiting dilutions and infection of human PBMC, as described previously (19). The 50% tissue culture infectious dose (TCID₅₀) of virus stocks generated in human PBMC was determined by limiting dilution and infection of human or pig-tailed macaque PBMC.

Young adult pig-tailed macaques used in this study were housed in accordance with Animal Welfare Act guidelines. Macaques 147F, 87-011, 82-64, and 82-67 were housed at the University of Alabama at Birmingham. Tissues and plasma from macaques 275, 276, 541, 542, 543, and 544 were obtained in collaboration with researchers at the New England Primate Research Center. Animals were bled for serology and hematology prior to virus inoculation and for virus isolation and quantitation at specified times after infection. SIV-seronegative and clinically normal animals were inoculated intrave-

nously with 0.5- or 1-ml aliquots of SIV-PBj14-bcl3 (3.2×10^4 TCID₅₀s per ml, as determined by titration in human PBMC). Two animals (82-64 and 82-67) were inoculated intravenously with 1.2×10^5 cryopreserved mesenteric lymph node cells from a pig-tailed macaque (147F) that died on day 8 after SIV-PBj14-bcl3 infection. The number of infectious cells in this dose was equivalent to the cell-free virus TCID₅₀ of 3.2×10^4 , as determined by titration with pig-tailed macaque PBMC.

The monkeys were observed daily, and physical examinations were performed at each blood collection. Animals in extremis were euthanatized if they failed to respond to supportive therapy. Following exsanguination under anesthesia, necropsies were performed on all animals. Lymph nodes and spleens were removed aseptically, minced, and passed through a 1-mm² stainless steel wire mesh to prepare single-cell suspensions. PBMC and plasma were obtained by density gradient centrifugation of heparinized peripheral blood in Ficoll-sodium diatrizoate (LSM; Organon Teknika, Durham, N.C.). No tissues were available from macaque 82-67 because it died during the night of day 7 postinfection.

Determinations of virus load in plasma and tissues. Tenfold serial dilutions of plasma samples and fivefold serial dilutions of CD4⁺ cell populations were made in complete RPMI 1640 medium (10% heat-inactivated fetal bovine serum, 1% L-glutamine, 1% sodium bicarbonate, 0.8% amphotericin B [Fungizone; Gibco Laboratories], 55 μ g of gentamicin per ml) containing IL-2 (8 U/ml) and DEAE-dextran (8 μ g/ml). One-hundred-microliter aliquots of plasma or 1-ml aliquots of cell suspensions were added to duplicate wells of 12-well plates containing 3×10^6 concanavalin A (Boehringer Mannheim, Indianapolis, Ind.)-stimulated PBMC from an uninfected pig-tailed macaque. The TCID₅₀ was determined from the number of positive wells at each dilution. Aliquots of cell-free culture supernatant were removed every 5 to 6 days and ultracentrifuged to concentrate virus for determination of reverse transcriptase (RT) activity (54). Cultures were refed with complete medium alone or medium containing 1.5×10^6 concanavalin A-stimulated pig-tailed macaque PBMC and were monitored for 6 weeks.

SIV p27 antigen levels in plasma or serum were determined with an SIV-p27 enzyme-linked immunosorbent assay (ELISA) kit (Coulter, Hialeah, Fla.) and microtiter plate reader, according to the manufacturer's instructions.

