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Vaccine-Induced Virus-Neutralizing Antibodies and Cytotoxic T Cells Do Not Protect Macaques from Experimental Infection with Simian Immunodeficiency Virus SIVmac32H (J5)

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To gain further insight into the ability of subunit vaccines to protect monkeys from experimental infection with simian immunodeficiency virus (SIV), two groups of cynomolgus macaques were immunized with either recombinant SIVmac32H-derived envelope glycoproteins (Env) incorporated into immune-stimulating complexes (iscoms) (group A) or with these SIV Env iscoms in combination with p27^{gag} iscoms and three Nef lipopeptides (group B). Four monkeys immunized with recombinant feline immunodeficiency virus Env iscoms served as controls (group C). Animals were immunized intramuscularly at weeks 0, 4, 10, and 16. Two weeks after the last immunization, monkeys were challenged intravenously with 50 monkey 50% infectious doses of virus derived from the J5 molecular clone of SIVmac32H propagated in monkey peripheral blood mononuclear cells. High titers of SIV-neutralizing antibodies were induced in the monkeys of groups A and B. In addition, p27^{gag}-specific antibodies were detected in the monkeys of group B. Vaccine-induced cytotoxic-T-lymphocyte precursors against Env, Gag, and Nef were detected on the day of challenge in the monkeys of group B. Env-specific cytotoxic-T-lymphocyte precursors were detected in one monkey from group A. In spite of the observed antibody and T-cell responses, none of the monkeys was protected from experimental infection. In addition, longitudinal determination of cell-associated virus loads at weeks 2, 4, 6, 9, and 12 postchallenge revealed no significant differences between vaccinated and control monkeys. These findings illustrate the need to clarify the roles of the different arms of the immune system in conferring protection against primate lentivirus infections.

Simian immunodeficiency virus (SIV) has a genomic organization similar to that of human immunodeficiency virus (HIV) and can induce a disease in certain macaques similar to human AIDS. Therefore, SIV infection of macaques is widely used as a model for HIV vaccine research. Previous observations have provided evidence that whole inactivated SIV vaccines can induce protective immunity in macaques (10, 13, 18, 29, 30, 40, 41), although in some of these studies the immune responses against cellular components of the vaccine proved to be at the basis of the observed protection (3, 12, 53). Immunization of rhesus macaques with live attenuated SIV has provided further evidence that the induction of protective immunity may be achieved by vaccination (16, 35). However, concerns about the overall safety of this approach may limit its potential for the development of HIV type 1 (HIV-1) vaccines.

Subunit vaccines have been proposed as a safe alternative to inactivated or live attenuated virus vaccines, with the advantage that only antigens relevant for the induction of protective immunity may be included. In this respect, much attention has been paid to the envelope glycoprotein (Env) as the major target of virus-neutralizing (VN) antibodies. Furthermore, Env has been shown to be recognized by cytotoxic T lymphocytes (CTL) in both HIV-infected humans and SIV-infected macaques (59). A role for CTL in controlling virus replication has been suggested from the observation that the clearance of viremia during primary infection is coincident with the emergence of HIV-1-specific CTL and prior to the development of detectable VN antibodies (34). Immunization of cynomolgus macaques with live vaccinia virus expressing Env and a subsequent booster injection with recombinant Env did confer protection against homologous challenge with SIVmne (25). However, similar immunization protocols did not induce protection against SIVmac infection (22, 51), although decreased virus loads were observed in some studies (1, 27).

The relatively conserved Gag protein is another candidate for inclusion in SIV subunit vaccines. Gag is known to be a major target for CTL responses in both HIV-1 and SIV infections (31, 59), in which Gag-specific CTL are believed to play a role in delaying the onset of disease (9).

Regulatory proteins expressed early in viral replication are also interesting components of candidate SIV subunit vaccines, since CTL directed against these proteins may kill infected cells before the expression of structural proteins and before the release of infectious virus particles. In this respect, Nef appears to be of particular interest, since it is abundantly expressed early in infection and is able to induce CTL responses in both humans and macaques (14, 15, 58). In HIV-1-infected individuals, several CTL epitopes have been identified in the central

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region of Nef and shown to be recognized in the context of a broad range of major histocompatibility complex (MHC) haplotypes (14, 15). In SIV-infected macaques, three Nef peptides, with a sequence highly homologous to that of the central region of the HIV-1 Nef protein, have been shown to contain CTL epitopes (7).

The system used for delivery of the selected antigens may play a crucial role in the generation of adequate B- and T-cell responses. Particular attention should be given to the induction of MHC class I-restricted $CD8^+$ CTL, because it requires antigen processing via the endogenous route, which is usually not accomplished by nonreplicating antigen presentation forms (for a review, see reference 37). However, proteins incorporated into immune-stimulating complexes (iscoms), as well as peptides bound to a lipid tail, have been shown to induce $CD8^+$ MHC class I-restricted CTL in vitro and in vivo (6, 17, 36, 39, 50, 55, 57). Interestingly, previous studies have suggested that gp120-enriched SIVmac32H iscoms are effective in inducing protection against challenge with cell-associated SIV (19, 23, 42). Furthermore, HIV-2 iscoms have been shown to induce long-lasting protective immunity against challenge with cell-free HIV-2 (43).

In this study, two groups of monkeys were immunized with either recombinant SIV Env incorporated into iscoms (group A) or with SIV Env iscoms combined with $p27^{gag}$ iscoms and three Nef lipopeptides containing the CTL epitopes previously identified in the central region of Nef (amino acids 108 to 123, 155 to 169, and 164 to 178) (7) (group B). Despite the induction of VN antibody responses and CTL responses against Env, Gag, and Nef, none of the monkeys proved to be protected against intravenous challenge with the J5 molecular clone of SIVmac32H.

