Vaccine-Induced Neutralizing Antibodies Directed in Part to the Simian Immunodeficiency Virus (SIV) V2 Domain Were Unable To Protect Rhesus Monkeys from SIV Experimental Challenge

KATIA SCHLIENGER,[1] DAVID C. MONTEFIORI,[2] MARYLINE MANCINI,[1] YVES RIVIÈRE,[3] PIERRE TIOLLAIS,[1] AND MARIE-LOUISE MICHEL[1]*

Unité de Recombinaison et Expression Génétique, Institut National de la Santé et de la Recherche Médicale U163,[1] and Unité de Virologie et Immunologie Cellulaire, Centre National de la Recherche Scientifique URA 1157,[3] Institut Pasteur, 75724 Paris Cedex 15, France, and Center for AIDS Research, Department of Surgery, Duke University Medical Center, Durham, North Carolina 27710[2]

The potential of the simian immunodeficiency virus (SIV) variable 2 (V2) domain as an effective region to boost SIV-neutralizing antibodies and to protect against live SIV challenge was tested in rhesus macaques. In this study, two rhesus macaques were primed with vaccinia virus recombinants expressing the surface glycoprotein gp140 of SIVmac and were given booster injections with the SIVmac V2 domain presented by a highly immunogenic carrier, the hepatitis B surface antigen (HBsAg). The two vaccinated macaques exhibited SIV-neutralizing antibodies after primer injections that were enhanced by the V2/HBsAg injections. Part of these SIV-neutralizing antibodies were directed specifically to the V2 region, as shown by neutralization-blocking experiments. However, despite having consistent SIV-neutralizing antibody titers, animals were not protected against homologous challenge with BK28, the molecular clone of SIVmac251. No SIV envelope-specific cellular cytotoxic response was detected throughout the immunization protocol, suggesting that neutralizing antibodies directed to SIV envelope gp140 and especially to the V2 domain were unable on their own to protect against SIV challenge. Furthermore, the vaccinees seemed to have higher viral loads than control animals after challenge, raising the question of whether neutralizing antibodies induced by vaccination and directed to the SIV envelope selected viral escape mutants, as shown previously in SIV-infected macaques. This mechanism is certainly worthy of intensive investigation and raises some concern for SIV envelope-targeted immunization.

The human immunodeficiency virus (HIV) is the causative agent of AIDS, a sexually transmitted disease that can also be transmitted through blood and perinatally. Efforts to develop a safe and effective AIDS vaccine have been facilitated by the development of several animal models. For example, chimpanzees can be infected by certain strains of HIV (1, 16) although they do not develop AIDS. In contrast, macaques infected with the simian immunodeficiency virus (SIV), a lentivirus closely related to HIV, develop a disease similar to AIDS in humans (12, 37) and represent the best current models of HIV pathogenesis. Challenge of immunized macaques with SIV also permits the evaluation of vaccines which may be applied to the prevention of AIDS in humans.

Protection from HIV type 1 (HIV-1) infection by vaccination in the chimpanzee model has correlated with induction of HIV-1-neutralizing antibodies directed to the V3 region of the envelope glycoprotein (6, 7, 20). Furthermore, passive infusion of antibodies directed to the HIV-1 V3 domain or of neutralizing serum with high titers to the HIV-2 V3 domain has successfully protected monkeys from HIV-1 and HIV-2 challenge, respectively (15, 54). In the SIV-macaque model, no correlation has been established between a component of the immune response and protection. Human cell-grown whole

inactivated virus vaccines were shown to successfully protect macaques against experimental intravenous infection with SIV (9, 13, 28, 30, 50, 52, 62). However, this protection was linked to immune response directed to human cellular antigens present on the viral particles used for vaccination (3, 63). When the challenge SIV was grown on monkey cells, no protection was observed (10, 36), though the appearance of simian AIDS was significantly delayed (24). Again, no correlation was noted between suppression of virus replication and the immune responses induced. More recently, the successful protection of rhesus monkeys by an attenuated strain of SIV against high-dose challenge with pathogenic SIV provided strong evidence that protective immunity by vaccination is feasible (11), but the mechanisms that were responsible for the observed protective immunity are not known. Furthermore, the use of an attenuated strain of HIV for vaccination in humans raises a great deal of concern about safety, due to the difficulty of evaluating reversion to virulence and the potential of the attenuated virus to integrate and become tumorigenic. Subunit vaccines, such as peptides or proteins, are now being developed with the rationale of eliciting an equally efficacious immune response without the risks associated with live vaccines. Immunization with homologous recombinant Env (26–28) or Env peptides (60) partially protected macaques challenged with an SIV molecular clone, and neutralizing antibodies were present at the time of challenges. Furthermore, passively transferred antibodies were shown to protect macaques in some cases from an SIV challenge (38, 54).

* Corresponding author. Mailing address: Unité de Recombinaison et Expression Génétique, Institut Pasteur, 28 rue du Dr. Roux, 75724 Paris Cedex 15, France. Phone: (33-1) 45 68 88 49. Fax: (33-1) 45 68 89 43.

