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An Adenovirus-Simian Immunodeficiency Virus *env* Vaccine Elicits Humoral, Cellular, and Mucosal Immune Responses in Rhesus Macaques and Decreases Viral Burden following Vaginal Challenge

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Six female rhesus macaques were immunized orally and intranasally at 0 weeks and intratracheally at 12 weeks with an adenovirus type 5 host range mutant (Ad5hr)-simian immunodeficiency virus SIV_{sm} *env* recombinant and at 24 and 36 weeks with native SIV_{mac251} gp120 in Syntex adjuvant. Four macaques received the Ad5hr vector and adjuvant alone; two additional controls were naive. In vivo replication of the Ad5hr wild-type and recombinant vectors occurred with detection of Ad5 DNA in stool samples and/or nasal secretions in all macaques and increases in Ad5 neutralizing antibody in 9 of 10 macaques following Ad administrations. SIV-specific neutralizing antibodies appeared after the second recombinant immunization and rose to titers >10,000 following the second subunit boost. Immunoglobulin G (IgG) and IgA antibodies able to bind gp120 developed in nasal and rectal secretions, and SIV-specific IgGs were also observed in vaginal secretions and saliva. T-cell proliferative responses to SIV gp140 and T-helper epitopes were sporadically detected in all immunized macaques. Following vaginal challenge with SIV_{mac251}, transient or persistent infection resulted in both immunized and control monkeys. The mean viral burden in persistently infected immunized macaques was significantly decreased in the primary infection period compared to that of control macaques. These results establish in vivo use of the Ad5hr vector, which overcomes the host range restriction of human Ads for rhesus macaques, thereby providing a new model for evaluation of Ad-based vaccines. In addition, they show that a vaccine regimen using the Ad5hr-SIV *env* recombinant and gp120 subunit induces strong humoral, cellular, and mucosal immunity in rhesus macaques. The reduced viral burden achieved solely with an *env*-based vaccine supports further development of Ad-based vaccines comprising additional viral components for immune therapy and AIDS vaccine development.

Human immunodeficiency virus (HIV) can be transmitted by parenteral exposure to blood transfusions and unsterilized needles (25, 28, 52) and perinatally from infected mothers to their newborns (28, 52). However, the predominant mode of transmission is by heterosexual or homosexual intercourse (25, 50–52). Epidemiological studies show that about 70 to 80% of all AIDS cases worldwide result from heterosexual transmission (2, 13, 15, 19, 21). The rising incidence of heterosexual transmission of HIV in North America and its high prevalence in Africa (90% of all AIDS cases) (9, 27, 46, 51) highlight the need for developing vaccine strategies that will prevent transmission of HIV across the genital mucosa.

Mucosal and systemic immune responses are elicited and regulated with a considerable degree of independence (3, 10, 39). Induction of an immune response in one of these systems does not necessarily lead to a response in the other. Peripheral immunization induces poor mucosal immunity. Mucosal immunization, on the other hand, offers an advantage in that some delivery systems induce both mucosal and systemic immunity (38). It is of paramount importance that a potential HIV vaccine induce protective immunity at mucosal surfaces of the genital tract as well as in the systemic compartment.

Adenoviruses (Ads), which replicate in the gut, may prove to be effective vaccine vehicles for development of mucosal immunity. Human Ads have received increasing attention as potential vectors for recombinant viral vaccines. Ad-vectored recombinant vaccines have been constructed by using key viral genes from a variety of animal viruses, including genes encoding hepatitis B virus surface antigen (8, 12), vesicular stomatitis virus glycoprotein (8, 56), HIV Env (14, 43, 45) and Gag antigens (43, 48), herpes simplex virus glycoprotein B (44), human cytomegalovirus glycoprotein B (44), respiratory syncytial virus F and G glycoproteins (44), rabies virus glycoprotein (44), and parainfluenza virus type 3 glycoproteins F and HN (44). Advantages of recombinant Ad-vectored vaccines include safety (11, 17, 55) and ease of administration. Human Ad vaccines (containing Ad types 4 and 7 [Ad4 and Ad7]) have proven safe as well as efficacious during use in military recruits for over 30 years (55). Moreover, the ability to orally immunize by means of enteric coated capsules should prove helpful for use worldwide. The antigenic diversity of Ads (over 45 serotypes described), which may allow exploitation of antigenically distinct vectors for multiple sequential booster immunizations if necessary, also offers an advantage.

Initial immunogenicity studies evaluated Ad–HIV type 1 (HIV-1) *env* and *gag* recombinants which were effective in

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eliciting both cell-mediated immune responses and strong humoral responses. Serum antibodies to HIV-1 p24 were generated in mice and rhesus macaques immunized by recombinant Ad–HIV-1 *gag* vaccine (48). The dog model is nonpermissive for Ad replication but permits recombinant antigen expression (8). In this model, recombinant human Ads (types 4, 5, and 7) expressing the HIV-1 Env membrane glycoprotein (gp160) were shown to elicit strong humoral responses directed at the Env antigen as well as high-titer neutralizing antibodies when administered intratracheally (43). The host range restriction of human Ads has hindered development of the approach in animal models. In chimpanzees, sequential oral delivery of Ad7-, Ad4-, and Ad5-vectored vaccines in enteric coated capsules resulted in poor enteric replication of viruses and induction of only low HIV neutralizing antibody responses (45). Later, however, the intranasal route of administration for primary and subsequent Ad-HIV booster inoculations of chimpanzees was shown to result in extensive replication of these viruses (34) and has allowed evaluation of Ad-vectored vaccines in an animal model infectable by HIV-1. The inoculation of chimpanzees with Ad-HIV by the intranasal route combined with a subunit gp160 booster was shown to elicit substantial immune responses at secretory sites, neutralizing antibodies at levels previously associated with protection against intravenous cell-free HIV challenge (4, 49), and cellular immune responses (34).

The most recent studies in the chimpanzee model have pursued the combination prime-boost vaccine regimen using Ad4-, Ad5-, or Ad7-based HIV-1_{mn} gp160 recombinants as priming immunogens and CHO-expressed gp120 as the subunit booster. This approach has elicited persistent antibodies capable of neutralizing both lab-adapted and primary isolates and significant cytotoxic T-lymphocyte (CTL) activity and has resulted in long-lasting protection against high-dose HIV challenge following a minimal number of immunizations (35, 59).

To pursue this promising vaccine strategy, we have turned to the simian immunodeficiency virus (SIV)-rhesus macaque model. While Ad recombinants suitable for use in humans cannot be assessed in this system, the model offers the advantage of evaluating protection against disease as well as against infection in proof-of-concept studies. Further, a variety of challenge stocks, including those titered vaginally and rectally, are available, and so protection elicited by a vaccine approach which should stimulate mucosal immunity can be assessed for protection against transmission across mucosal barriers. Finally, the lesser expense of the model means that larger numbers of animals can be used to provide more statistically meaningful results.

Here we establish that the rhesus macaque model is suitable for assessing Ad-based SIV vaccines. Using a combination protocol involving (i) priming with an Ad5 host range mutant (Ad5hr)-SIV_{sm} *env* recombinant that efficiently replicates in monkey cells and expresses the SIV_{sm} envelope gene (7) and (ii) boosting with a native subunit, SIV_{mac251} gp120, we demonstrate that humoral, cellular, and mucosal immune responses are elicited in rhesus macaques. We further show that this approach, in which only SIV envelope was used as immunogen, resulted in significantly decreased viral burdens following infection with the virulent SIV_{mac251} isolate by the vaginal route. Inclusion of additional SIV components in the vaccine should result in greater protective efficacy. The availability of this new rhesus macaque model will expedite evaluation of additional Ad recombinants in this highly promising combination vaccine approach and will facilitate testing of protective efficacy of the Ad-based vaccine strategy against multiple routes of viral transmission.

