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Inoculation of Baboons and Macaques with Simian Immunodeficiency Virus/Mne, a Primate Lentivirus Closely Related to Human Immunodeficiency Virus Type 2

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A primate lymphotropic lentivirus was isolated on the human T-cell line HuT 78 after cocultivation of a lymph node from a pig-tailed macaque (*Macaca nemestrina*) that had died with malignant lymphoma. This isolate, originally designated *M. nemestrina* immunodeficiency virus (MnIV) and now classified as simian immunodeficiency virus (SIV/Mne), was inoculated intravenously into three juvenile rhesus monkeys (*Macaca mulatta*), three juvenile pig-tailed macaques (*M. nemestrina*), and two juvenile baboons (*Papio cynocephalus*). All six macaques became viremic by 3 weeks after inoculation, whereas neither of the baboons developed viremia. One pig-tailed macaque died at 15 weeks with suppurative peritonitis secondary to ulcerative, necrotizing colitis. Immunologic abnormalities included a marked decrease in $CD4^+$ peripheral blood lymphocytes. Although five macaques mounted an antibody response to SIV/Mne, the animal that died at 15 weeks remained antibody negative. Three other macaques (two rhesus and one pig-tailed) died 66 to 87 weeks after inoculation after exhibiting progressive weight loss, anemia, and diarrhea. Histopathologic findings at necropsy included various manifestations of immune deficiency, nephropathy, subacute encephalitis, pancreatitis, adenocarcinoma, and lymphoid atrophy. SIV/Mne could be readily isolated from the spleens and lymph nodes of all necropsied macaques, and from the cerebrospinal fluid, brains, bone marrow, livers, and pancreas of some of the animals. SIV antigens were localized by avidin-biotin immunohistochemistry to pancreatic islet cells and to bone marrow endothelial cells. The data suggest that African baboons may be resistant to infection by SIV/Mne, whereas Asian macaques are susceptible to infection with this pathogenic primate lentivirus.

Two distinct molecular classes of retroviruses have been isolated from patients with acquired immune deficiency syndrome (AIDS). The viruses isolated in the United States, Europe, and central Africa have been designated human immunodeficiency virus type 1 (HIV-1) (1, 34), and those from West Africa have been designated HIV-2 (7, 8). Retroviruses partially related to these human AIDS viruses have also been obtained from various nonhuman primate species. The original isolates were from rhesus monkeys with immunodeficiency or lymphoma and were named STLV-III (SIV) mac (9). Other isolates were subsequently obtained from asymptomatic African green monkeys (SIV/agm) (18), mangabey monkeys (SIV/SMM, SIV/Delta, SMLV) (12, 30, 29) as well as from a pig-tailed macaque with lymphoma (MnIV, SIV/Mne) (3). These SIV strains are partially related to HIV-1, sharing morphologic and cytopathic features, some immunological relatedness (3, 9, 12, 18, 30, 38; L. E. Henderson, R. E. Benveniste, R. Sowder, T. D. Copeland, A. M. Schultz, and S. Oroszlan, J. Virol., in press), and limited nucleic acid homology (16, 17, 22).

The SIV isolates are even more closely related to the West African AIDS isolate, HIV-2. SIV/Mne, for example, shares a 90% amino acid sequence identity with HIV-2 in the N-terminal portion of the major *gag* protein and only a 58% identity over this same region with HIV-1 (3, 14; Henderson et al., in press). The recent nucleic acid sequencing of SIV/mac (5) and SIV/agm (11) confirms this level of identity for the major *gag* proteins of these viruses. The two SIV isolates and HIV-2 share a 75% overall nucleotide sequence homology, but HIV-2 and SIV have only a 40% identity to HIV-1.

The homology among different SIV isolates is beginning to be determined; although they appear to be closely related to each other, they can be distinguished by their restriction enzyme patterns (22). An exception is the large homology observed between SIV/mac and SIV/agm. These two isolates are also nearly identical (22) to an isolate obtained from healthy West Africans (HTLV-IV) (19).

Macaques inoculated with SIV/mac develop an immune deficiency syndrome characterized by weight loss, opportunistic infections, and a decrease in $CD4^+$ blood lymphocytes (6, 27). Since SIV viruses are closely related to HIV-2, which has been associated with AIDS in West African patients (8), they may provide a valuable model for HIV infections. However, African primate species naturally infected with SIV (African green and mangabey monkeys) are apparently asymptomatic, whereas Asian macaques are often symptomatic when infected. This observation prompted us to further examine the effect of the host species on disease by inoculating SIV/Mne into two macaque species and into baboons, another African species.

MATERIALS AND METHODS

Animals. The primates inoculated with SIV/Mne included three juvenile (aged 6 to 10 months) colony-born rhesus macaques (*M. mulatta* A85033, A85034, and A85037), three

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juvenile (7 to 10 months) pig-tailed macaques (*M. nemestrina* T85056, M85026, and F85062), and two baboons (12 months old) (*P. cynocephalus* F84351 and T84335). Before inoculation, each animal was determined to be clinically healthy by physical examination and complete blood cell count. They were also tested for preexisting SIV/Mne and type D (40)-related retroviruses by cocultivating peripheral blood lymphocytes (PBL) with appropriate cell lines and assaying for reverse transcriptase activity. No viruses were detected, and none of the animals had circulating antibodies to the two classes of viruses before inoculation. Six age- and sex-matched animals (two from each species) were used as controls.

Throughout the study, the inoculated animals were housed in group cages by species in a negative-pressure, high-efficiency particulate air-filtered room (P-3 facility) at the University of Washington Regional Primate Research Center and fed monkey chow (Ralston Purina Co., St. Louis, Mo.) ad libitum. The eight inoculated animals and six controls were examined at regular intervals for changes in their clinical and immunological status and for the presence of SIV/Mne and type D retroviruses. When the animals became ill, they were given supportive treatment by the veterinary staff. Animals that were nonresponsive to treatment and terminally ill were euthanized.

Virus inoculation. The eight animals were inoculated with SIV/Mne isolated on HuT 78 cells from a pig-tailed macaque that had died with lymphoma in 1982. SIV/Mne was endpoint diluted on HuT 78 cells, subcultured for five additional passages, and used as the source of virus to inoculate into animals. Each animal was inoculated intravenously with 2.0 ml of filtered (pore size, 0.45 μm) cell culture fluid from the SIV/Mne-infected HuT 78 cells. This inoculum contained 10^3 tissue culture infectious doses (TCID) of virus as measured by reinfecting HuT 78 cells.

Virus isolation. Indicator cell lines used to test for the replication of SIV/Mne included the human T-cell line HuT 78 (American Type Culture Collection, Rockville, Md.) and normal human PBL (Program Resources, Inc., Frederick, Md.). Primate PBL were isolated from heparinized whole blood by centrifugation in Leucoprep tubes (Becton Dickinson, Lincoln Park, N.J.) and were stimulated with 1 μg of phytohemagglutinin (PHA) (Burroughs Wellcome, Dartford, England) per ml for 2 to 4 days at a concentration of 5×10^5 cells/ml of RPMI 1640 medium containing 10% fetal calf serum (FCS; Advanced Biotechnologies, Inc., Silver Spring, Md.) and 2 μg of Polybrene per ml. After centrifugation to remove the PHA, lymphocytes were added to 10^7 HuT 78 cells and cultured in the presence of 64 half-maximal units per ml of human interleukin-2 (IL-2) (Advanced Biotechnologies) for 1 week and then subcultured weekly without IL-2 for up to 12 weeks. For virus isolation from tissue samples, small portions of fresh or frozen tissue were minced, added to HuT 78 cells, and subcultured weekly. The presence of virus in plasma samples was detected by cocultivating 0.5 ml of plasma with HuT 78 cells in the presence of 25 U of preservative-free heparin (Eli Lilly & Co., Indianapolis, Ind.) per ml. Cell culture supernatant fluids were assayed every 2 weeks for reverse transcriptase activity. Then, 7 ml of culture fluid was clarified of cells, the virus was pelleted through a glycerol cushion, and the presence of reverse transcriptase was assayed with synthetic templates as previously described (3).