Cell surface staining and FACScan analysis. Surface expression of cellular markers of activation, which included major histocompatibility complex class II HLA-DR, CD25 (anti-TAC), and CD71 (transferrin receptor), and alterations in lymphocyte subsets were monitored with a panel of mouse anti-human monoclonal antibodies: CD4, leu3a; CD8, leu2a; and CD20, leu16 (Becton-Dickinson, Mountain View, Calif.). HLA-DR, CD25, and CD71 expression was evaluated because these antigens are known to increase in density on the surface of activated T lymphocytes (30, 48), and monoclonal antibody reagents that cross-react with these antigens on pig-tailed macaque cells are available. Lymphocytes were gated according to forward-scatter-versus-side-scatter characteristics, and Simultest Control γ 1/ γ 2a (immunoglobulin G1-fluorescein isothiocyanate/immunoglobulin G2a-phycoerythrin) antibodies were used to identify negative cell populations (Becton-Dickinson). Analyses were performed with a FACS-STAR flow cytometer (Becton-Dickinson). EDTA-treated blood samples were stained by a whole-blood lysis procedure, according to the manufacturer's instructions. Some results are reported as the percentages of each lymphocyte subset displaying a particular surface antigen.

Infection of resting macaque lymphocytes. PBMC from

normal pig-tailed macaques were aliquoted into 25-cm² flasks (1.5×10^7 cells in 10 ml of complete RPMI 1640 medium without IL-2 and DEAE-dextran) and inoculated with 6.4×10^5 TCID₅₀s of either SIVsmm9 or SIV-PBj14 (multiplicity of infection, 0.04); uninfected control cultures were maintained in medium alone. Aliquots of lymphocytes were removed from cultures and stained for expression of activation markers on day 0 and at various intervals after infection. Virus production (assayed by RT activity) and cell number and viability were determined every 5 to 6 days. Aliquots of culture supernatants were frozen at -80°C until tested for the presence of cytokines.

Proliferation assays. The ability of SIV-PBj14 to induce proliferation of resting pig-tailed macaque PBMC or purified CD4⁺ and CD8⁺ lymphocytes was evaluated by incubating 10^5 cells with 10 ng (as determined by SIV p27 ELISA) of infectious or heat-inactivated virus (56°C for 1 h), 0.1% PHA, or medium alone for 7 days in a total volume of 0.2 ml of complete RPMI 1640 medium containing 10% human AB serum in 96-well round-bottom plates (41). Cells were pulsed on day 6 with 1 μCi of [³H]thymidine (DuPont NEN Research Products, Boston, Mass.) and harvested after an 18-h incubation by using a Combi cell harvester (Skatron Inc., Sterling, Va.). Incorporation of radioactivity into cellular DNA was determined by liquid scintillation counting with a beta counter. Purified populations of CD4⁺ and CD8⁺ lymphocytes were obtained by positive selection with antibody-coated magnetic beads (Dynabeads; Dynal Inc., Great Neck, N.Y.). The purities of the cell populations were 96 to 98% for CD4⁺ cells and 80 to 87% for CD8⁺ cells, as determined by FACScan analysis; contaminating cell populations were identified as CD8⁺ or CD20⁺ for the CD4⁺-enriched cells and CD20⁺ for the CD8⁺-enriched cells.

Cell proliferation in 10-ml cultures of macaque PBMC in which SIVsmm9 or SIV-PBj14 replicated was assessed by removing PBMC from uninfected or infected cultures and immediately pulsing the cells with 1 μCi of [³H]thymidine for 18 h. Stimulation indices were determined by dividing the counts per minute incorporated by lymphocytes incubated with virus or PHA by the counts per minute incorporated by lymphocytes incubated in medium alone.

Cytokine assays. TNF- α and IL-6 concentrations in plasma, serum, or culture supernatants were determined with commercial ELISA kits (Innotest hTNF- α [Innogenetics, Antwerp, Belgium] and the Predicta IL-6 kit [Genzyme Corp., Cambridge, Mass.]), according to the manufacturers' instructions. IL-2 in infected culture supernatants was detected by incorporation of [³H]thymidine into murine CTLL cells (22).

