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Rhesus Macaques Previously Infected with Simian/Human Immunodeficiency Virus Are Protected from Vaginal Challenge with Pathogenic SIVmac239

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Nontraumatic vaginal inoculation of rhesus macaques with a simian/human immunodeficiency virus (SIV/HIV) chimera containing the envelope gene from HIV-1 89.6 (SHIV 89.6) results in systemic infection (Y. Lu, B. Brosio, M. Lafaile, J. Li, R. G. Collman, J. Sodroski, and C. J. Miller, J. Virol. 70:3045–3050, 1996). A total of five rhesus macaques have each been infected by exposure to at least three intravaginal inoculations of SHIV 89.6. The SHIV 89.6 infection is characterized by a transient viremia that evokes humoral and cellular immune responses to HIV and SIV antigens, but disease does not develop in animals infected with SHIV 89.6. To determine if a previous infection with SHIV 89.6 by vaginal inoculation could protect animals from vaginal challenge with pathogenic SIV, all five animals were intravaginally inoculated twice with pathogenic SIVmac239. After challenge, all of the SHIV-immunized animals had low or undetectable viral RNA levels in plasma compared to control animals. Three of the five of the SHIV-immunized animals remained virus isolation negative for more than 8 months, while two became virus isolation positive. The presence of SIV Gag-specific cytotoxic T lymphocytes in peripheral blood mononuclear cells and SIV-specific antibodies in cervicovaginal secretions at the time of challenge was associated with resistance to pathogenic SIV infection after vaginal challenge. These results suggest that protection from sexual transmission of HIV may be possible by effectively stimulating both humoral and cellular antiviral immunity in the systemic and genital mucosal immune compartments.

Human immunodeficiency virus type 1 (HIV-1) is transmitted primarily by sexual contact, and to stop the HIV pandemic, a vaccine capable of preventing sexual transmission is required. The SIV/rhesus macaque model of HIV sexual transmission is being used to investigate the interaction between the virus and the host genital mucosa and to test vaccines designed to prevent the heterosexual transmission of HIV (22, 24, 27, 28, 31–34). The nature of the immune response that is required to prevent vaginal transmission of SIV remains to be determined. However, it has been shown that systemic immunization with a live-attenuated SIV, which protects rhesus macaques from systemic challenge with SIV, does not provide protection from vaginal inoculation with virulent SIV (20). This has led to the supposition that local immune responses in the genital tract may be required for protection from genital challenge (33). Numerous investigators have reported the presence of anti-HIV and -SIV antibodies in the genital tract secretions of infected women (2–4, 18) and female rhesus macaques (26). We recently reported that SIV-specific cytotoxic T lymphocytes (CTL) are present in the vaginal mucosa of rhesus macaques that were vaginally infected with SIV (17). Thus, both humoral and cellular components of the SIV-specific immune response are present in the female genital tract of vaginally infected animals. By using a vaccine capable of eliciting the full range of both local and systemic immune responses, it may be possible to prevent vaginal transmission of SIV and, by inference, HIV.

In rhesus macaques, an SIV/HIV-1 chimeric virus (SHIV) containing the *env* gene from HIV-1 89.6 is capable of initiating a systemic infection following vaginal inoculation (19). As previously reported, five animals were inoculated intravaginally several times with SHIV 89.6, and they all developed a transient viremia and strong serum antibody responses to SIV and HIV Env antigens (19). These animals did not develop any clinical signs or laboratory abnormalities associated with the infection. We reasoned that because the animals had been infected with the SHIV by vaginal inoculation, it was likely that genital mucosal immune responses had been generated. To determine if a previous infection with SHIV 89.6 by the vaginal route could protect animals from vaginal challenge with pathogenic SIV, all five animals were vaginally inoculated with a virus stock derived from the pathogenic molecular clone SIVmac239 (13). After challenge, SIV *env* gene sequences could be detected in peripheral blood mononuclear cells (PBMC) of only two of the five animals which had previously been infected with SHIV. In addition, all five animals that were previously infected with SHIV had much lower viral RNA levels in plasma than did controls and three of five of the animals remained virus isolation negative for more than 8 months. Thus, previous infection with SHIV and the accompanying immune response to SHIV protected three of five animals and conferred resistance to vaginal challenge with pathogenic SIV in the two remaining animals.

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MATERIALS AND METHODS

Animals. All animals used in this study were captive-bred, mature, cycling female rhesus macaques (*Macaca mulatta*) from the California Regional Primate Research Center. Prior to their initial use, the animals were negative for antibodies to HIV-2, SIV, type D retrovirus, and simian T-cell leukemia virus type 1

TABLE 1. Oligonucleotides used as primers in the nested PCR to detect SIV *gag*, SIV *env*, or HIV *env*

Region of amplification	Sequence of:	
	Outer primers	Inner primers
SIV *gag*	5′-ACTGTCTGCGTCATCTGGTGC 5′-GTCCCAATCTGCAGCCTCCTC	5′-CACGCAGAAAAAGTGAAAC 5′-CTCTGATAATCTGCATAGCCGC
SIV *env*	5′-TTATGGTGTACCAGCTTGGAGGAATGC 5′-CCAAACCAAGTAGAAGTCTGTGTCTCCATC	5′-CGATACTTGGGGAACAACTCAGTGCCTAC 5′-GAGACCACCACCTTAGAACATTTAGGC
HIV *env*	5′-CCCAACCCACAAGAAGTAGTATTGG 5′-ACAAATGCTCTCCCTGGTCCTC	5′-GTAAAATTAACCCCACTCTGTGTT 5′-TGTGTACATTGTACTGTGGTGACAT
β-Actin	5′-GCCTCAGGGCAGCGGAACCGCTCA 5′-GCCTCAGGGCAGCGGAACCGCTCA	

(STLV-1). The animals which were previously infected with SHIV will be referred to as SHIV-immunized animals. The virology and immunology of the SHIV infection in these animals were described in detail in a previous report (19). At least three intravaginal inoculations with SHIV 89.6 were required to infect the animals in this study (19). The animals were housed in accordance with American Association for Accreditation of Laboratory Animal Care standards. When necessary, the animals were immobilized with 10 mg of ketamine HCl (Parke-Davis, Morris Plains, N.J.) per kg injected intramuscularly.

Virus stocks and animal inoculations. A detailed explanation of the rhesus PBMC-grown SHIV 89.6 stock that was used to infect the animals has been published previously (19). SHIV 89.6 contains functional HIV-1 *vpu*, *tat*, *rev*, and *env* genes engineered to replace their SIV counterparts within the SIVmac239 proviral DNA. The virus was constructed by using the *env* gene from HIV-1 HXBc2 except for the *Kpm*I (nucleotide 5925)-*Bam*HI (nucleotide 8053) fragment, which encodes the ectodomain of the gp120 and gp41 envelope glycoproteins. This *env* fragment in SHIV 89.6 was derived from HIV-1 89.6 (7). The challenge virus stock was generated by expanding SIVmac239 (a generous gift from R. Desrosiers) in CEMx174 cells. The stock contained 10^5 50% tissue culture infective doses per ml. A description of the technique used for intravaginal inoculation has been published (32). For this vaginal challenge study, the SHIV-immunized and control animals were inoculated intravaginally with 1 ml of SIVmac239 twice in a 4-h period. We have previously shown that multiple intravaginal inoculations increase the efficiency of vaginal SIV transmission (29, 30). This high dose of challenge virus was used to ensure that the control animals became infected. No inoculations were performed during menses, and seminal plasma was not used in the inoculations.

Virus isolation. Virus was isolated from heparinized whole blood obtained from the SIV-inoculated rhesus macaques. PBMC were isolated by Ficoll gradient separation (lymphocyte separation medium; Organon Teknika, West Chester, Pa.) and cocultured with CEMx174 cells (12) (provided by James A. Hoxie, University of Pennsylvania, Philadelphia) as previously described (15). A total of 5×10^6 PBMC were cocultivated with 2×10^6 to 3×10^6 CEMx174 cells. Aliquots of the culture media were assayed regularly for the presence of SIV major core protein (p27) by antigen capture enzyme-linked immunosorbent assay (ELISA) (15). Cultures were considered positive if they were antigen positive at two consecutive time points. A detailed description of the technique and criteria used to determine if the culture medium was antigen positive has been published (23). All cultures were maintained for 8 weeks and tested for SIV p27 by ELISA before being scored as virus negative. Blood samples for virus isolation were collected at the times indicated in Table 3.