TABLE 1. Ad5hr-SIVenv recombinant priming and SIVgp120 subunit boosting of rhesus macaques

Macaque	Inoculation[a]			
	0 wk	12 wk	24 wk	36 wk
353	Ad5hr-SIVenv	Ad5hr-SIVenv	SIV gp120	SIV gp120
354	O + IN	IT	IM	IM
355	O + IN	IT	IM	IM
356	O + IN	IT	IM	IM
357	O + IN	IT	IM	IM
358	O + IN	IT	IM	IM
359	Ad5hr-WT	Ad5hr-WT	Syntex	Syntex
360	O + IN	IT	IM	IM
361	O + IN	IT	IM	IM
362	O + IN	IT	IM	IM
363	None	None	None	None
364	None	None	None	None

[a] O + IN, 5×10^8 PFU/500 μl of PBS was administered orally, and 5×10^8 PFU/500 μl of PBS was administered intranasally. Oral administration was given by stomach tube following administration of bicarbonate solution. For intranasal administration, 250 μl of the inoculum was placed in each nostril. IT, 5×10^8 PFU/500 μl of PBS was administered intratracheally. IM, 100 μg of native SIVgp120 in an equal volume of Syntex adjuvant was administered intramuscularly. The total volume was 400 μl. Control animals received Syntex adjuvant diluted 1:1 with PBS in a total volume of 400 μl.

MATERIALS AND METHODS

Animals. Twelve adult multiparous female rhesus macaques, aged 6 to 17 years, were used in this study. They were negative for SIV, simian retrovirus type D, and simian T-cell leukemia virus prior to use.

Immunogens. An Ad5hr mutant able to infect rhesus macaque cells was used to construct an SIV_{sm} envelope recombinant (Ad5hr-SIVenv) as described previously (7). Native SIV_{mac251} gp120 protein was purified from a productive tissue culture medium by disruption and affinity chromatography as previously described for HIV-1 gp120 (22).

Immunization of macaques. As shown in Table 1, six macaques were primed orally and intranasally at 0 weeks and intratracheally at 12 weeks with Ad5hr-SIV recombinant carrying the envelope gene of SIV_{sm}. The macaques were subsequently boosted intramuscularly at 24 and 36 weeks with Syntex-adjuvanted SIV_{mac251} gp120. Four control macaques were similarly immunized with the Ad5hr vector only, followed by Syntex adjuvant alone. Two additional naive controls brought the study group to 12. Blood and secretory samples were collected every 2 to 4 weeks for monitoring immune responses. Fecal samples were collected several days after Ad5hr immunizations to monitor replication of the wild-type (WT) vector or recombinant. Peripheral blood mononuclear cells (PBMCs) were separated from heparinized blood by centrifugation on lymphocyte separation medium (Organon Teknika Corp., Durham, N.C.). PBMCs were used fresh or were frozen viably in liquid nitrogen in a freezing medium made up 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 made up of phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (Sigma) 0.01% thinersal and 750 Kallikrein inhibitor units of aprotinin/ml. Vaginal swabs were not taken when the animals were menstruating. An occasional nasal swab showed contamination with blood and was not included in the analyses. Saliva specimens were collected by placing a syringe without a needle 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. Stool specimens 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.

Evaluation of Ad5hr recombinant and Ad5hr WT replication. The presence of Ad5hr DNA in stool specimens was measured by PCR. PCR amplification was performed with a Perkin-Elmer Cetus (Norwalk, Conn.) PCR kit. Briefly, 500 μl of stool specimen was heated at 95°C for 5 min and clarified by centrifugation. Ten microliters of the supernatant was amplified in the following reaction mixture: 10 μl of 10× buffer; 2 μl each of dATP, dCTP, dGTP, and dTTP; 2.5 μl of each oligomer (at 1 μg/μl), 0.5 μl of native *Taq* DNA polymerase, and 66.5 μl of water. The mixtures were amplified on a Perkin-Elmer Cetus DNA Thermal Cycler for 30 cycles, each consisting of 1 min of denaturing at 94°C, 1.5 min of primer annealing at 60°C, and 2-min of extension at 72°C. A second round of PCR (nested PCR) was performed with a 2.5-μl aliquot of the first PCR product as a DNA template and a corresponding set of nested primers. After 30 cycles of amplification, 10 μl of the reaction product was run on a 1.2% agarose gel (GIBCO BRL, Gaithersburg, Md.) and electrophoretically separated by size.

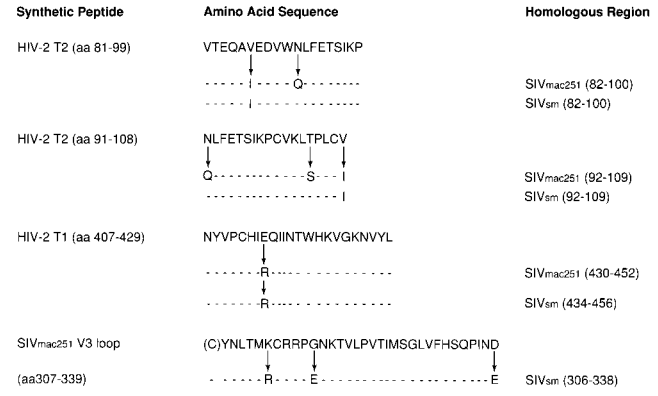

FIG. 1. HIV-2_{SBLISY} synthetic peptides used for T-cell proliferation assays. The homology between HIV-2_{SBLISY} T-helper epitopes and the corresponding regions in SIV_{mac251} and SIV_{sm} envelope proteins is illustrated. A comparison of the SIV_{mac251} V3 loop with that of SIV_{sm} is also provided. aa, amino acids.

DNA bands were visualized on a UV box after staining with ethidium bromide. The amplified DNA was further analyzed by Southern blotting and hybridization to a ^{32}P-labeled Ad5 fiber gene probe. Primers were selected from regions of the Ad5 fiber gene and were obtained from Midland Certified Reagent Co., Midland, Tex. Primer sequences for the first PCR amplification were as follows: (Ad5 positions 57 to 80; sense), 5′-GAAACCGGCCCTCCAACTGTGCC-3′; and Ad5 (561 to 584; antisense), 5′-GCTCTTTCAAGTCAATGCCCAAGC-3′. Nested primer sequences were as follows: Ad5 (137 to 161; sense), 5′-TACTCTCTTTGGACCTATCCGAACC-3′; and Ad5 (538 to 560; antisense), 5′-TACCAGTGGCAGTAGTTAGAGGG-3′. The Ad5 (372 to 391; sense) probe sequence was 5′-GCAACACACTCACCATGCAA-3′. The assay sensitivity was 10^{-1} PFU/ml of DNA. A positive control (containing 10^2 PFU of Ad5hr DNA/ml) and a negative control were run concurrently with the test samples. Nested PCR was performed on 10-μl aliquots of pharyngeal and nasal swab samples as described above. The samples were heated at 95°C for 5 min prior to use.

Serologic responses to the Ad5hr vector. Anti-Ad5 neutralization titers were determined by microneutralization analysis as described previously (8), using serial twofold dilutions of macaque sera. Endpoint titers were defined as the reciprocal of the last serum dilution at which Ad5 cytopathic effect was not observed.

