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# A Partially Attenuated Simian Immunodeficiency Virus Induces Host Immunity That Correlates with Resistance to Pathogenic Virus Challenge

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**Three infectious, attenuated molecular clones of simian immunodeficiency virus (SIVmac) were tested for viral and host determinants of protective immunity. The viruses differed in degree of virulence from highly attenuated to moderately attenuated to partially attenuated. Levels of immune stimulation and antiviral immunity were measured in rhesus macaques inoculated 2 years previously with these viruses. Monkeys infected with the highly attenuated or moderately attenuated viruses had minimal lymphoid hyperplasia, normal CD4/CD8 ratios, low levels of SIV-specific antibodies, and cytotoxic T-lymphocyte activity against $p55^{gag}$ (Gag) or $gp160^{env}$ (Env). Monkeys infected with the partially attenuated virus had moderate to marked lymphoid hyperplasia, normal CD4/CD8 ratios, high levels of SIV-specific antibodies, and cytotoxic T-lymphocyte activity against both Gag and Env. After pathogenic virus challenge, monkeys immunized with the partially attenuated virus had 100- to 1,000-fold-lower viral load in peripheral blood mononuclear cells and lymph node mononuclear cells than naive control animals. One of four monkeys immunized with the highly attenuated virus and two of four monkeys immunized with the moderately attenuated virus developed similarly low viral loads after challenge. These three attenuated strains of SIV induced a spectrum of antiviral immunity that was inversely associated with their degree of attenuation. Only the least attenuated virus induced resistance to challenge infection in all immunized monkeys.**

Attenuation is the process of reducing the ability of a pathogen to cause disease (30). Attenuated viruses replicate in the host to a lesser extent than the virulent parental virus, induce immune responses like the virulent virus does, but do not cause serious disease. This is the basis of effective live-attenuated viral vaccines (1, 34). The nature of protective immune responses induced by vaccination is not known for most viruses. In a suitable animal model, attenuated viruses can provide a tool for the study of viral determinants and host factors involved in protective immunity.

The immune deficiency associated with human immunodeficiency virus (HIV) infection has a complex pathogenesis that usually results in death of the host, in spite of strong antiviral immunity (28). Simian immunodeficiency virus (SIV) infection of rhesus macaques is a practical animal model for HIV infection because the two viruses are genetically related and some strains of SIVmac cause an immunodeficiency in monkeys, simian AIDS, that is similar to human AIDS (10, 15). SIVmac1A11 (22) and SIVmac239Δnef (14) are two molecularly cloned, attenuated viruses that have been tested as live-virus vaccines. The infection of rhesus monkeys by these viruses differs in a number of ways, including the duration of viremia following intravenous (i.v.) inoculation and the outcome of pathogenic virus challenge. SIVmac1A11 causes a transient viremia of 6- to 8-wk duration, and monkeys immunized by this virus are protected from parenteral challenge with a low dose, 1 to 10 animal infectious doses, but not a high dose of pathogenic SIVmac (25, 26). SIVmac239Δnef causes a more persistent viremia of approximately 1-year duration, and immunized monkeys are protected against challenge with both low and high doses (9). Thus, there appears to be a minimum duration of attenuated-virus replication in the host that is needed to stimulate strong protective immunity to SIVmac.

Infections with three genetically related, attenuated molecular clones of SIV that differ in duration of viremia and disease potential were used to test this hypothesis. For a previous study (24), recombinant viruses were constructed with reciprocal exchanges of genetic regions between the pathogenic molecular clone, SIVmac239 (13), and the nonpathogenic molecular clone, SIVmac1A11 (22). These two parental viruses differ by only 2% in nucleotide sequence (21). The biological properties of viral load over time, host immune response, and the time to progression to simian AIDS in monkeys inoculated with the recombinant viruses are intermediate to those of the parental viruses. In general, the more of one parental genome a recombinant contains, the more in vivo properties it shares with the parental virus (24). Reciprocal exchanges of only the gp120 domain of the viral envelope attenuates SIVmac239 and lengthens the duration of viremia of SIVmac1A11. Both of these recombinants are attenuated because infection with either virus did not result in simian AIDS after 2 years, but one of four monkeys infected with the recombinant virus that contained mostly the SIVmac239 genome had persistent viremia and eventually developed simian AIDS 30 months postinfection.

Here we show that rhesus macaques inoculated 2 years previously with SIVmac1A11 or the two gp120 recombinant

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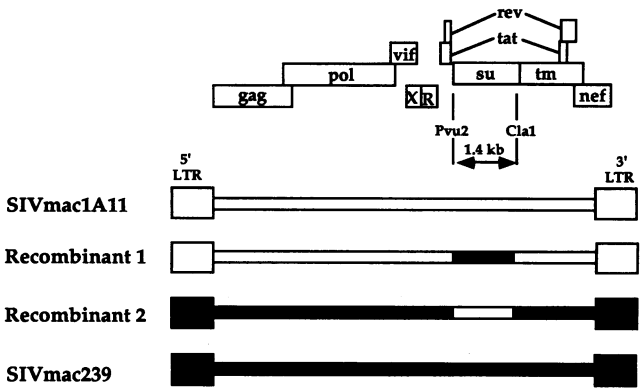


FIG. 1. Genomic organization of SIVmac239, SIVmac1A11 and the two recombinant viruses. The double-headed arrow indicates the 1.4-kb fragment containing the SU domain of the *env* gene. tm, transmembrane domain; LTR, long terminal repeat. (Modified from reference 24 with permission.)

viruses generate different levels of immune system activation and antiviral immune response. These properties of host immunity correlate with the duration of viremia of each vaccine virus and with viral load after pathogenic virus challenge. Animals immunized with the least attenuated molecular clone generated the strongest immune responses and had the lowest viral loads after challenge.