Other cells used to detect virus replication included normal human PBL which were grown in the continuous presence of IL-2 after being stimulated for 3 days with PHA (1 μg/ml); fresh human PBL (10×10^6 to 20×10^6 cells) were added to the cultures every 2 weeks. Type D primate retroviruses (40) were detected by cocultivating tissues, plasma, or lymphocytes with a human lung carcinoma cell line, A549 (28), and by assaying for reverse transcriptase activity. Virus isolation was performed approximately monthly from inoculation until death or euthanasia of the animals.

Virus neutralization assay. Filtered (pore size, 0.45 μm) culture fluids from SIV/Mne-infected HuT 78 cells were stored at −70°C and thawed once before use. The viral titer was 10^3 TCID/ml on HuT 78 cells (measured by reverse transcriptase assay), but 10^6 total viral particles per ml were present (measured by determining the protein concentration and the total amount of p28 antigen by radioimmunoassay). Plasma was heat inactivated (56°C, 30 min) and diluted in RPMI 1640 medium containing 10% FCS and IL-2. SIV/Mne stocks were diluted 1:10, mixed with equal volumes of plasma, and incubated at 37°C for 1 h. Virus-plasma mixtures (2.0 ml) were added to 20×10^6 human PBL (PHA stimulated) in a total volume of 4.0 ml of RPMI 1640–10% FCS–IL-2 for 4 h at 37°C. Cells were then washed three times with RPMI 1640 and suspended in 10 ml of RPMI 1640–10% FCS–IL-2. Reverse transcriptase activity was measured 2 and 3 weeks later.

Gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on 10 to 20% gradient gels by the method of Laemmli (24). Proteins were visualized by staining with Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Richmond, Calif.).

Immunoblotting. SIV/Mne was grown in HuT 78 cells by Advanced Biotechnologies, harvested, banded on 15 to 60% (wt/vol) sucrose gradients and concentrated 1,000-fold as previously described (3). After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, viral proteins were transferred to 0.45-μm nitrocellulose paper (Schleicher & Schuell, Inc., Keene, N.H.) by electrophoresis (30 V, 16 h) in a Trans-Blot cell (Bio-Rad) in the presence of 0.025 M Tris–0.192 M glycine–20% methanol (41). After transfer, the nitrocellulose paper was washed for 2 to 4 h in 0.01 M Tris (pH 7.3)–5×10^{-3} M EDTA–0.15 M NaCl–0.25% gelatin (solution A) containing 0.1% Triton X-100, cut into 4-mm strips, and then stored at –20°C until used. Each 4-mm strip represented the transfer of approximately 12 μg of viral proteins.

Serum samples were incubated for 1.5 h at 37°C in a tenfold excess of FCS to adsorb proteins that often yielded nonspecific antibody responses. They were then added at various dilutions to nitrocellulose strips in the presence of solution A containing 1% Triton X-100 and incubated for 1.5 h at 37°C. The nitrocellulose strips were washed six times at room temperature (10 min each) in 5×10^{-3} M Tris (pH 7.3)–0.15 M NaCl–0.1% Triton X-100. Secondary antibody (rabbit anti-human immunoglobulin G [IgG], peroxidase conjugated) (Sigma Chemical Co., St. Louis, Mo.) or rabbit anti-monkey IgG, peroxidase conjugated (U.S. Biochemical Corp., Cleveland, Ohio), was added to the strips in the presence of solution A containing 1% Triton X-100 and incubated for 1.5 h at 37°C. The strips were washed three times (10 min each) with 5×10^{-3} M Tris (pH 7.3)–0.15 M NaCl–0.1% Triton X-100, and three times with 5×10^{-3} M Tris (pH 7.3)–0.15 M NaCl. Color development was performed with 4-chloro-1-naphthol (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) activated by hydrogen peroxide (H_2O_2).

Enzyme-linked immunosorbent assay (ELISA). Sucrose-

banded and concentrated SIV/Mne virus was disrupted in 1% sodium dodecyl sulfate and diluted to 1 μg of viral protein per ml in carbonate-bicarbonate buffer, pH 9.6. Low-bind microtest polystyrene plates (Nunc, Roskilde, Denmark) were coated with this antigen for 16 h at 4°C and washed with phosphate-buffered saline (PBS) containing 0.1% Tween 20. Primate sera were diluted in 10 volumes of FCS for 16 h at 4°C, and further dilutions were made in PBS–0.1% Tween 20–1 mg of gelatin per ml across the antigen-coated plates. The plates were incubated overnight at 4°C, washed with PBS–0.1% Tween 20, and reincubated for 2 h at 37°C with horseradish peroxidase-conjugated anti-human IgG (Sigma) diluted 1:1,000 in PBS–0.1% Tween 20–1 mg of gelatin per ml. The plates were washed again and developed in the dark at room temperature with orthophenylenediamine in phosphate-acetate buffer, pH 5.0, activated with H_2O_2. The reaction was stopped after 30 min with 8 M H_2SO_4, and the optical density at 490 nm was read on a multiscan spectrophotometer (Flow Laboratories, Inc., McLean, Va.). Sera which yielded optical densities over 0.25 at a dilution of 1:32 were considered positive; this criterion was based on parallel immunoblot results.

In vitro proliferation of PBL. Peripheral blood mononuclear cells were cultured in a microsystem (10^5 cells per 0.2 ml) in the presence of optimal concentrations of PHA, concanavalin A, or pokeweed mitogen for 3 days in RPMI with 10% FCS and added streptomycin-penicillin. DNA synthesis was estimated by [^{3}H]thymidine incorporation (31).

Histopathology and immunocytochemistry. Tissue samples from all euthanized animals were fixed in 10% neutral buffered Formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histological examination. Immunocytochemical studies were performed primarily on lymph nodes, spleen, heart, liver, kidney, lung, brain, pancreas, and bone marrow tissues that had been fixed for 2 to 7 days. Sections of Formalin-fixed tissues were incubated with a rabbit polyclonal antibody to whole disrupted SIV/Mne (3) as previously described for localization of HIV and SIV antigens in fixed tissue sections (43) by using avidin-biotin complex (ABC) immunocytochemistry with Vectastain kits (Vector Laboratories, Inc., Burlingame, Calif.). Controls included the omission of the primary antibody, use of normal rabbit serum, and testing of similar tissues from noninfected animals.