Assessment of cell-mediated immune responses. T-cell proliferative responses were monitored by culturing 3×10^5 PBMCs in 200 μl of RPMI 1640 containing 10% fetal calf serum and penicillin-streptomycin (R10 medium) in the presence of 1 μg of SIV protein or synthetic peptides in microtiter plates for 5 days at 37°C. [^{3}H]thymidine (1 μCi/well) was added, and the plates were incubated for another 24 h. The cells were harvested onto filter mats by using a Skatron cell harvester, and thymidine incorporation was determined with a Beta plate counter (Wallac, LKB Diagnostics Inc., Gaithersburg, Md.). Results of triplicate determinations were expressed as the stimulation index (SI), defined as incorporation in the presence of test antigen relative to incorporation in the presence of control peptide or R10 medium alone. Proliferations with an SI of 1.5 or greater were considered positive. Data were expressed by dividing the SI of each immunized macaque by the mean SI of controls. Antigens used as stimuli included: recombinant SIV gp140 (produced in the baculovirus system) and synthetic peptides corresponding to T2 helper epitopes of the HIV-2_{SBLISY} envelope, the T1 HIV-2 helper epitope, and the V3 loop of SIV_{mac251} as shown in Fig. 1. The HIV-2 peptides were used because of their significant homology with corresponding regions of SIV_{mac251} and SIV_{sm}.

To assay CTL activity, 5×10^6 effector PBMCs were stimulated in vitro for 5 days in 10 ml of R10 medium containing 5 μg of concanavalin A (Sigma) and 10 U of interleukin 2 (Boehringer Mannheim, Indianapolis, Ind.) per ml. Autologous target B cells for each macaque were obtained by transforming macaque PBMCs with herpesvirus papio. On day 5 of the in vitro stimulation, target cells were infected with vaccinia virus constructs at a multiplicity of infection of 10 for 1 h at 37°C. Infected cells were washed and labeled with ^{51}Cr (10 μCi/10^6 cells/ml) overnight at 37°C. Vaccinia virus recombinants used included vaccinia virus expressing SIV_{sm}H4 Env and Gag (Wyeth-SIV [vJS-11]) (18), vaccinia virus expressing SIV_{mac251} Env (V194) (24), and control vaccinia virus expressing β-galactosidase (VSC-8) (6). CTL activity was assessed by mixing effector and vaccinia virus-infected cells at various effector-to-target ratios in duplicate, beginning at 100:1, and incubating them for 4 to 6 h at 37°C. Controls included target wells with no effectors for determining spontaneous release and target cells in the presence of 1% Nonidet P-40 for determining total release. Supernatant medium was harvested with a Skatron harvester, and ^{51}Cr released was counted in a gamma counter. Specific percent lysis was calculated according to the following formula:

$$\frac{\text{cpm/experimental release} - \text{cpm/spontaneous release}}{\text{cpm/total release} - \text{cpm/spontaneous release}} \times 100$$

CTL activities for two immunized animals (353 and 357) and for one control (361) were not assessed owing to inability to transform autologous B cells.

Assessment of humoral immune responses. Antibodies capable of binding the SIV envelope protein were assessed by enzyme-linked immunosorbent assay (ELISA), using whole disrupted sucrose banded SIV_{mac251} (50 ng/well) supplemented with purified SIV_{mac251} gp120 (20 ng/well) as previously described (16). Sera were screened at 1:25 or 1:50 dilution.

Neutralization assays for cell-free SIV were carried out as described previously (53). Briefly, H9 cells (47) were used as targets for infection, and a frozen titered stock of SIV produced by K1W cells (23) was the infecting virus. Serum samples were heat inactivated at 56°C for 30 min prior to all assays. Twenty microliters each of appropriately diluted virus and threefold serial serum dilutions, beginning at 1:10, were incubated for 1 h at 4°C. Ten microliters of Polybrene-treated H9 cells (4×10^6/ml) was added to each well, and the virus-serum-cell mixtures were incubated for another hour at 37°C. Subsequently, 15-μl aliquots of each mixture were plated into 200 μl of R10 medium and cultured for 5 to 6 days. The cells were washed, spotted onto Teflon-coated multiwell microscope slides, and fixed with methanol-acetone (1:1) for 10 min at room temperature. Virus infection was determined by immunofluorescence, using a mouse monoclonal antibody to SIV p27 (ABI, Rockville, Md.) and a goat anti-mouse immunoglobulin G (IgG)-fluorescein isothiocyanate conjugate (Tago Inc., Burlingame, Calif.) as the secondary antibody. Endpoint titers were defined as the reciprocal of the serum dilution at which infectivity levels were 60% of control values after normalization of the data to control infectivity levels in the presence of normal macaque serum.

Assessment of mucosal immune responses. Mucosal samples of saliva and vaginal, rectal, and nasal secretions were collected (see above) and assayed for SIV gp120-specific IgA, IgG, and IgM antibodies by ELISA. Briefly, 100 μl of a 5-μg/ml solution of native SIV gp120 (ABL, Kensington, Md.) in carbonate-bicarbonate buffer (pH 9.6) was adsorbed overnight at 4°C onto wells of Immulon 1 plates (Dynatech Ltd., Chantilly, Va.). The plates were washed three times with water and blocked for 30 min at room temperature with 5% bovine serum albumin in PBS. The plates were again washed with water, and 80 μl of sample plus 20 μl of 1% normal goat serum in PBS was added. Following incubation at 37°C for 1 h, the plates were washed three times with PBS containing 0.05% Tween 20 (PBS-Tween). For assay of SIV-specific IgG and IgM, 100 μl of peroxidase-conjugated goat anti-human IgG or IgM (Kirkegaard & Perry Laboratories, Inc. Gaithersburg, Md.) was added to wells and incubated for 1 h at room temperature. Following two washes with PBS-Tween and one wash with PBS, the plates were developed for 20 min with substrate solution containing 0.05% *o*-phenylenediamine dihydrochloride and 0.005% hydrogen peroxide in citrate buffer (pH 5). The reaction was stopped by the addition of 50 μl 4 N sulfuric acid, and the absorbance was read at 492 nm. Assays for SIV gp120-specific IgA were carried out with biotinylated anti-rhesus IgA (Lederle-Praxis Biologicals, West Henrietta, N.Y.). Bound IgA was detected by streptavidin-alkaline phosphatase conjugate (Boehringer Mannheim). This reaction was developed for 15 to 20 min with 2,2′-azinobis (ethylbenzylthrazolinesulfonic acid) (ABTS) substrate (Kirkegaard & Perry Laboratories), and the absorbance was read at 405nm.

Intravaginal challenge of macaques and assessment of SIV_{mac251} infection. Macaques were challenged at week 40 after initial immunization with a single inoculation of SIV_{mac251} (>10^5 50% tissue culture infective doses [$TCID_{50}$] in 1 ml) delivered via a tuberculin syringe into the vaginal canal as previously described (40, 41). Macaques were sufficiently anesthetized so as to remain immobile for 20 to 30 min postinoculation. Blood samples were collected periodically following challenge for assessment of virological status of the monkeys.

Virus isolation was carried out by coculturing 2×10^6 to 3×10^6 macaque PBMCs with 5×10^5 CEMx174 cells or phytohemagglutinin-activated human PBMCs. Culture supernatants were tested for virus expression by using SIV p27 antigen capture kits (Coulter). Virus isolation was scored as positive if two successive antigen capture assays were positive.