## MATERIALS AND METHODS

**Animals and viruses.** Colony-bred, adult rhesus macaques (*Macaca mulatta*), seronegative for simian type D retroviruses, simian T-cell leukemia virus, and SIV, were housed in accordance with the American Association for Accreditation of Laboratory Animal Care guidelines. The investigators adhered to the guidelines of the Committee on Care and Use of Laboratory Animals, National Resources Council. Four monkeys were inoculated i.v. with $10^4$ 50% tissue culture infectious doses ($TCID_{50}$) of one of three previously described molecular clones of SIV (24): SIVmac1A11, SIVmac1A11/239env/1A11 (here designated recombinant 1), or the reciprocal clone SIVmac239/1A11env/239 (here designated recombinant 2). Recombinants 1 and 2 contained exchanges of gp120 from SIVmac239 and SIVmac1A11, as shown in Fig. 1. Monkeys had been infected 2 years (recombinants 1 and 2) and 2.5 years (SIVmac1A11) prior to challenge. Eleven immunized monkeys and two naive controls were challenged i.v. with 1 to 10 animal infectious doses of cell-free SIVmac251, an uncloned biological isolate propagated in rhesus peripheral blood mononuclear cells (PBMC), and titered by i.v. inoculation of rhesus macaques (18; provided by R. Desrosiers, New England Regional Primate Research Center).

**Lymph node histology and SIV-specific in situ hybridization.** Axillary or inguinal lymph nodes were obtained by transcutaneous biopsy under ketamine HCl anesthesia (10 mg/kg, injected intramuscularly; Parke-Davis, Morris Plains, N.J.). Tissues were examined histologically for changes in lymphoid architecture, and in situ hybridization was used to detect the presence of viral nucleic acids. The tissue was fixed in 10% phosphate-buffered formalin and paraffin-embedded for in situ hybridization and hematoxylin counterstaining. In situ hybridization was performed by a procedure previously described (16), with modifications. The probe was full-length SIVmac1A11 proviral DNA (3, 22) labeled with $^{35}S$ (Amersham, Arlington Heights, Ill.). Tissues were mounted on Superfrost Plus slides (Fisher Scientific Co., Santa Clara, Calif.). The hybridization mixture, which contained 50% deionized formamide (Sigma Chemical Co., St. Louis, Mo.), 10% dextran sulfate (Sigma), 50 mmol of $NaH_2PO_4$ (Sigma) per liter, 0.6 mol of NaCl (Sigma) per liter, 8 mmol of EDTA (Sigma) per liter, 1× Denhardt's solution (Sigma), 75 μg of *Escherichia coli* tRNA (Sigma) per ml, 100 μg of salmon sperm DNA (Sigma) per ml, and the $^{35}S$-labeled probe, was heated at 95°C for 8 min and then cooled on ice, after which 20 mmol of dithiothreitol (Sigma) per liter was added. One hundred microliters of the mixture was dispensed onto each slide. The slides were then denatured for 7 min at 95°C, cooled on ice, and placed in a humidified chamber to hybridize overnight at 37°C. After hybridization, the slides were washed in 2× standard saline citrate with 50% formamide (Sigma), dehydrated in graded ethanols with 0.3 M ammonium acetate (Sigma), then coated with radiographic emulsion (Kodak Nuclear Track B2; Fisher), and exposed at 4°C. Positive and negative control slides were included in every reaction, and the autoradiographic exposure was prolonged from 5 to 12 days in order to detect weakly positive signal.

**SIV-specific antibodies in serum as detected by whole-virus ELISA and an antigen-reduction neutralization assay.** Levels of SIV-specific antibodies in serum were determined by enzyme-linked immunosorbent assay (ELISA), using sucrose-purified SIVmac propagated in Hut78 cells (American Type Culture Collection, Rockville, Md.) as previously described (23). Serial twofold dilutions of test, positive control, and negative control sera were plated from 1:100 to 1:204,800. The titer was defined as the serum dilution at or above 50% of the maximal optical density from the linear portion of the positive control curve.

A virus neutralizing assay based on SIV p27 core antigen production from the CEMx174 cell line (38; provided by J. A. Hoxie, University of Pennsylvania, Philadelphia) was used as described previously (19), with the following modifications: a reduction in the volumes of serum and virus in the incubation step from 100 to 50 μl, and an increase in the amount of virus from 4 to 10 $TCID_{50}$. Serial fourfold dilutions of sera were made from 1:5 to 1:5,120. Culture supernatants were collected after a 5-day incubation for quantification of SIV p27 antigen by antigen capture ELISA (19). The neutralization titer was defined as the greatest serum dilution that reduced p27 antigen production to 2 standard deviations below the antigen production in control wells containing serum from an SIV-seronegative monkey.

**SIV-specific CTL. (i) Preparation of effector cells.** Two methods were used to stimulate effector cells: antigen-specific stimulation and mitogenic stimulation. Antigen-specific stimulators were prepared from $10^6$ autologous $CD4^+$ T cells enriched by immunomagnetic bead (M450 anti-CD8 Dynabeads) depletion of $CD8^+$ lymphocytes, as instructed by the manufacturer (Dynal, Great Neck, N.Y.), from PBMC isolated from heparinized blood by density gradient centrifugation (4) (Lymphocyte Separation Medium; Organon Teknika, West Chester, Pa.). $CD4^+$-enriched cells were infected with SIVmac1A11, at a multiplicity of infection of 0.1, for 1 h at 37°C, washed in phosphate-buffered saline, and then stimulated with 0.5 μg of Staphylococcal enterotoxin A (Toxin Technologies, Madison, Wis.) per ml in 1 ml of complete medium, consisting of RPMI 1640 (JRH Biosciences, Lenexa, Kans.), 100 U of penicillin per ml, 100 μg of streptomycin (GIBCO, Grand Island, N.Y.) per ml, and 10% fetal calf serum (Intergen, Purchase, N.Y.) supplemented with 20 U of human recombinant interleukin-2 (IL-2; provided by Cetus Corpora-

tion, Emeryville, Calif.) per ml. On day 4 of culture, in order to slow the growth of the stimulator cells, the cells were resuspended in complete medium without IL-2, incubated overnight, and then washed prior to the addition of responder cells. In preliminary experiments, there was often high lytic activity against mock-infected and vaccinia virus-infected target cells if the $CD4^+$ stimulator cells were pulsed with mitomycin (Sigma) to arrest cell division. Fresh, autologous PBMC were mixed with the stimulator cells at a responder-to-stimulator ratio of 10:1 and cultured at $2 \times 10^6$ cells per ml in complete medium without exogenous IL-2. After 48 h, 5% human lymphocyte conditioned medium (human IL-2; Schiapparelli Biosystems, Inc., Columbia, Md.) was added. The culture medium was changed on day 4, and on days 7 and 10, the medium was changed and further supplemented with 20 U of human recombinant IL-2 per ml. Cytotoxic T-lymphocyte (CTL) cultures were assayed for cytotoxic activity on days 12 to 16.

