JOURNAL OF VIROLOGY, Sept. 1999, p. 7430–7440 Vol. 73, No. 9
0022-538X/99/$04.00+0

Factors Associated with Slow Disease Progression in Macaques Immunized with an Adenovirus-Simian Immunodeficiency Virus (SIV) Envelope Priming-gp120 Boosting Regimen and Challenged Vaginally with SIVmac251

SUZAN L. BUGE,[1] LALITA MURTY,[1] KAMALPREET ARORA,[1] V. S. KALYANARAMAN,[2] PHILLIP D. MARKHAM,[2] ERSELL S. RICHARDSON,[1] KRISTINE ALDRICH,[1] L. JEAN PATTERSON,[1] CHRISTOPHER J. MILLER,[3] SHEAU-MEI CHENG,[4] AND MARJORIE ROBERT-GUROFF[1]*

Basic Research Laboratory, National Cancer Institute, Bethesda, Maryland 20892[1]; Advanced BioScience Laboratories, Inc., Kensington, Maryland 20895[2]; California Regional Primate Research Center, University of California at Davis, Davis, California 95616[3]; and Wyeth-Ayerst Research, Radnor, Pennsylvania 19087[4]

Received 2 March 1999/Accepted 15 June 1999

Rhesus macaques were immunized with a combination vaccine regimen consisting of adenovirus type 5 host range mutant-simian immunodeficiency virus envelope (Ad5hr-SIVenv) recombinant priming and boosting with native SIV gp120. Upon intravaginal challenge with SIVmac251, both persistently and transiently viremic animals were observed (S. L. Buge, E. Richardson, S. Alipanah, P. Markham, S. Cheng, N. Kalyan, C. J. Miller, M. Lubeck, S. Udem, J. Eldridge, and M. Robert-Guroff, J. Virol. 71:8531–8541, 1997). Long-term follow-up of the persistently viremic immunized macaques, which displayed significantly reduced viral burdens during the first 18 weeks postchallenge compared to controls, has now shown that one of four became a slow progressor, clearing virus from plasma and remaining asymptomatic with stable CD4 counts for 134 weeks postchallenge. Reboosting of the transiently viremic macaques did not reactivate latent virus. Rechallenge with two sequential SIVmac251 intravaginal exposures again resulted in partial protection of one of two immunized macaques, manifested by viral clearance and stable CD4 counts. No single immune parameter was associated with partial protection. Development of a strong antibody response capable of neutralizing a primary SIVmac251 isolate together with SIV-specific cytotoxic T lymphocytes were implicated, while $CD8^+$ T-cell antiviral activity and mucosal immune responses were not associated with delayed disease progression. Our data show that even a third immunization with the same Ad5hr-SIVenv recombinant can elicit significant immune responses to the inserted gene product, suggesting that preexisting Ad antibodies may not preclude effective immunization. Further, the partial protection against a virulent, pathogenic SIV challenge observed in two of six macaques immunized with a vaccine regimen based solely on the viral envelope indicates that this vectored-vaccine approach has promise and that multicomponent vaccines based in the same system merit further investigation.

While human immunodeficiency virus (HIV) can be transmitted horizontally by blood and vertically from infected mother to child, by far the most prevalent mode of transmission worldwide occurs sexually, across genital mucosal surfaces. In fact, heterosexual transmission accounts for 70 to 80% of all occurrences (1, 19, 21, 25, 31). Therefore, the need for AIDS vaccines capable of eliciting effective immunity at mucosal sites, including the vagina and rectum, is crucial. Simian immunodeficiency virus (SIV) and SIV-HIV (SHIV) chimera infections of macaques have provided valuable models for vaccine studies in nonhuman primates. These viruses can infect macaques vaginally and rectally, and the consequences of these infections with regard to pathogenesis and disease progression have been described (16, 29, 40, 48, 52). Such fundamental studies have provided the basis for investigations of prophylactic vaccines aimed at preventing infection via these routes. Various degrees of success have been achieved.

Vaccine approaches, including targeted iliac lymph node immunization (37), infection with live, attenuated SIVmac251 with a deletion in the *nef* gene (17), exposure to naturally attenuated HIV type 2 (HIV-2) (53) or SHIV (55), and immunization with NYVAC-SIV recombinant vaccines, in the presence or absence of cytokine adjuvants (6), have shown various degrees of protection against subsequent SIV intrarectal challenges. Psoralen- and formalin-inactivated SIV preparations, in some cases encapsulated as microspheres, have shown a degree of protection against both intrarectal and intravaginal challenges (15, 43, 44, 64). However, the degree to which human cellular antigens present in the inactivated viral preparations and on the surface of the challenge viruses contributed to this protection is not clear. Following oral immunization with an attenuated SHIV with deletions in accessory genes, 10 of 12 macaques were able to control virus replication following intravaginal challenge with a pathogenic SHIV isolate, although sterilizing immunity was not achieved (30). In addition, macaques immunized vaginally with an attenuated SHIV were protected from intravaginal challenge with pathogenic SIV (49).

The inability of the vaccine candidates tested to date to elicit greater protective efficacy against highly virulent and pathogenic SIV and SHIV isolates indicates that while protection via these mucosal challenge routes is possible, the immunization strategies used so far are not optimal. Attenuated live virus vaccines have been most effective, as has also been observed following intravenous challenges (2, 18, 67); however, the safety

* Corresponding author. Mailing address: Basic Research Laboratory, National Cancer Institute, Building 41, Room D804, 41 Library Dr. MSC 5055, Bethesda, MD 20892-5055. Phone: (301) 496-2114. Fax: (301) 496-8394. E-mail: guroffm@exchange.nih.gov.

of these vaccines remains a concern (3, 4). In the development of better strategies, the immune responses correlated with protective outcomes could indicate the direction to pursue for greater vaccine efficacy. In studies carried out to date, several immune responses have been implicated in controlling viral replication following mucosal transmission. These include local SIV p27-specific immunoglobulin A (IgA)-secreting cells, CD8-suppressor factor, and the chemokines RANTES and MIP-1β (37) as well as SIV-specific $CD8^+$ cytotoxic T lymphocytes (49, 53), in some cases observed in gut-associated lymph nodes (17). However, while cell-mediated immunity appears highly important in controlling viral replication following mucosal transmission, a strong antibody response is also critical. Rapid disease progression following either intravenous or mucosal exposure to pathogenic SHIV has been observed in animals who fail to develop virus-specific antibodies (40).

We have previously reported that after an initial oral plus intranasal immunization and subsequent intratracheal administration of an adenovirus type 5 host range mutant-SIV envelope (Ad5hr-SIVenv) recombinant vaccine followed by two boosts with native SIV gp120 in Syntex adjuvant, six immunized rhesus macaques developed SIV-specific humoral, cellular, and mucosal immune responses (8). After intravaginal challenge with over 10^5 50% tissue culture infective doses ($TCID_{50}$) of pathogenic SIVmac251, four of the immunized macaques and three control macaques became persistently viremic while two immunized macaques and two controls exhibited only transient viremia. A sixth control macaque showed no signs of infection. Among the persistently viremic animals, the immunized macaques exhibited lower viral burdens during the acute phase of infection than did the controls. To determine whether the initial immunization regimen exerted any long-lasting effect on the eventual disease outcome, we monitored the animals over the next 140 weeks. In addition, to further explore the effects of the combination vaccine regimen, the remaining transiently viremic immunized macaques were reboosted with the Ad5hr-SIVenv vaccine and native gp120 in alum 64 weeks after the initial challenge and rechallenged intravaginally 2 weeks later with twice the previous dosage of infectious SIVmac251. The transiently viremic control macaques, including the control animal which showed no evidence of infection upon initial challenge, were similarly inoculated with the Ad5hr wild-type vector (Ad5hr-WT) and alum alone and rechallenged. Postchallenge monitoring of these animals indicated that the two immunized animals displayed a better outcome than controls, with higher CD4-cell counts and lower viral loads. One of the two animals actually cleared the virus so that SIV RNA was no longer detected in the plasma. Thus, overall, two of six immunized macaques exhibited partial protection. Factors associated with the protective responses were investigated, and the results are reported here.

MATERIALS AND METHODS

Immunogens. A previously described SIVsm envelope recombinant (Ad5hr-SIVenv) based in an Ad5hr mutant capable of infecting rhesus macaque cells was used in these studies (10). Native SIVmac251 gp120 protein was purified from a productive tissue culture medium by disruption and affinity chromatography as previously described for HIV-1 gp120 (32).

Immunization and challenge of macaques. The initial immunization and challenge of 12 adult multiparous female rhesus macaques were reported previously (8) and are summarized in Fig. 1. Two immunized macaques, transiently viremic as a result of the first vaginal challenge, were subsequently reboosted intratracheally with 5×10^8 PFU of the Ad5hr-SIVenv recombinant and intramuscularly with 100 μg of native SIV gp120 in alum at week 104 post-initial immunization. Two transiently viremic control macaques and an additional control macaque with no evidence of infection following the initial challenge (no. 359) were administered the Ad5hr-WT vector and alum adjuvant alone. Two weeks later, all five macaques were challenged intravaginally with 2 doses of over 10^5 $TCID_{50}$ of SIVmac251 in 1 ml delivered via a tuberculin syringe into the vaginal canal as previously described (48, 49), once in the morning and once in the evening of the same day (Fig. 1). Macaques were sufficiently anesthetized so as to remain immobile for 20 to 30 min postinoculation. Macaque 359 again failed to become infected by the double challenge dose and is not considered further in this study. The basis for its resistance to vaginal infection by SIVmac251 is under investigation.

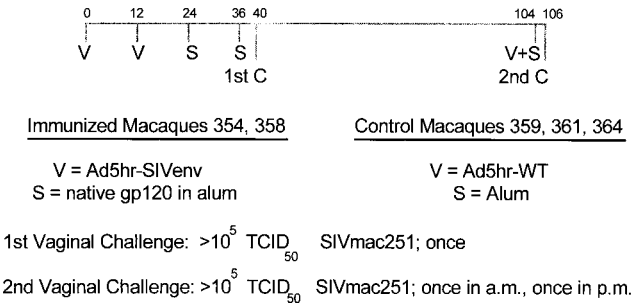

FIG. 1. Immunization and challenge protocol. Twelve macaques, six immunized (no. 353 to 358) and six controls (no. 359 to 364), were immunized and challenged according to the schedule shown at the top of the figure (in weeks) as reported previously (8). Subsequently the five macaques indicated were reboosted and rechallenged as described at the bottom of the figure. Macaque 359 did not become infected following the second challenge and was not included further in this study.