Statistical methods. PBMC from 18 clinically normal, uninfected pig-tailed macaques were examined by FACScan analysis to determine normal levels of cell surface activation markers on lymphocytes. These values were compared with percentages of activation markers on PBMC from SIV-PBj14-infected animals by one-way analysis of variance (Statistix version 4.0 program; Analytical Software, St. Paul, Minn.). Lymph node tissues were obtained from some animals prior to infection for comparison of expression of activation markers. The minimum number of PBMC containing one infectious CD4⁺ cell, at a confidence interval of 95%, was determined with the VacMan program (55). To compare virus replication with other parameters, SIV-PBj14-infected animals were ranked according to the concentration of p27 antigen in their peripheral blood at the time of death, and correlations (Pearson) were calculated with the Statistix version 4.0 program.

FIG. 1. CD4/CD8 ratios in peripheral blood ($n = 8$) and lymph node ($n = 5$) lymphocytes of SIV-PBj14-infected pig-tailed macaques before infection and at the time of death. Each symbol represents a different macaque.

RESULTS

Clinical course of SIV-PBj14 infection of macaques. Pig-tailed macaques were infected with SIV-PBj14-bcl3 not only to characterize lymphocyte activation *in vivo* and to establish a correlation, if any, with virus replication but also to determine whether there was any apparent phenotypic difference between lymphocytes in the peripheral blood and lymph nodes. Regardless of the dosage or form (cell-free or infected cells) of SIV-PBj14-bcl3 inoculated, macaques became acutely ill, with death occurring from days 7 to 10. Macaques 541 and 542 were euthanized on day 6, before acute disease was evident. Infected animals became depressed, anorexic, and dehydrated, with a loss of greater than 10% body weight, and exhibited signs of shock (weak, thready femoral pulse, decreased capillary refill time, cold extremities, etc.). Most animals showed signs of illness for only 24 h before death or euthanasia. In contrast to macaques infected with uncloned SIV-PBj14, as described previously (19, 35, 50), macaques inoculated with SIV-PBj14-bcl3 developed mild mucoid diarrhea with relatively small amounts of blood. Only macaque 275 developed a cutaneous erythematous, macular rash. Lymphopenia, generally involving all subsets of circulating lymphocytes, developed in most animals between days 4 and 6, which is consistent with earlier reports of SIV-PBj14-induced acute disease (19, 28, 35). Between the time of infection and death, CD4/CD8 ratios in PBMC and peripheral lymph nodes decreased by 50% or more in a majority of the animals (Fig. 1).

Virus replication and tissue burden in SIV-PBj14-infected macaques. To determine the kinetics of virus replication *in vivo*, plasma or serum samples obtained early after infection, before the onset of signs of acute disease, and at the time of death were assayed for the number of infectious virions and the concentration of SIV p27 antigen (Fig. 2). In all animals for which samples were available, low levels of p27 antigen and infectious virus were detected 4 to 6 days postinfection. During the subsequent 2 to 3 days before death, however, the production of virus increased rapidly. At death, six of eight animals had p27 levels of greater than 3,000 pg/ml (range, 3,466 to 8,168 pg/ml), which reflected increases of 20- to more than 600-fold; titers of infectious virus in plasma also increased as much as 10^2 - to 10^5 -fold during this time.

Because lymph nodes appear to be reservoirs for HIV-1 (13, 31, 44, 45), and cells in the periphery represent only 2% of total lymphocytes, virus load in various lymphoid compartments was also evaluated. The frequencies of infectious CD4⁺ cells in

FIG. 2. Plasma antigenemia (A) and viremia (B) in SIV-PBj14-infected pig-tailed macaques at death and at an interim time (days 4 to 6) between infection and death. The horizontal bars indicate the arithmetic (A) or the geometric (B) means. Symbols represent macaques as follows: ■, 543; ●, 544; ▲, 276; ◆, 147F; □, 82-64; ○, 87-011; △, 82-67; ◇, 275; ×, 542.

peripheral blood, the mesenteric lymph nodes, and the spleen were determined by limiting dilution and coculture with normal pig-tailed macaque PBMC (Table 1). The apparent frequency of infected CD4⁺ cells in peripheral blood (range, 1.8×10^{-3} to 1.0) was more variable than that obtained for the lymph nodes and spleen. Since SIV-PBj14 exhibits tropism for gut-associated lymphoid tissue, it was of interest to determine whether there was a difference in the frequencies of infected cells in peripheral nodes and mesenteric lymph nodes. The minimal number of infectious CD4⁺ cells in the peripheral nodes of macaques 147F and 82-64 was virtually identical to that of cells in the mesenteric lymph nodes (data not shown), suggesting that there was no inherent difference in the abilities of different lymph nodes to support SIV replication.