PCR-based detection of SIV *gag* and *env* and HIV *env*. To discriminate between SIV and SHIV genomes in PBMC, three sets of nested oligonucleotide primer pairs were used. The sequences of the nested primer pairs used to detect SIV *gag*, SIV *env*, or HIV *env* are presented in Table 1. The specificity of the two sets of *env* primers and the relative efficiency of all the primer sets were determined in a series of preliminary experiments (Fig. 1 and 2). Nested PCR was carried out on genomic PBMC DNA in a DNA thermal cycler (Perkin-Elmer Cetus, Emeryville, Calif.) by a modification of a previously described technique (41). Briefly, cryopreserved PBMC isolated from whole blood were washed three times in Tris buffer at 4°C and resuspended at 10^7 cells/ml. Then 10 μl of the cell suspension was added to 10 μl of PCR lysis buffer (50 mM Tris-HCl [pH 8.3], 0.45% Nonidet P-40, 0.45% Tween 20) with 200 μg of proteinase K per ml. The cells were incubated for 3 h at 55°C followed by 10 min at 96°C. Two rounds of 30 cycles of amplification were performed on aliquots of plasmid DNA containing the complete genome of SIVmac1A11 (21) (positive control) or aliquots of cell lysates under conditions described elsewhere (41). DNA from uninfected CEMx174 cells was amplified as a negative control in all assays to monitor potential reagent contamination. By using the primers listed in Table 1, β-actin DNA sequences were amplified by two rounds of PCR (30 cycles/round) from all PBMC lysates to detect potential inhibitors of *Taq* polymerase in cell lysates. Following the second round of amplification, a 10-μl aliquot of the reaction product was removed and run on a 1.5% agarose gel. Amplified products in the gel were visualized by ethidium bromide staining. Blood samples for PCR analysis were collected at the times indicated in Table 3.

bDNA quantitation of SIV RNA in plasma. Quantitative assays for the measurement of SIV RNA were performed by a branched-DNA (bDNA) signal amplification assay specific for SIV (8). This assay is similar to the Quantiplex HIV RNA assay (37), except that target probes were designed to hybridize with the *pol* region of the SIVmac group of primate lentiviruses including SIVmac251 and SIVmac239. SIV *pol* RNA in plasma samples were quantified by comparison with a standard curve produced by using serial dilutions of cell-free SIV-infected tissue culture supernatant. Quantitation of this standard curve was determined by comparison with purified, quantitated, in vitro-transcribed SIVmac239 *pol* RNA. SIV RNA associated with viral particles was measured after being pelleted from 1 ml of heparinized plasma (23,500 × *g* for 1 h at 4°C). The lower quantitation limit of this assay was 10,000 copies of SIV RNA per ml of plasma.

Anti-SIV and HIV env antibody responses in vaginal washes and serum before and after SIVmac239 challenge. Immuoblots were performed to detect serum antibody responses to specific SIV proteins by a previously described technique (39). Antibody responses to HIV antigens were assessed with a commercial HIV-1 Western blot kit (Dupont, Wilmington, Del.) as specified by the manufacturer.

Vaginal washes consist of a mixture of cervical and vaginal secretions (CVS) and were collected by vigorously infusing 6 ml of sterile phosphate-buffered saline (PBS) into the vaginal canal and aspirating as much of the instilled volume as possible. Care was taken to ensure that the cervical mucus was bathed in the lavage fluid and that no trauma to the mucosa occurred during the procedure. The samples were snap-frozen on dry ice and stored at −80°C until analysis. For analysis, the sample was thawed and centrifuged at 8,000 × *g* in a microcentrifuge for 10 min, and the supernatant was collected. Neomycin sulfate (200 μg/ml; ICN Biomedical Inc, Aurora, Ohio) and a cocktail (3%, vol/vol) of protease inhibitors

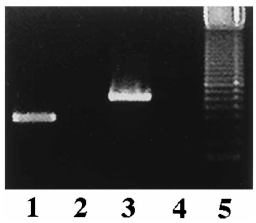

FIG. 1. Specificity of SIV *env* and HIV *env* PCR primers. To test the specificity of the oligonucleotide *env* primers used in this study (Table 1), total-cell genomic DNA was extracted from the spleen of a rhesus macaque infected with SHIV 89.6 and used as a template for nested PCR to amplify HIV-1 gp120 sequences with the HIV *env* primers (lane 1) or SIVmac239 gp120 sequences with the SIV *env* primers (lane 2). In addition, total-cell genomic DNA was extracted from the spleen of a rhesus macaque infected with SIVmac239 and used as a template for nested PCR to amplify SIVmac gp120 sequences with the SIV *env* primers (lane 3) or HIV-1 gp120 sequences with the HIV *env* primers (lane 4). Amplified product was visible only when the HIV *env* primers were reacted with DNA from the SHIV-infected animal (lane 1) or when the SIV *env* primers were reacted with DNA from the SIV-infected animal (lane 3). Lane 5 contains molecular weight standards. With either set of primer pairs, amplifications of gp120 sequences from DNA extracted from uninfected animals were negative.