In the second method, cultures for SIV-specific CTL were stimulated with concanavalin A (type IV; 10 μg/ml; Sigma) as previously described (29) in complete medium containing 5% human lymphocyte conditioned medium. Culture medium was changed on days 4, 7, and 10 as described above, and CTL lytic activity was tested on days 12 to 16.

**(ii) Preparation of target cells.** Autologous, herpesvirus papio-transformed B-cell lines were established for use as target cells as described previously (29). Briefly, PBMC were plated at $10^5$ per well in a 96-well flat-bottom plate (Fisher) in 100 μl of complete medium with 20% fetal calf serum and were incubated with 100 μl of culture supernatant from herpesvirus papio producer cell line, 594Sx1055 (provided by M. Sharp, Southwest Foundation for Biomedical Research, San Antonio, Tex.). Transformed cultures were evident by 4 weeks, and individual wells were expanded into a cell line grown in complete medium. B-cell lines were cryopreserved in aliquots and thawed 2 weeks prior to use. Sixteen hours prior to the CTL assay, $10^6$ cells were infected with one of three vaccinia viruses at a multiplicity of infection of 30 PFU per cell: the parental WR strain (vvWR) or a recombinant vaccinia virus containing the full-length open reading frame of either $p55^{gag}$ (vv-gag) or $gp160^{env}$ (vv-env) derived from the sequence of SIVmac239 (2; provided by L. Giavedoni and T. Yilma, University of California, Davis). Target cells were cultured overnight in a humidified 5% $CO_2$ incubator. The expression of vaccinia virus antigen was confirmed by indirect immunofluorescence using a rhesus anti-vaccinia virus serum followed by a goat anti-human immunoglobulin G serum conjugated with fluorescein isothiocyanate (FITC) (Tago Immunologicals, Burlingame, Calif.). For experiments to characterize the major histocompatibility virus (MHC) restriction of lytic cells, the murine cell line P815 (American Type Culture Collection) was either mock-infected or infected with vvWR, vv-gag, or vv-env (37).

**SIV-specific CTL assay.** A conventional chromium release assay was used to detect antiviral CTL activity. Prior to the assay, $10^6$ targets were labeled with 50 μCi of sodium chromate (250 to 500 mCi/mg; Amersham) for 1 h at 37°C, washed three times, resuspended in complete medium supplemented with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; GIBCO), and plated at $5 \times 10^3$ cells per well in a 96-well round-bottom plate (Fisher). Various concentrations of effector cells in a 100-μl volume were added to targets in triplicate. Spontaneous release wells contained 100 μl of target cells plus 100 μl of complete medium with HEPES buffer. Maximal release wells contained 100 μl targets plus 100 μl of detergent (37): 5% sodium dodecyl sulfate (Sigma) and 0.5% Triton X-100 (Sigma). Plates were centrifuged at 1,000 rpm for 1 min and then incubated for 4 to 5 h at 37°C in 5% $CO_2$. The supernatant was harvested for detection of counts per minute in a liquid scintillation counter (Microbeta 1450; Wallac Biosystems, Gaithersburg, Md.). Percent specific lysis was determined by the following formula: 100 × [(experimental release − spontaneous release)/(maximal release − spontaneous release)]. Specific lysis was considered positive if it was greater than two times the level of lysis of vvWR-infected cells and if it was greater than 10%. Data were normalized to lytic units (LU), where 1 LU is the number of effector cells per $10^7$ PBMC necessary to obtain 20% specific lysis in a unit of time (12). LU were calculated from the following formula: $10^7$/[(number of target cells per well) × (number of effector cells necessary to obtain 20% specific lysis)]. The lysis of uninfected target cells was routinely negative, and spontaneous chromium release was less than 30% of maximal release for all targets.

**Plasma SIV p27 antigen levels.** Plasma p27 antigenemia was measured by a commercial SIV core antigen capture ELISA (Coulter Corporation, Hialeah, Fla.) as instructed by the manufacturer.

**Quantification of cell-free and cell-associated viral load.** The concentrations of cell-free virus in plasma and cell-associated virus, latent or productive, in PBMC and lymph node mononuclear cells (LNMC) were measured in a limiting-dilution assay (41). Fresh plasma was centrifuged for 1 min at 10,000 rpm to pellet contaminating cells, diluted serially 10-fold in complete medium, and cocultured with CEMx174 cells in 1 ml in a 24-well plate (Fisher). Likewise, PBMC and LNMC, from axillary or inguinal lymph nodes teased into single-cell suspension, were washed, diluted serially 10-fold from $10^6$ cells per ml, and cocultured with CEMx174 cells. After 4 weeks, all negative wells at $10^6$ cells per well were pooled to a T25 tissue culture flask (Fisher) and cultured for an additional 4 weeks. In addition, $10^7$ PBMC or LNMC were cocultured with $2 \times 10^6$ CEMx174 cells in T75 tissue culture flasks (Fisher) and maintained for 8 weeks before terminating as negative. The $TCID_{50}$ of cell-free and cell-associated virus was calculated by the method of Reed and Muench (35). Negative cultures correspond to less than 1.0 $TCID_{50}$ per ml plasma or less than 0.1 $TCID_{50}$ per $10^6$ cells, the limits of detection for these assays.