Sample collection. Blood samples were collected periodically following boost and challenge for assessment of immunologic responses and the virological statuses of the monkeys. Peripheral blood mononuclear cells (PBMCs) were separated from heparinized blood by centrifugation on lymphocyte separation medium (LSM; Organon Teknika Corp., Durham, N.C.). PBMCs were used fresh or were frozen viably in liquid nitrogen in a freezing medium consisting of 8% dimethyl sulfoxide (Sigma Chemical Co., St. Louis, Mo.) in sterile fetal bovine serum. Serum samples were stored at −70°C prior to use.

Nasal, vaginal, and rectal secretions were sampled by gently swabbing mucous membrane surfaces with cotton-tipped applicator sticks, after which the applicators were stored in transfer medium consisting of phosphate-buffered saline (PBS) with 0.1% bovine serum albumin (Sigma), 0.01% thimerosal, and 750 Kallikrein inhibitor units of aprotinin/ml. Vaginal swab samples were not taken when the animals were menstruating. Secretions showing contamination with blood were not included in analyses. Saliva specimens were collected by placing a needleless syringe inside the mouth, close to the cheek. Two milliliters of saliva was collected and immediately added to 0.2 ml of 10× transfer medium. All samples were stored at −70°C until use.

Assessment of Ad5hr-WT and Ad5hr-SIVenv recombinant replication. For several days following immunization, fecal samples were collected as 10% suspensions in Dulbecco's modified Eagle medium containing gentamicin (200 μg/ml) and amphotericin (10 μg/ml) and stored at −70°C for further use. The presence of Ad5hr DNA in stool specimens was assessed by PCR, using a Perkin-Elmer Cetus (Norwalk, Conn.) PCR kit. The primers and reaction conditions used were exactly as previously described (8).

A microneutralization assay was used as previously described (11) for the assessment of neutralizing antibodies to the Ad5hr vector in serially diluted macaque sera. The assay was carried out with the Ad5hr-WT virus and A549 cells as targets for infection. The neutralizing titer was defined as the reciprocal of the last serum dilution at which a cytopathic effect was not evident.

Assessment of cell-mediated immune responses. Cytotoxic T-cell (CTL) activity was monitored following double in vitro stimulation of effector cells as described elsewhere (65). Briefly, autologous B cells, obtained by herpesvirus papio transformation of PBMCs, were infected with vaccinia virus expressing either SIVmac251 Env (V194) (35) or SIVsmH4 Env and Gag (Wyeth-SIV[vJS-11]) (24) at a multiplicity of infection of 10 for 2 h at 37°C. Infected cells were centrifuged, resuspended in 1 ml of RPMI 1640 medium containing 10% fetal calf serum and antibiotics (R10 medium), and incubated at 37°C overnight. The next day, the cells were pooled, pelleted, and resuspended in 1 ml of glutaraldehyde and then used as stimulators. The stimulators were washed three times with 1× PBS, resuspended in 1 ml of R10 medium plus 10% interleukin-2 (IL-2), and added to effector PBMCs at an effector-to-stimulator (E:S) ratio of 10:1 in approximately 5 ml of the same medium. The effector cells were incubated at 37°C for 7 days, separated on LSM, and resuspended in approximately 5 ml of R10 plus 10% IL-2. Washed stimulators were added again at an effector-to-stimulator ratio of 10:1, and the cultures were incubated at 37°C for another week. The effector cells were subsequently used in a conventional CTL assay, as described previously, with autologous cells infected with vaccinia virus-SIVmac251 *env* and -SIVsmH4 *env* and *gag* as targets (8). Vaccinia virus expressing β-galactosidase (VSC-8) (9) served as control.

The antiviral activity of $CD8^+$ T cells was assayed by an endogenous suppression assay (42) modified from a previously described method (38) by the inclusion of CD28 costimulation (5). Briefly, macaque effector $CD8^+$ T cells were positively selected by using anti-CD8 antibody-coated immunomagnetic beads according to the manufacturer's instructions (Dynal, Lake Success, N.Y.). The $CD8^+$ cells were stimulated for 3 days in culture medium (R10 plus 10% IL-2) with goat antimouse IgG immunomagnetic beads (Dynal) coated with 2 μg of anti-CD3 (33) and 2 μg of anti-CD28 (Immunotech, Westbrook, Maine) antibodies per 10^7 beads. Target $CD4^+$ T cells were negatively selected from PBMCs of an SIVmac251-infected animal by using anti-$CD8^+$ antibody-coated immunomagnetic beads and stimulated for 3 days with phytohemagglutinin (PHA; 3 μg/ml) in culture medium. After the 3-day stimulation, the effector and target cell populations were pelleted, resuspended in culture medium, and distributed into replicate wells of a microtiter plate so that each well contained 7×10^4 SIV-infected target cells and various numbers of $CD8^+$ effector cells to give effector-to-target (E:T) ratios of 4:1, 2:1, 1:1, 0:5:1 and 0.25:1 in a total volume of 200 μl. Wells containing no effector cells served as controls. The plates were incubated for up to 14 days, and supernatants were collected on days 5, 7, 10, and 14. Replacement medium was added to the wells as necessary. The level of p27 in culture supernatants was measured by antigen capture assay (Coulter, Westbrooke, Maine). The percent suppression of SIV replication was determined by comparing the amount of p27 antigen in control wells containing target cells alone with the amount of p27 antigen in wells containing effector-target cocultures.

Assessment of humoral and mucosal immune responses. Neutralization assays using cell-free, lab-adapted SIVmac251 were carried out as previously described (8). Titers were expressed as the reciprocal of the serum dilution at which infectivity was 50% of the control value following normalization of the data to control infectivity levels in the presence of normal macaque serum. Antibodies able to neutralize primary isolates were also evaluated by using an infectivity reduction assay, modified from that described by Mascola et al. (45) by using PBMC target populations depleted of $CD8^+$ T cells. Briefly, human PBMCs were obtained by Ficoll-Hypaque separation and depleted of $CD8^+$ cells by the use of anti-$CD8^+$ immunomagnetic beads (Dynal). The remaining cells were adjusted to a concentration of 1×10^6 to 2×10^6/ml in R10. PHA-M (GIBCO-BRL) was added to a final dilution of 1:100, IL-2 (Boehringer-Mannheim, Indianapolis, Ind.) was added to a final concentration of 10%, and the cells were cultured at 37°C for 48 to 72 h. A primary virus stock of SIVmac251 was serially diluted in R10 plus 10% IL-2, and 20-μl aliquots were plated into wells of a 96-well microtiter plate in quadruplicate. Twenty microliters of test serum, heat inactivated at 56°C for 30 min and diluted 1:10 in R10 plus 10% IL-2, was added to each well, and the plate was incubated for 30 min at 37°C or 1 h at room temperature. The stimulated, $CD8^+$ T-cell-depleted PBMCs were washed and adjusted to a concentration of 10^7/ml, and 10 μl of the cell suspension was added to each well along with 50 μl of R10 containing 10% IL-2. Following a 24-h incubation at 37°C, the cells were washed three times with RPMI 1640 and resuspended in 100 μl of R10 containing 10% IL-2. Twenty microliters of each of these cell suspensions was transferred into wells of another microtiter plate, each containing 200 μl of R10 plus 10% IL-2. Following a 7-day incubation, cell supernatants were tested for SIV p27 by antigen capture assay (Coulter). Fifty percent infective doses (ID_{50}) were calculated by using computer software and the statistical method of Spearman-Karber (62). Fold reduction was calculated by dividing the ID_{50} obtained in the presence of a prebleed serum by the ID_{50} obtained in the presence of test serum.

For pepscan analysis, a series of 20-mer overlapping peptides (with a 14-amino-acid overlap) of SIVmac251 gp120 were synthesized in solid phase on 96-well crowns (Chiron Corporation, Emeryville, Calif.). The peptides were cleaved from the crowns with 0.1% trifluoroacetic acid, which was subsequently removed from the peptides by evaporation under nitrogen. The peptides were reconstituted in 0.1% glacial acetic acid at a concentration of 2 mg/ml. Microtiter plates were coated with 100 μl of the peptides at a concentration of 20 μg/ml in 50 mM bicarbonate buffer containing 4 μg/ml of bovine serum albumin. After incubation at 4°C, the plates were aspirated and blocked with 200 μl of 1.25% dry milk in water/well for 1 h at room temperature. One hundred microliters of macaque serum diluted 1:100 in DILSIM (Organon Teknika) was added to each of the wells, and the plates were incubated at 37°C for 1 h. The plates were washed four times with PBS-Tween 20 (0.5%). One hundred microliters of horseradish peroxidase-conjugated goat anti-human IgG was added to each well, and the plates were incubated at 37°C for 1 h. The wells were again washed four times with PBS-Tween 20, and TMB-peroxidase substrate (100 μl; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) was added to each. After 30 min at room temperature, the reaction was stopped by the addition of 100 μl of sulfuric acid and the plates were read at 450 nm.

For titrations of peptide 52, serial twofold dilutions of macaque serum beginning at 1:50 were assayed, as described above, on microtiter plates coated with 1 μg of peptide 52/well. Endpoint titers were defined as the reciprocal of the serum dilution at which the absorbance of the test serum was equivalent to the absorbance of the prebleed serum diluted 1:50.

Mucosal immune responses in vaginal, rectal, and nasal secretions were assessed for SIV gp120-specific IgA, IgG, and IgM antibodies by enzyme-linked immunosorbent assay (ELISA) as previously described (8).

Virologic assays to assess SIV infection. Virus isolations from macaque PBMCs on human PHA-stimulated PBMCs or on CEMX174 cells, assessment of SIV proviral DNA in macaque PBMCs by nested PCR using *gag* primers and probe, and quantification of SIV RNA in plasma by the nucleic acid sequence based amplification (NASBA) technique were carried out as described previously (8).