MATERIALS AND METHODS

Formulation of vaccine preparations. (i) Preparation of SIV Env iscoms. Two Env glycoproteins, referred to as 8672-m and 8789-m, were produced and incorporated into iscoms as previously described (26). Briefly, peripheral blood mononuclear cells (PBMC) were derived from two rhesus macaques shortly after infection either with the 32H isolate of SIVmac251 (November 1988 pool) (macaque 8789) or with the same virus after it had undergone an 11-month in vivo passage (macaque 8672) (19). Two SIV Env genes were amplified from PBMC by PCR, and the putative primary and secondary cleavage sites were mutated by site-directed mutagenesis. These constructs were cloned in plasmid pSC65 (kindly provided by B. Moss, Bethesda, Md.) under the control of a synthetic early-late promoter. Recombinant vaccinia viruses (rVVs) were made by homologous recombination with vaccinia virus (WR strain). rVVs were used to infect baby hamster kidney cells. Twenty hours after infection, cells were harvested, and the rVV was inactivated by paraformaldehyde (PFA) fixation. Subsequently, the Env glycoprotein was solubilized from the cell membrane by using Rosenbuch-Tenside (Bachem, Bubendorf, Switzerland), purified by lentil-lectin chromatography, and eventually incorporated into iscoms. The Env protein of feline immunodeficiency virus (FIV) strain AM19 (45) was produced and incorporated into iscoms following the same procedure.

(ii) SIV Gag iscoms. The C-terminal part of p17 and p27 of the SIVmac251 *gag* gene was excised from plasmid pKA27 (kindly provided by N. Almond and P. Kitchin through the Medical Research Council) and cloned into the *Eco*RI and *Xba* sites of plasmid pMALc (New England Biolabs, Inc., Beverly, Mass.). As only hydrophobic proteins incorporate efficiently into iscoms, a sequence containing 29 amino acids of the hydrophobic part of the transmembrane sequence of HIV clone 320 (2) (amino acids 683 to 711 [WAGLWNWFSITNWLWYIKI FIMIVGGLVG]) was amplified by PCR and cloned into the C terminus of the $p27^{gag}$ gene. The resulting plasmid, pMALc-Gagp27, expresses in *Escherichia coli* a fusion product of maltose-binding protein which is linked to SIV $p27^{gag}$ by a factor Xa cleavage site. The fusion protein was first released from bacteria by sonication and subsequently purified from bacterial proteins through affinity chromatography by allowing its maltose-binding protein part to bind to an amylose column. The $p27^{gag}$ protein was released from the column by incubation with 12.5 μg of factor Xa (New England Biolabs) per ml in Xa buffer (20 mM Tris-HCl [pH 7.4], 100 mM NaCl, 2 mM $MgCl_2$) containing 0.25% MEGA-10, 10 μg of cholesterol per ml, 10 μg of phosphatidylethanolamine per ml, and 2 μg each of the of protease inhibitors aprotinin and leupeptin per ml. The $p27^{gag}$ protein was finally collected and incorporated into iscoms by a procedure similar to that described for the preparation of SIV Env iscoms. The amount of $p27^{gag}$ present in each iscom preparation was quantified by a commercial antigen capture enzyme-linked immunosorbent assay (ELISA) (Vironostika, HIV Antigen Microelisa system; Organon Teknika B.V., Boxtel, The Netherlands) according to the procedure recommended by the manufacturer. Recombinant $p27^{gag}$ (kindly provided by I. Jones, Oxford, United Kingdom) was used as a standard protein for quantification.

(iii) Nef lipopeptides. Three Nef lipopeptides were constructed according to sequences previously found to contain CTL epitopes in SIVmac-infected macaques (7). The following Nef peptide sequences were based on the J5 molecular clone of SIVmac32H (47): peptide 1 (amino acids 108 to 123), LRTMSYK LAVDMSHFI; peptide 2 (amino acids 155 to 169), DWQDYTSGPGIRYPK; peptide 3 (amino acids 164 to 178), GIRYPKTFGWLWKLV. These peptides were synthesized by standard Fmoc solid-phase methods (20), starting with Rink amide resin. The N-terminal lysine was coupled as Fmoc-Lys(Fmoc). After Fmoc deprotection, the N-terminal and the side chain amino group of lysine were palmitoylated with palmitic anhydride. Two serine residues were introduced between the N-terminal lysine and the Nef peptide sequence.

Animals and immunization protocol (see Table 1). This study included twelve colony-bred juvenile cynomolgus macaques (*Macaca fascicularis*) between 1.5 and 3.5 years old and weighing from 1.4 to 2.9 kg at the beginning of the study. All monkeys were seronegative for SIV, type D retrovirus, and simian T-lymphotropic virus. Macaques were allocated to three groups of four monkeys each, including two male and two female monkeys and different MHC haplotypes. Animals were immunized intramuscularly at weeks 0, 4, 10, and 16 with either 20 μg of SIV Env iscoms (8672-m Env and 8789-m Env at 10 μg each) (group A) or with 20 μg of SIV Env iscoms and 10 μg of $p27^{gag}$ iscoms in combination with the three Nef lipopeptides at 1 μg each (group B). Four monkeys vaccinated with recombinant FIV Env iscoms served as controls (group C).

SIV challenge. Two weeks after the fourth immunization, monkeys were infected intravenously with 50 monkey 50% infectious doses (MID_{50}) of the cell-free March 1992 challenge stock (J5M) of SIVmac32H (pJ5), a pathogenic molecular clone derived from SIVmac32H (November 1988 pool) (47). The challenge stock derived from this molecular clone had been propagated in rhesus macaque PBMC and titrated in vivo by intravenous inoculation of rhesus macaques (47). One monkey (K2 [group A]) died during recovery from anesthesia on the day of challenge.