However, B-cell epitopes of the SIV envelope are not yet fully characterized. In contrast to that of HIV-1, the V3 loop of SIV envelope by itself does not elicit neutralizing antibodies (29). Furthermore, no equivalent immunodominant neutralization determinant on SIV has yet been identified.

The second variable domain (V2) of SIV was predicted to have high potential to form an antigenic site (47) and has been shown to contain epitopes for neutralizing antibodies (4, 14, 31, 32). Several murine monoclonal antibodies (MAbs) displaying cell-free virus-neutralizing activity in vitro have been reported to recognize the V2 epitope (4, 14, 31, 32). One of them recognized V2 in two distinct isolates, SIVmac and SIVsm, suggesting that this epitope is conserved and is a likely candidate for a broadly active vaccine (14). Furthermore, V2 represents a serum antibody-binding region. Indeed, screening of SIV peptides with serum panels of infected macaques (5) and of infected but asymptomatic mangabeys and African green monkeys revealed that the V2 domain contains a group-specific epitope (47). In addition, antibodies to the V2 neutralizing region seem to play some role in the control of disease progression in SIVmac-infected macaques (5), and in SIVsm-infected macaques, serum reactivity to the V2 domain was higher in surviving monkeys than in animals with an early development of simian AIDS (57). However, in this last study, the recognition of the V2 peptide by the monkey sera seemed to be conformational. In another study, the sera from all animals immunized with formalin-inactivated SIV reacted with peptides from the V2 region (42). Finally, it should be noted that the V2 homologous domain of HIV-1 gp120 was also recognized as a target for neutralizing antibodies (17, 22, 25, 43, 49, 65).

We recently reported that immunization of rhesus monkeys with recombinant hepatitis B surface antigen (HBsAg) particles presenting the HIV-1$_{LAI}$ envelope principal neutralizing determinant on their surfaces (V3HIV-1/HBsAg) allowed the generation of proliferative T-cell responses, cellular cytotoxicity, and neutralizing antibodies (58). Because the gp140 V2 domain of SIVmac seems to be a good candidate for inclusion in a vaccine, we have presented the V2 domain of SIVmac251 gp140 on HBsAg particles. In order to maximize both the cellular and the humoral responses (19, 26, 27), we used a combination immunization regimen that included a live recombinant vaccinia virus/gp140 (vacc/gp140) for priming and the hybrid V2/HBsAg particle for boosting. Animals were then challenged with BK28, a nonpathogenic molecular clone of SIVmac251 (35). Despite high titers of SIV-neutralizing antibodies induced by this immunization protocol, vaccinated macaques were not protected from infection.

MATERIALS AND METHODS

Construction of antigens. The wild-type vaccinia virus from the Copenhagen strain (wt vacc) and the live recombinant vaccinia virus expressing the modified SIVmac251 *env* gene (vacc/gp140) were kindly provided by M. P. Kieny (Transgène, Strasbourg, France). In the recombinant vacc/gp140 (VV TG 4177), derived from the plasmid pBK28, the cleavage site between gp130 and gp32 (amino acids [aa] 546 and 547) was mutated and the hydrophobic transmembrane domain (aa 166 to 189 of gp32) was deleted.

Hybrid V2/HBsAg were obtained by insertion of a synthetic oligomer encoding aa 176 to 189 of the V2 domain from SIVmac251 gp140 into the pSV2S plasmid (45). This plasmid was derived from a previously described HBsAg expression vector, pSVS (46), in which the pre-S2 region has been deleted and replaced with a polylinker. The pre-S1 domain was com-

pletely removed in order to avoid intracellular retention of hepatitis B virus (HBV) envelope proteins. The resulting plasmid contains an SIV-HBV fusion gene under the control of the simian virus 40 early promoter. The polyadenylation signal is provided by HBV untranslated sequences downstream of the S gene. Following transfection in animal cells, hybrid proteins were synthesized and assembled together with the HBsAg major proteins into 22-nm particles, which were secreted and could be collected in cell culture supernatants. These particles were purified on a cesium chloride gradient according to their density in a procedure that has been described previously (46).

Structure of the V2/HBsAg particles. (i) Immunoblots. Purified recombinant particles (40 ng) were boiled in Laemmli sample buffer, separated by electrophoresis on a sodium dodecyl sulfate–12.5% polyacrylamide gel, and electrotransferred onto Immobilon-P (Millipore Corp., Bedford, Mass.). Proteins were bound by an anti-V2 SIVmac251-specific MAb (MATG 2014, 15 μg/ml; Transgène) or by an anti-HBsAg-specific MAb (H166, 13 μg/ml) (53). Finally, immunoreactive proteins were detected by anti-mouse antibodies conjugated with peroxidase (Amersham, Little Chalfont, United Kingdom) diluted to 1:10,000 and revealed with the ECL Western blotting (immunoblotting) detection reagent (ECL; Amersham).