Activation of lymphocytes in SIV-PBj14-infected macaques. To determine whether lymphocytes were activated in vivo, and the subsets that were involved, PBMC from experimentally infected macaques were analyzed by dual-label immunofluorescence assays before infection (day 0) and at each subsequent blood collection. Both CD4⁺ and CD8⁺ lymphocytes exhibited increased expression of CD25 and CD71 as infection progressed (Fig. 3). Furthermore, greater expression of these activation markers was found on lymphocytes from the animals with higher concentrations of SIV p27 in plasma. When mean

TABLE 1. Frequency of infectious CD4⁺ cells in the peripheral blood and lymphoid organs of SIV-PBj14-infected pig-tailed macaques at death

Macaque ^a	Frequency in ^b :		
	PBMC	Mesenteric lymph nodes	Spleen
543	NA ^c	8.3×10^{-2}	NA
544	NA	5.0×10^{-1}	NA
276	1.8×10^{-3}	5.9×10^{-2}	8.3×10^{-2}
147F	1.4×10^{-1}	5.0×10^{-1}	8.3×10^{-2}
82-64	3.4×10^{-3}	8.3×10^{-2}	1.0
87-011	4.0×10^{-3}	NA	NA
82-67	1.0	NA	NA
275	1.7×10^{-2}	5.3×10^{-2}	8.3×10^{-2}
542 ^d	1.3×10^{-1}	8.3×10^{-2}	1.7×10^{-1}
541 ^d	NA	1.7×10^{-2}	NA

^a Animals are ranked according to p27 antigen concentration, from highest to lowest amounts, in peripheral circulation at the time of death.

^b Values were calculated from the minimum number of CD4⁺ cells required for a virus-positive culture as determined by end-point dilution and coculture with normal macaque PBMC. The calculation assumes that one infected cell is sufficient to transmit infection to indicator cells and, therefore, is a minimum estimate.

^c NA, not available.

^d Animals were euthanized on day 6, before signs of acute disease developed.

values for PBMC from infected animals were compared with those for PBMC from 18 uninfected animals, increases in expression of CD25 and CD71, but not of HLA-DR, on both CD4⁺ and CD8⁺ cells were statistically significant.

In contrast to lymphocytes in peripheral blood, CD4⁺ and CD8⁺ lymphocytes from lymph nodes did not display increases in activation markers, with the exception of those of macaque 275, which appeared to have a higher level of expression of CD25 on CD4⁺ cells (Fig. 4). Surprisingly, the only statistically significant change was a decrease in expression of HLA-DR on CD4⁺ lymphocytes. These results show that systemic measurements of various parameters may not reflect conditions in other tissues at the same time. In animals from which tissues were available prior to infection, there was a consistent decrease of approximately 50% in the CD4/CD8 ratios in lymph nodes, indicative of either a loss of CD4⁺ lymphocytes or an increase of CD8⁺ lymphocytes or both (Fig. 1).

Cytokine production in SIV-PBj14-infected macaques. Pig-tailed macaques infected with SIV-PBj14-bcl3 did not develop profuse diarrhea as previously observed in macaques inocu-

FIG. 3. Expression of cell surface activation antigens on peripheral blood lymphocytes from SIV-PBj14-infected pig-tailed macaques at death (■; $n = 8$). The mean of values for infected animals was compared with that for a pool of 18 normal uninfected animals (▨) and is reported as percentages of cells expressing a particular antigen for each subset. Asterisks indicate statistical significance; P values ranged from <0.0000 to 0.0005 .