Lymphocyte subset monitoring. A panel of mouse anti-human monoclonal antibodies previously shown to react with macaque and baboon lymphocytes (36, 37) was used in this study. The antibodies were conjugated with fluorescein-5-isothiocyanate (FITC) (13) or with the phycobiliprotein R-phycoerythrin (PE) as previously described (26). PBL from experimental and control animals were stained with the following combinations of antibodies for two-color analyses, which has the advantage over one-color analysis of increased sensitivity, resolution, and subset definition. The antibody combinations were: (i) PE-anti-CD4 (red) and FITC-anti-pan-leukocyte marker Lp220 (green) to monitor the two helper/inducer subsets $CD4^+$ $Lp220^+$ cells and $CD4^+$ $Lp220^-$ T cells; (ii) PE-anti-CD8 to monitor cytotoxic/suppressor $CD8^+$ T cells; (iii) PE-anti-CD20 (a pan B-cell marker) and FITC-anti-IgD to monitor resting $CD20^+$ IgD^+ and activated $CD20^+$ IgD^-, B cells; (iv) PE-anti-CD2 (E receptor) and FITC-anti-human lymphocyte antigen-DR to monitor resting (DR^-) and activated (DR^+) $CD2^+$ T cells; and (v) FITC-anti-CD16 (Fc IgG receptor) to monitor natural killer cells. After a 30-min incubation on ice with the various monoclonal antibody pairs, the cells were washed twice, and quantitative two-color analyses were performed using a FACStar cell sorter (Becton Dickinson) as previously described (26, 36). Forward- and right-angle scatter states were set on lymphocytes to exclude other leukocytes from the analyses. Nonspecific staining was assessed with control nonbinding isotype-matched antibodies. For each combination, 10^4 cells were examined. Results are presented as a percentage of a subset of the total population analyzed. In one experiment, T lymphocytes from an infected animal were stained with FITC-anti-CD4 and sorted into $CD4^+$ and $CD4^-$ (CD8-enriched) populations, using a FACStar cell sorter, as previously described (37).

RESULTS

Inoculation of macaques and baboons with SIV/Mne. SIV/Mne had previously been isolated from a pig-tailed macaque (*M. nemestrina* T76321) that had died with lymphoma in 1982 at the University of Washington Regional Primate Research Center by cocultivating frozen lymph node tissue with the human T-cell line HuT 78 (3). In order to examine its pathogenicity, SIV/Mne was endpoint diluted on HuT 78 cells and used as the source of virus to inoculate into colony-born primates. Three juvenile (aged 6 to 10 months) rhesus monkeys (*M. mulatta* A85033, A85034, and A85037), three juvenile (7 to 10 months) pig-tailed macaques (*M. nemestrina* T85056, M85026, and F85062), and two baboons (12 months) (*P. cynocephalus* F84351 and T84335) were inoculated intravenously with 2.0 ml of filtered (pore size, 0.45 μm) cell culture fluid from an SIV/Mne-infected HuT 78 cell line. This inoculum represented approximately 10^6 SIV/Mne particles, as indicated by reverse transcriptase activity and quantitation of p28 viral *gag* antigen by radioimmunoassay, but only 10^3 TCID of virus, as measured by reinfecting HuT 78 cells. This discrepancy between total particles and infectious particles has been noted previously and is substantiated by the lack of readily detectable high-molecular-weight 35S or 70S viral RNA in SIV/Mne virus preparations (3).

The eight primates were tested before inoculation for preexisting SIV/Mne and simian type D-related viruses in PBL by cocultivating with HuT 78 and A549 cells, respectively; the latter cell line replicates all known primate type C and type D retroviruses (40). No viruses were detected, and none of the animals had circulating antibodies to the two classes of viruses before virus inoculation as determined by immunoblotting.

Virus isolation in inoculated primates. The eight inoculated animals and six age- and sex-matched controls (two from each species) were examined at regular intervals for the presence of antibodies to type D retrovirus and SIV/Mne, for the presence of these viruses in plasma and PBL, and for changes in their clinical and immunologic status. The presence of SIV/Mne in PBL or plasma was detected by measuring for Mg^{2+}-dependent reverse transcriptase activity after cocultivating with HuT 78 cells or normal human PBL, as described in Materials and Methods. Although both baboons developed marked peripheral lymphadenopathy, no virus could be isolated from their PBL or from lymph node biopsy specimens obtained 19 weeks after inoculation. They also did not develop a detectable antibody response and were clinically normal when they were removed from the study 1 year post-inoculation.

In contrast, SIV/Mne could be readily isolated from the PBL of all six macaques, beginning approximately 3 weeks

TABLE 1. Isolation of SIV/Mne from PBL of inoculated primates

Animal	RT detected at indicated wk after inoculation[a]					Wk until death
	3	7	14	26	65	
M. mulatta						
A85033	455 (4)[b]	721 (7)	0	547 (10)	NT[c]	66
A85034	1,700 (9)	814 (10)	0	0	2,400 (5)	87
A85037	376 (9)	265 (10)	0	0	0	Alive
M. nemestrina						
T85056	781 (9)	1,500 (4)	274 (5)			15
M85026	453 (6)	764 (4)	459 (6)	843 (9)	1,300 (4)	Alive
F85062	327 (9)	NT	627 (8)	998 (7)	567 (5)	80
P. cynocephalus						
F84351	0	0	0	0	NT	Alive
T84335	0	0	0	0	NT	Alive

[a] PBL (3×10^6 cells) were isolated from heparinized whole blood and cocultivated with 10^7 HuT 78 cells as described in Materials and Methods. Reverse transcriptase (RT) activity in pelleted culture fluids was detected as previously described (3). Results are expressed as 10^3 counts per minute of [^{3}H]TMP incorporated into radioactive poly(dT) in a 60-min incubation experiment at 37°C; 10^5 cpm represent 1.2 pmol of TMP incorporated. Polymerase activity in uninfected HuT 78 cells varied from 2,700 to 9,000 cpm in these assays. Cocultivations were assayed every 2 weeks and were maintained 12 weeks before being discontinued.

[b] Numbers in parentheses represent the weeks of cocultivation required until reverse transcriptase activity was detected and are a crude estimate of viral titer in infected animals.

[c] NT, Not tested.

after inoculation and for most animals variably but continuously thereafter (Table 1). The exception was rhesus A85037, from which virus could no longer be isolated on HuT 78 cells after 7 weeks and which remained healthy 90 weeks after inoculation. After these cocultivations had been initiated, it was found that human PBL are a more sensitive indicator line for the growth of SIV/Mne than are HuT 78 cells. A retrospective cocultivation of frozen PBL (obtained at 65 weeks after inoculation) from rhesus A85037 revealed that this animal did possess low levels of virus in its PBL. The amount of virus present in PBL can be quantitated approximately by cocultivating a fixed number of primate PBL with a fixed number of actively growing human PBL or HuT 78 cells and measuring the length of time until reverse transcriptase activity is detectable in pelleted cell culture fluids. The number of weeks of cocultivation that elapsed until reverse transcriptase activity was detectable is also indicated in Table 1. Generally, virus titers tended to be highest a few weeks before the death of the animal.