SIV_{mac251} proviral DNA in macaque PBMCs was assessed by PCR using a nested set of SIV *gag* primer pairs and a GeneAmp DNA amplification reagent kit (Perkin-Elmer, Branchburg, N.J.). Briefly, 1 μg of DNA extracted from macaque PBMCs was amplified in the following reaction mixture: 5 mM $MgCl_2$, 10 μl of PCR buffer II, 1 mM each deoxynucleoside triphosphate, 2.5 μl of each primer (1 μg/μl), 0.5 μl of *Taq* polymerase (0.5 U/100 ml), and sterile distilled water to 100 μl. Each cycle consisted of 1 min of denaturing at 94°C, 1 min of primer annealing at 58°C, and 1.25 min of extension at 72°C. The oligonucleotide primers from conserved regions of the *gag* gene (numbered according to the SIV_{mac251} sequence) and specific for SIV_{mac251} and SIV_{sm} were as follows: SIV gag 1 (sense, 257; 5′-GGTGCATTCACGCAGAAGAGAAAG-3′) and SIV gag 2 (antisense, 532; 5′-TCATAGGGAGTGCAGCCTTCTGA-3′) and nested primers SIV gag 3 (sense, 323; 5′-TAGTGGTGGAAACAGGAACAGCAG-3′) and SIV gag 4 (antisense, 508; 5′-AGTGCTTGAAATCCTGGCACTAC-3′). Primers 2 and 4 each had one nucleotide different from the SIV_{mac251} sequence.

TABLE 2. Replication of Ad5hr WT vector and Ad5hr-SIVenv recombinant in rhesus macaques

Macaque	Latest day of Ad shedding[a] in:					Adenovirus neutralizing antibody titer[b]	
	Stool samples		Pharyngeal swabs, post 1st	Nasal secretions			
	Post 1st	Post 2nd		Post 1st	Post 2nd	Post 1st	Post 2nd
Ad5hr-SIV env immunized							
353	4	0	0	14	0	—	—
354	0	0	0	0	14	—	32
355	0	1	0	0	14	—	64
356	4	0	0	7	0	—	32
357	1	0	0	0	0	4	16
358	4	0	0	0	14	—	32
Ad5hr WT controls							
359	1	0	0	21	14	—	32
360	8	0	0	0	14	—	8
361	8	0	0	0	0	—	16
362	4	0	0	0	28	—	16

[a] The latest day that Ad5hr DNA shedding was detected in stool samples, pharyngeal swabs, and nasal secretions following first and second administrations (post-1st and post-2nd) of Ad constructs. Shedding was detected by nested PCR amplification of Ad DNA.

[b] Reciprocal of the serum dilution at which Ad5hr plaque formation was completely inhibited in a microtiter assay. —, no inhibition was observed at 1:2 serum dilutions. Assays were carried out two to three times. Sera or plasma samples obtained 2 and 4 weeks after each Ad5hr administration were tested; peak mean titers obtained are reported. The two naive control macaques exhibited no Ad shedding or Ad neutralizing antibodies.

After 30 cycles, 10 μl of the amplified DNA was removed and amplified for an additional 30 cycles, using the nested primer pair. Twenty microliters of the first- and second-round amplified products were electrophoresed on 1.5% agarose gels. DNA bands were visualized by staining with ethidium bromide and transferred by Southern blotting to nitrocellulose membranes. Hybridization was carried out with a ^{32}P-labeled SIV *gag* probe (sense, 388; 5′-GGCAGA GGAGGAAATTACCCAGT-3′).

SIV_{mac251} RNA in plasma was quantitated by nucleic acid sequence-based amplification (NASBA) (54). Briefly, RNA was isolated from 100 μl of plasma by the extraction method of Boom et al. (5), subjected to isothermal enzymatic amplification by using primers which target SIV *gag* sequences, and quantified by electrochemiluminescence chemistry by using an internal standard coextracted and coamplified along with the WT SIV RNA present in the plasma sample. Quantitation was valid down to 5×10^3 RNA copies/input volume.

RESULTS

Ad replication following immunizations. In order for Ad recombinants to express inserted genes for induction of immune responses, they must initially replicate in the respiratory tract, gut, and/or lymphoid or other tissue of the infected host animal. Therefore, replication of Ad5hr recombinant and WT vector was first assessed by examining the shedding of Ad5hr DNA in stool samples of immunized and control macaques. As described in Materials and Methods, nested DNA PCR specific for the Ad5 fiber gene was carried out on stool samples obtained following Ad immunizations. Following the first Ad5hr-SIVenv intranasal and oral immunization at week 0, Ad5 DNA was detected in stools of four of six immunized animals for 1 to 4 days, with no further detection (Table 2). Four of four control animals similarly immunized with the Ad5hr WT vector exhibited shedding for 1 to 8 days. Ad5hr DNA was not detected in pharyngeal swab samples taken 1 and 4 days following these first Ad5hr WT and recombinant administrations. However, Ad5 DNA was detected in the nasal secretions of two immunized macaques and one control macaque for 1 to 3 weeks (Table 2).

Following the second Ad5hr-SIVenv immunization, given intratracheally at week 12, Ad5 DNA was detected in stool samples of only one immunized animal (355), at 1 day postimmunization. Shedding of Ad5 DNA in stools by the control macaques following intratracheal administration of the Ad5hr vector was not observed. Pharyngeal swabs were not available for analyses following these second Ad5hr recombinant and WT administrations. However, examination of nasal secretions showed that Ad5 DNA was present in three immunized macaques for 2 weeks and in three control macaques for 2 to 4 weeks.

To obtain further evidence that the Ad5hr recombinant and WT vectors replicated in the macaques, Ad5 serum neutralizing antibodies were assessed. Following the initial Ad administrations, low-titer Ad neutralizing antibodies were detected only in macaque 357 immunized with the Ad5hr-SIVenv recombinant (Table 2). Following intratracheal administrations at week 12, however, all macaques developed moderate to high Ad neutralizing titers with the exception of immunized macaque 353, which exhibited no anti-Ad5hr neutralizing antibody activity.

Cell-mediated immune responses. T-cell proliferative responses in immunized macaques were assessed over the immunization course by thymidine incorporation assays as described in Materials and Methods. Four of six immunized macaques showed proliferative responses after the first Ad5hr-SIVenv immunization, while all six showed proliferative responses after the second (Fig. 2). T-cell proliferative responses were sporadic and were mostly to recombinant SIV gp140 and the HIV-2 T1 peptide. However, immunized animals 354 and 355 showed proliferative responses to all test antigens.

Although a live vector was used to induce cell-mediated immunity, significant SIV-specific CTL activity greater than 10% was not observed in any of the immunized macaques over the immunization course regardless of whether the vaccinia virus-SIV_{sm} (Wyeth-SIV [vJS-11]) or vaccinia virus SIV_{mac251} (V194) construct was used in preparing target cells. One control animal (359) which received the Ad5hr WT vector showed consistent CTL responses of approximately 10%. Thus, low-level CTL activity observed in immunized macaques was not meaningful. Macaque 359 was retested and found negative for simian retrovirus type D.