**Lymphocyte phenotyping.** PBMC were stained with anti-human monoclonal antibodies to CD4 (phycoerythrin-conjugated OKT4; Ortho Diagnostic Systems, Inc., Raritan, N.J.) or to CD8 and CD16 (Leu 2a-FITC and Leu 11a-FITC; Becton Dickinson Immunocytometry Systems, San Jose, Calif.) as instructed by the manufacturers, and immunofluorescence was measured with a dual-laser flow cytometer (FACSCAN; Becton Dickinson).

## RESULTS

**Three levels of attenuation of SIVmac.** Monkeys experimentally infected for 2 years with three attenuated clones of SIV were chosen for this study because they had no clinical signs of simian AIDS and they were not persistently viremic (24). In addition, all of the monkeys had age-dependent CD4/CD8 ratios in the normal range, from 0.59 to 1.59 (7). Monkeys infected with SIVmac1A11 were viremic for only 4 to 6 weeks postinoculation (p.i.). Monkeys infected with recombinant 1 had a primary viremia for 6 to 8 weeks and were intermittently viremic for 1 year p.i. Monkeys infected with recombinant 2 had a primary viremia for 27 weeks and were thereafter intermittently viremic. One monkey from this group was not challenged because of persistent viremia; it later developed simian AIDS at 30 months p.i.

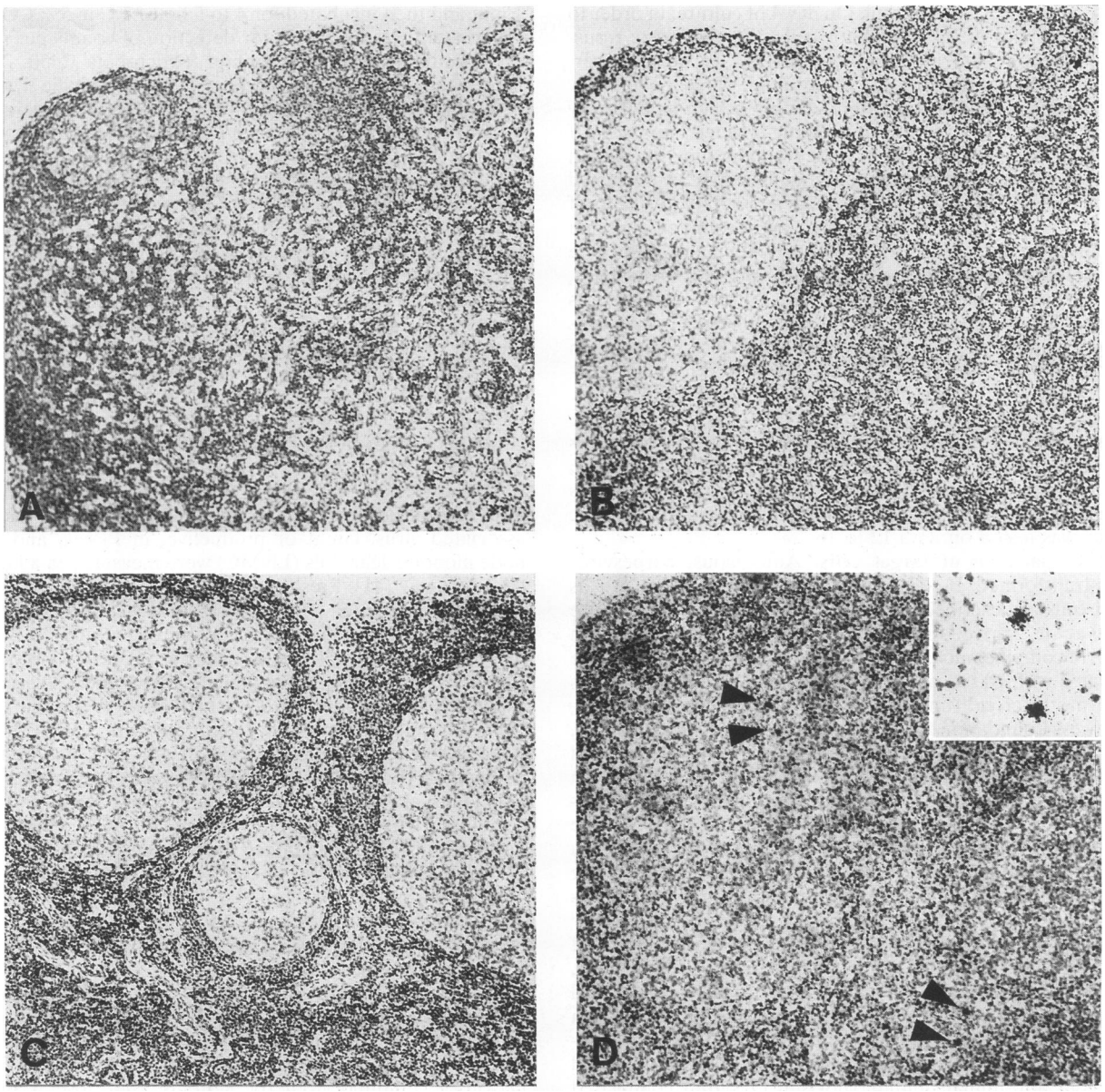


FIG. 2. SIV-specific in situ hybridization and histology of lymph nodes from monkeys immunized with attenuated SIV clones. In situ hybridization was performed with a $^{35}$S-labeled SIVmac1A11 DNA probe as described in Materials and Methods. (A) A normal lymph node from a monkey immunized with SIVmac1A11 with a primary and a secondary follicle. SIV-infected cells were not detected in this lymph node sample. (B) Mild follicular hyperplasia in a lymph node from a monkey immunized with recombinant 1. SIV-infected cells were not detected in this lymph node sample. (C) Moderate follicular hyperplasia in a lymph node from a monkey immunized with recombinant 2. SIV-infected cells were not detected in this lymph node sample. (D) Lymph node from a monkey persistently infected with recombinant 2 showing marked follicular hyperplasia and four in situ positive cells (arrowheads) in enlarged germinal centers. Total magnification, ×300. The inset in panel D shows the lower two in situ positive cells at a total magnification of ×600.