RESULTS

Outcome of first vaginal challenge with SIVmac251. As previously reported (8), following immunization with an Ad5hr-SIVenv recombinant priming-SIV gp120 boosting regimen and intravaginal challenge with a single dose of $>10^5$ $TCID_{50}$ of SIVmac251, both persistent and transient viremia were observed among immunized and control macaques. Transient viremia following intravaginal exposure to both SIV and SHIV has been well described (48, 49), and it complicated the interpretation of the vaccine challenge results. Nevertheless, it was clear that among persistently viremic animals, immunized macaques exhibited significantly lower viral burdens during the acute infection period than did the controls (8). To evaluate whether these lower viral loads would translate into delayed disease progression, the persistently viremic macaques were monitored long-term, up to 140 weeks postchallenge. As illustrated in Fig. 2, one of the four immunized macaques, no. 353, became a slow progressor. By week 10 postchallenge, SIV RNA was rarely detected in its plasma, and only at low levels. Virus could be isolated from the PBMCs of macaque 353 until week 28 but subsequently was isolated only once (at week 94) during the entire follow-up period (data not shown). The CD4-cell number remained stable until recently, when it dropped at week 134, signaling the onset of disease. In contrast, the other persistently viremic macaques all expressed SIV RNA continuously in plasma and exhibited significantly decreased CD4-cell numbers within a year of viral challenge. The remaining three immunized macaques (no. 355, 356, and 357) have died, as have two of the control macaques (no. 362 and 363). The remaining control animal (no. 360) has exhibited CD4-cell counts of 100 or less for over a year. Overall, immunized macaque 353 has exhibited a much slower disease course than the other persistently viremic animals.

Occult infection of macaques following vaginal infection with SIV has recently been reported (47), raising the possibility that the slow disease progression of macaque 353 was simply a manifestation of this phenomenon rather than a consequence of vaccination-induced immune responses. This does not seem to be the case, since in animals with natural occult infections, detection of virus or viral nucleic acids is very rare. In transiently viremic animals, virus can only be intermittently isolated during the first 10 weeks postexposure, and detection of proviral DNA by PCR techniques is generally limited to this initial period as well (48). Moreover, transiently viremic animals rarely, if ever, seroconvert (47, 48). In contrast, macaque 353 exhibited a solid acute SIV infection characterized by the presence of SIV RNA in the plasma for the first 6 weeks of the acute infection period and intermittently thereafter. Moreover, proviral DNA was consistently detected in PBMCs through week 32, and virus could be isolated for the first 28 weeks postchallenge (8). In addition, an increase in the neutralizing-antibody response was seen following challenge, as was also observed in the other immunized and persistently viremic macaques (Fig. 3). Two of the three persistently viremic control animals also developed neutralizing antibodies within 4 to 6 weeks, although due to the absence of immunization the titers were approximately 10-fold lower. The third control, macaque 363, failed to develop an antibody response within 10 weeks

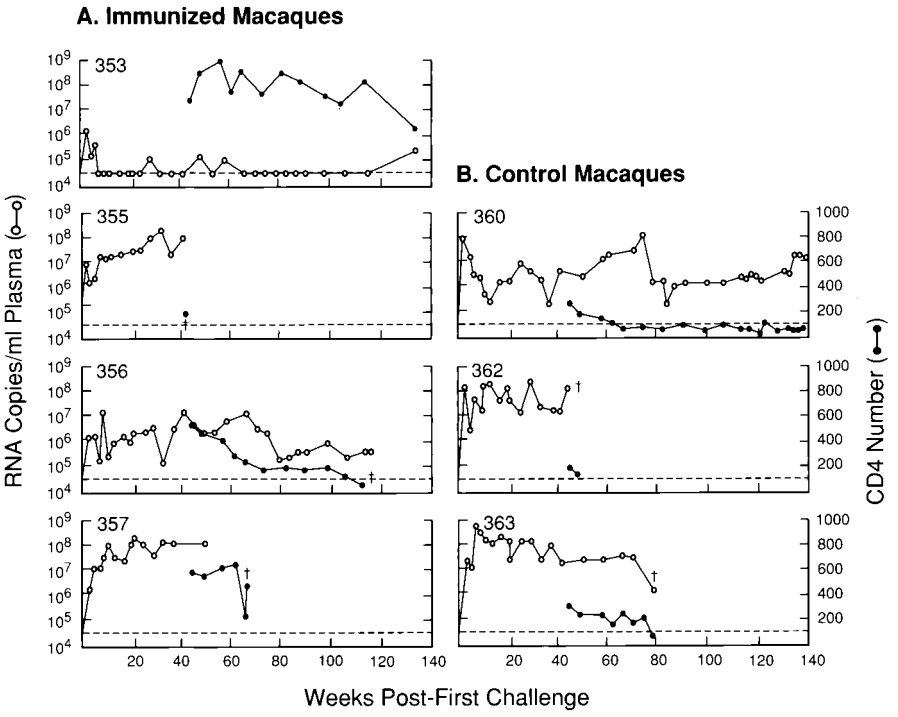

FIG. 2. Long-term follow-up of persistently viremic macaques following the first vaginal challenge with SIVmac251. The death of a macaque is indicated by a dagger symbol. The lower limit of detection of RNA by the NASBA technique is indicated by the dashed line. After its CD4-cell count had been approximately 100 for over 50 weeks, macaque 360 was treated with a peptide polymer modeling the CD4 binding site on SIV gp120 on four occasions (weeks 113, 117, 123, and 128) with no apparent effect on CD4-cell number or viral load (51a). Macaque 356 was similarly treated once at week 113 when its CD4-cell count was already below 100. It was euthanized at week 116.

but quickly exhibited a high viral burden of nearly 10^9 copies of RNA/ml of plasma (Fig. 2 and 3).

In contrast to the persistently viremic animals, immunized macaques 354 and 358 and control macaques 361 and 364 fulfilled the criteria of occult infection, exhibiting signs of viral infection on only one or two occasions, as previously reported (8). None of these four animals developed an increase in neutralizing antibodies following virus challenge. In fact, the immunized macaques possessed neutralizing titers of over 1,000 at the time of challenge but did not exhibit an anamnestic response following intravaginal virus exposure (Fig. 3).

Humoral immune responses at first challenge. Immune responses in the persistently viremic, immunized macaques at the time of the first vaginal challenge were compared in an attempt to determine if the slower disease progression in macaque 353 was associated with a particular immunological parameter. As shown in Table 1, there was no clear correlation between titers of neutralizing antibodies and disease progression. In fact, the slow progressor macaque 353 had the lowest neutralizing-antibody titer for lab-adapted SIVmac251 among the persistently infected macaques. Serum of macaque 353 did exhibit the greatest ability to inhibit infection of the SIV251 primary isolate; however, the difference in infectious titer compared to the other progressor macaques was minimal.

To further compare the humoral immune responses of the persistently viremic, immunized macaques, we carried out a pepscan analysis, reacting macaque sera against a series of overlapping peptides of SIVmac251 gp120 by ELISA. As shown in Fig. 4A, the slow progressor macaque 353 exhibited a broad pattern of reactivity, but the linear envelope epitopes recognized were not appreciably different from those observed in progressing macaques. The only correlation observed between two persistently viremic macaques which progressed rapidly (macaques 355 and 357) compared to the slow progressor 353 and macaque 356, which exhibited a moderate rate of progression and survived 116 weeks postchallenge, was in reactivity to peptide 52. Macaques 355 and 357, which died quickly, had poor reactivity to peptide 52, exhibiting low serum antibody titers to this peptide, while the other two macaques had approximately four- to sixfold higher titers (Fig. 4A; Table 1). Peptide 52, with the amino acid sequence PVTIMSGLVF HSQPINDRPK, represents the central region of the V3 loop, which in SIV is highly conserved among isolates (36).

Cellular immune responses at first challenge. Unfortunately, autologous B lymphocytes of macaque 353 could not be transformed by herpesvirus papio. Therefore, a possible contribution of a strong CTL response at the time of the first challenge to development of slow disease progression could not be assessed in comparison to the other persistently viremic animals. Assays for $CD8^+$ T-cell suppressor activity also were not carried out at this time. However, T-cell proliferative responses against a variety of SIV and HIV-2 antigens were measured and, as summarized in Table 1, were seen in all persistently viremic, immunized macaques over the immunization course prior to challenge. The animal with the broadest proliferative response, however, was macaque 355, which died the earliest. Thus, T-cell proliferation could not be correlated with slow disease progression.

Mucosal immune responses at first challenge. Antibody reactivities in secretory fluids were evaluated as a measure of mucosal immunity. As previously reported (8), virus-specific IgG was observed in nasal, rectal, and vaginal secretions of the immunized macaques over the course of the immunization period. Virus-specific IgA was observed only in nasal and rectal

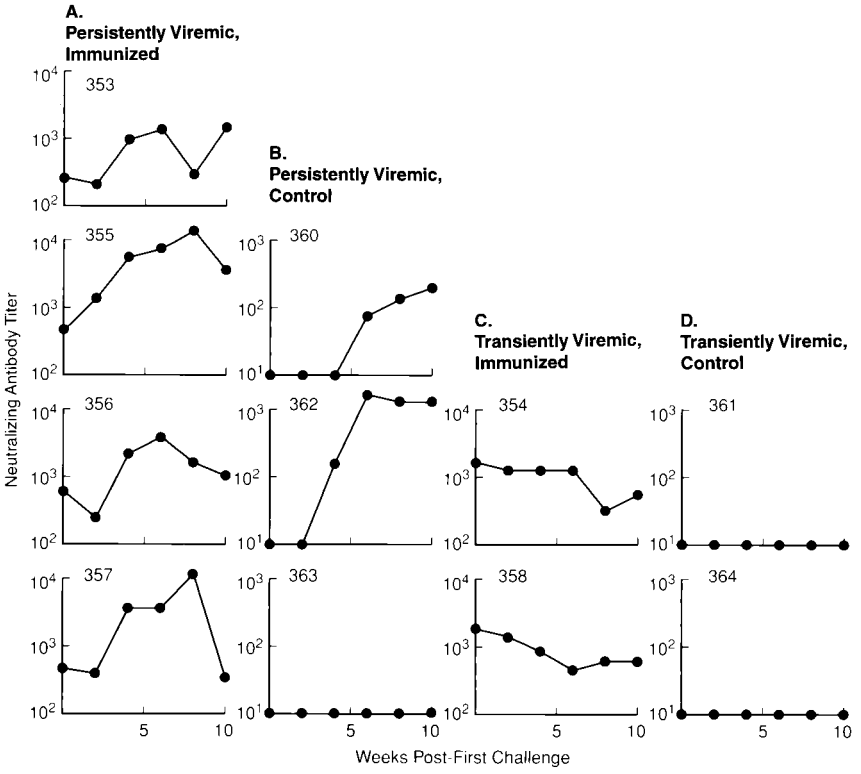

FIG. 3. Neutralization of lab-adapted SIVmac251 by macaque sera after the first vaginal challenge. Neutralizing-antibody assays were carried out over 10 weeks postchallenge as described in Materials and Methods.

secretions, never in vaginal fluids. As summarized in Table 1, at the time of the first vaginal challenge, only macaques 356 and 354 had significant anti-SIVenv IgG in their vaginal secretions. Macaque 353 had poor secretory immune responses at the time of challenge, which therefore did not correlate with the slow disease progression of this animal.