Humoral immune responses. Specific SIVgp120 serum antibodies were elicited by the immunization regimen and were first detected in immunized macaques after the second Ad5hr-SIVenv immunization (Fig. 3A). The antibody levels gradually declined until the administration of the first gp120 subunit, when they were significantly though transiently boosted. Anti-

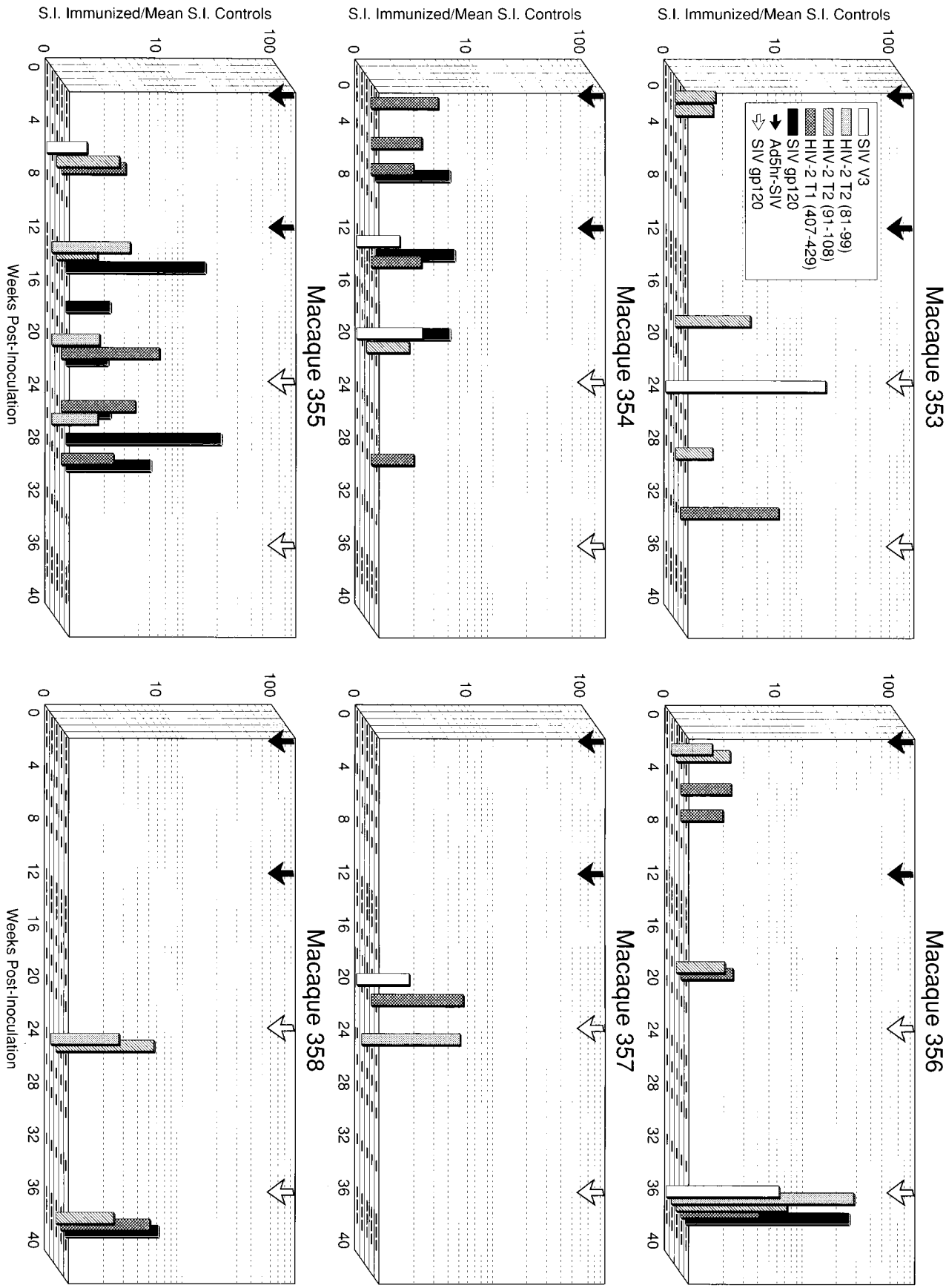

FIG. 2. T-cell proliferative responses in immunized macaques. Arrows indicate time of immunizations. T-cell proliferative responses were determined by thymidine incorporation as described in Materials and Methods. SI values shown were normalized by dividing SI of each immunized macaque with the mean SI of controls. Control SI values therefore are not shown.

SIV antibodies were still somewhat elevated at 36 weeks, when the second gp120 subunit was administered. This boost resulted in an increase of serum antibodies, although not to previous levels, suggesting that a longer time span between subunit administrations may have resulted in better boosting of the overall humoral immune response (Fig. 3A).

Strong neutralizing antibody responses to SIV_{mac251} were also first observed after the second Ad5hr-SIVenv immunization (Fig. 3B). They gradually declined and subsequently were boosted with each envelope administration. Mean SIV_{mac251}-specific neutralizing titers reached nearly 1,000 2 weeks after the first SIVgp120 administration and peaked at over 10,000 following the second SIVgp120 boost. Control macaques showed no neutralizing antibodies.

Mucosal antibody responses. Both IgG and IgA SIV-specific antibody responses developed at several mucosal sites over the course of immunization. Transient IgM responses were detected only in nasal secretions of two of the six immunized macaques following the first protein boost (not shown). The predominant mucosal antibodies detected were of the IgG isotype (Fig. 4). Two administrations of Ad5hr-SIVenv recombinant were necessary for development of weak SIV-specific IgG responses in nasal and vaginal secretions (Fig. 4a and b). The administration of the first protein boost resulted in induction of an IgG response in rectal fluids and a significant increase of IgG responses in nasal and vaginal secretions. IgG antibody levels declined nearly to baseline during the weeks between protein boosts. The second envelope immunization did not boost the IgG responses to higher levels, although nasal and rectal IgG responses returned to previous peak levels. In addition, moderate levels of IgG responses were observed in saliva of five of six immunized macaques 2 weeks after the administration of this second protein boost (data not shown). SIV-specific IgG responses in saliva were not assessed at other time points.

Like the SIV-specific IgG responses seen in nasal and vaginal secretions, SIV-specific IgA responses in nasal and rectal secretions were first detected following the second Ad5hr-SIVenv administration (Fig. 5). Administration of the protein subunit induced higher SIV-specific IgA levels, and the response in nasal secretions was more sustained than either the rectal IgA response or the IgG responses at multiple mucosal sites. While the IgA responses were readily observed in nasal and rectal secretions, no SIV-specific IgA was detected in vaginal swabs or saliva over the entire immunization course.

Results of intravaginal challenge. Four weeks following the second protein booster immunization, the 12 macaques were challenged intravaginally with a single inoculation of $>10^5$ $TCID_{50}$ of the virulent SIV_{mac251}. As reported previously (40), following virus exposure by the vaginal route, both transient and persistent infection resulted. As summarized in Table 3, four of the immunized macaques (353, 355, 356, and 357) and three of the control macaques (360, 362, and 363) developed persistent infection, as shown by the isolation of virus from their PBMCs, the detection of proviral DNA by nested PCR, and the demonstration of viral RNA in plasma for nearly a year following challenge. In contrast, two immunized macaques (354 and 358) and two control macaques (361 and 364) exhibited transient infection, with only rare detections of infectious virus or proviral DNA in PBMCs and viral RNA in plasma. No detectable signs of infection were observed in one control macaque (359). Of interest is the fact that this macaque had exhibited cross-reactive CTL activity with SIV-specific target cells (see above), although evidence of infection with another retrovirus could not be verified.

Because transient or no infection was observed in both immunized and control groups, it is not possible to attribute transient infection of immunized macaques 354 and 358 to the vaccine regimen. However evaluation of all the persistently infected macaques revealed that lesser viral burdens developed during the primary infection period in the immunized macaques compared to the controls (Fig. 6). During the first 6 weeks following challenge, mean viral burdens ranged from 1 to 2 logs lower in the persistently infected immunized macaques than in the persistently infected controls. Moreover, throughout the following period from 8 to 18 weeks postchal lenge, a log reduction in viral burden was consistently maintained in the immunized macaques. By week 20 and thereafter, the two groups of macaques exhibited similar mean viral burdens.