PBMC isolation. Heparinized blood samples were collected at two-, three-, or four-week intervals. PBMC were isolated by density gradient separation with a 68:32 (vol/vol) solution composed of 6% dextran (Sigma, St. Louis, Mo.) in distilled water and 32.8% sodium metrizoate (Nycomed, Oslo, Norway). Plasma samples were cryopreserved until used. PBMC were used immediately or stored at −135°C until used.

Quantification of SIV Env-specific serum antibodies by ELISA. Ninety-six-well plates (Costar, Cambridge, Mass.) coated with concanavalin A (Pharmacia LKB, Uppsala, Sweden) were incubated with 50 μl of 100-ng/ml lentil-lectin-purified Env derived from SIVmac32H-infected C8166 cells (19) in phosphate-buffered saline (PBS) containing 1% Triton X-100. Virus-derived SIVmac32H was used as immobilized antigen to confirm that the Env-specific antibodies detected after immunization with vaccinia virus-produced Env glycoprotein recognized the native protein. Quantification of SIV Env was performed as previously described (16). Uninfected C8166 cells were used as the negative control. After 16 h of incubation at room temperature (RT), wells were blocked with PBS containing 0.1% Tween 20 and 1% bovine serum albumin (PTB) supplemented with 10% fetal calf serum (FCS) and 0.5% nonfat dry milk. Subsequently, plates were incubated for 2 h at RT with 50 μl of twofold dilutions of plasma in PTB containing 4% FCS. After being washed, bound antibody was detected by using a biotin-conjugated goat anti-human immunoglobulin preparation (Amersham) and subsequently incubated with horseradish peroxidase-conjugated streptavidin (Amersham). For substrate reactions, 3,3′,5,5′-tetramethyl-benzidine was used (5). Endpoint titers were calculated by using a cutoff value three times above the respective dilution of the preimmune serum at an optical density at 450 nm. One-way analysis of variance (Minitab, Inc., State College, Pa.) was used to compare antibody titers between groups. The level of significance was $P < 0.05$.

Quantification of SIV Gag-specific serum antibodies by inhibition ELISA. Gag-specific antibody titers were measured in inhibition ELISAs with biotinylated polyclonal antiserum from an SIVmac-infected monkey. Ninety-six-well plates (Costar) were coated with 100 μl of a 1:200 dilution in PBS of sonicated bacterial culture of pMALc-Gagp27 containing about 10 μg of $p27^{gag}$ per ml. After incubation at RT for 16 h, wells were incubated for 30 min with PTB containing 10% FCS and then for 60 min at RT with 100 μl of twofold dilutions of monkey plasma. Fifty microliters was discarded from each well before 50 μl of biotinylated polyclonal serum in PTB containing 4% FCS was added. This polyclonal serum was derived from an SIVmacBK28-infected rhesus macaque (kindly provided by J. Heeney, TNO, Rijswijk, The Netherlands) and was shown to have a high titer of Gag-specific antibodies by immunoblotting. It was diluted to give an absorbance at an optical density at 450 nm equal to 60% of the maximum absorbance in a direct SIV ELISA. After incubation for 2 h at RT, wells were washed and incubated with horseradish peroxidase-conjugated streptavidin. For substrate reactions, 3,3′,5,5′-tetramethyl-benzidine was used (5). Inhibition titers were defined as the dilutions of monkey plasma inhibiting 50% of the absorbance at an optical density at 450 nm without the addition of plasma.

SIV neutralization assay. The SIV neutralization assay used in these experiments was performed as previously described with SIVmac32H (19). Briefly, 10 μl of serial dilutions of heat-inactivated sera were incubated in four replicate wells with 10 μl of SIVmac32H diluted to give 10 infectious particles per 10 μl. After a 30-min incubation at 37°C, 200 μl of medium containing 2×10^5 C8166 cells was added to each well. Plates were incubated for 7 days at 37°C, after which the cells from each well were transferred to poly-L-lysine-coated flat-bottom microtiter plates, fixed in methanol, and examined for the presence of SIVmac-infected cells by an SIV-specific immunoperoxidase assay. Wells containing no infected cells were scored as positive, and the number of positive wells was used to determine the 50% neutralizing dose endpoint for the relative serum. Each time point was tested twice. The differences in titers among the different groups at each time point were evaluated by Student's *t* test. The threshold of significance was $P < 0.05$.

Determination of frequencies for CTL precursors (CTLp). (i) Preparation of antigen-presenting cells. Lymphoblastoid B-cell lines (B-LCL) were established by the incubation of PBMC with supernatant from the S594 cell line producing herpesvirus papio (44) (kindly provided by R. Bontrop, Rijswijk, The Netherlands). Autologous B-LCL were infected for 18 h with rVV expressing either Env (v8672-m and v8789-m) (26), $p55^{gag}$ (SIVmac32H) (kindly provided by A. McMichel, Oxford, United Kingdom), or Nef (SIVmac32H clone pJ5) (47a) at a multiplicity of infection of 10 and then fixed in 1.5% PFA.