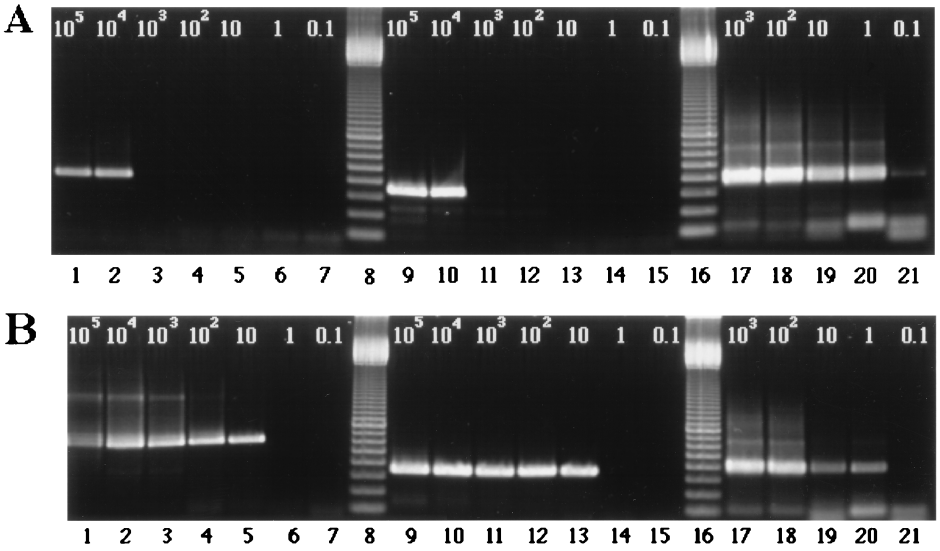

FIG. 2. Relative efficiency of SIV *gag*, SIV *env*, and HIV *env* PCR primers used to detect proviral sequences in cellular DNA. The relative efficiency of the oligonucleotide PCR primers used in this study (Table 1) was tested as follows. (A) cellular genomic DNA was extracted from the spleen of a rhesus macaque infected with SHIV 89.6 and used as a template for nested PCR to amplify HIV-1 gp120 sequences with the HIV *env* primers (lanes 1 to 7), SIV *gag* sequences with the SIV *gag* primers (lanes 9 to 15), or β-actin sequences with β-actin primers (lanes 17 to 21). The concentration of the DNA was determined with a spectrophotometer, and dilutions of the DNA corresponding to 10^5, 10^4, 10^3, 10^2, 10, 1, or 0.1 cellular DNA equivalent were loaded into the lanes. The amount of cellular DNA in each lane is noted in the figure. Lanes 8 and 16 are molecular weight standards. By using either the HIV *env* or SIV *gag* primer, amplified product was detected in reaction mixtures containing as little as 10^4 cellular DNA equivalents of genomic DNA extracted from the spleen of the SHIV 89.6-infected animal. Thus, to within an order of magnitude, the SIV *gag* and HIV *env* primer sets were equally efficient at detecting SHIV sequences in tissues of infected animals. (B) Cellular genomic DNA was extracted from the spleen of a rhesus macaque infected with SIVmac239 and used as a template for nested PCR to amplify SIVmac gp120 sequences with the SIV *env* primers (lanes 1 to 7), SIV *gag* sequences with the SIV *gag* primers (lanes 9 to 15), or β-actin sequences with β-actin primers (lanes 17 to 21). The concentration of the DNA was determined with a spectrophotometer, and dilutions of the DNA corresponding to 10^5, 10^4, 10^3, 10^2, 10, 1, or 0.1 cellular DNA equivalent were loaded into the lanes. The amount of DNA in each lane is noted in the figure. Lanes 8 and 16 are molecular weight standards. By using either the SIV *env* or the SIV *gag* primers, amplified product was detected in reaction mixtures containing as little as 10 cellular DNA equivalents of genomic DNA extracted from the spleen of the SIVmac239-infected animal. Thus, to within an order of magnitude, the SIV *gag* and SIV *env* primer sets were equally efficient at detecting SIV sequences in tissues of infected animals. The β-actin primers detected genomic DNA in the tubes containing 1 cellular DNA equivalent of genomic DNA from both of the spleen preparations. These studies demonstrate that both the SIV *env* and HIV *env* oligonucleotide primers are as efficient as the SIV *gag* primers at detecting proviral DNA sequences in genomic DNA.

[0.6 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 3 μg of aprotinin per ml, 30 μM leupeptin, 9.75 μM bestatin (Sigma Chemical Co., St. Louis, Mo.)] was added to the supernatant, and the ELISA was performed as described below.

The specificity and sensitivity of the total immunoglobulin A (IgA) ELISA and the antisera used in the anti-SIV isotype-specific ELISAs were determined in a series of preliminary experiments (Fig. 3 and 4). Prior to determining antibody titers, sera and vaginal secretions were screened for the presence of anti-SIV or anti-HIV-1 gp160 antibodies by using a 1:100 dilution of sera and a 1:4 dilution of vaginal washes in the same ELISAs that were used to determine antibody titers (described below). Results were calculated from changes in optical density (ΔOD), where ΔOD is defined as the difference between the mean OD of a dilution of sample tested in two antigen-coated wells and the mean OD of the same dilution of sample tested in two antigen-free (control) wells. For each antigen, the cutoff value (CO) was calculated as the mean ΔOD + 3 standard deviations of duplicate wells containing sera or vaginal washes from 12 randomly selected seronegative adult female rhesus macaques. If the Δ OD/CO ratio for a sample was greater than 2, the sample was considered to be positive.

To determine the concentration of IgA in CVS (Table 2), vaginal wash samples were diluted 1:10, 1:20, 1:40, and 1:80 and tested for the presence of IgA on the same plate and by the same assay used to produce the standard curve (Fig. 4). For each vaginal wash sample, the concentration of the IgA in the samples was determined by plotting the OD at each dilution against the standard curve. Only dilutions which produced OD values in the linear portion of the curve were used to calculate the IgA concentration in a sample.

To determine anti-SIV antibody titers in antibody-positive serum and CVS, 96-well microtiter plates (Nunc Immunoplate II Maxisorp; Applied Scientific, South San Francisco, Calif.) were coated with whole pelleted SIVmac251 (Advanced Biologics Inc., Columbia, Md.) at 5 μg/ml in 0.1 M Na_2CO_3–$NaHCO_3$ buffer (pH 9.6) and blocked with 4% nonfat powdered milk. Plasma and vaginal wash samples were serially diluted (1:4) in duplicate, and the plates were incubated overnight at 4°C. Antibody binding was detected with a 1:2,000 dilution of peroxidase-conjugated goat anti-monkey IgG(Fc) (100 μl per well) or a 1:1,000 dilution of peroxidase-conjugated goat anti-monkey IgA(Fc) (100 μl per well) (Nordic Laboratories, San Juan Capistrano, Calif.) for 1 h at 37°C. Plates were developed with *o*-phenylenediamine dihydrochloride (Sigma Chemical Co.) for 5 min and stopped with H_2SO_4 before the OD at 490 nm was read. For each serum or vaginal wash sample, the end point titer of anti-SIV antibodies was defined as the reciprocal of the last dilution giving a ΔOD greater than 0.2. Anti-HIV *env* antibodies in vaginal wash samples were detected by a similar ELISA, except that the wells were coated with recombinant HIV gp160 (5 μg/ml) derived from the envelope of HIV-1 LAI (Transgéne, Strasbourg, France). Due to the limited amount of this antigen, we were unable to determine the titer of anti HIV-1 gp160 in CVS samples.

Anti-SIV CTL responses in PBMC. At the time of challenge, the presence of SIV-specific CTL in PBMC was assessed. The details of the CTL assay have been reported previously (16). Briefly, lymphocytes were stimulated with autologous $CD4^+$ T cells infected with SIV and cultured for 14 days in RPMI 1640 medium supplemented with 10% fetal calf serum, antibiotics, 5% human lymphocyte-conditioned medium (human interleukin-2; Schiapparelli Biosystems, Inc. Columbia, Md.), and 20 U of recombinant human interleukin-2 (donated by Cetus Corp.) per ml. Autologous B lymphocytes were transformed by herpesvirus papio (594Sx 1055 producer cell line, provided by M. Sharp, Southwest Foundation for Biomedical Research, San Antonio, Tex.) and infected overnight with wild-type vaccinia virus or a recombinant vaccinia virus expressing the $p55^{gag}$ (vvgag) of SIVmac239 (provided by L. Giavedoni and T. Yilma, University of California, Davis, Calif.) and then labeled with 50 μCi of chromium-51 ($Na_2{}^{51}CrO_4$ [Amersham Holdings, Inc. Arlington Heights, Ill.]) per 10^6 cells. Effector and target cells were added simultaneously at multiple effector-to-target-cell ratios in a 4-h chromium release assay, and the percent specific lysis was calculated from supernatant chromium measured in a liquid scintillation counter (Microbeta 1450; Wallac Biosystems, Gaithersburg, Md.). Standard criteria were used for determining which cultures had SIV-specific lysis (see the legend to Fig. 5).

RESULTS

Anti-SIV and -HIV immune responses at the time of challenge. Of the five SHIV-immunized animals, three (animals 17376, 20429, and 21170) had detectable SIV-specific CTL in PBMC at the time of SIV challenge (Table 3; Fig. 5). One animal (animal 25726) was negative for SIV-specific CTL, and one animal (animal 22190) was not tested due to inability to transform her B cells (Table 3). At the time of challenge,