Antibody response in inoculated primates. The development of antibodies to SIV/Mne was determined by Western immunoblotting and by ELISA. Figure 1 shows the immunoblot results with sera obtained at various times after inoculation. The two inoculated baboons from which virus was not isolated also never mounted a detectable antibody response. Five of the six macaques had developed antibod-

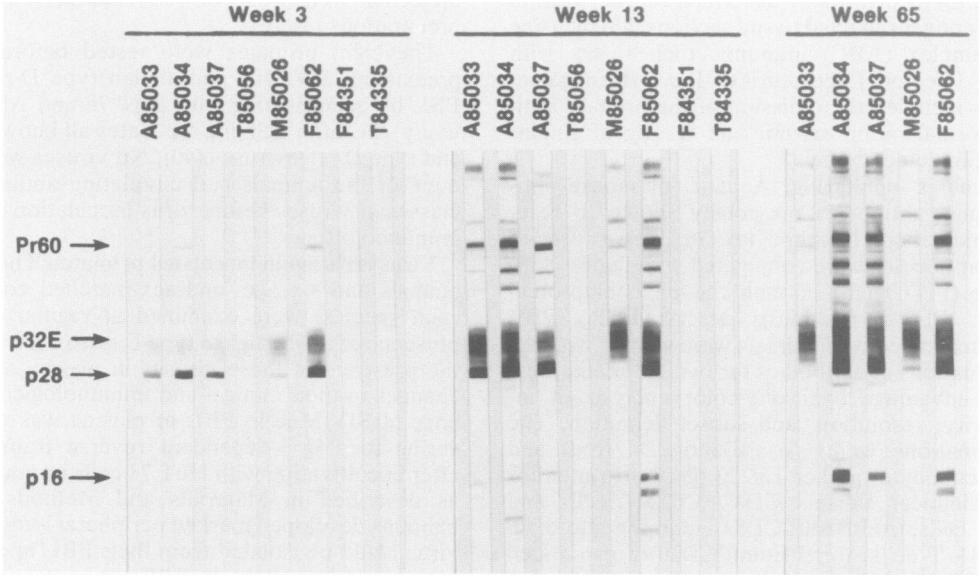

FIG. 1. Immunoblot analyses of antibodies to SIV/Mne in inoculated primates. Sucrose-banded SIV/Mne was disrupted, and the viral proteins were separated by electrophoresis through a 10 to 20% gel and transferred to nitrocellulose paper as described in Materials and Methods. Nitrocellulose strips were incubated with primate plasma (1:100 dilution), and the presence of antigen-antibody complexes was detected by incubation with peroxidase-conjugated anti-human IgG antiserum and development with chloronaphthol as described in Materials and Methods. Antibodies present in plasma samples obtained at 3, 13, and 65 weeks after inoculation are shown. Animal identifications are as in Table 1.

ies to the major internal *gag* protein p28 by 3 weeks and subsequently developed antibodies to other proteins. These included antibodies to the other *gag* proteins of SIV/Mne which include an N-terminal p16, the p28, and C-terminal peptides designated p2, p8 (the nucleic acid-binding protein), p1, and p6. In addition, macaques F85062 and A85034 developed antibodies to p14, which is a protein that has been purified from virus preparations and shown not to be a *gag*-encoded protein (L. Henderson, R. Sowder, T. Copeland, R. Benveniste, and S. Oroszlan, submitted for publication). Antibodies to the *gag* precursor, designated Pr60, were also detected. In addition, these five animals developed antibodies to high-molecular-weight proteins that by analogy to HIV-2 can be assumed to be envelope glycoproteins (Fig. 1). The wide band present above the p28 major *gag* protein is the transmembrane protein of SIV/Mne, designated p32E.

It is evident from the data in Fig. 1 that *M. nemestrina* T85056, although viremic (Table 1), never developed an antibody response. This animal was the first to exhibit clinical symptoms, characterized by weight loss, a cutaneous macular rash, anemia, and diarrhea, and was euthanized while moribund 15 weeks after inoculation. Subsequently, rhesus macaque A85033 died at 66 weeks, pig-tailed macaque F85062 died at 80 weeks, and rhesus A85034 died at 87 weeks postinoculation. As shown in Fig. 1, the serum examined 1 week before rhesus A85033 died (week 65) showed a loss of p28 and p16 *gag* antibodies, similar to the results seen in some AIDS patients during the terminal phase of their illness.

The antibody titers to SIV antigens (determined by ELISA) are shown in Fig. 2. The five macaques that developed an antibody response had titers that ranged from approximately 3 to 6 $\log_{10}$ dilutions. Maximal titers developed by 16 to 30 weeks and remained fairly constant. The time of death correlated approximately with antibody titers; the macaque with no antibodies died at 15 weeks, and animals with titers of 3.5, 4.8, and 5.5 $\log_{10}$ dilutions of sera died at 66, 80, and 87 weeks, respectively.

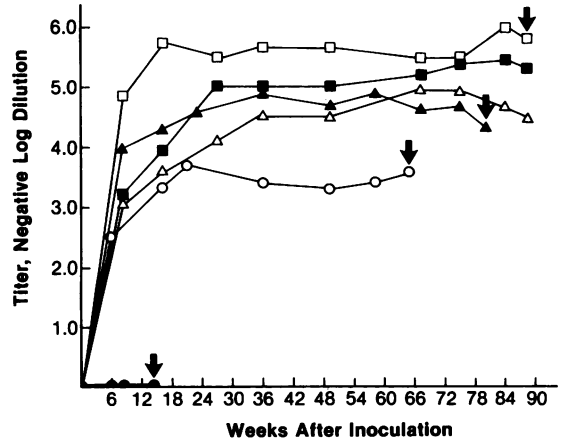

FIG. 2. Antibody titer to SIV/Mne in inoculated macaques. Sera from macaques inoculated with SIV/Mne were assayed for antibodies by ELISA as described in Materials and Methods. Titers are expressed as negative $\log_{10}$ dilutions, i.e., 4.0 equals 0.3 optical density at 490 nm at a 1 in 10,000 dilution. The arrows indicate the times at which moribund animals were necropsied. Inoculated primates included *M. mulatta* A85033 (○), A85034 (□), A85037 (△), and *M. nemestrina* T85056 (●), M85026 (■), and F85062 (▲). Not shown are data for the two baboons which did not become infected and remained seronegative.

In an attempt to correlate the presence of neutralizing antibodies with the clinical course of the animals, macaque sera were also tested for the ability to neutralize virus. Various dilutions of sera were incubated with 100 TCID (but 10^5 total particles) of SIV/Mne, and reverse transcriptase activity was measured 2 weeks after cocultivation with normal human PBL (data not shown). Most serum samples tested had low titers of neutralizing activity (45 to 90% inhibition of reverse transcriptase activity at a 1:100 dilution) that correlated approximately with the overall titers shown in Fig. 2. The sera from macaque T85056, as expected, had no detectable neutralizing activity. SIV/Mne could be readily isolated from the plasma of the antibody-negative macaque (T85056) but not from the plasma of the antibody-positive animals.

Immunologic status of infected animals. The responses of PBL to the mitogens concanavalin A, pokeweed mitogen, and PHA and the percentage of $CD4^+$, $CD8^+$, T-cell, and B-cell subsets were monitored in the inoculated animals and compared with base-line data obtained in age- and sex-matched control animals. The stimulation of PBL obtained from inoculated animals to all three mitogens was the same as that of PBL from control animals until just shortly (1 to 2 weeks) before death, when mitogen responses were markedly depressed to less than 10% of control levels (data not shown). These results might be explained, at least in part, by reduced levels of lymphocyte subsets in these animals (see below).