(ii) Limiting-dilution microcultures. Cryopreserved PBMC collected on the day of challenge and at week 9 or 12 postchallenge were thawed and seeded in parallel in serial dilutions ranging from 4×10^4 to 1×10^3 in RPMI 1640 (Gibco, Gaithersburg, Md.) containing 100 IU of penicillin per ml, 100 μg of streptomycin per ml, 2 mM L-glutamine, and 2×10^{-5} M β-mercaptoethanol (referred to as complete medium) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, Utah) (referred to as R-10). Cultures were set up in 24 replicate wells of 96-well round-bottom plates in a final volume of 100 μl per well. Autologous irradiated (2,500 rads) feeder PBMC were added at 10^4 per well. Responder cells were stimulated on days 0 and 7 of culture with autologous rVV-infected B-LCL expressing the appropriate SIV protein at 10^4 per well. Recombinant interleukin-2 at 10 U/ml was added to microcultures on days 3, 7, and 10. On day 14, cells from each well were resuspended and two 70-μl aliquots were screened for cytotoxicity in split-well CTL assays with either rVV expressing one of the SIV proteins or control wild-type vaccinia viruses (Copenhagen strain).

The phenotype of the expanded cell population was assessed on day 15 of culture by flow cytometry (fluorescence-activated cell sorter [FACS] analysis) with fluorescein isothiocyanate-conjugated anti-CD4 (Okt4; Ortho Diagnostics, Raritan, N.J.) and phycoerythrin-conjugated anti-CD8 (Leu 2a; Becton Dickinson, Etten-Leur, The Netherlands).

(iii) Cytotoxicity assay. Cytotoxicity was measured in standard 5-h sodium chromate (^{51}Cr) release assays. Autologous B-LCL infected for 16 h at a multiplicity of infection of 10 with either rVV expressing one of the SIV proteins or control wild-type vaccinia viruses (Copenhagen strain) were used as target cells. These were labelled for 1 h with 100 μCi of ^{51}Cr at 37°C in 5% CO_2, washed three times in complete medium, resuspended in R-10 at 10^5/ml, and added to effector cells in 50 μl (5,000 cells per well) in 96-well round-bottom plates (Costar). After a 5-h incubation at 37°C in 5% CO_2, supernatants were harvested with a Skatron harvester (Skatron, Oslo, Norway), and the release of ^{51}Cr was measured in a gamma counter. Maximum ^{51}Cr release was determined by detergent (5% Triton X-100) lysis of target cells. Spontaneous release was determined by the incubation of target cells in R-10 alone. Maximum ^{51}Cr release and spontaneous release were set up in 18 replicate wells. Spontaneous release was <25% of maximum ^{51}Cr release in all assays.

(iv) Calculation of CTLp frequencies. Individual wells were considered positive when the experimental release of specific targets but control targets exceeded spontaneous release by 3 standard deviations. CTLp frequencies were estimated by the method described by Strijbosch et al. (54). Frequencies were normalized to the number of CTLp per 10^6 plated cells.

Quantification of cell-associated virus load. Serial fivefold dilutions of freshly isolated PBMC in R-10 ranging from 1×10^6 to 1.6×10^3 were seeded in duplicate wells in the presence of 3 μg of concanavalin A per ml. PBMC were cocultured with the human T-cell line C8166 at 10^5 cells per ml. Recombinant interleukin-2 was added on day 3 at 10 U/ml. Cells were cultured for 6 weeks at 37°C in a humidified 5% CO_2 incubator. Cultures were refreshed twice a week. Supernatants were assayed regularly for $p27^{gag}$ by antigen capture ELISA (Organon Teknika). The number of infected cells was calculated from the highest positive dilution and expressed as the number of infected cells per 10^6 PBMC. The differences in the mean values of virus load among the different groups at each time point were evaluated by Student's *t* test. The threshold of significance was $P < 0.05$.

RESULTS

Antibody responses induced by the candidate SIV subunit vaccines. (i) SIV Env-specific serum antibodies. Monkeys were immunized intramuscularly with the different vaccine preparations at weeks 0, 4, 10, and 16 as outlined in Table 1. The first immunization induced Env-specific antibodies in three monkeys from group A and two monkeys from group B (Fig. 1A). The responses in all monkeys increased after the second and third immunizations but declined rapidly after each booster injection. After the fourth immunization, antibody titers returned to approximately the same levels as those reached after the third immunization, that is, at levels similar to those found after experimental infection. Env-specific antibody titers did not differ significantly between monkeys from groups A and B. No SIV Env-specific antibodies were found in the monkeys from control group C before challenge.

TABLE 1. Immunization schedule for cynomolgus macaques

Group	Monkeys	Immunogens[a]	μg/dose
A	769, K70, K81, K2	SIV Env iscom 8672-m	10
		SIV Env iscom 8789-m	10
B	K77, K80, K83, K88	SIV Env iscom 8672-m	10
		SIV Env iscom 8789-m	10
		SIV $p27^{gag}$ iscoms	10
		Nef lipopeptide 108–123 (LRTMSYKLAVDMSH)	1.0
		Nef lipopeptide 155–169 (DWQDYTSGPGIRYPK)	1.0
		Nef lipopeptide 164–178 (GIRYPKTGWLWKLV)	1.0
C	K66, K71, K73, K79	FIV Env iscoms	10

[a] Monkeys were immunized intramuscularly at weeks 0, 4, 10, and 16 and challenged intravenously at week 18 with 50 MID_{50} of the J5 molecular clone of SIVmac32H.

(ii) SIV Gag-specific serum antibodies. $p27^{gag}$-specific antibody levels were measured in two monkeys from group B after the first immunization and in all monkeys from group B after the second immunization (Fig. 1B). Titers increased after the second and third immunizations but declined rapidly after each booster injection. After the fourth immunization, antibody titers reached levels similar to those measured after the third immunization. No $p27^{gag}$-specific serum antibodies were found before challenge in monkeys from groups A and C.

(iii) VN serum antibodies. All monkeys from groups A and B developed VN antibodies to SIVmac32H upon immunization (Fig. 1C). These VN antibody titers were similar to or exceeded those observed after experimental SIVmac infection. VN antibody titers did not differ significantly between monkeys from groups A and B. In monkeys from control group C, no VN antibodies were found before challenge.