Reboost and rechallenge of the transiently viremic macaques. Transient viremia and occult SIV infection of macaques are generally observed following a low-dose virus exposure. Therefore, to further evaluate the protective efficacy of the combination vaccine regimen, the animals exhibiting transient viremia following the first virus challenge were rechallenged vaginally with a higher dose of SIVmac251. The two transiently viremic control macaques and the two transiently viremic immunized macaques were first reboosted either with the Ad5hr-WT vector and alum or with the Ad5hr-SIVenv recombinant and gp120 protein as outlined in Fig. 1. Replication of the Ad5hr-WT and Ad5hr-SIVenv recombinant vectors subsequently was assessed by examining the shedding of Ad5hr DNA in stool and nasal secretions of the macaques by nested DNA PCR specific for the Ad5 fiber gene as described in Materials and Methods. Ad5hr DNA could not be detected in stool samples. However, Ad5hr DNA was shed in the nasal secretions of immunized macaque 354 for a 4-day period. Shedding was not seen in any of the other macaques. Consistent with the poor replication of the Ad5hr vectors following this third administration, increases in Ad5 neutralizing-antibody titers of 1 doubling dilution or more were not seen at all in the control animals. Ad5 neutralizing-antibody titers in immunized macaque 354 increased from 32 at the time of booster immunization to 64 at the time of challenge, and in immunized macaque 358 they increased from 12 to 128 over the same time period.

To determine if the reboosting reactivated latent SIV in the transiently viremic animals, plasma samples obtained from each animal 1 and 2 weeks following the reboost were assessed for viral RNA by the NASBA technique. SIV RNA was not detected in any of the samples (data not shown), indicating that reactivation had not occurred.

Two weeks following the booster immunization and 66 weeks following the first single intravaginal SIV exposure, the transiently viremic macaques were rechallenged intravaginally with two sequential doses of $>10^5$ $TCID_{50}$ of the virulent SIVmac251, one in the morning and one in the evening. As summarized in Fig. 5, the four macaques became persistently viremic. Virus was isolated from their PBMCs, and viral RNA appeared in the plasma with typical kinetics. The animals were monitored for 70 weeks following this second challenge. As illustrated, macaque 354 maintained a stable CD4-cell number for nearly a year following infection, with a significant decline occurring only at week 48 after the second challenge. In line with this slower disease progression, virus could be isolated from the blood of this animal for 8 weeks postchallenge but not thereafter. In contrast, the other three macaques displayed immediate drops in CD4-cell numbers. Virus isolation was sporadic in macaque 358 (Fig. 5), while the control macaques, 361 and 364, exhibited more-consistent virus isolation and higher viral burdens. These control animals died at weeks 73 and 57 postchallenge, respectively.

Humoral immune responses at rechallenge. Factors associated with the slower disease progression of macaque 354 relative to the other animals were investigated. The two immu-

nized transiently viremic macaques, 354 and 358, had the highest overall neutralizing-antibody titers, including inhibition of a primary SIV251 isolate by using an infectivity reduction technique, at the time of the first challenge (Table 2). However, since transient viremia was also seen among the controls lacking antibody, this parameter could not be correlated with their development of occult infection. Neutralizing-antibody responses were subsequently assessed following the rebooster immunization. Both macaques 354 and 358 had persistent neutralizing antibody resulting from their previous immunization history. At the time of the reboost, macaque 354 had a neutralizing-antibody titer against lab-adapted SIVmac251 of 870 and macaque 358 had a titer of 510. Both animals displayed significant responses to the booster immunization, with approximate 10-fold increases in neutralizing-antibody titers 2 weeks later, at the time of the second challenge (Table 2). However, at the second challenge, macaque 354 was able to strongly neutralize primary SIVmac251, as shown in an infectivity reduction assay, whereas macaque 358 (as well as the control macaques) could not (Table 2).

Although the patterns of reactivity against linear SIV peptides in a pepscan assay for macaques 354 and 358 were similar (Fig. 4), a higher titer against peptide 52 was seen for macaque 354 (Table 2), in line with its slower disease progression as discussed above for persistently viremic animals 353 and 356.

Cell-mediated immune responses at rechallenge. CTL activity was assessed by chromium release assay following the reboost, as described in Materials and Methods, using double in vitro stimulation of effector cells. As summarized in Fig. 6, in spite of the relatively poor replication of the Ad5hr-SIVenv recombinant following the reboost, both immunized macaques developed significant CTL activities of 10 to 15% over background lysis by the day of challenge. Of interest is the fact that the activity was greatest against target cells infected with vaccinia virus-SIVsm rather than vaccinia virus-SIV251. The Ad5hr-SIVenv recombinant contains the SIVsm sequence. It is also notable that macaque 354, which exhibited the highest level of Ad5hr replication as indicated by shedding in nasal secretions, also displayed the highest CTL activity. Control macaque 364 was not able to lyse vaccinia virus-SIVsm-infected autologous target cells and showed only minimal killing of vaccinia virus-SIV251. CTL activity of control macaque 361 could not be assessed due to the inability of herpesvirus papio to transform autologous B cells.

The ability of macaque $CD8^+$ T cells to suppress SIV replication in macaque $CD4^+$ lymphocytes infected with SIV in vivo was also evaluated. As illustrated in Fig. 7, macaque 358 exhibited good $CD8^+$ T-cell antiviral activity, both at the time of challenge and following SIV challenge. In contrast, macaque 354 showed no $CD8^+$ T-cell suppressor activity at any of the three time points tested, and control macaque 364 exhibited only minimal inhibitory activity. Thus, the slower disease progression of macaque 354 could not be attributed to this non-major histocompatibility complex-restricted activity.

Mucosal immune responses at rechallenge. SIV-specific IgG antibody responses were seen at nasal and rectal sites of the two immunized macaques after the reboost, with macaque 354 exhibiting the highest level of response 1 week following the immunization and macaque 358 showing the highest level of response 2 weeks after the boost at the time of challenge (Fig. 8). Modest increases in SIV-specific IgA were also seen 2 weeks following the reboost in both animals. Overall, the more slowly progressing macaque 354 did not exhibit higher levels of mucosal immune response at challenge than macaque 358. Notably, SIV-specific antibodies, both IgG and IgA, were absent in vaginal fluids of both animals.

TABLE 1. Immune responses of persistently viremic, immunized macaques at the time of the first SIVmac251 challenge

Macaque no.	Disease outcome	Immune response							
		Humoral			Cellular	Mucosal[b]			
		Neutralizing antibody				IgG		IgA	
		Lab-adapted SIVmac251 (titer)	Primary SIVmac251 (fold reduction)	Binding antibody to Peptide 52 (titer)	T-cell proliferation[a] (relative SI)	Nasal (absorbance)	Rectal (absorbance)	Vaginal (absorbance)	Nasal (absorbance)	Rectal (absorbance)
353	Slow progressor	395	3.8	3,120	++	0	0	0	0.2	0
356	Progressor	795	2.1	2,000	+++	0.3	1.3	1.3	1.2	0
355	Rapid progressor	645	2.2	520	++++	0	1.0	0.2	0	0.7
357	Rapid progressor	580	1.4	400	+	0.7	0	0.6	0	bc[c]

[a] T-cell proliferation (8) is summarized as follows: +, 1 to 5 positive stimulation indices (SIs) over the immunization course; ++, 6 to 10 positive SIs; +++, 11 to 15 positive SIs; ++++, >15 positive SIs.
[b] Prebleed absorbance values have been subtracted from values obtained at challenge at mucosal sites.
[c] bc, blood contamination.