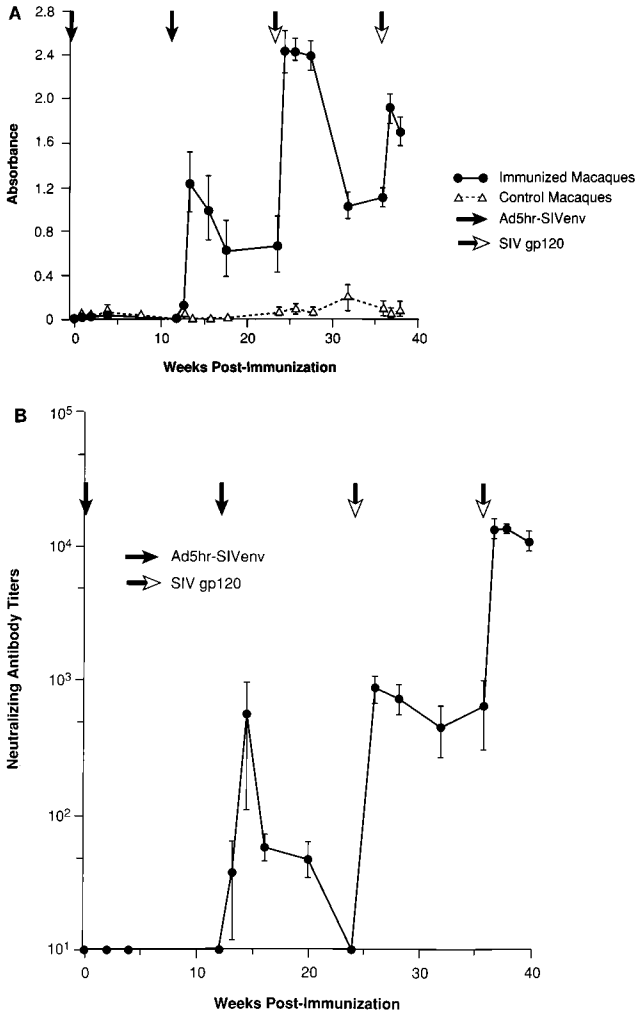

FIG. 3. Elicitation of SIV-specific humoral immune responses. Arrows indicate time of immunizations. (A) Detection of SIV antibodies by ELISA. Values plotted are mean absorbance values ± standard error of the mean. (B) SIV neutralizing antibodies in immunized macaques. Sera were tested for neutralizing activity against SIV_{mac251} grown in K1W cells. Plotted values are mean titers ± standard error of the mean. Neutralizing antibody titers of all control macaques were less than 10. Serum concentrations of greater than 1:10 were not tested.

DISCUSSION

The initial goal of this study was to evaluate the capacity of an Ad5hr-SIVenv recombinant vaccine to replicate in rhesus

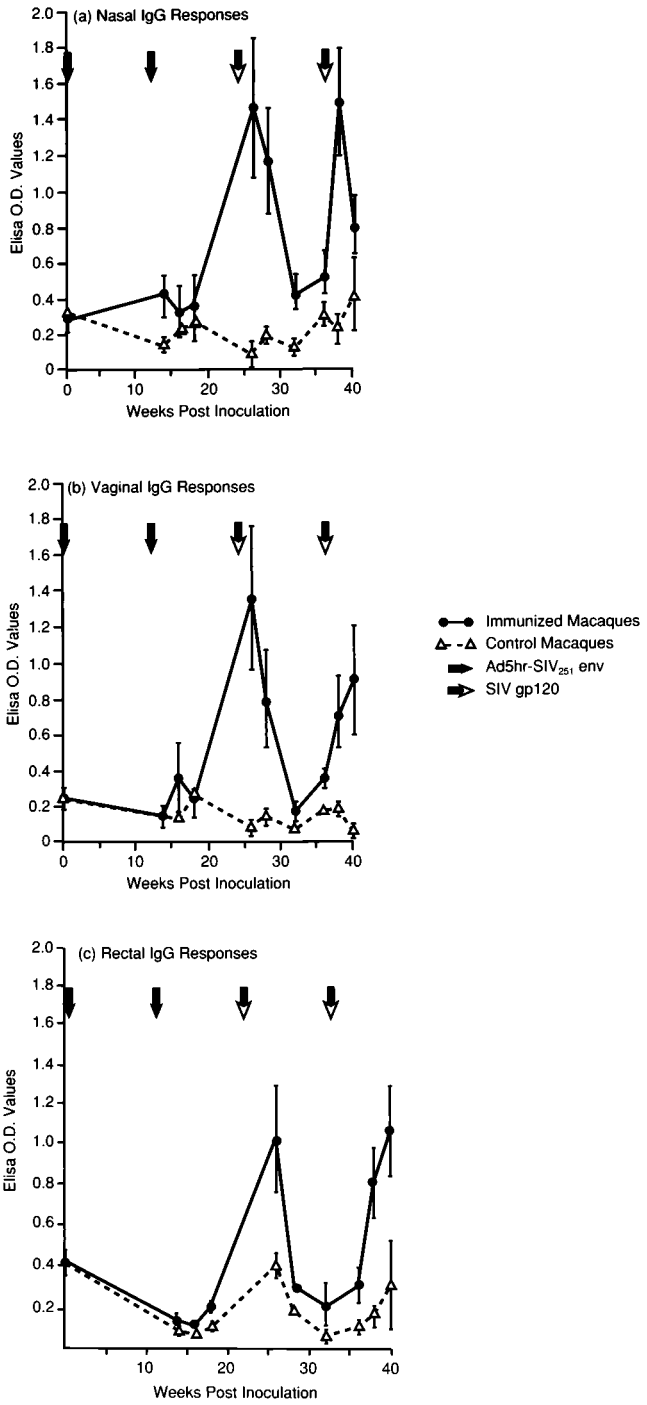

FIG. 4. SIV-specific mucosal IgG responses. Binding of IgG in nasal (a) vaginal (b) and rectal (c) secretions to native SIV gp120 was tested by ELISA. Mean absorbance values ± standard error of the mean are plotted. Arrows indicate time of immunizations. O.D., optical density.

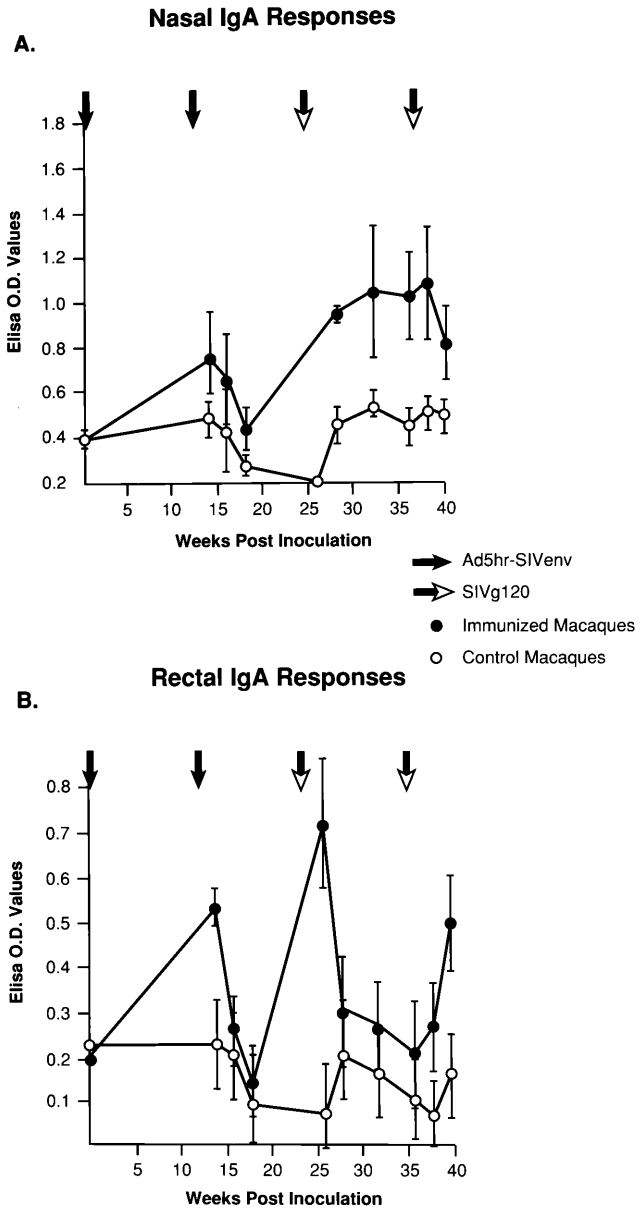

FIG. 5. SIV-specific mucosal IgA responses. Binding of IgA in nasal (A) and rectal (B) secretions to SIV gp120 was tested by ELISA. Mean absorbance values ± standard error of the mean are plotted. Arrows indicate time of immunizations. O.D., optical density.