CTLp frequencies on the day of challenge. The abilities of candidate SIV subunit vaccines to induce SIV-specific CTLp were studied by limiting-dilution analyses of PBMC collected on the day of challenge (Table 2). The CTLp frequencies of replicate experiments fell consistently within the same range, with largely overlapping 95% confidence intervals. More specifically, the coefficient of variation for 12 replicate experiments of both Env- and Gag-specific CTL responses showed a mean of 8.1%. Relatively high levels of CTLp specific for Env, Gag, and Nef were detected in PBMC from group B monkeys K80 and K83, ranging from 7 to 105/10^6 PBMC. The highest CTLp frequencies were found for Env (105 and 25/10^6 PBMC, respectively). These responses were clearly vaccine induced since no specific CTLp were detected in PBMC collected from these monkeys before immunization. The other two monkeys in group B, K77 and K88, showed CTLp against two of the three proteins (K77, Gag- and Nef-specific CTLp; macaque K88, Env- and Gag-specific CTLp), albeit at lower levels (2 to 15/10^6 PBMC). In the monkeys from group A, only macaque

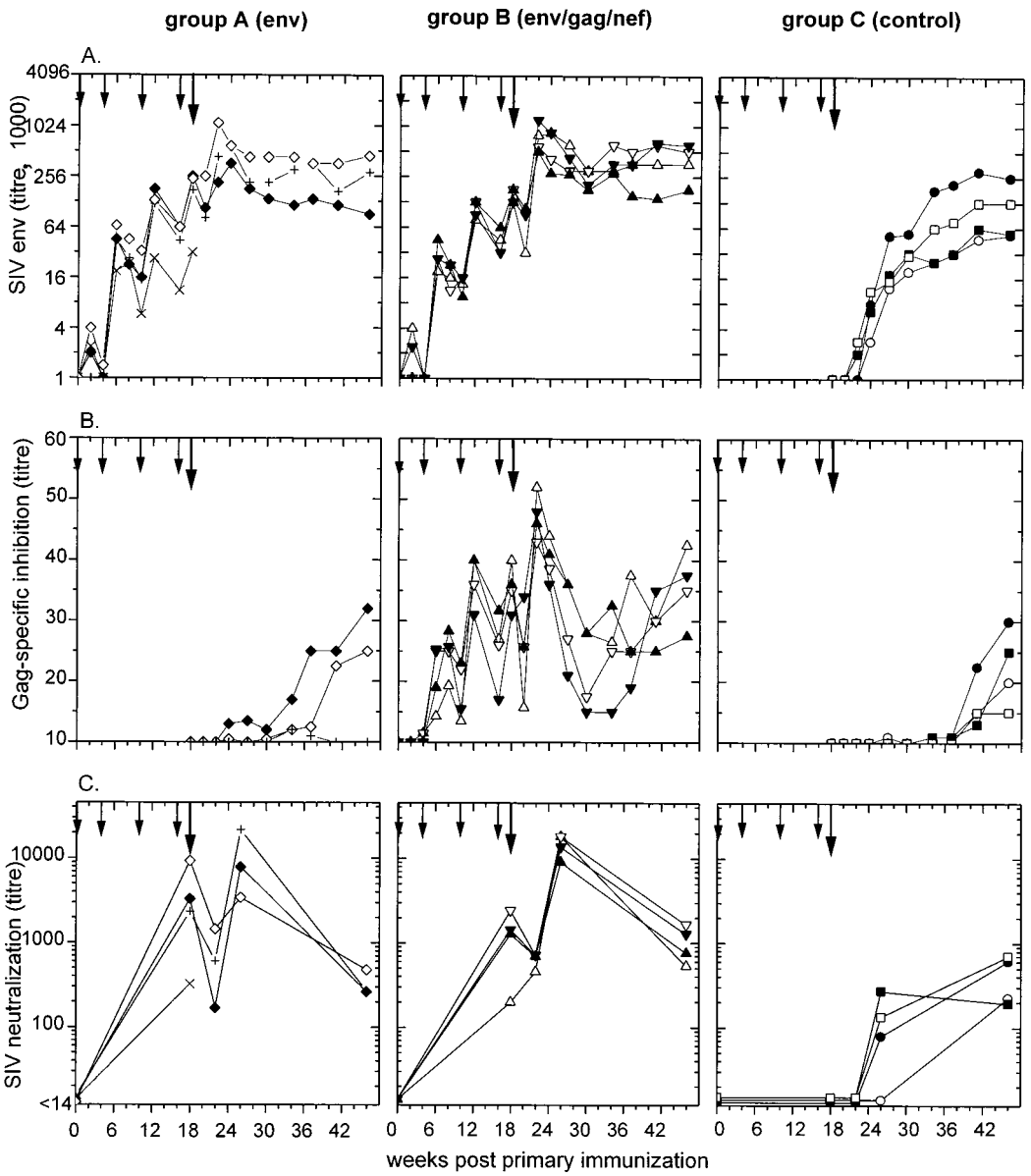

FIG. 1. Pre- and postchallenge SIV-specific antibody responses in group A, B, and C monkeys. (A) SIV Env-specific serum antibodies, as determined by indirect ELISA with SIVmac32H-derived Env as immobilized antigen. (B) SIV Gag-specific antibodies, as determined by inhibition ELISA. Bacterial $p27^{gag}$ was used as immobilized antigen. Biotinylated polyclonal serum of an SIVmac-infected monkey was used for inhibition. (C) SIVmac32H VN antibody responses. Serial dilutions of monkey plasma were incubated with SIVmac32H and added to C8166 cells. The number of negative wells was used to determine the 50% neutralizing dose endpoint titer for the relative plasma. The weeks of immunizations are indicated by small arrows. The day of challenge is indicated by a large arrow. Group A monkeys (Env immunized): ◆, 769; ◇, K70; +, K81; ×, K2. Group B monkeys (Env-Gag-Nef immunized): △, K77; ▽, K80; ▼, K83; ▲, K88. Group C monkeys (control): ○, K66; ■, K71; ●, K73; □, K79. Monkey K2 (group A) died during recovery from anesthesia on the day of challenge.