Prior to challenge, LNMC were cocultured with CEMx174 cells to detect infectious virus. SIV was not isolated from LNMC of monkeys inoculated 2 years previously with SIVmac1A11. However, with one exception in the group inoculated with recombinant 1, SIV was recovered from LNMC of all monkeys. In addition to culture, the extent of productive viral infection was determined by in situ hybridization. As shown in Fig. 2, in situ hybridization using a full-length viral probe and prolonged autoradiographic exposure revealed no detectable signal in the lymph nodes from any of the animals prior to challenge, with the exception of one monkey infected with SIVmac1A11 whose lymph node contained one or less in situ positive cell per high-power field (not shown). In contrast, the lymph node from the monkey infected with recombinant 2 that was not challenged contained zero to four in situ positive cells per high-power field (Fig. 2D). Thus, on the basis of viral culture and in situ hybridization results, monkeys inoculated with these attenuated viruses had a low level of latently infected cells in peripheral lymph nodes.

Figure 2 also shows a spectrum of lymphoid morphology

TABLE 1. Antiviral antibody titers from the day of challenge of monkeys immunized with SIVmac1A11 or one of two recombinant molecular clones of SIV

| Immunization | Neutralization titer[a] | ELISA titer[b] |
|---|---|---|
| SIVmac1A11 ($n$ = 4) | 20 | 400 |
| | 20 | <100 |
| | 80 | 200 |
| | 5 | 1,600 |
| Recombinant 1 ($n$ = 4) | 20 | 12,800 |
| | 5 | 3,200 |
| | 5 | 800 |
| | 20 | 800 |
| Recombinant 2 ($n$ = 3) | 5,120 | 25,600 |
| | 5 | 12,800 |
| | 1,280 | 6,400 |
| Naive ($n$ = 2) | <5 | <100 |
| | <5 | <100 |

[a] SIV neutralization titers were defined as the highest serum dilution that reduced p27 antigen production 2 standard deviations below the antigen production in wells containing serum from an SIV-seronegative monkey. Reciprocal data are shown.

[b] Anti-SIV antibody titers by whole-virus ELISA were defined as the serum dilution at or above 50% of the maximal optical density from the linear portion of the positive control curve. Reciprocal data are shown.

from normal (Fig. 2A), to mild (Fig. 2B) and moderate (Fig. 2C) follicular hyperplasia. The lymph node architecture in monkeys immunized with SIVmac1A11 or recombinant 1 ranged from normal to mild follicular hyperplasia (Fig. 2A and B), but all four monkeys immunized with recombinant 2 had mild to moderate follicular hyperplasia (Fig. 2B and C). The virulence potential of these viruses and the duration of primary viremia distinguish three levels of attenuation that is also reflected by the degree of chronic lymphoid hyperplasia.

**Antiviral antibody responses.** Both neutralizing and ELISA titers of anti-SIV antibodies were measured on the day of challenge (Table 1). Monkeys immunized with SIVmac1A11 had the weakest antibody response: neutralizing titers ranged from 1:5 to 1:80, and ELISA titers ranged from less than 1:100 to 1:1,600. Monkeys immunized with recombinant 1 had an intermediate antibody response, with neutralizing antibody titers from 1:5 to 1:20 and ELISA antibody titers from 1:800 to 1:12,800. The group immunized with recombinant 2 had the highest neutralizing and ELISA antibody titers. Neutralizing titers from monkeys in this group ranged from 1:5 to 1:5,120, and ELISA titers ranged from 1:6,400 to 1:125,600. These titers are similar to those from monkeys infected with pathogenic SIVmac239 (5).

**Antiviral CTL responses.** Cell-mediated immunity in monkeys immunized with the attenuated strains of SIV was measured by CTL activity against the antigens of two viral proteins, Gag and Env. Unlike monkeys infected with pathogenic SIVmac, monkeys immunized with attenuated SIV clones had weak or no detectable CTL responses following mitogenic stimulation of their PBMC (Fig. 3A). In contrast to mitogenic stimulation, antigen-specific stimulation of PBMC by using autologous $CD4^+$ lymphocytes infected in vitro with SIV resulted in stronger CTL activity (Fig. 3B). This result suggests that the precursor frequency of CTL memory cells is low in monkeys immunized with these attenuated clones. The results of antigen-specific stimulation of cytotoxic activity in immunized and control monkeys are shown in Table 2. Two of four monkeys immunized with SIVmac1A11 had positive CTL activity. All monkeys immunized with either recombinant 1 or recombinant 2 had CTL activity against one or both targets. One of two naive monkeys had weak CTL activity prior to challenge. Similar to SIV-specific antibody responses, the strength of CTL responses was inversely associated with the level of attenuation of the immunizing virus; SIVmac1A11 induced the weakest CTL response, while recombinant 2 induced the strongest response.

The nature of the cytotoxic effector cells was characterized by comparing lysis of autologous and xenogeneic target cells and by lymphocyte subset depletion of effector cells. Monkeys with Gag-specific cytotoxic activity directed against autologous targets had no detectable activity against murine P815 target cells expressing the same SIV antigen, a result consistent with MHC restriction of the cytolytic activity (37). Depletion of $CD8^+$ lymphocytes from the effector population also abrogated the Gag-specific lysis, but for some monkeys, Env-specific lysis was still detectable. Depletion of $CD4^+$ lymphocytes had no effect on Gag-specific lysis but decreased Env-specific lysis by approximately 50%. Effector cell populations contained few, if any, $CD16^+$ cells; together with the lack of lysis of xenogeneic target cells, this finding suggests that Env-specific lysis was not mediated by natural killer cells.