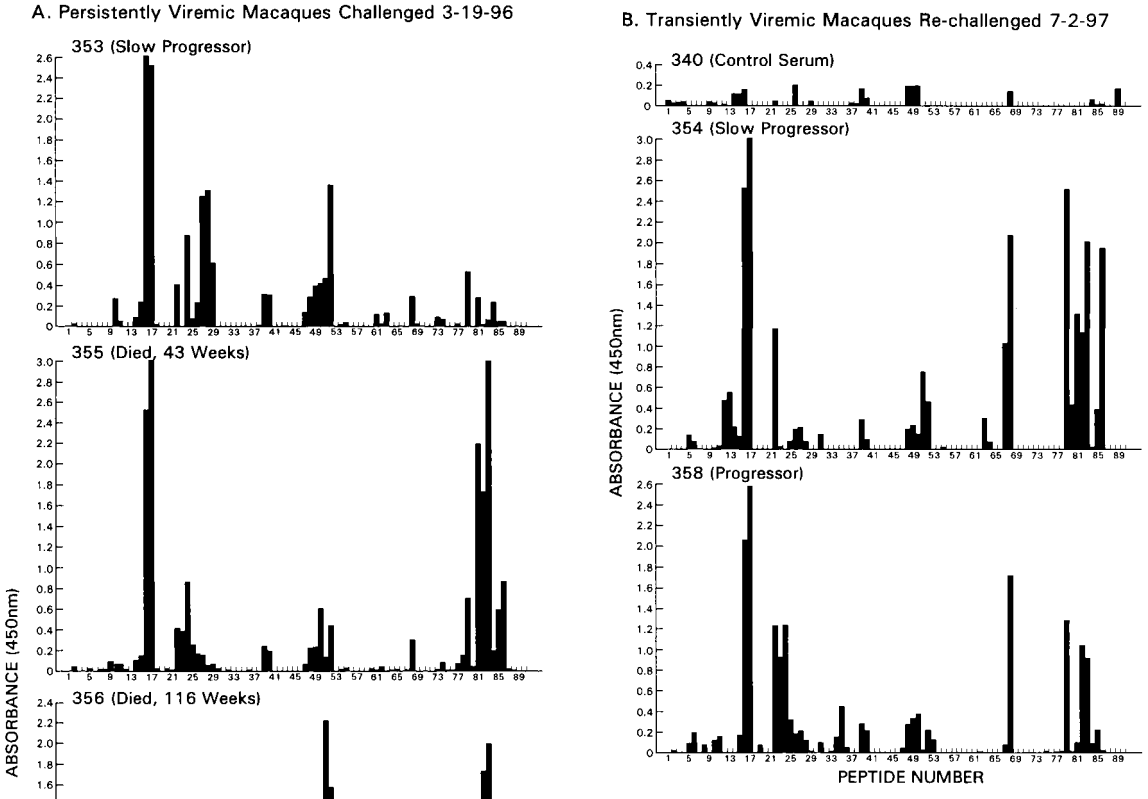

FIG. 4. Pepscan analysis of macaque sera, using SIVmac251 gp120. Sera of persistently viremic immunized macaques were subjected to an ELISA at the time of the first challenge (19 March 1996), and transiently viremic immunized macaques were assayed at the time of the rechallenge (2 July 1997).

DISCUSSION

We have previously reported that following an Ad5hr-SIV-env priming-gp120 boosting regimen, immunized macaques which became persistently viremic after intravaginal challenge nevertheless displayed significantly decreased viral burdens during the first 18 to 20 weeks postchallenge compared to control animals (8). To determine whether this decreased viral load would ultimately translate into delayed disease progression, the animals were followed long-term. One of the four immunized but persistently viremic animals, macaque 353, was a slow disease progressor, displaying stable CD4-cell counts and plasma viral RNA at the threshold of detection for 115 weeks postchallenge. CD4-cell counts of macaque 353 began to decline only at week 134, coincident with a modest rise in plasma RNA levels. In contrast, the control macaques exhibited falling CD4-cell numbers by 40 weeks postchallenge and sustained high viral burdens. Two of the control animals died, at weeks 47 and 79. The third control remains alive at the time of writing has displayed CD4-cell counts of approximately 100 for the previous 80 weeks. The other three immunized macaques have died, two rapidly at weeks 43 and 67 and one after a slower disease course at week 116. Thus, following the initial SIVmac251 challenge, one of four immunized animals exhibited a significant level of protection, even though sterilizing immunity was not obtained.

To elucidate the basis for the partial protection of macaque 353, we reexamined several immunologic parameters at the time of challenge and compared them with those of the three other immunized animals. Neutralization of lab-adapted SIV was not correlated with slow disease progression. Moreover, while macaque 353, among the four persistently viremic and immunized animals, exhibited the greatest ability to neutralize the SIVmac251 primary isolate, the fold reduction in infectious titer in the presence of macaque 353 serum was not quite twofold greater than that displayed by the other immunized animals. Therefore, it is debatable whether this difference translated into meaningful protection in vivo, where serum concentrations would be 10-fold higher. Of interest was the observation that macaque 353 as well as macaque 356, which exhibited a somewhat delayed disease progression, had higher antibody titers to a linear peptide, no. 52, in the V3 region of

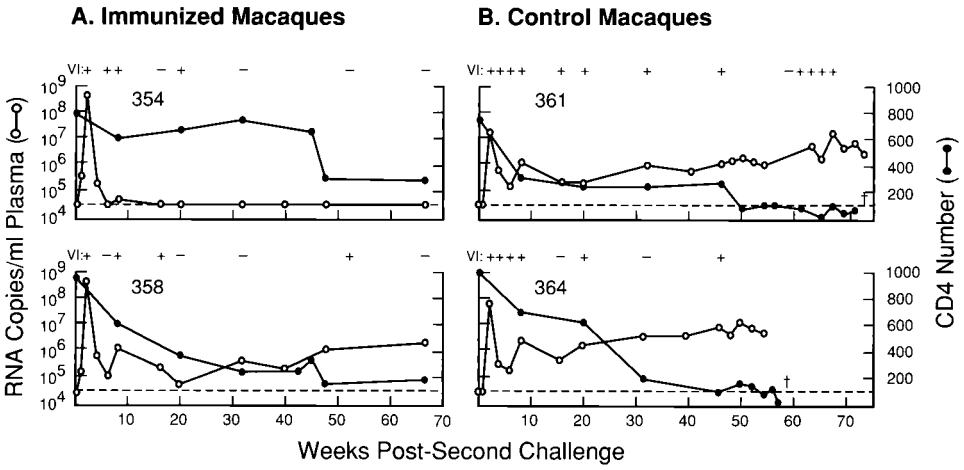

FIG. 5. Outcome of second vaginal challenge of macaques transiently viremic following the first challenge. Results of virus isolation attempts (VI) following the rechallenge are shown (+, virus isolation positive; −, virus isolation negative). Control macaques 364 and 361 were also treated with the peptide polymer modeling the CD4 binding site on SIV gp120, as outlined in the legend to Fig. 2, on three and four occasions, respectively (weeks 46, 50, 56, and 61), with no apparent benefit. The macaques subsequently were euthanized at weeks 57 and 73, respectively.

SIV gp120 than the two more rapidly progressing macaques. The V3 region of SIV was originally thought to lack any linear neutralizing epitopes (28, 56), unlike the V3 region of HIV, which is a major neutralization site (23, 46, 50, 59). However, weakly neutralizing monoclonal antibodies have been generated against a linear peptide that includes the amino acids of peptide 52 (34), and more recently a synthetic peptide with the sequence MSGLVFHSQPINDRPKQAWC was shown to elicit a broadly reactive neutralizing antibody with a high titer in goats (51). In this latter report, however, SIVmac251-infected macaques did not exhibit any antibodies that reacted with this peptide. Thus, this linear epitope appears to be weakly immunogenic in monkeys. Amino acids of the epitope, underlined in the peptide above, are also present in peptide 52 described in the present study, which has in addition the amino-terminal residues PVTI. It is possible that neutralizing antibody mediated by this epitope contributed to the slow progression of macaque 353 and the delayed death of macaque 356.

Unfortunately, CTL responses could not be evaluated in macaque 353, and $CD8^+$ T-cell-mediated suppressive activity was not assessed. The only possible comparison of cell-mediated immunity among the persistently viremic, immunized macaques, therefore, was T-cell proliferative responses. Such responses have been associated with long-term nonprogression in HIV-infected humans (58). Macaque 353 exhibited an intermediate level of T-cell proliferative response which could not be correlated with its slow disease progression. In addition, at the time of challenge, this animal displayed poor mucosal immune responses which also did not correlate with the slow progression. Therefore, while macaque 353 exhibited a good humoral immune response, the basis for its slow disease progression could not be determined.

Among the four transiently viremic macaques after the first SIVmac251 challenge, the immunized animals (no. 354 and 358) exhibited neutralizing-antibody activity which was higher than that seen in immunized animals which became persistently viremic (Table 1). In an effort to determine if macaques 354 and 358 could better resist intravaginal infection than mock-immunized controls, we conducted a second challenge experiment using two sequential SIVmac251 vaginal exposures. Prior to the rechallenge, the immunized macaques were reboosted and the full complement of immune responses, including functional CD8 T-cell-mediated activities, were evaluated. While both immunized macaques exhibited high levels of neutralizing-antibody activity against the lab-adapted SIV isolate, the more slowly progressing macaque, no. 354, was also able to strongly neutralize the primary SIV isolate. The serum

TABLE 2. Immune responses of transiently viremic, immunized macaques at the time of the first and second SIVmac251 challenges

Challenge	Macaque no.	Disease outcome	Immune response						
			Humoral			Cellular			
			Neutralizing antibody		Binding antibody to Peptide 52 (titer)	T-cell proliferation (relative SI)	CTL		$CD8^+$ T-cell suppression (%)[b]
			Lab-adapted SIVmac251 (titer)	Primary SIVmac251 (fold reduction)			SIV251 targets (% lysis)[a]	SIVsm targets (% lysis)[a]	
First	354	Transient viremia	2,255	8.0	ND[c]	+++	2.1	0	ND
	358	Transient	2,335	4.6	ND	+	1.6	0	ND
Second	354	Slow progressor	7,835	34.5	1,020	ND	1	15.1	0
	358	Slow progressor	4,415	1.4	235	ND	5.1	10.6	75

[a] Percent lysis by CTL is reported at E:T ratios of 100:1 following subtraction of percent lysis obtained using control target cells expressing β-galactosidase.
[b] Values of percent $CD8^+$-cell suppression are reported at E:T ratios of 1:1.
[c] ND, not done.