769 exhibited low levels of Env-specific CTLp ($1/10^6$ PBMC). To investigate whether the CTL responses found were MHC restricted, cells expanded by Env-specific stimulation of PBMC from monkey K80 were tested against autologous and MHC class I-mismatched rVV Env-infected target cells. Lysis of the autologous target cells but not of the MHC class I-mismatched target cells was detected, indicating that the CTL responses induced were indeed predominantly MHC class I restricted (data not shown). In addition, with cells from the same monkey, FACS analysis was performed on day 15 with cultures exhibiting Env-specific CTLp activity. The majority of expanded cells proved to be of the $CD8^+$ phenotype ($CD8^+$, 69%; $CD4^+$, 17%).

SIV-specific serum antibodies after challenge. Env-specific antibody titers decreased during the first 2 weeks after challenge in the monkeys from groups A and B but showed an anamnestic response that peaked at week 4 or 6 postchallenge (Fig. 1A). Env-specific antibodies were also detected in the monkeys from control group C, starting at week 4 or 6 after infection and increasing gradually afterwards. In all three groups of monkeys, Env-specific antibody titers eventually reached a plateau at about the same level. VN antibody development in the monkeys from groups A and B also showed an anamnestic response (Fig. 1C). In the monkeys from group C, VN antibodies were induced following infection. With the exception of monkey K88, in all of the monkeys from group B,

TABLE 2. Pre- and postchallenge Env-, Gag-, and Nef-specific CTLp frequencies[a]

Group	Macaque	Frequency (CTLp/10^6 PBMC)[b]					
		Day of challenge			Wk 9 or 12 postchallenge		
		Env	Gag	Nef	Env	Gag	Nef
A	769	1 (0–3)	ND	ND	8 (4–12)	2 (0–3)	0
	K70	0	ND	ND	3 (1–5)	1 (1–2)	0
	K81	0	ND	ND	13 (8–17)	10 (6–14)	0
B	K77	0	2 (0–5)	4 (0–10)	8 (0–16)	5 (0–10)	17 (10–24)
	K80	105 (80–130)	10 (5–15)	7 (3–11)	110 (96–151)	15 (9–20)	63 (49–76)
	K83	25 (16–34)	13 (4–22)	11 (5–17)	8 (4–12)	11 (6–16)	10 (4–16)
	K88	15 (4–26)	3 (0–8)	0	95 (74–116)	19 (7–30)	2 (0–6)

[a] Effector cells were obtained by specific stimulation with PFA-fixed autologous B-LCL infected with rVV expressing the respective SIV protein in the presence of recombinant interleukin-2. Split-well ^{51}Cr release assays were performed on day 14 of culture. Specific cytotoxicity was assayed with autologous B-LCL infected with either rVV expressing the SIV protein under investigation or wild-type control vaccinia virus. Data from limiting-dilution assays were analyzed with maximum likelihood and minimum chi-square methods.

[b] Env-, Gag-, and Nef-specific CTLp frequencies were also measured in the PBMC of K80, K83, and K88 (Env only) before immunization, and no CTLp were detected. The 95% confidence intervals are in parentheses. ND, not done.

$p27^{gag}$-specific plasma antibody titers declined 2 weeks after challenge but showed an anamnestic response that peaked at week 4 postchallenge (Fig. 1B). $p27^{gag}$-specific antibody responses were also induced after challenge in the monkeys from groups A and C.

SIV-specific CTLp frequencies after challenge. The frequencies of CTLp specific for Env, Gag, and Nef were measured at week 9 or 12 postchallenge in PBMC from group A and B monkeys (Table 2). Postchallenge CTLp measurements were carried out in parallel with those of prechallenge CTLp under identical culture and assay conditions. In monkey K80 (group B), the frequencies of Env- and Gag-specific CTLp were similar to those detected on the day of challenge, whereas the frequency of Nef-specific CTLp showed a marked increase. In monkey K83 (group B), the frequencies of Gag- and Nef-specific CTLp were similar to those measured on the day of challenge, whereas the frequency of Env-specific CTLp showed a slight decrease. Monkey K88 (group B) showed an increase in both Env- and Gag-specific CTLp after challenge and the induction of relatively low levels of Nef-specific CTLp. In monkey K77 (group B), Env-specific CTLp were induced after challenge. In the same monkey, Gag- and Nef-specific CTLp were detected, with a marked increase in the latter. Env-specific CTLp were detected in all of the monkeys from group A after challenge. In the same group, Gag-specific CTLp were detected at relatively low (monkeys 769 and K70) or high (monkey K81) frequencies. No Nef-specific CTLp were demonstrated in any monkey from group A.

Cell-associated virus loads. After challenge with 50 MID_{50} of the J5 molecular clone of SIVmac32H, all animals became infected, as illustrated by repeated isolation of SIV from their PBMC (Fig. 2). SIV was isolated from all monkeys at week 2 postchallenge. Longitudinal evaluation of cell-associated virus loads showed that the number of SIV-infected cells in circulation fluctuated over time, with a peak at 2 or 4 weeks after challenge. Furthermore, virus loads differed considerably within and among the groups. The mean value of virus load for the monkeys from control group C was consistently higher than that for the vaccinated groups, with the exception of the values at week 12. However, the differences in virus loads did not reach statistical significance.