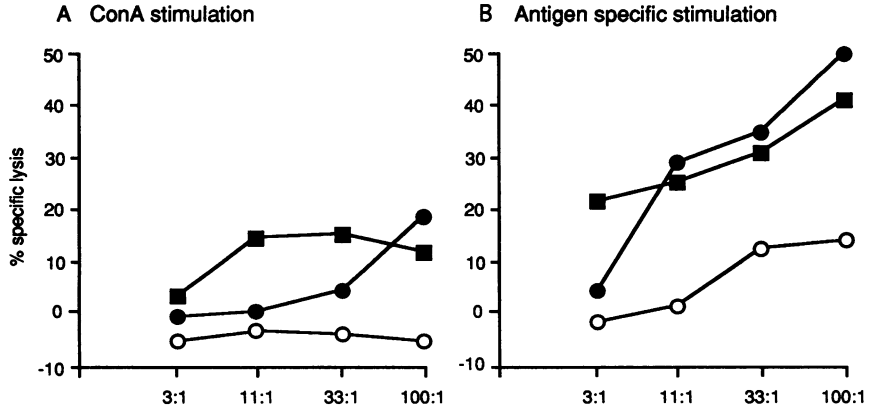


FIG. 3. SIV-specific cytotoxic activity in concanavalin A- and antigen-stimulated cultures from a monkey immunized with recombinant 1. PBMC were stimulated with either concanavalin A (ConA; A) or autologous SIV-infected $CD4^+$ T cells (B). Fourteen days after stimulation, cytotoxic activity was measured against autologous B-lymphoblastoid cells infected with the wild-type vvWR (open circles), vv-gag (closed circles), or vv-env (squares) as described in Materials and Methods.

TABLE 2. CTL activity immediately prior to challenge in monkeys immunized with SIVmac1A11 or one of two recombinant molecular clones of SIV[a]

| Immunization | LU/$10^7$ lymphocytes[b] | | |
|---|---|---|---|
| | vvWR | vv-gag | vv-env |
| SIVmac1A11 ($n$ = 4) | 18 | 9 | 12 |
| | <0.1 | 11 | 32 |
| | <0.1 | <0.1 | <0.1 |
| | 29 | 99 | 224 |
| Recombinant 1 ($n$ = 4) | <0.1 | 1 | 195 |
| | NT[c] | NT | NT |
| | <0.1 | 313 | 339 |
| | <0.1 | 47 | 0.1 |
| Recombinant 2 ($n$ = 3) | 3 | 37 | 18 |
| | 8 | 215 | 525 |
| | 120 | 440 | 881 |
| Naive ($n$ = 2) | 14 | 24 | 19 |
| | <0.1 | 18 | 1 |

[a] Autologous B-cell lines were infected with wild-type or recombinant vaccinia viruses as described in Materials and Methods.

[b] One LU was defined as the number of effector cells per $10^7$ lymphocytes necessary to obtain 20% specific lysis in a unit of time. Positive CTL activity was defined as at least 10 LU/$10^7$ lymphocytes and twice the number of LU necessary to lyse vvWR-expressing targets. Positive values are underlined.

[c] NT, not tested.

These results are consistent with classical $CD8^+$, MHC-restricted CTL lysis of target cells expressing Gag antigen and both $CD8^+$ and $CD8^-$ CTL lysis of target cells expressing the Env antigen. The $CD8^-$ effector cells generated in CTL cultures from 30% of the monkeys could be $CD4^+$ CTL, as described by Callahan et al. for HIV-1 (6). Another mechanism, syncytium formation with $CD4^+$ T cells in the CTL assay, could also contribute to chromium release from gp160-expressing target cells (17).

**Viral load following pathogenic virus challenge.** On the day of challenge, one monkey immunized with recombinant 2 had a viral load of 1 $TCID_{50}$ per $10^6$ PBMC and all the other monkeys were aviremic. Immunized monkeys and two naive controls were inoculated i.v. with 1 to 10 animal infectious doses of pathogenic SIVmac251. The viral load after challenge was quantified by measuring cell-associated virus in PBMC and LNMC, cell-free virus in plasma, and SIV p27 antigen in plasma. At 2 weeks postchallenge (p.c.), neither infectious cell-free virus nor viral antigen was detectable in the plasma from immunized monkeys, but plasma from the naive controls contained high levels of infectious virus ($10^3$ to $10^4$ $TCID_{50}$/ml) and p27 antigen (>2 ng/ml). Infectious cell-associated virus was isolated from most monkeys at 1 and 2 weeks p.c., but there was a significant reduction in viral load in some immunized monkeys compared with the controls (Fig. 4). The two naive controls, three of four monkeys immunized with SIVmac1A11, and two of four monkeys immunized with recombinant 1 had persistent high-level viremia, >10 $TCID_{50}$/$10^6$ PBMC. One monkey immunized with recombinant 1 and all monkeys immunized with recombinant 2 had low (≤10 $TCID_{50}$/$10^6$ PBMC) or undetectable levels of viremia after challenge.

The viral load in LNMC was measured in parallel with the load in PBMC at 12 and 24 weeks p.c. With three exceptions, levels of virus in LNMC exceeded or were equal to the levels in PBMC. Several monkeys had no detectable viremia but still had significant levels of SIV in their lymph nodes. Additionally, there was a reduction of virus in LNMC at 24 weeks p.c. compared with the 12-week time point. At 12 weeks p.c., 8 of