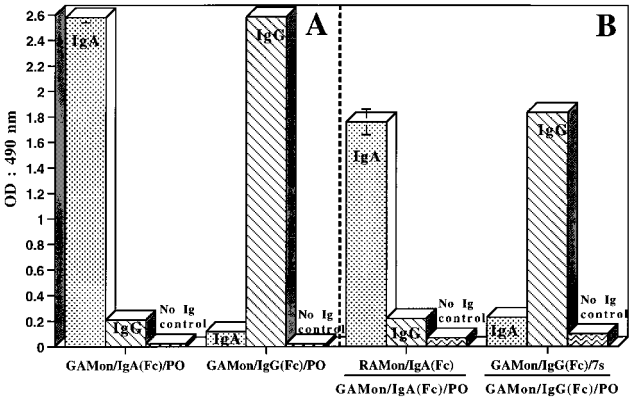

FIG. 3. Specificity of the isotype-specific anti-monkey IgG and IgA reagents used in the anti-SIV antibody ELISAs and the total IgA sandwich ELISA. (A) To determine the isotype-specificity of the detection antibodies used in these studies, ELISA plates were coated with 100 μl of purified rhesus IgG or secretory (S-IgA) IgA (HRP Inc., Denver, Penn.) in PBS (pH 7.2) at a concentration of 2 μg/ml. This concentration was previously found to produce OD values near the top of the linear portion of a curve that was generated by titrating the coating antigen while keeping the concentration of the detection or capture antibodies constant (data not shown). Then 100 μl of the detection antibody [1:2,000 peroxidase-conjugated goat anti-monkey IgG(Fc) or 1:1,000 peroxidase-conjugated goat anti-monkey IgA(Fc); Nordic Laboratories] was added to the wells. The blocking and conjugate development steps were as described in Materials and Methods. All these studies were performed in triplicate. The isotype-specific detection antibodies did not cross-react with the other Ig isotype tested. (B) The specificities of the total IgA and total IgG ELISAs were determined by coating ELISA plates with 100 μl of rabbit anti-monkey IgA(Fc) (1:3,000) or goat anti-monkey IgG(Fc)/7s (6 μg/ml) in PBS (pH 7.2). Then 100 μl of purified rhesus IgG or S-IgA (HRP Inc.) at a concentration of 2 μg/ml was added to the wells. To detect the captured S-IgA or IgG, peroxidase-conjugated goat anti-monkey IgG(Fc) (1:2,000) or peroxidase-conjugated goat anti-monkey IgA(Fc) (1:1,000) was added to the wells. The blocking and conjugate development steps were as described in Materials and Methods. All these studies were performed in triplicate. Under the *x* axis, the detection antibody is the reagent listed below the line, while the capture antibody is the reagent listed above the line. These results demonstrate that the isotype-specific sandwich ELISA did not cross-react significantly with the other immunoglobulin isotype tested.

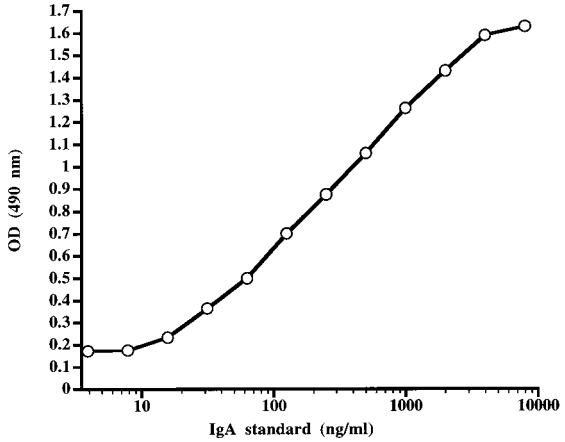

FIG. 4. Quantitation of rhesus macaque IgA by an IgA-specific sandwich ELISA. The sensitivity of the IgA-specific sandwich ELISA was tested by coating plates with rabbit anti-monkey IgA(Fc) (1:3,000 in PBS; pH 7.2). The plates were blocked with 4% nonfat milk. Purified rhesus macaque S-IgA (HRP Inc.) was serially diluted from 8,000 to 1 ng/ml and added, in duplicate, to the wells. The plates were washed, and peroxidase-conjugated goat anti-monkey IgA (Fc) (1:1,000) was added. The plates were developed as described in Materials and Methods, and a standard curve was generated. Purified S-IgA was not added to two negative control wells. The limit of detection for the assay was defined as the minimum concentration of S-IgA giving an OD that was 3 standard deviations above the mean OD of the S-IgA-free (control) wells. Based on this definition, 20 ng of IgA per ml of sample was the lower limit of detection for this sandwich ELISA.

anti-HIV-1 gp160 and anti-SIV antibodies were present in the serum of all the immunized animals (Fig. 6 to 8). The titers of the anti-SIV IgG antibodies in serum (Fig. 6) ranged from 1,000 to 4,000. Three of the five animals had anti-SIV antibodies and four of the five animals had HIV-1 gp160 antibodies in CVS at the time of challenge (Fig. 9). Titers of SIV-specific IgG in the secretions of the three animals (animals 20429, 21170, and 25726) that had SIV-specific antibodies ranged from 1:8 to 1:32 (data not shown). Although IgA was present in the vaginal wash samples (Table 2), virus-specific IgA antibodies were not detected in the vaginal secretions of any animal at the time of challenge.

Virus isolation after challenge with SIVmac239. At the time of challenge, the SHIV-immunized animals had been virus isolation negative for periods ranging from 12 to 52 weeks (Table 3). The challenge inoculum consisted of 1 ml of SIVmac239 (10^5 50% tissue culture infective doses) delivered via a tuberculin syringe into the vaginal canal. Two doses of the virus inoculum were given to each animal, with a 4-h interval between the inoculations. It was not possible to isolate virus from the PBMC of three of the SHIV-immunized animals (animals 17376, 20429, and 21170) after challenge (Table 3). All three of the virus isolation-negative animals had SIV-specific CTL in PBMC, and two of them (animals 20429 and 21170) had anti-SIV IgG antibodies in vaginal secretions at the time of challenge. Virus was isolated from the PBMC of two of the SHIV-immunized animals (animals 22190 and 25726), although at the time of challenge these animals had the highest titers of SIV-specific IgG in serum in the group of immunized animals. After SIV challenge, one of these animals (animal 25726) became only intermittently virus isolation positive. At the time of challenge, this animal had a high level of SIV-specific and HIV Env-specific IgG in vaginal secretions but no SIV-specific CTL in PBMC. One of the immunized animals (animal 22190) was more consistently virus isolation positive after challenge. This animal had no detectable SIV or HIV-specific IgG in vaginal secretions at the time of SIVmac239 challenge. Because the B cells from this animal could not be transformed, it was not possible to determine if the animal had SIV-specific CTL in PBMC at the time of challenge. The p27 antigen capture ELISA used to detect viral antigen in the cultures cannot discriminate between SIV and SHIV 89.6. Thus, it was not possible to determine if the virus isolated from the two SHIV-immunized animals was SHIV or challenge virus. Both of the

TABLE 2. Concentration of IgA in vaginal wash samples of SHIV-immunized monkeys before and after challenge with SIVmac239

Animal	Total IgA concn[a] (μg/ml) in vaginal wash samples at time (wk) after SIVmac239 challenge:			
	0	1	4	8
17376	3.44	1.00	1.70	1.10
20429	0.52	0.21	0.46	0.22
21170	0.64	0.58	0.40	0.25
22190	0.84	0.80	0.65	1.00
25726	14.59	5.00	6.60	9.00

[a] Total IgA in vaginal wash samples measured by sandwich ELISA (Fig. 4). Due to potential variability, the dilution of vaginal secretions which occurs during sample collection was not considered when calculating these values. We estimate that at least a 10-fold dilution of vaginal secretions occurs during the vaginal wash procedure.

TABLE 3. Virus isolation from PBMC of SHIV-immunized and control rhesus macaques after intravaginal challenge/exposure with SIVmac239

Animal	Time (wk) since:		CTL present at challenge	Virus isolation results at time (wk) after SIVmac239 challenge:											% Positive[a]
	SHIV inoculation	SHIV isolation		1	2	4	6	8	12	16	20	24	28	32	
SHIV immunized															
17376[b]	64	52	Yes	−	−	−	−	−	−	−	−	−	−[b]		0
20429	63	16	Yes	−	−	−	−	−	−	−	−	−	−	−[c]	0
21170	63	32	Yes	−	−	−	−	−	−	−	−	−	−	−[c]	0
22190	28	12	ND[d]	+	+	−	−	−	+	+	−	+	−	+	55
25726	28	20	No	−	−	+	−	−	−	−	+	−	−	−	18
Control															
26313				−	−	+	+	+	−	−	−	−	+	−	36
26438				−	+	+	+	+	+	+	+	+	+	+	91

[a] Of the 11 PBMC cocultures performed over the 8-month observation period, this is the percentage of cultures from which virus could be isolated.

[b] This animal (17376) was killed at 25 weeks postchallenge for clinical signs related to endometriosis. At necropsy, PBMC and mononuclear cell suspensions obtained from spleen, axillary, inguinal, iliac, and mesenteric lymph nodes were cocultivated with CEMx174 cells as described for PBMC in Materials and Methods. All cultures were virus isolation negative.

[c] In addition to the standard PBMC coculture technique described in Materials and Methods, $CD8^+$ T cells were depleted from the PBMC of these samples by using immunomagnetic beads (Dynal Inc., Lake Success, N.Y.). The $CD8^+$ T-cell-depleted PBMC were then cocultured with CEMx174 cells as described for PBMC in Materials and Methods. All $CD8^+$ T-cell-depleted cultures were virus isolation negative.