FIG. 4. Expression of cell surface activation antigens on lymph node lymphocytes from SIV-PBj14-infected pig-tailed macaques at death (■; $n = 8$). The mean of values for infected animals was compared with that for a pool of seven normal uninfected animals (▨) and is reported as percentages of cells expressing a particular antigen for each subset. The asterisk indicates statistical significance ($P = 0.0104$).

lated with uncloned SIV-PBj14 (19). The animals did, however, consistently develop a shock-like syndrome. Plasma samples from these animals were assayed to explore the possibility that acute disease and death might be related to elaboration of cytokines by activated lymphocytes and other cells of the immune system. Not only are TNF- α and IL-6 products of immune activation (8, 25, 27, 56), but also human recombinant TNF- α can induce shock and acute death when administered in large doses (49, 57, 58). At death, the concentration of TNF- α in plasma samples was elevated in all animals compared with concentrations in plasma obtained before infection or at intermediate times (Table 2). The IL-6 concentration was elevated in most animals at the time of death, but the concentration appeared to be more variable than that of TNF- α . IL-6 was not detected in macaques with the lowest concentrations of SIV p27 antigen, which included the macaques that were euthanatized before the onset of acute disease. The amounts of both TNF- α and IL-6 in the peripheral circulation correlated well with SIV p27 antigen concentration, especially if the one IL-6 outlier value for macaque 87-011 was omitted (Fig. 5). That the cytokines were produced in detectable amounts only at death, that is, concomitantly with rapid increases in virus load, indicates that cytokine production is linked directly to virus replication. Macaques 541 and 542 were euthanatized on day 6, before acute disease was evident; however, the presence of small amounts of virus and TNF- α in peripheral blood on day 6 suggests that they also would have developed signs of acute disease in 2 to 4 days.

TABLE 2. Levels of TNF- α and IL-6 in plasma of SIV-PBj14-infected pig-tailed macaques at death

Macaque	Level (pg/ml) of ^a :		
	SIV p27	TNF- α	IL-6
543	8,168	700	420
544	5,394	90	<35
276	5,015	350	200
147F	4,902	700	300
82-64	4,868	160	70
87-011	3,466	700	1,600
82-67	2,059	180	70
275	1,576	300	<35
542 ^b	598	55	<35
541 ^b	108	<25	<35

^a Minimum levels of detection were 25 pg/ml for TNF- α and 35 pg/ml for IL-6. Values were obtained with commercial ELISA kits.

^b Animals were euthanatized on day 6, before signs of acute disease developed.

Expression of activation markers following SIV-PBj14 infection of resting macaque lymphocytes in vitro. Although it was clear that macaque lymphocytes proliferated in association with SIV-PBj14 replication in vitro, the extent of activation and proliferation of CD4⁺ and CD8⁺ populations was not known (17). Therefore, at various times following in vitro infection, PBMC were analyzed by dual-label immunofluorescence and FACS analysis to identify lymphocyte subsets expressing specific cell surface activation markers. Expression of CD25 and CD71 on both CD4⁺ and CD8⁺ cells in SIV-PBj14-infected cultures increased (Fig. 6), but expression of HLA-DR did not change significantly (data not shown). Although the percentages of CD4⁺ cells displaying CD25 increased with time in the uninfected control and SIVsmm9-infected cultures, the percentages of CD25⁺ cells were always substantially higher in the SIV-PBj14-infected cultures. RT activity in SIV-PBj14-infected cultures peaked approximately on day 13, after which time the percentages of cells expressing CD25 and, subsequently, CD71 increased. The apparently lower proportion of activated CD4⁺ cells in the SIV-PBj14-infected cultures, compared with that of activated CD8⁺ cells, may be an artifact due to virus replication in and subsequent lysis of CD4⁺ cells. The number of B cells decreased with time in all cultures, with a more rapid decline in those infected with SIV-PBj14; B cells did not appear to be activated during SIV-PBj14 infection.