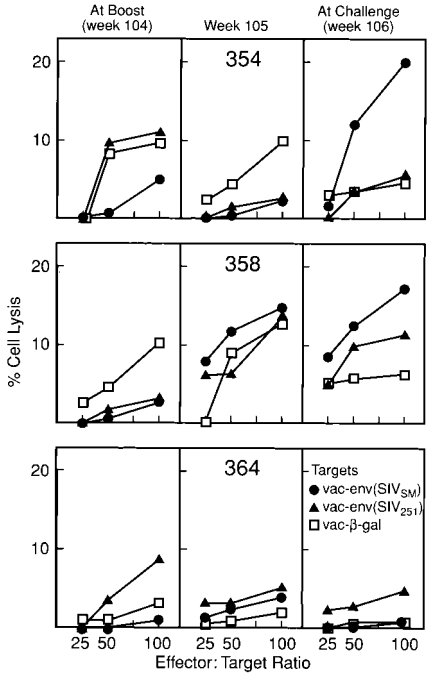

FIG. 6. CTL activity after reboost and at the time of the second vaginal challenge. Assays were carried out on viably frozen lymphocytes. vac, vaccinia virus recombinant containing the indicated gene insert.

of this animal also exhibited a higher binding titer against peptide 52. Taken together, the results indicated that this macaque possessed a strong, broad antibody response which likely contributed to its slower disease progression. Antibody responses have been shown to play a role in protection of chimpanzees against HIV infection (7, 14, 20, 41, 57, 68). In the macaque system, they have been shown to participate in more-rapid viral clearance (27). Moreover, passive transfer of immune serum has protected macaques from infection (12, 39, 54, 60). Primary-isolate neutralization and the development of a mature antibody response have also been correlated with protection of macaques against SIV infection (13, 67). In contrast, macaques unable to mount an antibody response follow-

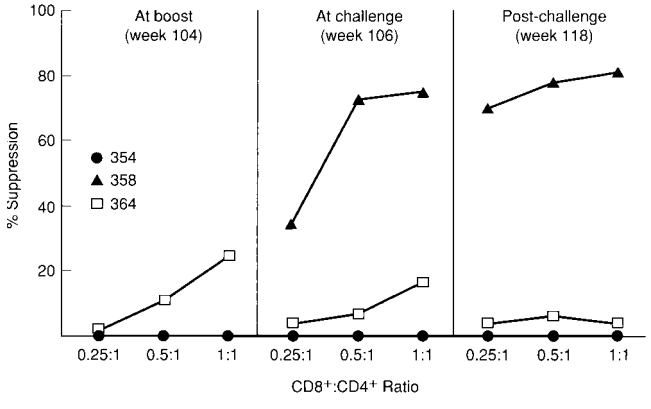

FIG. 7. $CD8^+$ T-cell antiviral activity pre- and post-second challenge. Assays were carried out on viably frozen lymphocytes. Suppression by macaque 361 PBMCs could not be determined because control levels of infection in the endogenous assay were too low. Additional PBMCs of macaque 361 were not available for reassay.

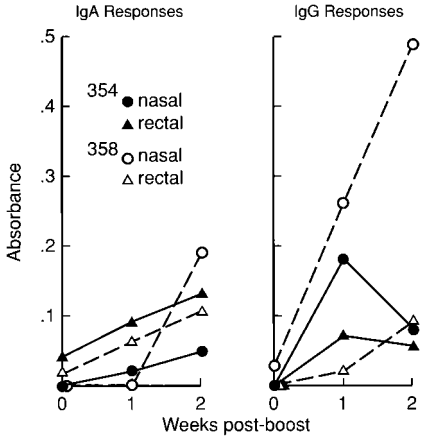

FIG. 8. Antibody reactivity of macaque sera to SIV gp120 in secretory fluids at the time of the reboost and rechallenge. No binding antibodies were detected in vaginal fluids.

ing exposure to SIV or SHIV quickly develop virulent infections and progress rapidly to AIDS and death (40, 61, 63).

Both immunized macaques exhibited significant CTL activity following the reboost. This is notable in view of the fact that both had previously received the same Ad5hr-SIVenv recombinant on two occasions. Thus, preexisting antibody to the recombinant vector did not preclude a boosting effect, since a cellular immune response to the inserted gene product was elicited. Sequential boosting with Ad recombinants based in different serotypes should theoretically induce better immune responses as a result of better recombinant replication. This remains to be determined in future experiments. The viral specificity of the CTL response elicited was evidence by the fact that SIVsm target cells rather than SIV251 target cells were recognized and killed. The SIVenv gene present in the Ad5hr recombinant immunogen was derived from the SIVsm virus.

Macaque 354 exhibited a slightly higher level of CTL killing than macaque 358, providing some correlation with the slower progression of disease in the former animal than in the latter. Overall, both CTL responses likely contributed to the greater longevity of the immunized macaques compared to the controls. Previous studies have associated development of CTL with protection against HIV and SIV in chimpanzees and macaques, respectively (17, 22, 41, 53).

In contrast to strong humoral and CTL responses, assessments of $CD8^+$-T-cell suppressive activity and mucosal immune responses did not demonstrate any correlation with slower disease progression in these reboosted and rechallenged animals. We have previously shown that high levels of antiviral activity present postchallenge correlate with decreased viremia and a slow rate of disease progression and that the viral burden is lower and the eventual disease outcome is better if prechallenge activity levels are also high (38). While this study lacked the statistical power to investigate such an association, macaque 358 likely benefited from both effects. Such antiviral activity, together with β-chemokines, has been associated with protection of macaques against SIV challenge (37, 66).

With regard to mucosal immunity, it is again notable that antibody levels at nasal and rectal sites were boosted, albeit modestly, following the third immunization with the Ad5hr SIVenv recombinant and native gp120, suggesting the occurrence of effective boosting at mucosal inductive sites in spite of

minimal recombinant replication (Fig. 8). However, neither immunized animal exhibited vaginal SIV-specific IgA or IgG at the time of challenge. One would expect mucosal immune responses to exert their effect at the time of viral exposure, and in fact, SIV-specific antibody in vaginal secretions has been associated with protection from a vaginal SIV challenge (44). However, sterilizing immunity was not seen here, perhaps due in part to a lack of antibodies at the vaginal site. It is not surprising that SIV-specific antibodies present at other secretory sites did not influence initial infection. Once systemic infection was established, these immune responses would be expected to have little effect on the rate of progression to AIDS and death.

Overall, we have demonstrated that following immunization with a combination vaccine regimen based on priming with an Ad-SIV envelope recombinant and boosting with an envelope protein, reduction in viral load and slow disease progression were achieved in two of six immunized macaques. No single immune response was associated with the better outcome. Rather, both strong antibody and CTL responses to the SIV envelope immunogens were observed. Others have shown that vaccines incorporating core viral components in addition to the envelope have greater protective efficacy (26). Subsequent studies will assess the utility of such multicomponent Ad-vectored vaccines in eliciting better protection.

ACKNOWLEDGMENTS

We thank Michael Rosenzweig for generously providing anti-CD3 monoclonal antibody reactive with rhesus CD3 and Ruth Woodward for carrying out the macaque experiments.

This study was supported in part by a Cooperative Research and Development Agreement (no. 0016) between the National Cancer Institute and Lederle-Praxis Biologicals Division, American Cyanamid Company.