DISCUSSION

In this paper, we have shown that SIV subunit vaccines consisting of Env glycoproteins incorporated into iscoms either

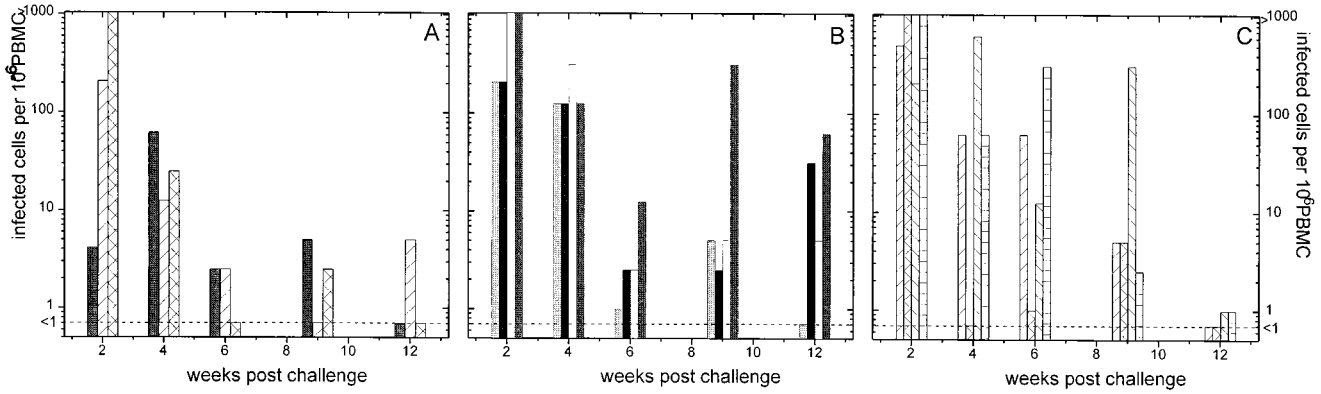

FIG. 2. Cell-associated virus loads in the PBMC of SIV-vaccinated (groups A [A] and B [B]) and control (group C [C]) monkeys during a 12-week follow-up period. Serial fivefold dilutions of PBMC ranging from 10^6 to 960 for week 2 postchallenge and from 10^6 to 1,600 for all other time points were incubated with C8166 cells for 6 weeks. The number of infected cells per 10^6 PBMC was calculated from the highest dilution that was positive in a $p27^{gag}$ antigen capture ELISA. Group A (Env) monkeys: ■, 769; ⊠, K70; ▨, K81. Group B (Env-Gag-Nef) monkeys: ▭, K77; ■, K80; □, K83; ▬, K88. Group C (control) monkeys: ▨, K66; ▨, K71; ▨, K73; ▨, K79.

alone or in combination with $p27^{gag}$ iscoms and Nef lipopeptides failed to generate protection against intravenous SIVmac challenge, despite the induction of VN antibodies and CTL responses.

The two Env glycoproteins used for immunization, 8789-m and 8672-m, shared 98.6 and 97.0% amino acid sequence homology, respectively, with the Env protein of the J5 molecular clone of SIVmac32H used for challenge (26). Because most of the VN antibodies detected in sera from infected monkeys recognize conformational epitopes (28), it is considered important that recombinant Env proteins are presented to the immune system in a form that most closely resembles the native conformation. In fact, using a panel of 15 monoclonal antibodies recognizing both conformational and linear epitopes, we have recently shown that the antigenicity of the recombinant Env proteins used in this study is similar to that of virus-derived Env protein (26). Furthermore, we found here that the VN antibody titers induced by the two recombinant Env proteins incorporated into iscoms in group A and B monkeys were similar to or exceeded those found after experimental SIVmac infection.

VN antibodies were measured with the 32H strain of SIVmac cultured in C8166 cells. It has previously been shown that antibodies which neutralize SIVmac32H also efficiently neutralize the J5 molecular clone derived from this virus (11a). The VN antibody titers on the day of challenge did not show inverse correlations with the levels of virus load measured after challenge. This is in agreement with previous studies carried out with the SIV macaque model, which failed to demonstrate a correlation between VN antibody titers and resistance to experimental SIV infection (13, 18, 24, 40). It should be pointed out, however, that in all of these studies, VN assays were based on the neutralization of virus propagated in T-cell lines. There are clear indications that the neutralization of HIV and FIV propagated in susceptible cell lines is more easily accomplished than neutralization of virus propagated on primary lymphocyte cultures (4, 48, 52). In light of these observations, one may speculate that the biological significance of the VN antibodies measured in our vaccinated monkeys is limited. The development of VN assays with monkey PBMC and non-cell-line-adapted SIV might help to demonstrate biologically more significant antibodies.

The method we used for the determination of CTLp frequencies was established in studies carried out with HIV-1-infected individuals (21, 33, 56) as well as SIVmac-infected monkeys (20a). The frequencies of SIV Env-, Gag-, and Nef-specific CTLp were measured in parallel assays of PBMC from the day of challenge and from week 9 or 12 postchallenge. Replicate experiments showed good reproducibility of CTLp frequency estimates.

The use of both iscoms and lipopeptides proved to be effective in inducing SIV-specific CTL responses in the monkeys from group B. As can be expected for analysis of an outbred population of monkeys, the frequencies of SIV-specific CTLp differed considerably among similarly immunized monkeys. The highest CTLp frequencies were those for the Env protein and appeared to be similar to those previously observed for SIVmac251-infected macaques (58). The vaccine-induced Gag- and Nef-specific CTLp frequencies were lower but comparable to those found by Yasutomi et al. in both vaccinated and SIVmac-infected macaques (60).