[d] ND, not done.

control animals inoculated intravaginally with SIVmac239 became virus isolation positive.

bDNA quantitation of SIV RNA in plasma. The SIV *pol* RNA level in plasma was quantitated by the bDNA assay on heparin anti-coagulated samples from 1 to 40 weeks after SIVmac challenge (Fig. 10). In the two naive control animals, a distinct peak of SIV *pol* RNA in plasma was observed at 4 (animal 26438) and 6 (animal 26313) weeks after challenge. Peak levels of greater than or equal to 200,000 viral RNA copies per ml of plasma were observed in these samples. In marked contrast, viral RNA was detected in plasma only intermittently and at much lower levels (<30,000 RNA copies per ml of plasma) in the SHIV-immunized animals. In fact, peak viral RNA levels in plasma below 30,000 RNA copies/ml correlated with SHIV immunization (Fisher's exact test; $P < 0.05$). The bDNA assay cannot distinguish between SHIV and SIV, and thus, it is not possible to determine if the SIV *pol* RNA sequences in the SHIV-immunized animals were from SIV or SHIV virions. To address this issue, a reverse transcription PCR assay could be used to amplify virus-specific *env* sequences from plasma viral RNA, but unfortunately these samples were consumed by the bDNA analysis. The significant difference between the viral RNA levels in the plasma of naive control and SHIV-immunized animals provides evidence that replication of the challenge virus was significantly reduced as a result of immunization. There was no apparent difference in viral RNA levels in plasma between animals which were virus isolation positive and those which were virus isolation negative.

PCR-based detection of SIV and HIV proviral genes in DNA from PBMC. To determine if the SHIV-immunized animals had become infected with SIV, nested PCR was carried out on cellular DNA isolated from PBMC collected at the time points indicated in Table 4. The three sets of primer pairs (Table 1) were used independently on separate aliquots of DNA from each PBMC sample analyzed. The results of the PCR analysis are summarized in Table 4. At the time of SIV challenge, SHIV-immunized animals were positive for proviral SIV *gag* and HIV *env* but negative for SIV *env* sequences (data not shown). After SIVmac239 challenge, SIV *gag* DNA was detected in the PBMC of all the animals, including controls, at all time points tested. With the exception of two samples, the SHIV-immunized animals were consistently HIV *env* PCR positive. This pattern of proviral sequences is consistent with the presence of SHIV proviral DNA in PBMC. The virus isolation-negative, SHIV-immunized animals were negative for SIV *env* sequences at all time points. Thus, the PCR analysis of PBMC DNA provided no evidence of SIV infection in these three animals. SIV *env* was detected in the virus isolation-positive, SHIV-immunized animals (animals 25726 and 22190) after SIV challenge. This is clear evidence that these two animals became infected with SIV. The naive control animals

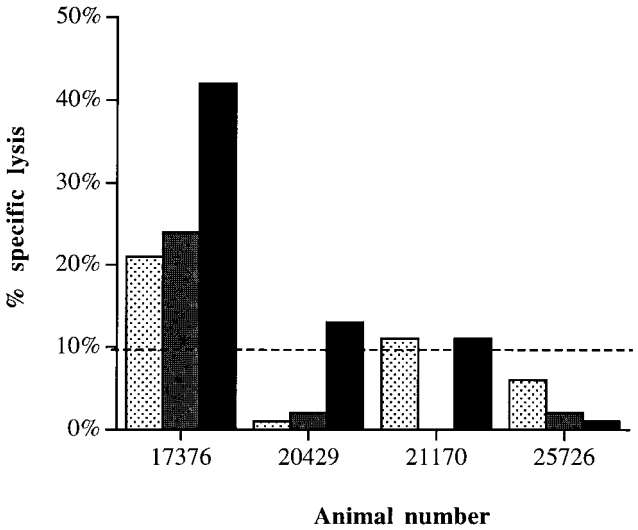

FIG. 5. Anti-SIV Gag CTL responses in SHIV-immunized rhesus monkeys prior to vaginal challenge with SIVmac239. PBMC taken at 30-day intervals in the 3 months immediately prior to challenge (represented by stippled, shaded, and black bars) were stimulated in culture as described in Materials and Methods. The percent specific lysis of autologous B cells infected with a recombinant vaccinia virus expressing the $p55^{gag}$ of SIVmac239 is shown. Lysis was considered positive if it was at least 10% and twofold greater than the lysis of the negative vaccinia virus control target. The horizontal line indicates 10% lysis.

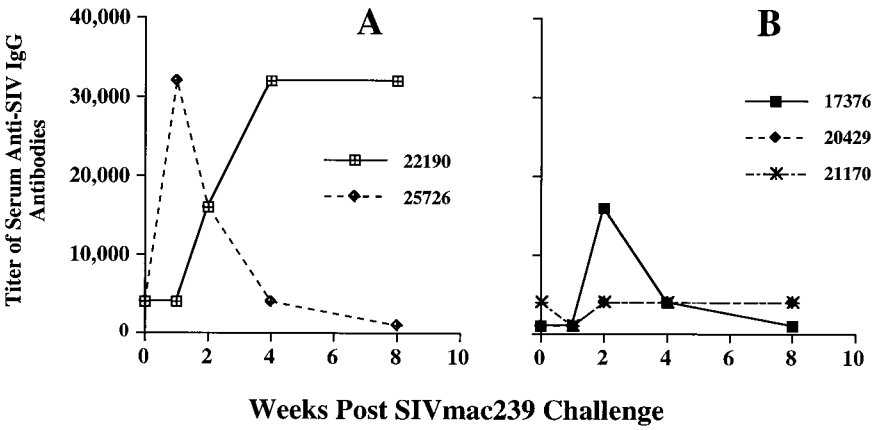

FIG. 6. Serum anti-SIV IgG titers in SHIV-immunized animals before and after intravaginal challenge with SIVmac239. (A) The two animals that were virus isolation positive after challenge rapidly developed high anti-SIV IgG antibody titers after challenge. This antibody response pattern is characteristic of an anamnestic immune response. One of the animals maintained this high titer, suggesting continued exposure to SIV antigens. (B) One of the three animals that were virus isolation negative after challenge (animal 17376) had a substantial increase in antibody titer after challenge, but the maximum titer in this animal was less than half the maximum titer in the virus isolation-positive animals. One virus isolation-negative animal (animal 20429) had a slightly increased antibody titer postchallenge. Although the third animal (animal 21170) initially had a transient decrease in titer, the anti-SIV IgG titer subsequently increased to prechallenge levels and remained there. For this analysis, serum was diluted from 1/1,000 to 1/32,000.

were consistently SIV *gag* and SIV *env* positive after SIVmac239 challenge, consistent with SIV infection.

SIV antibody responses after SIV challenge. The titers of anti-SIV serum antibodies are shown in Fig. 6. Two of the three immunized animals (animals 21170 and 20429) that were virus isolation negative after challenge had relatively stable anti-SIV serum antibody titers up to 8 weeks after challenge. This pattern of response is consistent with a limited exposure