macaques and, together with native SIV gp120 in a combination prime-boost approach, induce humoral, secretory, and cellular immune responses. Successful demonstration of induced immunity would provide a new nonhuman primate model for assessment of promising AIDS vaccine approaches utilizing Ad-based vaccine vehicles. Ad–HIV-1 recombinants have been tested in chimpanzees (34, 35, 45), and in a prime-boost vaccine regimen they have been shown to induce long-lasting protection against high-dose intravenous challenge (35). Availability of an SIV-rhesus macaque model would allow evaluation of protection against disease as well as assessment of protection against multiple routes of transmission in a less expensive animal model.

An Ad5hr-SIVenv recombinant was previously developed and shown to replicate efficiently in rhesus macaque cells and to express the inserted SIV envelope gene (7). This recombinant was used to infect rhesus macaques, first orally and intranasally. High doses of the recombinant, as well as the Ad5hr mutant, were administered at both sites, as the ability of these Ad5hr vectors to replicate in macaques was unknown. Four of six immunized macaques exhibited brief shedding of Ad5hr-

TABLE 3. SIV_{mac251} detection following intravaginal challenge

Wk post-challenge	Detection in immunized macaques[a]																	
	353			354			355			356			357			358		
	VI	DNA	RNA	VI	DNA	RNA	VI	DNA	RNA	VI	DNA	RNA	VI	DNA	RNA	VI	DNA	RNA
2	+	+	1.1×10^6	−	−	−	+	+	8.5×10^6	+	+	1.5×10^6	+	+	2.1×10^6	−	−	−
4	+	+	2.3×10^5	−	−	−	+	+	1.0×10^6	+	+	1.5×10^6	+	+	1.0×10^7	−	−	−
6	+	+	6.5×10^5	−	−	−	+	+	2.0×10^6	+	+	1.5×10^5	+	+	1.1×10^7	−	−	−
8	+	+	−	−	+	−	+	+	1.9×10^7	+	+	1.2×10^7	+	+	5.1×10^7	−	+	−
10	+	+	−	−	−	−	+	+	1.1×10^7	+	+	3.3×10^5	+	+	9.8×10^7	−	−	−
12	+	+	−	−	−	−	+	+	1.2×10^7	+	+	8.5×10^5	+	+	5.3×10^7	−	−	−
16			−			−			2.5×10^7			1.5×10^6			4.1×10^7			−
20	−	+	−	−	−		+	+	3.8×10^7	+	+	3.4×10^6	+	+	1.7×10^8	−	−	−
24	+	+	−	−	−	−	+	+	4.5×10^7	+	−	3.1×10^6	+	+	1.2×10^8	−	−	−
28	+	+	1.1×10^5	−	−	−	+	+	8.8×10^7	+	+	5.3×10^6	+	+	6.6×10^7	−	−	−
32	−	+	−	−		−		+	1.4×10^8	+	+	1.3×10^5	+	+	1.3×10^8		−	−
36	−		−			−	+		2.6×10^7	+		5.3×10^6	+		1.6×10^8	−		−
40			−			−			8.8×10^7			1.0×10^7						
48	−		1.9×10^5	−		−	+			+		3.4×10^6	+		1.2×10^8	−	−	−

[a] VI, virus isolation; DNA, detection of proviral DNA in PBMCs by nested PCR; RNA, = SIV_{mac251} plasma RNA (copies/milliliter) detected by NASBA; −, $<5 \times 10^4$ copies/ml.

SIVenv recombinant in stool samples, reflecting limited replication of the virus in the gut. Oral administration of the control vector was slightly more effective, with shedding of the Ad5hr WT vector observed for a longer period of time in stool samples of two of four control macaques. Intranasal administration of the Ad5hr vectors also resulted in limited viral replication in the upper respiratory tract. Only three macaques (two immunized and one control) had detectable Ad5 DNA in nasal secretions, while the pharyngeal swabs were consistently negative. In contrast, intratracheal immunization resulted in better replication of both the Ad5hr recombinant and Ad5 WT vector itself. Following the Ad5hr administrations by the intratracheal route, virus shedding in nasal secretions was observed in five additional macaques (Table 2). Notably, following this second immunization, Ad5hr recombinant and WT replication occurred at levels sufficient to induce detectable Ad neutralizing antibodies in 9 of 10 macaques.

The vaccine regimen was able to induce specific-SIV gp120 antibodies in the serum of immunized macaques, which first appeared after the second Ad5hr-SIVenv immunization, and were associated with neutralizing antibody activity. As a priming immunogen for humoral immune responses, the Ad5hr-SIVenv recombinant compares favorably with other live vector approaches. The prime boost approach used here elicited mean peak neutralizing antibody titers of 13,800. In contrast, two priming immunizations with attenuated vaccinia virus recombinants followed by two native SIV envelope subunit boosts induced neutralizing antibody responses of only 1,800 following the first subunit boost and 450 following the second (1). Neutralizing antibody titers achieved following similar combination protocols using WT vaccinia virus-based recombinants followed by subunit boosting have also been greater than 10,000 (20), while macaques immunized with multiple doses of a modified vaccinia virus Ankara followed by boosting with inactivated SIV achieved peak neutralizing titers of no more than 1,000 over the immunization course (18).

The Ad5hr-SIVenv/gp120 combination vaccine approach also elicited cellular immune responses. Sporadic low level proliferative responses were observed in the immunized macaques and were mostly to SIV gp140, reflecting response to multiple envelope epitopes. The few amino acid differences between the synthetic peptide stimulators and the sequence of the priming immunogen apparently did not preclude recognition by macaque T cells, although low-affinity responses may not have been detected. Significant CTL activity, however, was not detected over the immunization course. This was in part due to high activity of control macaque 359 against SIV-specific targets, thereby negating low CTL activity levels observed in immunized macaques. It is more likely, however, that the in vitro stimulation regimen using concanavalin A was not optimal. Voss et al. have shown that two rounds of in vitro stimulation using fixed autologous cells expressing viral antigens greatly enhances detection of viral antigen-specific CTLs in macaques (58).

Notably, mucosal immune responses were induced by the combination prime-boost regimen. SIV-specific secretory responses appeared at local sites of immunization (nasal) and also at distant mucosal sites (vagina and rectum). Both IgG and IgA antibody responses were observed, although the IgG responses were somewhat higher, and SIV-specific IgA was not detected in vaginal secretions. While the protective role of IgA responses is thought to exceed that of IgG responses at mucosal surfaces, evidence suggests that IgG comprises an important part of the protective response at mucosal sites. Most studies show that genital tract secretions contain IgA-to-IgG ratios of less than 1 (57), although mucosal immunization would be expected to increase this ratio. The role of IgA in vaccine protection in the rhesus macaque system remains to be clarified. A recent report has shown that the production of IgA serum antibodies in the presence of low IgG antibodies in vaccinated persons exacerbates rather than protects against infection after exposure to HIV by enhancing proliferation and dissemination of the virus (26).