REFERENCES

1. **Alexander, N. J.** 1990. Sexual transmission of human immunodeficiency virus: virus entry into the male and female genital tract. Fertil. Steril. **54:**1–18.
2. **Almond, H., K. Kent, M. Cranage, E. Rud, B. Clarke, and E. J. Stott.** 1995. Protection by attenuated simian immunodeficiency virus in macaques against challenge with virus-infected cells. Lancet **345:**1342–1344.
3. **Baba, T. W., Y. S. Jeong, D. Pennick, R. Bronson, M. F. Greene, and R. M. Ruprecht.** 1995. Pathogenicity of live, attenuated SIV after mucosal infection of neonatal macaques. Science **267:**1820–1825.
4. **Baba, T. W., V. Liska, A. H. Khimani, N. B. Ray, P. J. Dailey, D. Pennick, R. Bronson, M. F. Greene, H. M. McClure, L. N. Martin, and R. M. Ruprecht.** 1999. Live attenuated, multiply deleted simian immunodeficiency virus causes AIDS in infant and adult macaques. Nat. Med. **5:**194–203.
5. **Barker, E., K. N. Bossart, S. H. Fujimura, and J. A. Levy.** 1997. CD28 costimulation increases $CD8^+$ cell suppression of HIV replication. J. Immunol. **159:**5123–5131.
6. **Benson, J., C. Chougnet, M. Robert-Guroff, D. Montefiori, P. Markham, G. Shearer, R. C. Gallo, M. Cranage, E. Paoletti, K. Limbach, D. Venzon, J. Tartaglia, and G. Franchini.** 1998. Recombinant vaccine-induced protection against the highly pathogenic simian immunodeficiency virus SIV_{mac251}: dependence on route of challenge exposure. J. Virol. **72:**4170–4182.
7. **Berman, P. W., T. J. Gregory, L. Riddle, G. R. Nakamura, M. A. Champs, J. P. Porter, R. M. Wurm, R. D. Hershberg, E. K. Cobb, and J. W. Eichberg.** 1990. Protection of chimpanzees from infection by HIV-1 after vaccination with recombinant gp120 but not gp160 glycoproteins. Nature **345:**622–625.
8. **Buge, S. L., E. Richardson, S. Alipanah, P. Markham, S. Cheng, N. Kalyan, C. J. Miller, M. Lubeck, S. Udem, J. Eldridge, and M. Robert-Guroff.** 1997. An adenovirus-simian immunodeficiency virus *env* vaccine elicits humoral, cellular, and mucosal immune responses in rhesus macaques and decreases viral burden following vaginal challenge. J. Virol. **71:**8531–8541.
9. **Chakrabarti, S., K. Brechling, and B. Moss.** 1985. Vaccinia virus expression vector: coexpression of β-galactosidase provides visual screening of recombinant virus plaques. Mol. Cell Biol. **5:**3403–3409.
10. **Cheng, S.-M., S.-G. Lee, M. Ronchetti-Blume, K. P. Virk, S. Mizutani, J. W. Eichberg, A. Davis, P. P. Hung, V. M. Hirsch, R. M. Chanock, R. H. Purcell, and P. R. Johnson.** 1992. Coexpression of the simian immunodeficiency virus Env and Rev proteins by a recombinant human adenovirus host range mutant. J. Virol. **66:**6721–6727.
11. **Chengalvala, M., M. D. Lubeck, A. R. Davis, S. Mizutani, K. Molnar-Kimber, J. Morin, and P. P. Hung.** 1991. Evaluation of adenovirus type 4 and type 7 recombinant hepatitis B vaccines in dogs. Vaccine **9:**485–489.
12. **Clements, J. E., R. C. Montelaro, M. C. Zink, A. M. Amedee, S. Miller, A. M. Trichel, B. Jagerski, D. Hauer, L. N. Martin, R. P. Bohm, and M. Murphey-Corb.** 1995. Cross-protective immune responses induced in rhesus macaques by immunization with attenuated macrophage-tropic simian immunodeficiency virus. J. Virol. **69:**2737–2744.
13. **Cole, K. S., J. L. Rowles, B. A. Jagerski, M. Murphey-Corb, T. Unangst, J. E. Clements, J. Robinson, M. S. Wyand, R. C. Desrosiers, and R. C. Montelaro.** 1997. Evolution of envelope-specific antibody responses in monkeys experimentally infected or immunized with simian immunodeficiency virus and its association with the development of protective immunity. J. Virol. **71:**5069–5079.
14. **Conley, A. J., J. A. Kessler II, L. J. Boots, P. M. McKenna, W. A. Schleif, E. A. Emini, G. E. Mark III, H. Katinger, E. K. Cobb, S. M. Lunceford, S. R. Rouse, and K. K. Murthy.** 1996. The consequence of passive administration of an anti-human immunodeficiency virus type 1 neutralizing monoclonal antibody before challenge of chimpanzees with a primary virus isolate. J. Virol. **70:**6751–6758.
15. **Cranage, M., A. Baskerville, L. A. E. Ashworth, M. Dennis, N. Cook, S. Sharpe, G. Farrar, J. Rose, P. A. Kitchin, and P. J. Greenaway.** 1992. Intrarectal challenge of macaques vaccinated with formalin-inactivated simian immunodeficiency virus. Lancet **i:**273–274.
16. **Cranage, M. P., A. Baskerville, L. A. E. Ashworth, M. Dennis, N. Cook, R. Cook, S. A. Sharpe, J. Rose, P. Kitchin, and P. J. Greenaway.** 1992. Mucosal infection and vaccine studies with macaque SIV. Vaccine Res. **1:**311–318.
17. **Cranage, M. P., A. M. Whatmore, S. A. Sharpe, N. Cook, N. Polyanskaya, S. Leech, J. D. Smith, E. W. Rud, M. J. Dennis, and G. A. Hall.** 1997. Macaques infected with live attenuated SIVmac are protected against superinfection via the rectal mucosa. Virology **229:**143–154.
18. **Daniel, M. D., F. Kirchhoff, S. C. Czajak, P. K. Sehgal, and R. C. Desrosiers.** 1992. Protective effects of a live-attenuated SIV vaccine with a deletion in the *nef* gene. Science **258:**1938–1941.
19. **DeSchryver, A., and A. Meheus.** 1990. Epidemiology of sexually transmitted diseases: the global picture. Bull. W.H.O. **68:**639–654.
20. **Emini, E. A., W. A. Schleif, J. H. Nunberg, A. J. Conley, Y. Eda, S. Tokiyoshi, S. D. Putney, S. Matsushita, K. E. Cobb, C. M. Jett, J. W. Eichberg, and K. K. Murthy.** 1992. Prevention of HIV-1 infection in chimpanzees by gp120 V3 domain-specific monoclonal antibody. Nature **355:**728–730.
21. **Forrest, B. D.** 1992. The need for consideration of mucosal immunity in vaccine approaches to AIDS. Vaccine Res. **1:**137.
22. **Gallimore, A., M. Cranage, N. Cook, N. Almond, J. Bootman, E. Rud, P. Silvera, M. Dennis, T. Corcoran, J. Stott, A. McMichael, and F. Gotch.** 1995. Early suppression of SIV replication by $CD8^+$ nef-specific T cells in vaccinated macaques. Nat. Med. **1:**1167–1173.
23. **Goudsmit, J., C. Debouck, R. H. Meloen, L. Smit, M. Bakker, D. M. Asher, A. V. Wolff, C. J. Gibbs, Jr., and C. Gajdusek.** 1988. Human immunodeficiency virus type 1 neutralization epitope with conserved architecture elicits early type-specific antibodies in experimentally infected chimpanzees. Proc. Natl. Acad. Sci. USA **85:**4478–4482.
24. **Hirsch, V. M., T. R. Fuerst, G. Sulter, M. W. Carroll, L. C. Yang, S. Goldstein, M. Piatak, Jr., W. R. Elkins, W. G. Alvord, D. C. Montefiori, B. Moss, and J. D. Lifson.** 1996. Patterns of viral replication correlate with outcome in simian immunodeficiency virus (SIV)-infected macaques: effect of prior immunization with a trivalent SIV vaccine in modified vaccinia virus Ankara. J. Virol. **70:**3741–3752.
25. **Hospedales, J.** 1989. Heterosexual spread of HIV infection. Rev. Infect. Dis. **11:**663–665.
26. **Hu, S. L., P. Polacino, V. Stallard, J. Klaniecki, S. Pennarthu, B. M. Travis, L. Misher, H. Kornas, A. J. Langlois, W. R. Morton, and R. E. Benveniste.** 1996. Recombinant subunit vaccine as an approach to study correlates of protection against primate lentivirus infection. Immunol. Lett. **51:**115–119.
27. **Igarashi, T., C. Brown, A. Azadegan, N. Haigwood, D. Dimitrov, M. A. Martin, and R. Shibata.** 1999. Human immunodeficiency virus type 1 neutralizing antibodies accelerate clearance of cell-free virions from blood plasma. Nat. Med. **5:**211–216.
28. **Javaherian, K., A. J. Langlois, S. Schmidt, M. Kaufmann, N. Cates, J. P. M. Langedijk, R. H. Meloen, R. C. Desrosiers, D. P. W. Burns, D. P. Bolognesi, G. J. LaRosa, and S. D. Putney.** 1992. The principal neutralization determinant of simian immunodeficiency virus differs from that of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA **89:**1418–1422.
29. **Joag, S. V., I. Adany, Z. Li, L. Foresman, D. M. Pinson, C. Wang, E. B. Stephens, R. Raghavan, and O. Narayan.** 1997. Animal model of mucosally transmitted human immunodeficiency virus type 1 disease: intravaginal and oral deposition of simian/human immunodeficiency virus in macaques results in systemic infection, elimination of $CD4^+$ T cells, and AIDS. J. Virol. **71:**4016–4023.
30. **Joag, S. V., Z. Q. Liu, E. B. Stephens, M. S. Smith, A. Kumar, Z. Li, C. Wang, D. Sheffer, F. Jia, L. Foresman, I. Adany, J. Lifson, H. M. McClure, and O. Narayan.** 1998. Oral immunization of macaques with attenuated vaccine