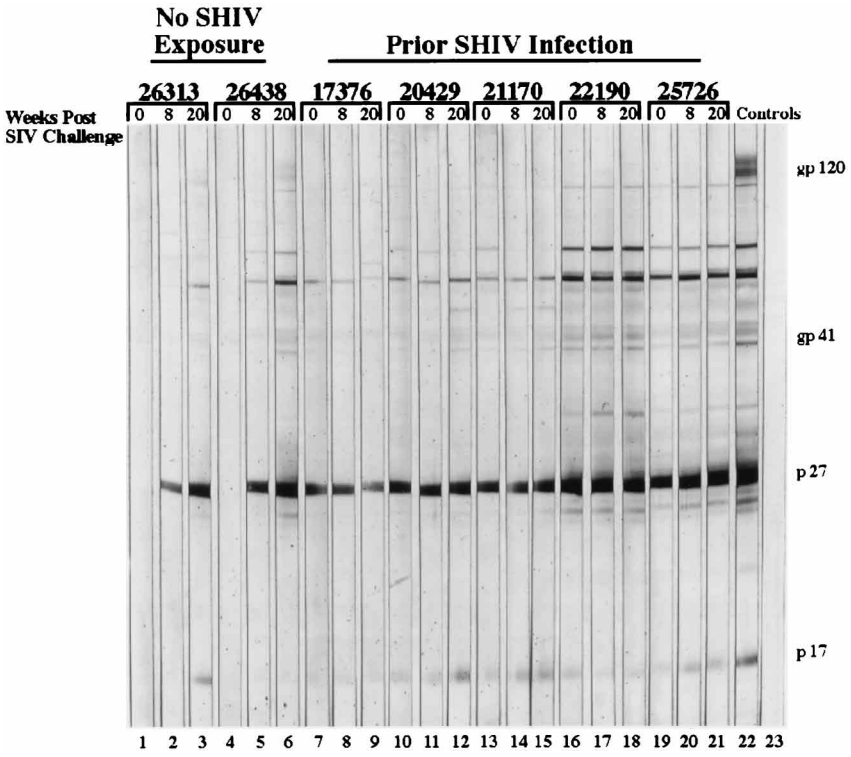

FIG. 7. Reaction of serum from SIV-challenged animals to SIV antigens in a Western blot assay. All the immunized animals were seropositive to SIV *gag* and *pol* antigens (p66, p55, p27, and p17) at the time of challenge. The sera of the three immunized animals that remained virus isolation negative after challenge (animals 17376, 20429, and 21170) did not develop stronger reactivity to SIV antigens or detectable anti-SIVgp120 antibodies after SIV challenge. Although SIV *env* sequences were detected in the PBMC DNA from the two virus isolation-positive animals (animals 22190 and 25726), anti-SIVgp120 antibody responses were not detected in the sera of these animals. The naive control animals (animals 26313 and 26438) seroconverted by 8 weeks postchallenge. Note that by 20 weeks p.i., faint reactivity to SIVenv antigens was detectable. The results obtained with sera collected at three time points (prechallenge and weeks 8 and 20 postchallenge) from each animal are shown: lanes 1 to 3, control monkey 26313; lanes 4 to 6, control monkey 26438; lanes 7 to 9, monkey 17376; lanes 10 to 12, monkey 20429; lanes 13 to 15, monkey 21170; lanes 16 to 18, monkey 22190; lanes 19 to 21, monkey 25726; lane 22, positive control serum from a known SIV-seropositive rhesus macaque; lane 23, negative control serum from a naive rhesus macaque.

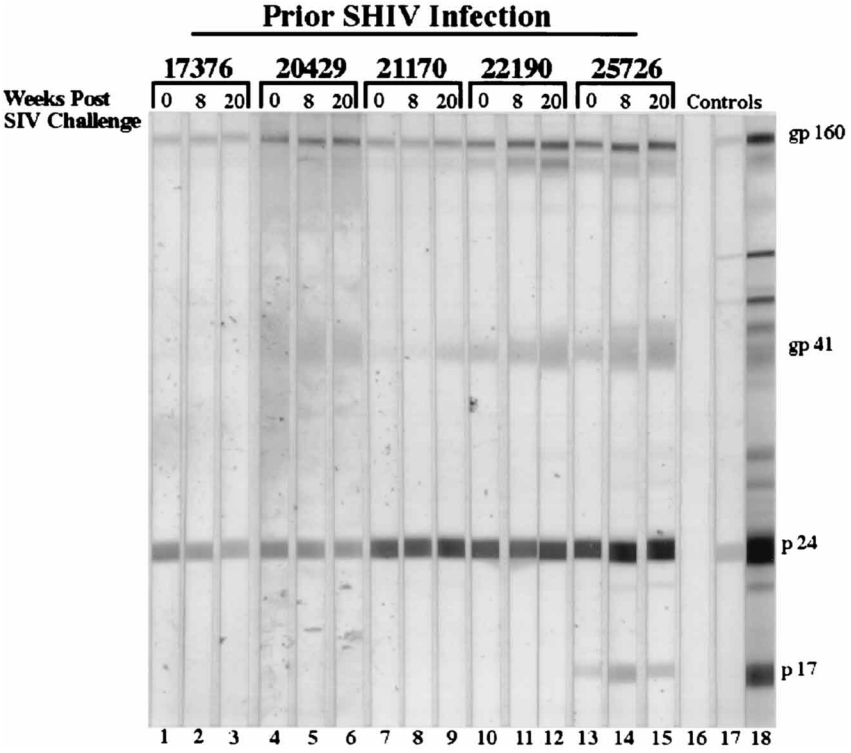

FIG. 8. Reaction of serum from SHIV-immunized animals to HIV antigens in a Western blot assay. All animals that had been previously SHIV infected and then challenged with SIV were seropositive to HIV *gag* and *env* antigens (gp160 and p24) at the time of challenge. The immunoreactivity of sera from SHIV-infected animals to HIVp24 antigen has been previously reported (19). The sera of the three immunized animals that remained virus isolation negative after challenge (animals 17376, 20429, and 21170) had slowly declining reactivity to HIV antigens after SIV challenge. Sera from the SHIV-immunized animals (animals 22190 and 25726) that became virus isolation positive after SIV challenge developed increased anti-HIV antibody reactivity. The naive control animals (animals 26313 and 26438) were not tested. The results obtained with sera collected at three time points (prechallenge and weeks 8 and 20 postchallenge) from each animal are shown: lanes 1 to 3, monkey 17376; lanes 4 to 6, monkey 20429; lanes 7 to 9, monkey 21170; lanes 10 to 12, monkey 22190; lanes 13 to 15, monkey 25726; lane 16, negative control serum supplied in the kit; lane 17, weak HIV-positive control serum supplied in the kit; lane 18, strong HIV-positive control serum supplied in the kit.

to SIV antigens following SIV challenge. The other virus isolation-negative animal (animal 17376) had a 16-fold increase in anti-SIV antibody titer at 2 weeks postchallenge, but the titer quickly declined below prechallenge levels (Fig. 6). This is consistent with a brief exposure to SIV antigens. The immunized animal (animal 25726) that became intermittently virus isolation positive after challenge had a similar pattern of antibody response: a spike in antibody titer at 1 week postchallenge and a gradually declining antibody titer until, at 4 weeks postchallenge, the titer was below prechallenge levels. The immunized animal (animal 22190) that became most consistently virus isolation positive after SIV challenge had a rapid and sustained increase in anti-SIV antibody titer (Fig. 6). The relatively rapid and large increase in antibody titer in the two animals (animals 25726 and 22190) that became virus isolation positive is characteristic of an anamnestic immune response. Anti-SIV gp120 antibodies were not detected in the Western blot assay. In general, the changes in the anti-SIV IgG antibody titers in vaginal secretions paralleled the changes in antibody titers in serum (data not shown). The two naive control animals developed steadily increasing serum anti-SIV antibody reactivity consistent with productive SIV infection (Fig. 7). By 20 weeks postchallenge, weak anti-SIV antibody reactivity was apparent in the serum samples from the control animals.

DISCUSSION

The results demonstrate that infection with virulence-attenuated SHIV 89.6 via vaginal inoculation can protect rhesus macaques from subsequent vaginal challenge with pathogenic SIVmac239. At the time of challenge, all the SHIV-immunized animals had SIV *gag* and HIV *env* SHIV proviral sequences in PBMC but were virus isolation negative. After vaginal challenge with SIVmac239, three of the SHIV-immunized animals remained virus isolation negative and SIVenv PCR negative. Based on all available criteria, these animals were protected from the challenge virus. After challenge, two animals were virus isolation positive and SIV *env* PCR positive. This outcome is consistent with a productive infection with the SIV challenge strain. Thus, of the five SHIV-immunized animals, three had no evidence of productive SIV infection and hence were protected from vaginal challenge with SIVmac239.