Proliferation of lymphocytes in vitro in response to SIV-

FIG. 5. Correlations between SIV p27 antigenemia and TNF- α (▲) and IL-6 (●) in plasma samples from SIV-PBj14-infected pig-tailed macaques at death.

FIG. 6. Expression of cell surface activation antigens on quiescent pig-tailed macaque lymphocytes on days 0 and 16 following in vitro infection with SIV-PBj14. Numbers in quadrants 1, 2, and 4 represent the percentages of total cells in the lymphocyte gate that expressed the indicated antigens. Values for the uninfected control culture were essentially the same as those for a culture infected with SIVsmm9. The results are representative of those from multiple experiments.

FIG. 7. Proliferation of quiescent pig-tailed macaque PBMC (▨) and purified CD4⁺ (■) and CD8⁺ (▤) lymphocytes induced by SIV-PBj14 in vitro. Stimulation indices were obtained by dividing counts per minute incorporated by cells incubated with either PHA or 10 ng of virus by counts per minute of control lymphocytes in medium alone. Stimulation index values greater than 2 are considered positive. HI, heat-inactivated virus.

PBj14 infection. We previously showed that in vitro infection of macaque lymphocytes with SIV-PBj14 results in cell proliferation and increased numbers of viable cells compared with those of uninfected or SIVsmm9-infected PBMC (17). To confirm and extend these results, two types of experiments were performed. First, resting macaque PBMC were incubated with 10 ng of infectious SIV-PBj14 or SIVsmm9 for 7 days (41). Proliferation was demonstrated by incorporation of [³H]thymidine in PBMC populations incubated with SIV-PBj14 but not with SIVsmm9 (Fig. 7). In addition, purified CD4⁺ lymphocytes were stimulated to proliferate by infectious SIV-PBj14, whereas CD8⁺ lymphocytes proliferated minimally. The finding that heat-inactivated virus also induced limited proliferation of either PBMC or purified CD4⁺ cells, but not CD8⁺ cells, suggests that replication of SIV-PBj14 is not required for but may enhance the process.

Second, cultures of quiescent PBMC were infected with SIV-PBj14 or SIVsmm9. At various times, aliquots of cells were removed and immediately tested for incorporation of [³H]thymidine. As the RT activity in the SIV-PBj14-infected cultures increased, larger amounts of [³H]thymidine were incorporated into cellular DNA, while uninfected control or SIVsmm9-infected cells, regardless of RT activity, showed little or no incorporation. At the time of peak RT activity, stimulation indices ranged from 0.7 to 2.0 (SIVsmm9-infected cultures) or 18 to 74 (SIV-PBj14-infected cultures).

Cytokine production in SIV-PBj14-infected cultures. Since in this study and others (2, 16) cytokines associated with acute inflammation were detected in plasma of infected animals, supernatants from cultures of infected macaque PBMC were assayed for the presence of TNF- α and IL-6 to determine if these cytokines were also produced in vitro in response to SIV-PBj14 infection. There appeared to be a trend toward larger amounts of TNF- α in SIV-PBj14-infected cultures (range, 85 to 160 pg/ml) than in SIVsmm9-infected or uninfected controls (range, <35 to 65 pg/ml); however, the differences were not consistent or significant. Concentrations of IL-6 in both SIVsmm9- and SIV-PBj14-infected cultures were variable, with the SIVsmm9-infected cultures producing a lesser or equivalent amount of IL-6 (range, 100 to 210 pg/ml) compared with that in the SIV-PBj14-infected cultures. Small amounts of IL-2 (0.04 to 0.1 U/ml) were detected in SIV-PBj14-infected

cultures on days 10 to 12, near the time of peak RT activity, but not in SIVsmm9-infected or uninfected control cultures. These data suggested a direct correlation between SIV infection and cytokine production, but this correlation did not appear to be specific for SIV-PBj14. It is possible that differences in cytokine production elicited by the two viruses were obscured by cellular reutilization as infection progressed.