The percentages of total $CD4^+$, total $CD8^+$, and activated B cells ($CD20^+$ IgD^-) were monitored in inoculated and control macaques (Fig. 3). Control animals monitored longitudinally for 20 months did not deviate significantly from normal subset frequencies (data not shown). In contrast, all four macaques that died showed a marked depletion of $CD4^+$ cells. The antibody-negative macaque (T85056) showed a decline in $CD4^+$ cells (including $Lp220^+$ and $Lp220^-$ $CD4^+$ cells) from the time of inoculation until death at 15 weeks. *M. mulatta* A85033, which died at 15 months, had low but close to normal levels of $CD4^+$ cells, normal levels of $CD8^+$ cells, and occasionally increased levels of B cells during the first 12 months after inoculation with SIV/Mne (Fig. 3). Approximately 3 months before death and 2 weeks before overt clinical signs, the frequency of $CD4^+$ cells began to decrease and $CD8^+$ cells began to dramatically increase. Close to the time of death both $CD4^+$ and $CD8^+$ cells were depleted and $CD20^+$ cells were increased. The increase in $CD20^+$ cells was seen principally in the IgD^- subset, which are activated B cells (23), and not in IgD^+ $CD20^+$ B cells, suggesting that the high frequency of $CD20^+$ B cells near the time of death was not due solely to the absence of other subsets. Neither $CD16^+$ natural killer cells nor $CD2^+$ DR^+-activated T cells fluctuated significantly from normal limits in infected or control groups (data not shown). The macaques that died at 18 and 20 months also displayed decreases in $CD4^+$ and $CD8^+$ subsets and increases in IgD^- B cells near the time of death. The two infected macaques that remained clinically normal did not have significant fluctuations in lymphocyte subset levels (Fig. 3). The inoculated baboons did not differ from the baboon controls in any subset distribution.

$CD4^+$ lymphocytes and $CD8^+$ lymphocytes were isolated from *M. nemestrina* M85026 6 months after inoculation, using a cell sorter. SIV/Mne was readily isolated on HuT 78 cells from the $CD4^+$ subset but not from the $CD8^+$-rich population. These data suggest that SIV/Mne infects $CD4^+$ lymphocytes preferentially.

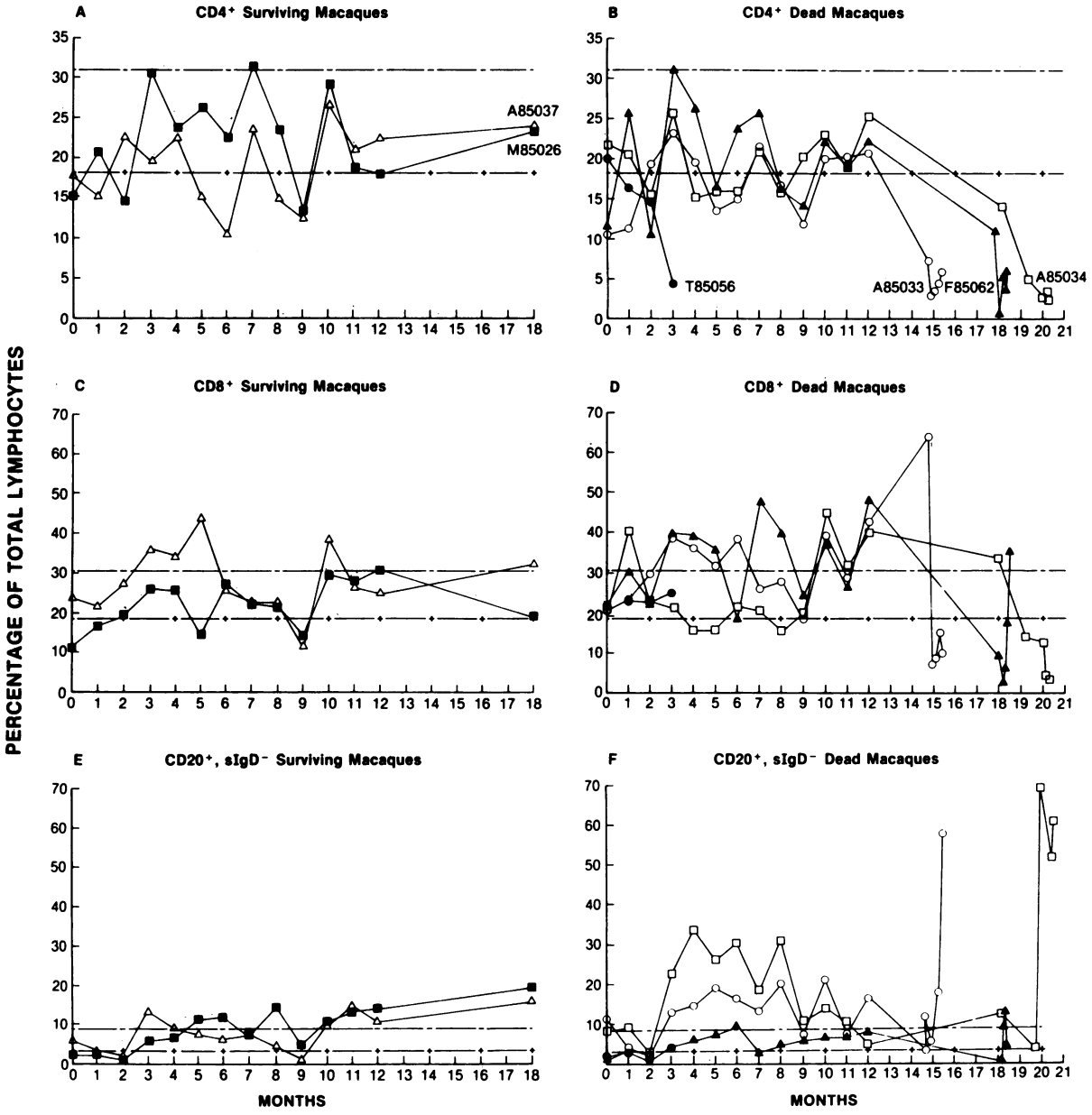

FIG. 3. Variations in blood levels of T helper/inducer cells ($CD4^+$), T suppressor/cytotoxic cells ($CD8^+$), and B cells among SIV/Mne-inoculated macaques. Normal limits of subset frequencies (differing by 1 standard deviation from the mean) in age- and sex-matched control macaques are shown (− and +); control animals did not deviate significantly from normal subset frequencies. The percentages of $CD4^+$ T helper/inducer cells, $CD8^+$ T suppressor/cytotoxic cells, and $CD20^+$, IgD^--activated B cells in the two macaques still alive 21 months after inoculation are shown in panels A, C, and E, respectively. The percentages of $CD4^+$, $CD8^+$, and $CD20^+$, IgD^- in the four macaques that died after inoculation are shown in panels B, D, and F, respectively.

Pathologic changes in SIV-inoculated macaques. The clinical and pathologic findings in the four macaques (two rhesus, two pigtailed) that died after inoculation with SIV/Mne are summarized in Table 2. All inoculated macaques developed peripheral lymphadenopathy starting at 3 weeks after inoculation that persisted for the remainder of the life of the animal. A macular erythematous skin rash, which was most pronounced in *M. nemestrina* T85056, started at 1 week after inoculation and lasted approximately 2 months. Rhesus A85037 1 year after inoculation also had a similar rash which lasted for 2 weeks. Although photographs of *M. nemestrina* and *M. mulatta* with this rash were not obtained, Fig. 4 shows a characteristic example of the rash from a *Macaca fascicularis* inoculated with SIV/Mne in a subsequent study. The erythematous areas were confined primarily to the medial aspects of the thigh, the inguinal and lower abdominal area, the medial aspect of the upper arm and the neck.