To achieve in vitro expansion of SIV-specific CTLp, autologous B-LCL infected with rVV expressing the respective SIV proteins and fixed in PFA were used for specific antigen stimulation. This protocol has previously been shown to selectively expand MHC class I-restricted $CD8^+$ CTL against the Gag protein of HIV-1 (56). The same approach has now also proved to selectively expand MHC class I-restricted $CD8^+$ CTL against SIV antigens (20a). In agreement with previous findings, the Env-specific cytotoxic response of monkey K80, which showed the highest frequency after vaccination, appeared to be predominantly directed against autologous targets but not MHC class I-mismatched targets, suggesting that the killing was mediated by MHC class I-restricted CTL rather than by NK cells (46). In addition, in the same monkey the majority of cells expanded by Env-specific antigen stimulation showed a $CD8^+$ phenotype. To our knowledge, this is the first demonstration of the induction of CTL responses in primates with antigens incorporated into iscoms.

In the monkeys from group A (immunized with Env iscoms alone), only one showed low-level Env-specific CTLp responses on the day of challenge. However, the ability of these monkeys to mount a CTL response against the Env protein was demonstrated by the detection of Env-specific CTLp after challenge. Although the number of monkeys in group A is too small to allow drawing any further conclusions, it may be speculated that in the monkeys from group B, simultaneous immunization with different SIV antigens may have improved the induction of Env-specific CTL, for example, by a mechanism of increased virus-specific T-helper-cell activity (49).

After challenge, an increase in SIV-specific CTLp was observed in some cases. This variability is in line with observations that the frequencies and kinetics vary considerably among infected monkeys (20a). This observation, also made for HIV-1-infected individuals (33), may reflect various degrees of homogeneity of CTL populations induced by vaccination or infection (11, 32, 38).

Upon immunization with the three Nef lipopeptides, Nef-specific CTLp were found in three of four monkeys. These monkeys had not been selected on the basis of their MHC haplotypes. Similarly, Bourgault et al. (6) noted that 6 of 12 unselected macaques showed CTL responses after immunization with a set of peptides also derived from the central region of Nef. This indicates that this region, like the corresponding region of HIV-1 Nef (14, 15), is recognized by CTL in the context of a range of different MHC class I haplotypes, stressing its potential for vaccine development purposes.

Nef has previously been shown to be an efficient CTL target in SIV-infected macaques; 8 of 12 monkeys displayed Nef-specific CTL responses (58). In our study, a similar number (four of seven monkeys) was found to display Nef-specific CTL activity after infection. Interestingly, no Nef-specific CTLp were detected at 12 weeks postchallenge in the monkeys from group A, whereas in the monkeys from group B (which had been immunized with the Nef lipopeptides), Nef-specific CTLp frequencies were either highly increased (K77 and K80), maintained (K83), or induced (K88) following infection. This finding allows speculation of a priming effect exerted by immunization with the three Nef lipopeptides in the monkeys from group B.

In SIV-infected monkeys, the presence of SIV-specific CTL has been shown to correlate with a better clinical outcome of infection, suggesting that vaccine-induced CTL contribute to protective immunity (8, 58). In one of our previous vaccine studies, four of eight monkeys appeared to be protected from challenge with cell-associated SIVmac (19). MHC class I-associated protection was observed; all of the animals protected against cell-associated virus challenge shared the MHC class I allele Mamu-A26 with the monkey cells used for challenge (24). This suggested that virus-specific CTL directly recognized the SIV-infected donor cells in an MHC-restricted manner. In this light, it is interesting that SIV-vaccinated Mamu-A26-pos-

itive monkeys, like the monkeys in this study, were not protected from challenge with cell-free, monkey-cell-grown SIVmac. In agreement with our observations, Yasutomi et al. have recently shown that a vaccine-elicited CTL response specific for a single viral epitope does not protect macaques from SIVmne challenge (60). They proposed the presence of CTL with only one specificity and the absence of VN antibodies as an explanation for the lack of protection. In this study, however, vaccine-induced VN antibodies and CTLp specific for one regulatory and two structural proteins apparently did not protect monkeys from experimental SIVmac infection. If CTL responses do play a significant role in mediating protection against SIV infection, it may be argued that CTLp frequencies should reach very high levels to exert a protective effect. On the other hand, CTL responses may have a beneficial effect on the course of infection despite their inability to clear SIV infection. In fact, although all monkeys were still clinically healthy 1 year after challenge, preliminary data suggest that the absence of CTLp early in infection may indeed correlate with a more rapid decrease in total CD4 counts later in infection.

An evaluation of the kinetics of cell-associated virus loads showed no significant differences among the respective groups of monkeys during a 12-week follow-up period. Indeed, we found that the virus loads fluctuated considerably over time. In fact, a higher mean virus load was measured in the PBMC of control monkeys than in those of the monkeys from groups A and B, especially at week 6 postchallenge. However, the opposite was true at week 12 postchallenge. This indicates that kinetic studies rather than measurements at one time point are required when virus loads are considered as a parameter for protection.

Taken together, our results show that candidate SIV subunit vaccines based on iscoms and lipopeptides efficiently induced specific VN antibodies and CTLp. However, the presence of VN antibodies and CTLp specific for multiple SIV proteins on the day of challenge proved to be insufficient to protect monkeys from intravenous SIVmac challenge. These findings indicate that the type of immunity needed to prevent infection may be quite different from that thought to control persistent infection with primate lentiviruses.

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