After intravaginal challenge, viral RNA levels in plasma were much lower in all SHIV-immunized animals than in control animals. Because the bDNA assay detects SIV *pol* RNA sequences, it does not discriminate between the SHIV strain and the SIV challenge strain used in this study. It is likely that in the SIV *env* PCR-negative SHIV-immunized animals, the low viral RNA level in plasma was due to the presence of SHIV virions in plasma. Even if the assumption is made that the viral RNA level in plasma was due to infection with the SIV challenge strain, all the SHIV-immunized animals, including the two SHIV-immunized animals which were virus isolation and SIV *env* positive after SIV challenge, had significantly lower viral RNA levels in plasma than the control animals did. The low viral RNA levels in plasma in the virus isolation and SIV *env* PCR-positive animals demonstrate that these animals had

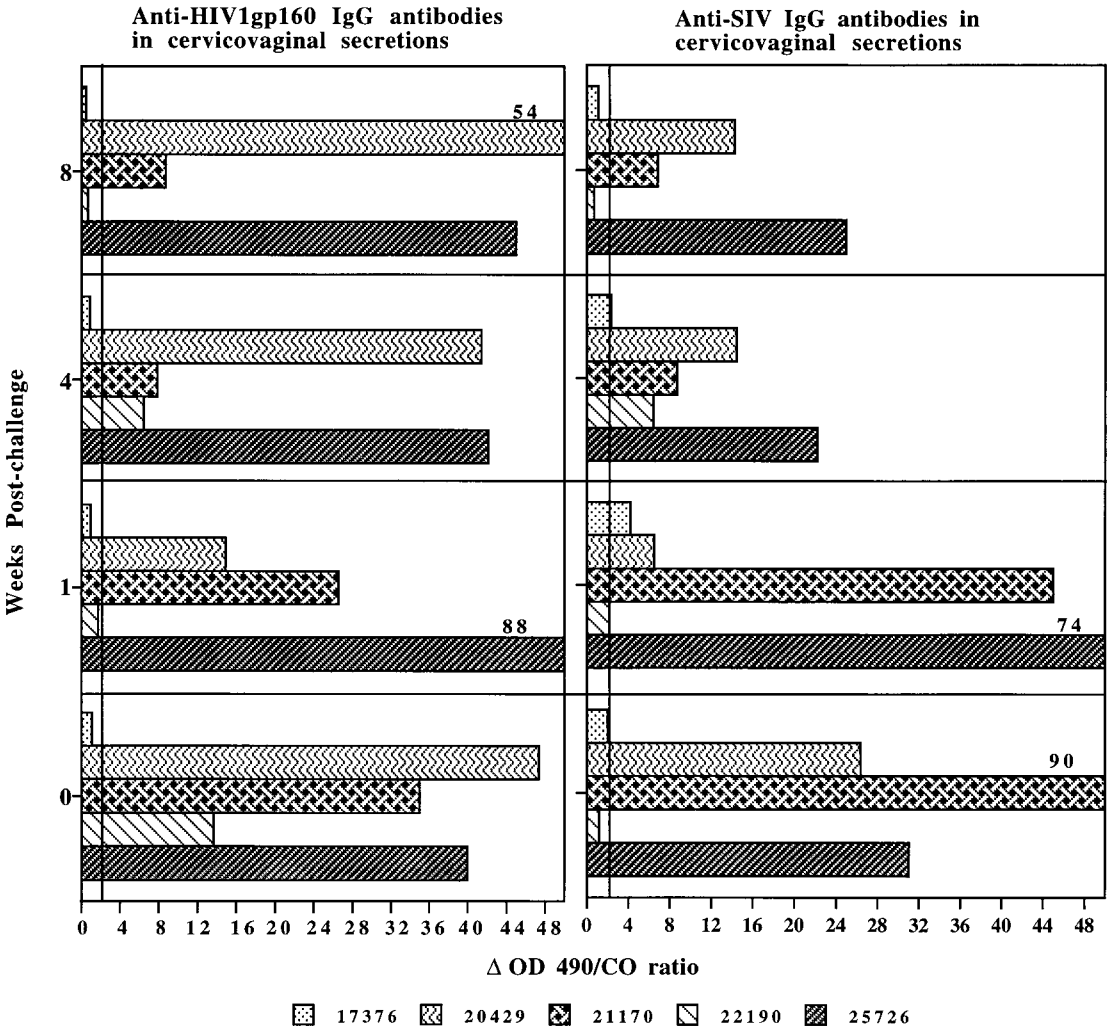

FIG. 9. Anti-HIV-1 gp160 and anti-whole SIV antibody levels in CVS of SHIV-immunized rhesus macaques before and after vaginal challenge with SIVmac239. Three of the SHIV-immunized animals (animals 20429, 21170, and 25726) had IgG antibodies to both HIV-1 gp160 and SIV in CVS at the time of challenge. After challenge, all the animals had anti-SIV IgG antibodies in vaginal secretions. After vaginal challenge with the SIVmac239, anti-HIV-1 gp160 IgG was present in the vaginal secretions of the animals that had been positive before challenge and anti-SIV antibodies were detected in CVS in at least one sample collected from every animal after challenge. The numerical value shown is the ratio of the difference in optical density (ΔOD, defined in the test) of the sample to the CO. The ΔOD/CO ratios of samples which had values greater that 48 are noted numerically above the relevant horizontal bar. The vertical line indicates a ΔOD/CO ratio of 2 (the point at which the sample was considered to be positive).

some resistance to the challenge inoculum. Several groups have recently reported that the level of plasma viremia in HIV-infected individuals is the best predictor of progression to AIDS. Individuals with low levels of viral RNA in plasma have a high probability of long-term AIDS-free survival (9, 25, 36). A similar relationship between virus load in plasma and outcome of SIV infection has been found in rhesus macaques (11, 16). At 9 months postinfection, all the animals in the study remain clinically healthy, with the exception of one of the virus isolation-negative animals (animal 17376), which was killed at 6 months postinfection due to clinical complications associated with severe endometriosis. Endometriosis is a condition which occurs in many captive female rhesus macaques and is unrelated to SIV infection. Lymph nodes (mesenteric, iliac, axillary, and inguinal) and spleen taken at necropsy from this animal were uniformly virus isolation and SIV *env* PCR negative (data not shown). Because all the SHIV-immunized animals, including the two animals from which virus was isolated, had very low viral RNA loads in plasma, it is unlikely that the SHIV-immunized animals will develop clinical disease. The clinical outcome following exposure to virus is the best measure of vaccine effectiveness, and we will continue to monitor all the animals in this study to characterize the long-term clinical outcome of the SIVmac239 challenge.

Others have reported that increased time of vaccination with live-attenuated SIV is associated with increased protection from intravenous challenge with virulent SIV (43). Although the number of animals used in the present study was small, a similar trend was observed. The three SHIV-immunized animals that remained virus isolation and SIV *env* PCR negative were infected with SHIV for more than 1 year prior to the challenge, while the two animals that were virus isolation and SIV *env* PCR positive after SIV challenge had been infected with SHIV for 24 weeks prior to the challenge. As previously pointed out (43), the time-dependent nature of this protection is most consistent with immune-mediated control of virus rep-

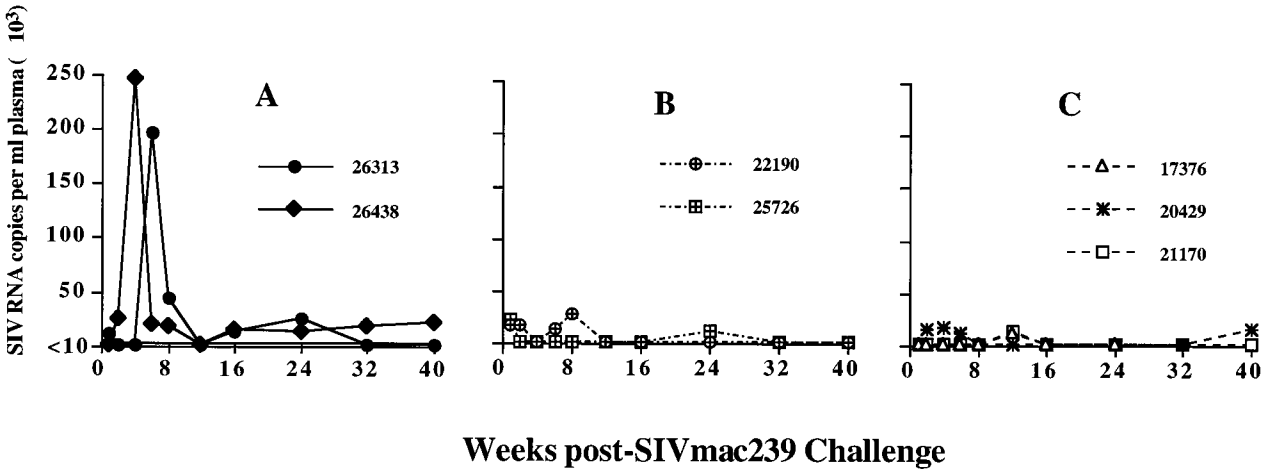

FIG. 10. Viral RNA levels in plasma of SHIV-immunized animals after vaginal challenge with SIVmac239. Viral RNA levels in plasma were determined by bDNA assay in rhesus monkeys after challenge with SIVmac239. (A) Nonimmunized control animals; (B) SHIV-immunized animals which became virus isolation positive after vaginal challenge with SIVmac239; (C) SHIV-immunized animals which were virus isolation negative after vaginal challenge with SIVmac239. Note that because the assay detects SIV *pol* sequences, it cannot distinguish between SHIV 89.6 and the SIV challenge strain.

lication rather than with viral interference with virus replication.