Shortly after inoculation, the two baboons developed marked peripheral lymphadenopathy which persisted for 8 months. In addition, baboon T84335 developed a macular erythematous rash at 2 weeks which persisted for an additional 5 weeks. Although similar in appearance to the rash seen in the macaques, it was less extensive, involving only the lower abdominal area. As stated previously, no virus was

TABLE 2. Clinicopathologic features in four macaques that died after inoculation with SIV/Mne[a]

Feature	*M. nemestrina*		*M. mulatta*	
	T85056	F85062	A85033	A85034
Death (wk)	15	80	66	87
Candidiasis	+++	−	−	−
Campylobacter spp.	−	+	+	+
Cryptosporidia spp.	−	−	−	+++
Syncytial giant cells in lymph nodes	+	−	−	−
Lymphoid atrophy	++	++	+	+
Bone marrow hyperplasia	NT	+	+++	+
Hemosiderosis	+++	++	+++	−
Subacute encephalitis	+	−	−	+
Enteritis	+++	+	+++	+++
Peritonitis	+++	−	−	−
Nephropathy	−	+++	−	+
Pancreatitis	−	−	++	+++
Neoplasia	−	−	−	+[b]
Anemia	++	+	+++	+

[a] The extent or severity of the abnormal findings is listed as follows: +++, severe; ++, moderate; +, mild; −, absent. NT, Not tested.
[b] Gastric adenocarcinoma.

isolated from plasma, PBL, or lymph node biopsies (by cocultivating with HuT 78 cells or human PBL) and no antibodies to SIV/Mne were detected. The baboons remained clinically and immunologically normal and were removed from the study 1 year after inoculation.

The first macaque to die, *M. nemestrina* T85056, had acute peritonitis secondary to severe ulcerative, necrotizing colitis. In addition, there was oral candidiasis and multinucleated giant cells in lymph nodes (Fig. 5). *M. mulatta* A85033 revealed marked lymphoid and bone marrow hyperplasia as well as pancreatitis at necropsy, whereas *M. nemestrina* F85062 had marked lymphoid atrophy and severe interstitial nephritis, tubular necrosis, and focal and segmental glomerulonephritis (Fig. 6). *M. mulatta* A85034 died with severe cryptosporidiosis, enteritis, pancreatitis, a gastric adenocarcinoma, and subacute encephalitis similar to that seen in animal T85056. All four macaques exhibited progressive weight loss and anemia (three animals also had accompanying diarrhea) 2 weeks before death.

Immunocytochemistry for viral antigens and virus isolation from tissues obtained at necropsy. Attempts were made to

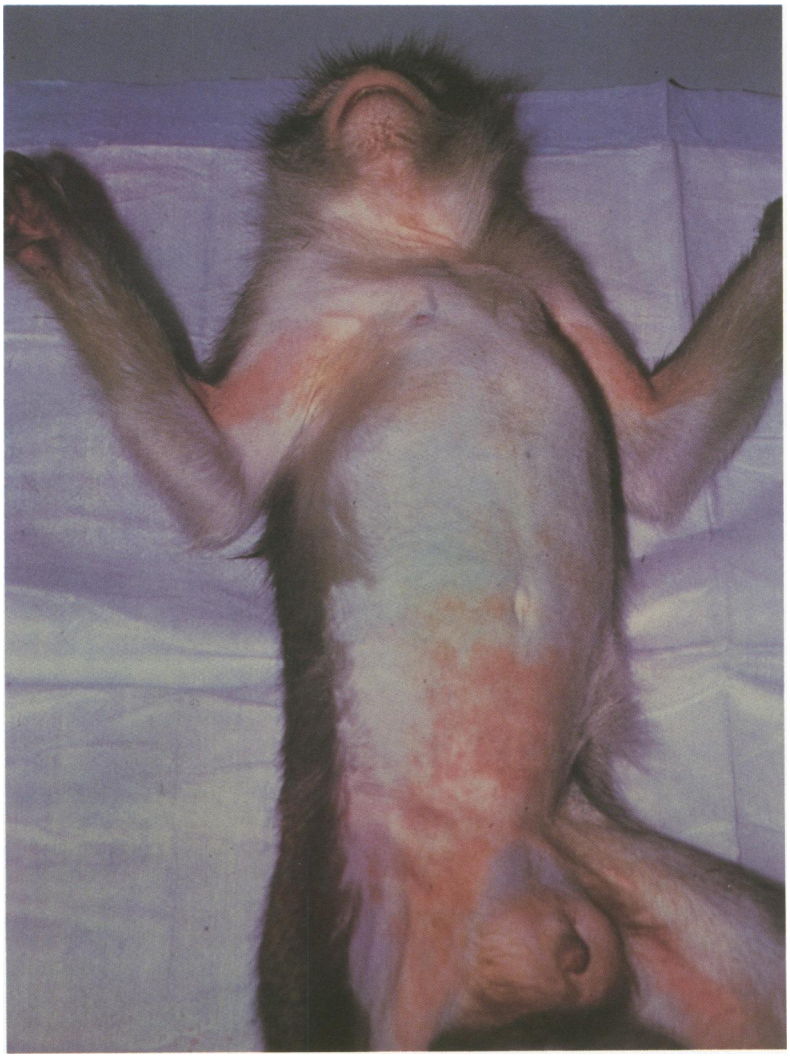

FIG. 4. Erythematous macular rash in *M. fascicularis* after inoculation with SIV/Mne. This rash is identical in appearance to that seen in *M. mulatta* and *M. nemestrina* in the present study.

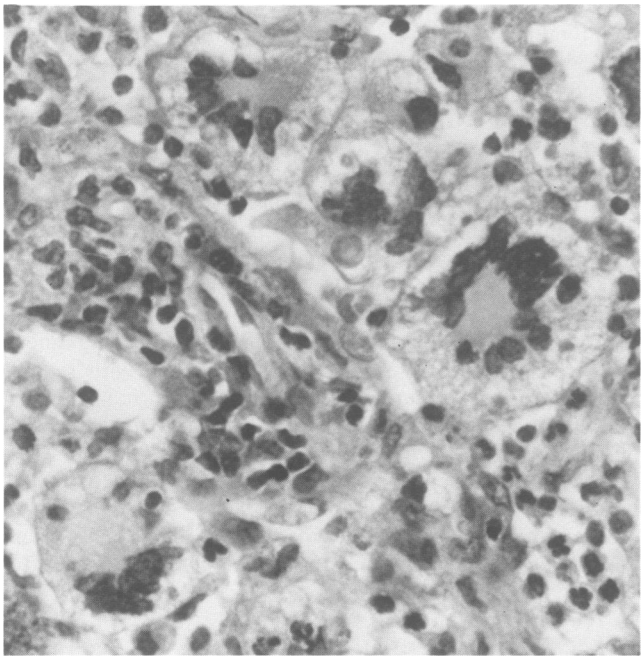

FIG. 5. Multinucleated giant cells in the lymph node of *M. nemestrina* T85056. Hematoxylin and eosin stain; magnification, ×300.

reisolate SIV/Mne from tissues obtained at necropsy from all four macaques by cocultivating with HuT 78 cells or normal human PBL and assaying for reverse transcriptase activity. Virus could be isolated on HuT 78 cells only from spleen and lymph node tissues obtained from all four macaques. By using human PBL as the indicator cell line, virus was also obtained from samples of bone marrow (F85062 and A85034), pancreas (F85062), liver (A85034), and cerebrospinal fluid (F85062). Virus was also isolated from the plasma of T85056 (the only antibody-negative macaque) and therefore not surprisingly from various tissues of that animal (brain, liver, kidney, heart, and lung) that may have had small amounts of plasma present at the time of cocultivation.