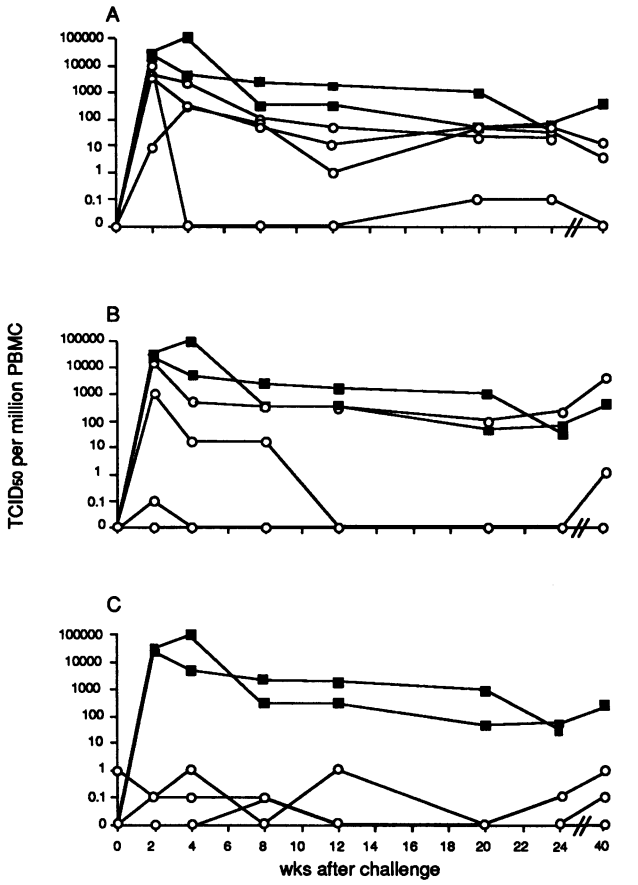


FIG. 4. Cell-associated viremia after pathogenic virus challenge in monkeys immunized with SIVmac1A11 (A), recombinant 1 (B), and recombinant 2 (C). The naive controls (squares) are the same in each panel. The limit of detection of this assay is 0.1 $TCID_{50}$/$10^6$ PBMC. See Results for details.

11 immunized monkeys had a high viral load (>10 $TCID_{50}$ per $10^6$ LNMC), whereas at 24 weeks p.c., the number of monkeys with a high viral load had decreased to five (Fig. 5A versus Fig. 5B). The control monkeys had high virus levels in both PBMC and LNMC at both time points, while all monkeys immunized with recombinant 2 had PBMC and LNMC viral loads of less than 10 $TCID_{50}$/$10^6$ cells. At 52 weeks p.c., viral load in PBMC and LNMC was essentially unchanged from the levels at 24 weeks p.c. (data not shown). The reduced viral load over time observed in all monkeys immunized with the partially attenuated strain of SIV (recombinant 2), in two monkeys immunized with recombinant 1, and in one monkey immunized with SIVmac1A11 suggests a process of slow viral clearance.

**Clinical outcome of pathogenic virus challenge.** Two monkeys, one immunized with SIVmac1A11 and one naive control, developed clinical signs of simian AIDS, and they were euthanized 27 and 35 weeks p.c., respectively. These two monkeys had high levels of SIV in PBMC and LNMC at 24 weeks p.c. At 52 weeks p.c., all remaining animals were healthy, with the exception of one monkey immunized with SIVmac1A11 that succumbed to anesthetic death during phlebotomy. This monkey had no detectable virus measured by coculture of PBMC, LNMC, spleen, or thymus. Peripheral blood CD4/CD8 ratios of the surviving monkeys were within the normal range with

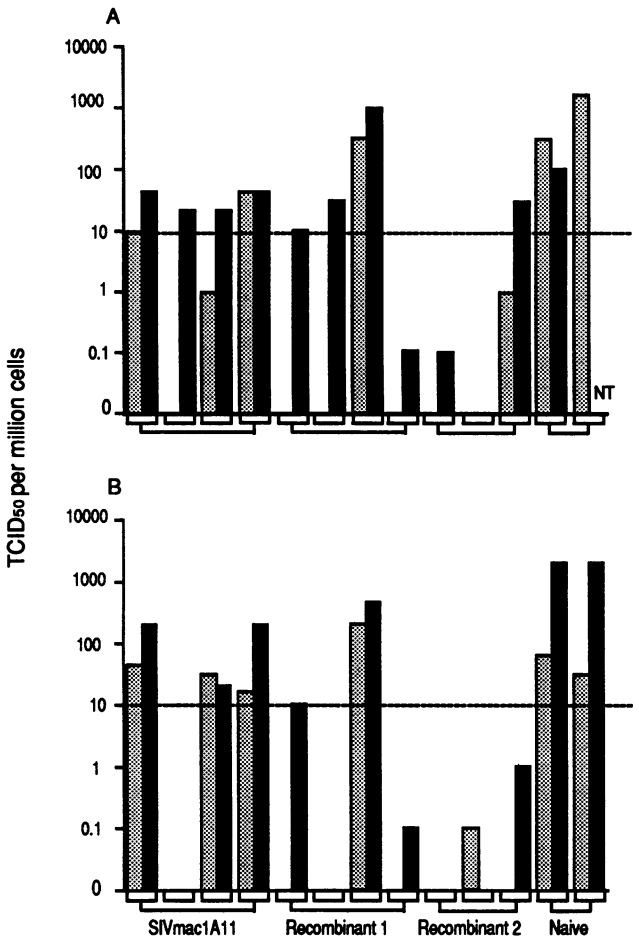


FIG. 5. Cell-associated viral load in blood and peripheral lymph nodes at 12 weeks (A) and 24 weeks (B) after pathogenic virus challenge. Stippled bars represent viral load in PBMC, and solid bars represent the load in LNMC. The dashed line at 10 $TCID_{50}/10^6$ cells is an arbitrary cutoff between high and low viral load. NT, not tested.

two exceptions: a ratio of 0.25 in a persistently viremic monkey immunized with recombinant 1, and a ratio of 0.27 in the surviving unimmunized control monkey. Both of these monkeys had consistently high viral loads after challenge.