DISCUSSION

SIV-PBj14 is unique among lentiviruses because of its ability to induce acute disease and death in macaques in 7 to 10 days (19, 35, 50, 51), in contrast to other SIV which induce an AIDS-like illness in months to years (10, 34, 36, 47). Equally remarkable is the ability of SIV-PBj14 to replicate in and induce activation and proliferation of resting macaque lymphocyte populations (17). The in vitro biologic properties of SIV-PBj14 appear to be related, at least in part, to its acute pathogenicity in pig-tailed macaques (16, 41). Identification of specific interactions between SIV-PBj14 and macaque lymphocytes in vitro, including pathways of activation, may provide insight into the pathogenic mechanisms of SIV-PBj14 in vivo. Because in vivo and in vitro findings in biologic systems do not always correlate, it was important to demonstrate that similar phenomena occurred in vivo and in vitro in specific populations of pig-tailed macaque lymphocytes. Our results indicate that in vitro infection of macaque PBMC by SIV-PBj14 accurately reflects in vivo activation of both CD4⁺ and CD8⁺ lymphocytes. Furthermore, not only is cellular activation in vitro temporally correlated with maximum virus production, but also there are temporal correlations between virus load and both cellular activation and production of TNF- α and IL-6 in infected macaques.

One possible explanation for the extensive activation of lymphocytes and production of cytokines is that SIV-PBj14 has acquired properties similar to those of a superantigen. Superantigens induce their effect by binding simultaneously to a major histocompatibility complex class II molecule and all T-cell receptors encoded by one or more V β gene segments (63). The T cells responding to superantigens are generally CD4⁺ cells, and in vivo administration of superantigens, such as bacterial toxins, leads first to expansion and then to depletion of CD4⁺ lymphocytes expressing particular V β genes (29). Recent in vivo and in vitro studies have demonstrated that SIV-PBj14 infection is followed by a preferential expansion of pig-tailed macaque CD4⁺ and CD8⁺ lymphocytes expressing V β 7 and V β 14 genes, which appears to be polyclonal relative to the T-cell receptor complementarity-determining region 3 (5). CD4⁺ cells might be directly activated by the virus in this manner, with activation of CD8⁺ cells resulting either from interactions with cytokines or from direct interaction with the virus. It is possible that differential V β usage among macaque species (rhesus, cynomolgus, and pig-tailed macaques) might account for some of the observed species variation in acute pathogenicity induced by SIV-PBj14 (19, 35, 50).

The lack of concordance between lymphocytes in lymph nodes and the peripheral blood, relative to expression of activation antigens, is puzzling. A priori one might expect cells in lymph nodes to express higher levels of activation markers given the relatively closed environment and the large numbers of infected cells supporting replication of virus. However, activated CD4⁺ cells could be eliminated fairly rapidly by virus replication and subsequent cell lysis. Furthermore, it seems reasonable to assume that the concentration of cytokines is higher within the microenvironment of the lymph node than in the periphery, which might result in direct cellular toxicity. In

fact, using electron microscopy, we observed large numbers of apoptotic cells in lymph nodes of macaques that died from SIV-PBj14 infection (data not shown). Since apoptosis can be induced by TNF- α in cells chronically infected with feline immunodeficiency virus (42), it is likely that TNF- α is the mediator of similar cytopathic effects in SIV-PBj14-infected tissues.