The protection provided by previous infection with SHIV 89.6 was associated with the presence of CTL. Virus was never isolated from the three animals that had SIV-specific CTL in their PBMC at the time of SIV challenge. We have previously shown that in rhesus macaques infected with SIVmac251 by vaginal inoculation, the presence of anti-SIV CTL in PBMC was always associated with SIV-specific CTL in the vaginal epithelium (17). Thus, it is likely that in the present study, the animals that had CTL in PBMC also had SIV-specific CTL in the vaginal epithelium. SIV-specific antibody, although present in the vaginal secretions of two of the three virus isolation- and SIV *env* PCR-negative animals, did not appear to be necessary for protection from vaginal SIV challenge. One of the animals (animal 17376) that was protected had no detectable anti-SIV antibody in vaginal secretions at the time of challenge. The virus isolation- and SIV *env* PCR-positive animal that had no CTL activity in PBMC (animal 27526) did have high levels of anti-SIV IgG in vaginal secretions. Although this animal did become SIV infected after challenge, the infection may have been blunted, because virus was isolated from PBMC collected at only two time points and the viral RNA level in plasma after challenge was low. Thus, both local anti-SIV IgG and local and systemic SIV-specific CTL may be involved in mediating protection from vaginal challenge, but, of the two immune effector mechanisms, CTL seem to be more important.

The SHIV-induced protection against SIV challenge was apparently not mediated by antibody responses to variable epitopes of the SIV envelope glycoprotein. The sequences of the SIV and HIV *env* gene are highly divergent (6). At 16 weeks after SHIV inoculation, three of the immunized animals in this study (animals 21170, 22190, and 25726) did have serum antibodies that cross-reacted with SIV gp120 as determined by Western blotting (19); however, we did not detect anti-SIV gp120 reactivity in the sera of any SHIV-immunized animals at the time of or after challenge. A number of studies have shown that protection from lentivirus infection can be induced with

TABLE 4. PCR-based detection of SIV *gag*, SIV *env*, and HIV *env* gene sequences in PBMC of SHIV-immunized and control rhesus macaques after intravaginal challenge/exposure with SIVmac239

Animal no.	Result of PCR analysis of PBMC DNA obtained at time (wk) after SIVmac239 challenge:																	
	2			4			6			8			16			20		
	SIV *gag*	SIV *env*	HIV *env*	SIV *gag*	SIV *env*	HIV *env*	SIV *gag*	SIV *env*	HIV *env*	SIV *gag*	SIV *env*	HIV *env*	SIV *gag*	SIV *env*	HIV *env*	SIV *gag*	SIV *env*	HIV *env*
SHIV immunized																		
17376[a]	+	−	+	+	−	+	+	−	+	+	−	−	+	−	+	ND[b]	−	+
20429	+	−	+	+	−	+	+	−	+	+	−	+	+	−	+	ND	−	+
21170	+	−	+	+	−	+	+	−	+	+	−	−	+	−	+	ND	−	+
22190	+	ND	+	+	−	+	+	ND	+	+	−	+	+	+	+	ND	+	+
25726	+	−	+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	+
Controls																		
26313	+	−	−	+	+	−	ND	ND	ND	+	+	−	+	+	−	ND	+	ND
26438	+	+	−	+	+	−	ND	ND	ND	+	+	−	+	+	−	ND	+	ND

[a] This animal (17376) was killed at 25 weeks postchallenge for clinical signs related to endometriosis. At necropsy, DNA was extracted from the spleen, and analyzed by PCR for the presence of SIV *gag*, SIV *env*, and HIV *env* proviral sequences as described for PBMC in Materials and Methods. The genomic DNA from the spleen of the animal was positive for SIV *gag* and HIV *env* proviral sequences but negative for SIV *env* proviral sequences.

[b] ND = not done.

vaccines which contain envelope antigens which are only distantly related to the challenge virus (5, 10, 38). A recent serologic analysis of HIV-1 isolates from different clades (35) demonstrated that antibody responses in sera from HIV-1-infected individuals can react to the envelope of HIV-1 isolates which are genetically very divergent from the virus which infects the individual. These nonhuman primate studies and serotyping analysis suggest that despite the genetic complexity of the HIV-1 envelope, it will be possible to develop a vaccine which is effective against most HIV-1 isolates.

In a previously published study, SHIV-infected animals were challenged with pathogenic SIV but were not solidly protected from the challenge virus. After intravenous challenge with SIV, the cynomolgus macaques which had previously been infected with SHIV HXBc2 became viremic but had reduced virus loads compared to naive control animals (14). In contrast, we report here that after vaginal challenge with pathogenic SIV, all of the animals immunized with SHIV 89.6 had low viral RNA loads in plasma and three of five remained virus isolation and PCR negative for SIV *env* proviral sequences. The weakness of SHIV HXBc2 as a vaccine compared to SHIV 89.6 may be because it replicates relatively poorly in rhesus macaques. Using a series of SIV clones that have different levels of attenuation (23), we have previously shown that the degree of protection conferred by an attenuated lentivirus vaccine is related to the ability of the vaccine virus to replicate in the host (16). Attenuated SIV strains which replicate well provide more robust protection from challenge with virulent SIV than do attenuated viruses that replicate poorly. SHIV 89.6 replicates better in macaques than SHIV HXBc2 does, and this may explain why the animals in our study appear to have been more solidly protected from SIV challenge than the animals immunized with SHIV HXBc2. An even more significant difference in the two studies may be the route of virus challenge. Vaginal inoculation of rhesus macaques with a pathogenic SHIV results in delayed virus replication compared to intravenous inoculation as measured by the appearance of p27 in serum (31a). Thus, delayed dissemination of SIV following vaginal challenge may provide the opportunity for a primed immune system to control acute virus replication and dissemination.

Currently available antiviral vaccines do not prevent infection; rather, the vaccine-induced immune responses control the replication of a challenge virus, leading to clearance of the infection (42). Although desirable, sterilizing immunity may not be a practical goal with lentivirus vaccines. However, there have been numerous reports that SIV disease progression can be delayed or even prevented by vaccines that do not elicit sterilizing immunity (1, 11, 16). Several recent studies suggest that it will be possible to develop a vaccine that can prevent disease and perhaps infection after HIV exposure by sexual contact. We have demonstrated that animals that have been immunized perivaginally with SIVmac1A11 and boosted with whole killed SIV have lower virus loads after intravaginal challenge with SIVmac251 than do naive control animals (31b). Rhesus macaques immunized intratracheally with microencapsulated killed SIV grown in human cells are protected from intravaginal challenge with SIVmac251 grown in human cells (24). Individuals who are infected with HIV-2 through heterosexual contact are resistant to becoming infected with HIV-1 (40). In the present study, we have demonstrated that vaginal infection with an attenuated SHIV strain can provide solid protection from vaginal challenge with pathogenic SIV. The immune system mechanisms which confer this protection will be the subject of future studies, but based on these results, we believe that developing a vaccine capable of preventing systemic HIV infection by sexual transmission may be possible. Obviously, such a vaccine would profoundly impact the course of the HIV pandemic.

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