## DISCUSSION

The ability of an attenuated clone of SIVmac to induce protective immunity appears to be directly related to its ability to produce a prolonged, disease-free initial viremia and a secondary persistent lymphoid hyperplasia, as seen with the three monkeys immunized with recombinant 2. SIVmac1A11, the most avirulent vaccine virus used in this study, produced a viremia lasting only 4 to 6 weeks. The recombinant 1 virus, containing mostly the SIVmac1A11 genome, produced a primary viremia lasting 6 to 8 weeks, while rhesus monkeys inoculated with recombinant 2, containing a large portion of the pathogenic SIVmac239 genome, were viremic for an average of 27 weeks. The propensity for each of these vaccine strains to induce simian AIDS was also proportional to the duration of primary viremia. All eight monkeys inoculated with SIVmac1A11 or recombinant 1 were aviremic at the time of challenge at 2 years. In contrast, one of the four monkeys inoculated with recombinant 2 was persistently viremic and was not challenged. This monkey developed simian AIDS at 30 months p.i. Thus, although recombinant 2 induced the best resistance to the pathogenic challenge virus, it retained the ability to cause lethal disease in one of four monkeys. This narrow line between attenuation and vaccine efficacy is not present in successful live-virus vaccines. For example, vaccine strains of measles virus, progressively attenuated to reduce the incidence of rash and fever, retained immunogenicity and efficacy (34).

The ability of the recombinant 2 virus to induce protective immunity appeared to require more than its capacity to maintain long-term primary viremia. After 2 years, all animals immunized with recombinant 2 had significant lymphoid follicular hyperplasia, without evidence of replicating virus as detectable by in situ hybridization, with the exception of the one animal that was not challenged and that later developed simian AIDS (Fig. 2). Although lymphoid follicular hyperplasia is not diagnostic of SIV or HIV infection, in the absence of other clinically apparent infections in these animals, we propose that it is an anatomical correlate of latent infection (in situ hybridization negative, coculture positive) and chronic antigenic stimulation. To our knowledge, lymphoid follicular hyperplasia in the absence of detectable virus replication is not seen with infections by pathogenic SIVmac in rhesus monkeys or HIV-1 in humans (32, 36). Monkeys infected with SIVmac239Δnef for 1 year had no evidence of lymphoid hyperplasia (14). This chronic antigenic stimulation is probably due to the trapping and maintenance of viral antigen-antibody complexes by follicular dendritic cells long after productive infection has ceased (1, 40).

We also studied the nature of resistance to challenge infection. All of the immunized monkeys developed some level of virus-specific immunity that persisted for 2 years prior to challenge. Antibody responses were detected in all monkeys soon after immunization, and the titers were in direct proportion to the length of primary viremia (24). However, only monkeys immunized with the least attenuated recombinant 2 virus maintained titers of neutralizing and ELISA antibodies comparable to those in monkeys infected with pathogenic SIVmac. Two weeks after challenge, animals immunized with SIVmac1A11 and recombinant 1 developed high anamnestic antibody titers (data not shown). The absence of cell-free plasma viremia in all immunized monkeys after challenge could be attributed to these high-titer antibody responses. Thus, high-titer antibody prior to challenge or anamnestic antibody responses do not prevent challenge infection but significantly modify the acute phase of infection with pathogenic SIVmac.

The activation of vaccine-generated, antiviral CTL memory cells requires productive virus infection. Strong CTL responses during the acute phase of infection should limit the spread of infection to new target cells, reducing the viral load during persistent infection to a very low level and perhaps eventually clearing the virus. All monkeys immunized with recombinant 2 had relatively strong CTL responses to Gag and Env antigens prior to challenge (Table 2). Individual monkeys in the other two groups had similar responses. However, the presence of an SIV-specific CTL response does not predict resistance to challenge infection in individual animals (27). One monkey immunized with SIVmac1A11 and another immunized with recombinant 1 had detectable CTL responses prior to challenge, but they maintained high viral loads at 12 and 24 weeks p.c. Therefore, a threshold level of response to multiple SIV antigens may be required for viral clearance or the effective control of persistent infection.

Other mechanisms that we have not studied may confer

resistance to challenge with pathogenic SIVmac. At the cellular level, virus interference, mediated by cell surface expression of viral envelope proteins, is a mechanism of resistance to superinfection (42). This mechanism has been reported effective for HIV-1 and HIV-2 in in vitro assays (11). At the level of the organism, classical virus interference would require that a majority of the cellular targets of infection be productively infected. In the present study, there was no evidence of productive viral infection, as measured by in situ hybridization, in peripheral lymph nodes prior to challenge (Fig. 2). Also, the level of latent infection in the lymph nodes of these animals 1 and 2 years after inoculation, as detected by coculture with permissive cells or by PCR, was very low (24). However, it is possible that infection with attenuated viruses could indirectly mediate resistance to virulent virus infection by other mechanisms such as altered cytokine expression.

What will be the fate of the immunized animals that are still alive 1 year after challenge? Those monkeys that developed simian AIDS after challenge had high virus levels in PBMC and LNMC and a rapid decline in CD4/CD8 T-cell ratios. This is consistent with the association between high long-term viral load and the progression to AIDS that has been suggested for SIV and HIV-1 (14, 20, 24, 39). Conversely, monkeys with low viral loads in PBMC and LNMC have remained healthy. We predict that the monkeys immunized with recombinant 2 will not develop simian AIDS.

The development of a safe and effective HIV vaccine is a formidable challenge (31). Indeed, other experimental lentivirus vaccines have enhanced infection or disease (8, 33). The induction of antiviral CTL responses by vaccination with a live-attenuated SIV provides protection from disease, if not infection, by limiting the degree of viral replication during the acute phase of infection. However, it is not clear that for SIV a degree of attenuation that provides both efficacy and safety can be achieved. Our data indicate that a live-attenuated molecular clone of SIV that induces protective immunity also retains virulence. There are two possible strategies to overcome this problem. One possibility is that viral persistence, and hence immunogenicity, can be genetically separated from virulence. Deletion of the *nef* gene from SIVmac239 attenuates the virus, producing a long-term viremia without causing disease in monkeys (9, 14). If the *nef* gene function is required for virulence but not for persistence, then SIVmac239Δnef would be a safer vaccine virus than recombinant 2. Another possibility is that immune responses that differ from those induced by infection would be more protective against lentiviruses. Novel immunization strategies are required to test this hypothesis.

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