Of all the mucosal sites examined, the highest antibody levels were observed in nasal secretions. This reflects the localized intranasal and intratracheal inoculations which resulted in viral replication lasting, on average, for 2 weeks. In contrast, Ad shedding in stool samples was of much shorter duration, suggesting that the method of oral administration was not optimal in spite of delivery of the vaccine in buffered solution. An alternate procedure to deliver the Ad5hr-SIVenv recombinant to the small intestine, such as the use of enteric coated capsules, might be advantageous in stimulating immune responses in the target regions of the vagina and rectum. A vaccine vehicle such as Ad which naturally replicates in sites able to stimulate mucosal immunity would greatly facilitate immuni-

TABLE 3—*Continued*

Detection in control macaques[a]																	
359			360			361			362			363			364		
VI	DNA	RNA	VI	DNA	RNA	VI	DNA	RNA	VI	DNA	RNA	VI	DNA	RNA	VI	DNA	RNA
−	−	−	+	+	9.0×10^7	−	+	2.0×10^6	+	+	1.2×10^8	+	+	2.7×10^7	−	−	−
−	−	−	+	+	1.8×10^7	−	−	−	+	+	5.4×10^6	+	+	1.1×10^7	−	−	−
−	−	−	+	+	4.9×10^6	−	−	7.2×10^6	+	+	7.4×10^7	+	+	6.6×10^8	−	−	−
−	−	−	+	+	3.5×10^6	−	−	−	+	+	3.3×10^7	+	+	4.9×10^8	+	−	−
−	−	−	+	+	7.4×10^5	−	−	−	+	−	2.0×10^8	+	+	1.9×10^8	−	−	−
−	−	−	+	+	5.2×10^5	−	−	−	+	+	3.0×10^8	+	+	1.5×10^8	−	−	−
		−			2.3×10^6			−			7.3×10^7			2.6×10^8			−
−	−		−	+	3.1×10^6	−	+	3.1×10^7	+	+	6.7×10^7	+	+	3.9×10^7	−	−	−
−	−	−	+	+	9.6×10^6	−	−	−	+	+	1.9×10^7	+	+	1.4×10^8	−	−	−
−	−	−	+	+	7.1×10^6	−	+	−	+	+	4.0×10^8	+	+	1.3×10^8	−	−	−
−	−	−	−	+	4.0×10^6	−	−	−	−	+	4.3×10^7	+	+	3.7×10^7	−	−	−
		−	−		3.6×10^5	−		−	+		2.7×10^7	+		8.5×10^7	−		−
					7.6×10^6									2.6×10^7			
−		−	−		5.2×10^6	−		−	+		2.5×10^7			4.1×10^7	−		−

zation. Localized immunization can induce immune responses in the urogenital area (29–31, 33), and in some cases, the responses are higher than those elicited by other immunization routes. However, targeted urogenital inoculation techniques are arduous and not yet suitable for general use.

A secondary goal of this investigation was to evaluate protection against mucosal challenge afforded by the vaccine regimen used. The results of the intravaginal challenge are difficult to interpret, due to the transient infection obtained in two macaques of both the immunized and control groups and the apparent lack of infection of one of the control macaques. The single intravaginal dose of $>10^5$ $TCID_{50}$ has infected 10 of 10 rhesus and 6 of 6 cynomolgus monkeys in other studies (39a). However, this dose was insufficient to achieve persistent infection of all animals in the present study. Although macaques are outbred animals, the macaques used here were from a different source than those used in the other experiments, and the vaginal inoculations were performed by different individuals. It is possible that slight differences in genetic backgrounds of the animals or differences in the inoculation techniques contributed to the different susceptibility to the SIV_{mac251} intravaginal challenge observed here.

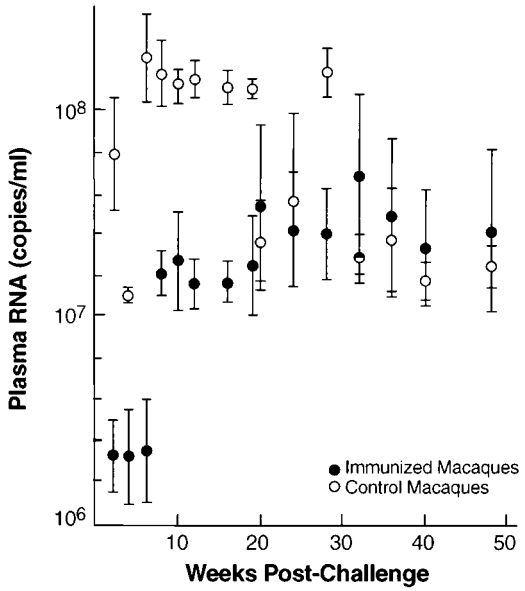

FIG. 6. Viral burden following vaginal challenge with SIV_{mac251}. PBMCs were obtained from macaques at biweekly intervals through week 12 postchallenge and at monthly intervals thereafter. Plasma SIV RNA levels were determined by the NASBA technique as described in Materials and Methods. Mean levels of plasma SIV RNA (± standard error of the mean) in persistently infected immunized or control macaques were calculated by using a value of 5×10^4 for samples scored negative in which SIV RNA was $<5 \times 10^4$ copies/ml.

Although it is not possible to determine if the immunization regimen contributed to transient infection in two of the immunized macaques, a valid comparison between persistently infected immunized and control macaques can be made. This comparison revealed a significant reduction in viral burden during the primary infection period following intravaginal challenge of the immunized macaques (Fig. 6). Plasma viremia has been shown to be associated with eventual disease outcome in the SIV-rhesus macaque model (18, 32). In fact, the initial level of plasma viremia following intravenous SIV challenge is crucial with regard to eventual disease progression (18). Low plasma viremia during the primary infection period together with subsequent restriction of virus replication is correlated with nonprogression to disease. Determination of whether the initial low viral burden seen here following intravaginal challenge influences disease outcome will await results of long-term follow-up of the persistently infected macaques. In the absence of sterilizing immunity, the ability to elicit protective immune responses sufficient to control viral burden and maintain low or undetectable levels of virus is a realistic goal which would have great impact on the morbidity and mortality resulting from HIV infection.

The reduction in initial viral burden seen here was achieved with a vaccine regimen based only on the SIV envelope. Previously, inoculation of macaques intravaginally with attenuated SIV (36) or recombinant SIV/HIV (41) has been shown to confer a degree of protection against subsequent intravaginal infection with pathogenic SIV. In addition, partial protection against subsequent intravaginal challenge has also been achieved by intramuscular immunization with inactivated SIV (36) or intramuscular immunization followed by mucosal boosting with an inactivated SIV vaccine administered in microspheres (37). An association between the development of CTLs to the Gag protein and protection from intravaginal challenge has also been observed (41). Therefore, inclusion of additional SIV components in the Ad-SIV recombinant vac-

cine would be expected to improve vaccine efficacy significantly.

This study has successfully demonstrated the utility of the SIV-rhesus macaque system for exploration of Ad-based AIDS vaccines. As a vaccine vehicle, the Ad5hr-SIVenv recombinant together with native gp120 boosting elicited high-titered neutralizing antibodies, cellular immune responses, and mucosal immunity including IgA at mucosal sites. Subsequent intravaginal challenge with the virulent SIV_{mac251} isolate resulted in significantly decreased viral burdens during the primary infection period of immunized persistently infected macaques. This system can now be further developed to examine effects of inclusion of additional viral genes and assessment of vaccine efficacy against multiple routes of infection and protection against disease progression.

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