virus induces protection against vaginally transmitted AIDS. J. Virol. **72:** 9069–9078.
31. **Johnson, A. M.** 1988. Heterosexual transmission of human immunodeficiency virus. Br. Med. J. **296:**1017–1020.
32. **Kalyanaraman, V. S., V. Rodriguez, F. Veronese, R. Rahman, P. Lusso, A. L. DeVico, T. Copeland, S. Oroszlan, R. C. Gallo, and M. G. Sarngadharan.** 1990. Characterization of the secreted, native gp120 and gp160 of the human immunodeficiency virus type 1. AIDS Res. Hum. Retroviruses **6:**371–380.
33. **Kawai, T., J. Wong, J. MacLean, A. B. Cosimi, and S. Wee.** 1994. Characterization of a monoclonal antibody (6G12) recognizing the cynomolgus monkey CD3 antigen. Transplant. Proc. **26:**1845–1846.
34. **Kent, K. A., E. Rud, T. Corcoran, C. Powell, C. Thiriart, C. Collignon, and E. J. Stott.** 1992. Identification of two neutralizing and 8 non-neutralizing epitopes on simian immunodeficiency virus envelope using monoclonal antibodies. AIDS Res. Hum. Retroviruses **8:**1147–1151.
35. **Koenig, S., V. M. Hirsch, R. A. Olmsted, D. Powell, W. Maury, A. Rabson, A. S. Fauci, R. H. Purcell, and P. R. Johnson.** 1989. Selective infection of human $CD4^+$ cells by simian immunodeficiency virus: productive infection associated with envelope glycoprotein-induced fusion. Proc. Natl. Acad. Sci. USA **86:**2443–2447.
36. **Korber, B., B. Hahn, B. Foley, J. W. Mellors, T. Leitner, G. Myers, F. McCutchan, and C. Kuiken.** 1997. Human retroviruses and AIDS. Los Alamos National Laboratory, Los Alamos, N.Mex.
37. **Lehner, T., Y. Wang, M. Cranage, L. A. Bergmeier, E. Mitchell, L. Tao, G. Hall, M. Dennis, N. Cook, R. Brookes, L. Klavinskis, I. Jones, C. Doyle, and R. Ward.** 1996. Protective mucosal immunity elicited by targeted iliac lymph node immunization with a subunit SIV envelope and core vaccine in macaques. Nat. Med. **2:**767–775.
38. **Leno, M., L. Carter, D. J. Venzon, J. Romano, P. D. Markham, K. Limbach, J. Tartaglia, E. Paoletti, J. Benson, G. Franchini, and M. Robert-Guroff.** 1999. $CD8^+$ lymphocyte antiviral activity in monkeys immunized with SIV recombinant poxvirus vaccines: potential role in vaccine efficacy. AIDS Res. Hum. Retroviruses **15:**461–470.
39. **Lewis, M. G., W. R. Elkins, R. E. McCutchan, R. E. Benveniste, C. Y. Lai, D. C. Montefiori, D. S. Burke, G. A. Eddy, and A. Shafferman.** 1993. Passively transferred antibodies directed against conserved regions of SIV envelope protect macaques from SIV infection. Vaccine **11:**1347–1355.
40. **Lu, Y. C., C. D. Pauza, X. S. Lu, D. C. Montefiori, and C. J. Miller.** 1998. Rhesus macaques that become systemically infected with pathogenic SHIV 89.6-PD after intravenous, rectal, or vaginal inoculation and fail to make an antiviral antibody response rapidly develop AIDS. J. Acquired Immune Defic. Syndr. Hum. Retrovirol. **19:**6–18.
41. **Lubeck, M. D., R. J. Natuk, M. Myagkikh, N. Kalyan, K. Aldrich, F. Sinangil, S. Alipanah, S. C. S. Murthy, P. K. Chanda, S. Nigida, P. D. Markham, S. Zolla-Pazner, K. Steimer, M. Wade, M. S. Reitz, Jr., L. O. Arthur, S. Mizutani, A. Davis, P. Hung, R. C. Gallo, J. Eichberg, and M. Robert-Guroff.** 1997. Long-term protection of chimpanzees against high-dose HIV-1 challenge induced by immunization. Nat. Med. **3:**651–658.
42. **Mackewicz, C., and J. A. Levy.** 1992. $CD8^+$ cell anti-HIV activity: nonlytic suppression of virus replication. AIDS Res. Hum. Retroviruses **8:**1039–1050.
43. **Marthas, M. L., C. J. Miller, S. Sutjipto, J. Higgins, J. Torten, B. L. Lohman, R. E. Unger, R. A. Ramos, H. Kiyono, J. R. McGhee, P. A. Marx, and N. C. Pedersen.** 1992. Efficacy of live-attenuated and whole-inactivated simian immunodeficiency virus vaccines against vaginal challenge with virulent SIV. J. Med. Primatol. **21:**99–107.
44. **Marx, P. A., R. W. Compans, A. Gettie, J. K. Staas, R. M. Gilley, M. J. Mulligan, G. V. Yamshchikov, D. Chen, and J. H. Eldridge.** 1993. Protection against vaginal SIV transmission with microencapsulated vaccine. Science **260:**1323–1327.
45. **Mascola, J. R., M. K. Louder, S. R. Surman, T. C. VanCott, X. F. Yu, J. Bradac, K. R. Porter, K. E. Nelson, M. Girard, J. G. McNeil, F. E. McCutchan, D. L. Birx, and D. S. Burke.** 1996. Human immunodeficiency virus type 1 neutralizing antibody serotyping using serum pools and an infectivity reduction assay. AIDS Res. Hum. Retroviruses **12:**1319–1328.
46. **Matsushita, S., M. Robert-Guroff, J. Rusche, A. Koito, T. Hattori, H. Hoshino, K. Javaherian, K. Takatsuki, and S. Putney.** 1988. Characterization of a human immunodeficiency virus neutralizing monoclonal antibody and mapping of the neutralizing epitope. J. Virol. **62:**2107–2114.
47. **McChesney, M. B., J. R. Collins, D. Lu, X. Lu, J. Torten, R. L. Ashley, M. W. Cloyd, and C. J. Miller.** 1998. Occult systemic infection and persistent simian immunodeficiency virus (SIV)-specific $CD4^+$-T-cell proliferative responses in rhesus macaques that were transiently viremic after intravaginal inoculation of SIV. J. Virol. **72:**10029–10035.
48. **Miller, C. J., M. Marthas, J. Torten, N. J. Alexander, J. P. Moore, G. F. Doncel, and A. G. Hendrickx.** 1994. Intravaginal inoculation of rhesus macaques with cell-free simian immunodeficiency virus results in persistent or transient viremia. J. Virol. **68:**6391–6400.
49. **Miller, C. J., M. B. McChesney, X. Lü, P. J. Dailey, C. Chutkowski, D. Lu, P. Brosio, B. Roberts, and Y. Lu.** 1997. Rhesus macaques previously infected with simian/human immunodeficiency virus are protected from vaginal challenge with pathogenic SIVmac239. J. Virol. **71:**1911–1921.
50. **Palker, T. J., M. E. Clark, A. J. Langlois, T. J. Matthews, K. J. Weinhold, R. R. Randall, D. P. Bolognesi, and B. F. Haynes.** 1988. Type-specific neutralization of the human immunodeficiency virus with antibodies to *env*-encoded synthetic peptides. Proc. Natl. Acad. Sci. USA **85:**1932–1936.
51. **Palker, T. J., A. J. Muir, D. E. Spragion, H. F. Staats, A. Langlois, and D. C. Montefiori.** 1996. The V3 domain of SIVmac251 gp120 contains a linear neutralizing epitope. Virology **224:**415–426.
51a. **Patterson, L. J., F. A. Robey, and M. Robert-Guroff.** Unpublished data.
52. **Pauza, C. D., M. Malkovsky, and M. S. Salvato.** 1994. SIV transmission across the mucosal barrier. AIDS Res. Hum. Retroviruses **10:**7–10.
53. **Putkonen, P., B. Mäkitalo, B. Böttiger, G. Biberfeld, and R. Thorstensson.** 1997. Protection of human immunodeficiency virus type 2-exposed seronegative macaques from mucosal simian immunodeficiency virus transmission. J. Virol. **71:**4981–4984.
54. **Putkonen, P., R. Thorstensson, L. Ghavamzadeh, J. Albert, K. Hild, G. Biberfeld, and E. Norrby.** 1991. Prevention of HIV-2 and SIVsm infection by passive immunization in cynomolgus monkeys. Nature **352:**436–438.
55. **Quesada-Rolander, M., B. Mäkitalo, R. Thorstensson, Y. J. Zhang, E. Castanos-Velez, G. Biberfeld, and P. Putkonen.** 1996. Protection against mucosal SIVsm challenge in macaques infected with a chimeric SIV that expresses HIV type 1 envelope. AIDS Res. Hum. Retroviruses **12:**993–999.
56. **Robert-Guroff, M., K. Aldrich, R. Muldoon, T. L. Stern, G. P. Bansal, T. J. Matthews, P. D. Markham, R. C. Gallo, and G. Franchini.** 1992. Cross-neutralization of human immunodeficiency virus type 1 and 2 and simian immunodeficiency virus isolates. J. Virol. **66:**3602–3608.
57. **Robert-Guroff, M., H. Kaur, L. J. Patterson, M. Leno, A. J. Conley, P. M. McKenna, P. D. Markham, E. Richardson, K. Aldrich, K. Arora, L. Murty, L. Carter, S. Zolla-Pazner, and F. Sinangil.** 1998. Vaccine protection against a heterologous, non-syncytium-inducing, primary human immunodeficiency virus. J. Virol. **72:**10275–10280.
58. **Rosenberg, E. S., J. M. Billingsley, A. M. Caliendo, S. L. Boswell, P. E. Sax, S. A. Kalams, and B. D. Walker.** 1997. Vigorous HIV-1-specific $CD4^+$ T cell responses associated with control of viremia. Science **278:**1447–1450.
59. **Rusche, J. R., K. Javaherian, C. McDanal, J. Petro, D. L. Lynn, R. Grimaila, A. Langlois, R. C. Gallo, L. O. Arthur, P. J. Fischinger, D. P. Bolognesi, S. D. Putney, and T. J. Matthews.** 1988. Antibodies that inhibit fusion of human immunodeficiency virus-infected cells bind a 24-amino acid sequence of the viral envelope, gp120. Proc. Natl. Acad. Sci. USA **85:**3198–3202.
60. **Shibata, R., T. Igarashi, N. Haigwood, A. Buckler-White, R. Ogert, W. Ross, R. Willey, M. W. Cho, and M. A. Martin.** 1999. Neutralizing antibody directed against the HIV-1 envelope glycoprotein can completely block HIV-1/SIV chimeric virus infections of macaque monkeys. Nat. Med. **5:**204–210.
61. **Sopper, S., U. Sauer, S. Hemm, M. Demuth, J. Müller, C. Stahl-Hennig, G. Hunsmann, V. ter Meulen, and R. Dörries.** 1998. Protective role of the virus-specific immune response for development of severe neurologic signs in simian immunodeficiency virus-infected macaques. J. Virol. **72:**9940–9947.
62. **Spouge, J. L.** 1993. Spouge ID-50 software, version 2.0 National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Md.
63. **Steger, K. K., M. Dykhuizen, J. L. Mitchen, P. W. Hinds, B. L. Preuninger, M. Wallace, J. Thomson, D. C. Montefiori, Y. Lu, and C. D. Pauza.** 1998. $CD4^+$-T-cell and $CD20^+$-B-cell changes predict rapid disease progression after simian-human immunodeficiency virus infection in macaques. J. Virol. **72:**1600–1605.
64. **Sutjipto, S., N. C. Pedersen, C. J. Miller, M. B. Gardner, C. V. Hanson, A. Gettie, M. Jennings, J. Higgins, and P. A. Marx.** 1990. Inactivated simian immunodeficiency virus vaccine failed to protect rhesus macaques from intravenous or genital mucosal infection but delayed disease in intravenously exposed animals. J. Virol. **64:**2290–2297.
65. **Voss, G., J. Li, K. Manson, M. Wyand, J. Sodroski, and N. L. Letvin.** 1995. Human immunodeficiency virus type 1 envelope glycoprotein-specific cytotoxic T lymphocytes in simian-human immunodeficiency virus-infected rhesus macaques. Virology **208:**770–775.
66. **Wang, Y. F., L. Tao, E. Mitchell, W. M. J. M. Bogers, C. Doyle, C. A. Bravery, L. A. Bergmeier, C. G. Kelly, J. L. Heeney, and T. Lehner.** 1998. Generation of CD8 suppressor factor and beta chemokines, induced by xenogeneic immunization, in the prevention of simian immunodeficiency virus infection in macaques. Proc. Natl. Acad. Sci. USA **95:**5223–5228.
67. **Wyand, M. S., K. H. Manson, M. Garcia-Moll, D. Montefiori, and R. C. Desrosiers.** 1996. Vaccine protection by a triple deletion mutant of simian immunodeficiency virus. J. Virol. **70:**3724–3733.
68. **Zolla-Pazner, S., M. Lubeck, S. Xu, S. Burda, R. J. Natuk, F. Sinangil, K. Steimer, R. C. Gallo, J. W. Eichberg, T. Matthews, and M. Robert-Guroff.** 1998. Induction of neutralizing antibodies to T-cell line-adapted and primary human immunodeficiency virus type 1 isolates with a prime-boost vaccine regimen in chimpanzees. J. Virol. **72:**1052–1059.