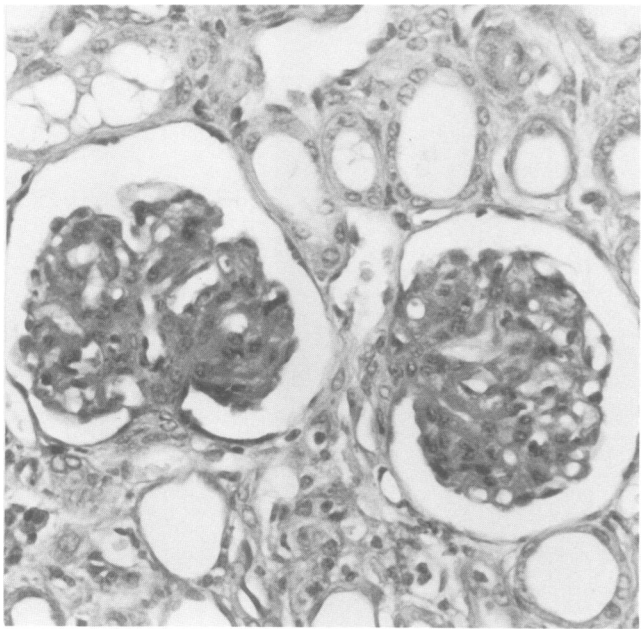

FIG. 6. Renal lesions in *M. nemestrina* F85062 showing focal and segmental glomerulonephritis. Periodic acid-Schiff stain; magnification, ×250.

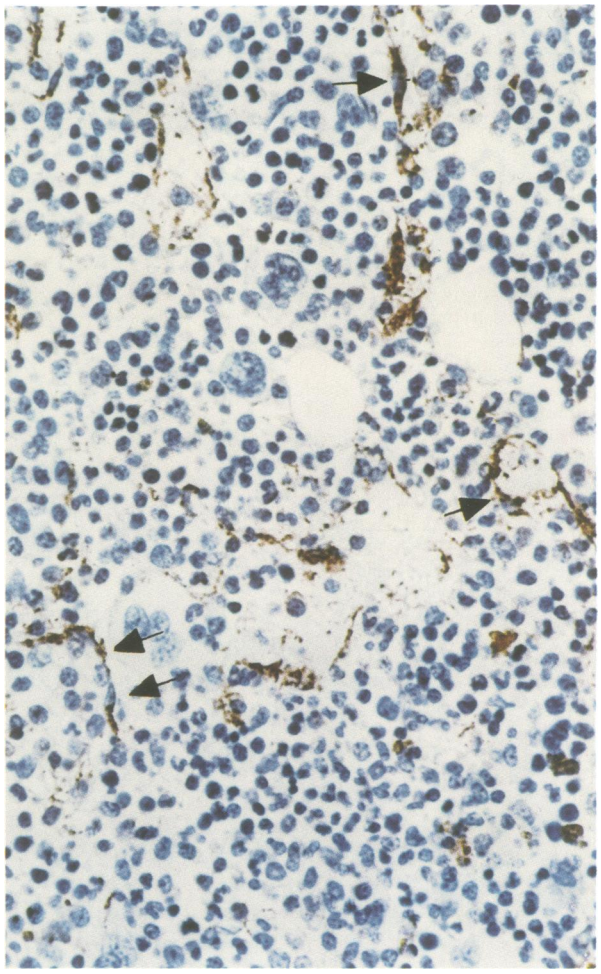

FIG. 7. Detection by immunocytochemistry of SIV/Mne antigens in bone marrow endothelial cells (arrows) of *M. nemestrina* F85062. The ABC technique is described in Materials and Methods. Hematoxylin stain; magnification, ×400.

Fixed autopsy specimens from these four macaques were screened for the presence of SIV/Mne antigens by ABC immunohistochemistry, using polyclonal antibodies to SIV/Mne as previously described (43). Viral antigens were detected in bone marrow endothelial cells (Fig. 7) of the macaque (F85062) from which virus was also isolated from bone marrow and in the pancreatic islet cells of another (Fig. 8), but antigen was not detected in various preparations of lymph node, spleen, or brain tissues.

DISCUSSION

The results presented here show that SIV/Mne, a lymphotropic lentivirus, is capable of inducing a fatal disease in macaques, but not in baboons. The disease in macaques was characterized by either a rapid clinical course (15 weeks) in the absence of viral antibodies or a more prolonged course (66 to >87 weeks) in animals with high titers of antibodies to

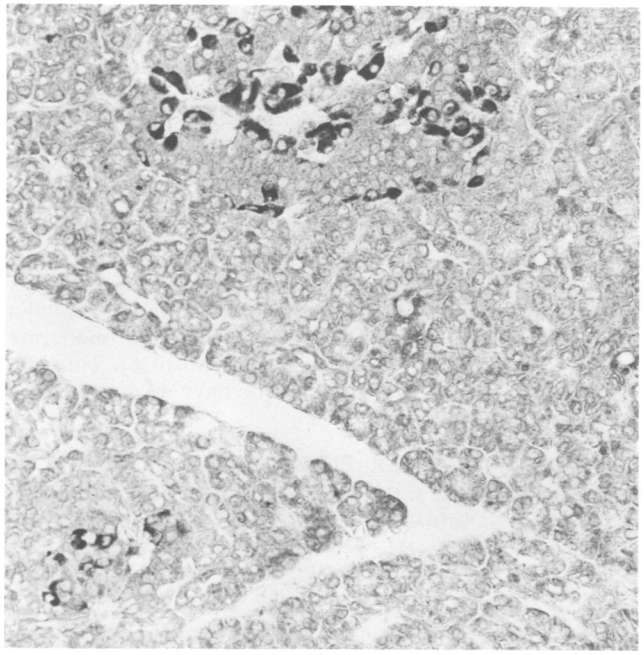

FIG. 8. Detection by immunocytochemistry of SIV/Mne antigens in pancreatic islet cells of *M. mulatta* A85033. Hematoxylin stain; magnification, ×250.

SIV antigens. Although this delayed onset of disease correlated with the presence of virus-neutralizing antibodies, there was only a weak neutralizing activity to SIV/Mne detected in primate plasma samples.

Macaques inoculated with the first SIV isolate (SIV/mac) at the New England Regional Primate Research Center develop a marked viral encephalitis characterized by perivascular infiltrates of macrophages with multinucleated giant cells present throughout the brain at necropsy (6, 27). Inoculations of macaques at other primate centers with tissue suspensions believed to contain SIV have also resulted in encephalitis (2, 4). Except for the mild subacute encephalitis seen in two of the six inoculated macaques in the present study, this was not a prominent feature of SIV/Mne inoculation.

All SIV strains, however, produce a wasting syndrome with secondary infections as contributing causes of death. These infections appear to differ in each primate colony. In the New England studies, an overwhelming adenovirus infection in multiple tissues, with inclusion bodies and necrosis, developed (27). In the Delta Primate Center studies, the inoculated macaques developed cytomegalovirus, *Candida* infections, or lymphoma. These latter animals were, however, inoculated with tissue homogenates and not with endpoint-diluted SIV (2). The macaques at the Washington Primate Center had several secondary lesions (caused by *Cryptosporidia*, *Candida*, *Campylobacter* infections) which varied for each animal except perhaps for a common enteritis of undetermined etiology. One of the macaques in the present study also developed a carcinoma of the stomach. Anal-rectal carcinomas and squamous cell carcinomas have been reported to occur at a higher frequency in AIDS patients (J. A. Levy, Proc. Am. Assoc. Cancer Res. **28**:474, 1987).