Cytokines and other mediators released during immune activation, such as acute inflammatory responses, appear to be major factors in induction of acute disease and death by SIV-PBj14. Development of shock, followed by death with no evidence of body fluid loss, is indicative of endotoxemia or production of a chemical mediator of shock (23). Injection of recombinant human TNF- α into mice elicits a clinical syndrome that includes diarrhea and shock, resulting in histopathologic changes that include necrosis and blunting of small intestinal villi with endothelial cell destruction (49), which is also characteristic of SIV-PBj14 histopathology (20). TNF- α in the sera of human patients with sepsis is indicative of a higher rate of mortality than that of patients with no detectable TNF- α (11); one study showed 100% mortality for patients in septic shock with greater than 100 pg of TNF- α per ml in their sera (60). Since all macaques that died acutely, except 544, had TNF- α levels exceeding 100 pg/ml, it is highly probable that overproduction of this cytokine was a primary factor in the acute death of these animals. That most of the animals in the present study failed to develop profuse diarrhea supports this idea.

Consistent with our results, Birx et al. (2) also concluded that SIV-PBj14-induced acute disease and death were due to acute-phase reactant products and shock. Although they detected high levels of IL-6 and IL-6 mRNA in sera and tissues in association with high viral loads, those investigators were unable to detect TNF- α . This discrepancy with our results might be explained by the different assay systems used. IL-6 alone, however, is unlikely to account for the severity of clinical signs in infected macaques, although it probably acts synergistically with TNF- α . A recent study showed that rhesus macaques injected daily with high doses of recombinant human IL-6 developed only mild acute changes, such as dehydration and decreased food consumption (52). In addition, because both TNF- α and IL-6 are paracrine and autocrine growth factors for T cells and macrophages, it is unlikely that one and not the other would be produced in large amounts. Since high levels of TNF- α were also detected in sera from mangabeys that died acutely from SIV-PBj14 infection (16), we conclude that both TNF- α and IL-6 are important mediators of SIV-PBj14 acute disease.

Changes in the SIVsmm9 genome responsible for the unique biologic properties of SIV-PBj14 and its derivatives have not been identified precisely. The *env* gene of SIV-PBj14 appears to be necessary for induction of lymphocyte proliferation (and by inference, production of acute disease), but other regions of the genome are also required (41). Among the SIVsmm/SIVmac/HIV-2 group of viruses, only the genome of SIV-PBj14 encodes two nuclear factor κ B (NF- κ B) binding sites in the enhancer region (11). Relative to this point, it is of interest that TNF- α mediates its effects by specifically inducing NF- κ B (43). Furthermore, TNF- α has been shown to induce replication of HIV-1 in chronically infected cell lines (15) and human PBMC (59) and replication of HIV and SIV in primary macrophages (38, 62). Despite indications that the two NF- κ B sites may confer a replicative advantage to SIV-PBj14 (12a) this duplication does not appear to be necessary for induction of acute disease (16, 41).

The acute disease syndrome induced by SIV-PBj14 in ma-

caques has many features in common with the syndrome seen in some people during primary HIV infection; these include, but are not limited to, fever, lethargy, anorexia, diarrhea, generalized lymphadenopathy, a macular erythematous truncal rash (7, 24), and high plasma virus titers (6, 9). In addition, acute HIV infection has been associated with an increase in IL-1 β and gamma interferon (40, 53), cytokines also important in inflammation and which can induce synthesis of TNF- α . The clinical, physiologic, virologic, and immunologic similarities between infection of humans with HIV-1 and macaques with SIV-PBj14 (and its cloned derivatives) validate the use of this simian model as a surrogate for HIV infection and disease. Furthermore, the demonstrated correlations between in vivo and in vitro events following SIV-PBj14 infection support the continued in vitro analysis of SIV-PBj14-lymphocyte interactions to provide insight into lentivirus pathogenesis, in general, and the extreme virulence of SIV-PBj14, in particular. Future studies should not only provide relevant information on the interrelationships between lentiviruses and cytokines that impact on pathogenesis but also suggest potential therapeutic approaches to prevent disease progression.

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