SIV/Mne was isolated from the $CD4^+$ but not the $CD8^+$ subset of lymphocytes. Previous studies have also shown that, like HIV-1, SIV/mac is tropic for $CD4^+$ lymphocytes (9, 21). These results are in contrast to the data obtained with SAIDS-D/Washington, the type D virus present at the Washington Primate Center which can be isolated equally from both $CD4^+$ and $CD8^+$ lymphocyte subsets (R. Benveniste and E. Clark, unpublished results). All four macaques that died after SIV/Mne inoculation had dramatically reduced $CD4^+$ cell levels as well as deficient responses to T-cell mitogens before death. Indeed, this was the principal immunologic abnormality seen in the animal that died 15 weeks after inoculation. These data agree with that previously reported for macaques dying shortly after SIV/mac infection (20, 27). The three animals that died after longer intervals (66 to 87 weeks) displayed increases in $CD8^+$ and $CD20^+$ IgD^- cells before their downward clinical course, followed by reduced levels of both $CD4^+$ and $CD8^+$ cells. These results suggest that during chronic SIV/Mne infection a complex set of interactions among cells of the immune system may be occurring. The increase in $CD8^+$ cells followed by a reduction in $CD8^+$ cell levels before death is noteworthy since $CD8^+$ cells have been reported to control HIV infection in vitro (42). Thus, a depletion in $CD8^+$ cells could contribute to the increased viral titers seen in dying animals. The effects of SIV/Mne on B-cell levels may be due to spontaneous B-cell activation because of loss or dysfunction of regulatory $CD4^+$ and/or $CD8^+$ cells that normally would control B-cell levels. Some patients with AIDS have hypergammaglobulinemia and B-cell hyperactivation (25, 45). The presence of activated IgD^- B cells in SIV/Mne-infected animals before death is consistent with B-cell activation. Several investigators have shown that HIV can stimulate normal human B cells (32, 39, 45) and infect Epstein-Barr virus-positive B-cell blasts, implying that lymphotropic lentiviruses can directly infect non-$CD4^+$ cells. Thus, another possibility is that SIV/Mne or one of its products acts directly on macaque B cells. Experiments are in progress to distinguish between these possibilities.

ABC immunohistochemical techniques have been previously employed with an SIV/Mne polyclonal rabbit antiserum to detect SIV/Delta antigens in sinus histiocytes and macrophages and multinucleated giant cells in lymph nodes and spleen from macaques inoculated with SIV/Delta (43). The results obtained with Formalin-fixed tissues from the SIV/Mne-infected macaques differ from those seen in these other SIV studies, since SIV antigens were not detected in those tissues (no giant cell lesions were evident). However, fusiform cells that appeared to be endothelial cells (erythrocytes were found within the capillary channels they formed) were shown to be highly immunoreactive to SIV antibodies in the bone marrow of one of the macaques. Viral infection of endothelial cells has been described in tissues from AIDS patients (15, 43, 44) and in endothelial cells infected with type C retrovirus (33) and may be important in the viremia and pathogenesis of the disease. We also found staining in the pancreatic islets of one macaque. Retroviruses have been previously found replicating within the pancreas of mice (10).

Normal human PBL seem to be a more sensitive indicator cell line than HuT 78 cells for the detection of SIV/Mne in PBL or tissues obtained from inoculated macaques. In fact, SIV/Mne replicates to higher titers in human PBL than it does in the same number of macaque PBL. These results may reflect a real in vitro virus preference for human lymphocytes or may simply reflect our inability to stimulate the differentiation of macaque lymphocytes to the same extent that human PBL can be stimulated.

The present studies show that although SIV/Mne is capable of causing a lethal disease in two species of macaques, some of the characteristics of this disease differ from those observed in other recent studies, particularly with respect to neurotropic lesions. These differences may reflect molecular heterogeneity among the various isolates. The SIV strains have recently been shown to be more closely related to HIV-2, the West African isolate, than to HIV-1. For example, the N-terminal portion of the major *gag* protein p28 of SIV/Mne was initially shown to be 90% identical to HIV-2 (3, 14) but only 58% identical to the same region of HIV-1 (3). A more complete comparison of 125 positively identified amino acids in the *gag* region of SIV/Mne reveals an 83% identity to the predicted amino acid sequence of HIV-2 *gag* proteins and 41% identity over this same region for HIV-1 (Henderson et al., in press). These similarities between HIV-2 and the SIV strains also extend to the remainder of the sequenced regions (5, 11).

The various SIV strains are beginning to be compared with each other. SIV/mac and SIV/agm have been molecularly cloned and sequenced (5, 11). These two isolates have a greater than 99% amino acid identity. We have used a 7-kilobase fragment of cloned SIV/agm, generously provided by James Mullins (Harvard University), to clone SIV/Mne. A comparison of a 1.5-kilobase sequenced 3′ region of SIV/Mne with SIV/mac reveals an approximately 9% difference between these isolates (R. Benveniste and G. Heidecker, submitted for publication). In the sequenced region of the *gag* genes (125 amino acids), 90% of the SIV/Mne amino acids were identical to predicted residues of SIV mac and SIV/agm (Henderson et al., in press).

SIV strains have been isolated from two African primate species, the African green monkey (*Cercopithecus* spp.) and the sooty mangabey (*Cercocebus atys*). In both these African genera, the virus, although present and eliciting an antibody response, causes no apparent disease. The present study shows that baboons, another primate whose habitat is Africa, is resistant to infection by SIV. Although one baboon in the present study developed a transient macular rash after inoculation similar to that reported in macaques infected with SIV/mac (35), no virus could be isolated. Baboon lymphocytes express CD4 receptors and can be infected in vitro by SIV/mac (21) and SIV/Mne (3) although they produce only low levels of virus. Thus, resistance in vivo to SIV/Mne infection cannot be attributed simply to the inability of baboon lymphocytes to bind and be infected by SIV/Mne. Other factors (genes?) influencing viral replication or host resistance may be present in baboons. Macaques, a species inhabiting Asia, seem to be uniformly susceptible to this class of viruses.

There is no adequate primate model at present for HIV-1-induced disease; although chimpanzees can be infected and seroconverted, no disease has been elicited in these animals. The primate viruses (SIV strains) cause a disease in macaques that is characterized by a rapid onset (less than 16 months) of weight loss, opportunistic infections, and depletion of $CD4^+$ lymphocytes that resembles in various respects AIDS in humans. Given the close homology between SIV and HIV-2, SIV infection of macaques is a valuable model for studying AIDS and its prevention and control.

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ADDENDUM

Of the six macaques inoculated with SIV/Mne, four had died after 15 to 87 weeks as reported in this article. The remaining two animals have since also died, A85037 at 96 weeks and M85026 at 120 weeks after inoculation. Both animals exhibited weight loss, diarrhea, and anemia, and both showed $CD4^+$ lymphopenia several weeks before they were euthanized while moribund. Histopathologic findings included severe necrotic ulceration of the external genitalia with multinucleated giant cells (A85037) and moderate nephropathy and subacute encephalitis (M85026). Thus, all six macques inoculated with SIV/Mne have died after 15 to 120 weeks.

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