(ii) ELISA. Reactivity of murine anti-SIV V2 MAbs MATG 2014 and MATG 2033 (4) and KK8, KK13, and KK54 (31, 32) with hybrid V2/HBsAg particles was assayed by a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA). MAb ascitic fluids diluted to 1:1,000 were used to capture twofold dilutions (starting from 20 ng/ml) of V2/HBsAg or native pre-S2/HBsAg as a control, for 16 h at room temperature. A rabbit polyclonal anti-HBsAg serum diluted to 1:500 was then added and incubated for 1 h at 37°C, and plates were revealed with a donkey anti-rabbit immunoglobulin-peroxidase conjugate (Amersham). Results were expressed as A_{450}.

Immunization of animals. Two rhesus macaques (*Macaca mulatta*) (animals 47004 and 47157) were immunized on day 0 with 5×10^7 PFU of recombinant vacc/gp140 intradermally in the deltoid region. They were reinoculated with 4×10^8 PFU of the same recombinant virus 2 months later (day 63). At months 7, 9, and 12 (days 215, 274, and 372, respectively), these macaques received booster injections with V2/HBsAg produced in CHO cells. In the first two booster injections, 60 μg of the immunogen was given intramuscularly without any adjuvant. However, in the third injection, which was given intradermally before the challenge, 30 μg of the particles was formulated in threonyl muramyl dipeptide with SAF-1 (Syntex, Palo Alto, Calif.) in order to increase the humoral response.

Two macaques from a previous study were used as controls: animal 42896 had received four injections of native HBsAg particles, and animal 42915 had received four injections of a hybrid V3 HIV-1/HBsAg particle (58). The latter animal was included as a control since it had been immunized with a hybrid HBsAg particle which did not contain SIV sequences. Both control animals received two intradermal injections with wt vacc on the same days and in the same doses as the experimental animals. Likewise, the control animals received booster injections on the same days, in the same doses, by the same route, and with the same adjuvant on the third booster injection as the experimental animals. Animal 42896 always received booster injections with native HBsAg particles, and animal 42915 always received booster injections with hybrid V3 HIV-1/HBsAg particles.

Animals were housed according to the official rules and recommendations of the European Economic Community and

were monitored for possible deleterious effects of immunization. They were anesthetized with ketamine before all inoculations and blood draws.

Cell-free challenge virus. The BK28 SIVmac challenge stock was kindly provided by A. M. Aubertin through the Agence Nationale de Recherche contre le SIDA (ANRS) program. Peripheral blood mononuclear cells (PBMC) of rhesus monkeys were transfected with recombinant plasmid pTG 668 (Transgène), and supernatants from two virus-producing cultures were mixed and filtered to constitute the challenge stock. Plasmid pTG 668 was modified from the molecular clone pBK28 of SIVmac251 (35) by deletion of cellular sequences flanking the viral genome and by mutation of the stop codon included in the gp41 sequence, allowing synthesis of a complete gp41.

Hematologic parameters. CD4+ and CD8+ cell counts were performed by FACScan analysis (FACScan flow cytometer; Becton Dickinson, Mountain View, Calif.) with mouse anti-human MAbs (CD8-fluorescein isothiocyanate [LeuTM-2a; Becton Dickinson] and CD4-phosphatidylethanolamine [Ortho-mune OKT4; Ortho Diagnostic Systems, Raritan, N.J.]) in a whole-blood lysis procedure (Lyse and Fix; Immunotech, Marseille, France) as described by the manufacturer.

Specific antibodies. Specific antibodies induced by vaccination were determined by ELISA with recombinant SIV gp140 (SIV rgp140; Repligen, Cambridge, Mass.) at 0.5 µg/ml, V2 peptide (aa 171 to 190; ANRS SP90454) at 5 µg/ml, and native pre-S2/HBsAg at 1 µg/ml, as coating antigens. Test sera were serially diluted (10-fold), and horseradish peroxidase-labelled anti-human immunoglobulin was used at 1:1,000 as the secondary antibody (Amersham). The titers were read as the reciprocal of the serum dilution giving greater than 2 times the optical density reading of the preimmune serum, with a minimum optical density of 0.050.

Virus neutralization assays. Virus-neutralizing titers were determined by inhibition of infectivity (assay 1) and cytopathic effect (CPE) reduction (assay 2) methods.

In the first assay, heat-inactivated serum samples (56°C, 30 min) and virus were diluted in RPMI 1640 with 10% heat-inactivated macaque serum. The virus used in this assay was SIVmacBK28 and was produced on rhesus monkeys' PBMC as described for the challenge virus. Serial dilutions of sera were incubated with 200 50% tissue culture infectious doses of virus at 37°C for 1 h and then added in quadruplicate to 2.5×10^4 CEMx174 cells (56) in RPMI 1640 with 10% heat-inactivated fetal bovine serum in a 96-well plate. Medium was added after 2 h and was changed at day 6. Culture supernatants were assayed for reverse transcriptase (RT) activity at day 7 (59). The percent inhibition of RT activity was expressed as the mean of each quadruplicate sample by comparison with preimmune serum of the same animal. Each result was the mean of 3 different experiments. Titers were defined as the reciprocal of the last dilution resulting in >50% RT inhibition.

In assay 2, CPE reduction titers were determined as described by Johnson et al. (30), with the CEMx174 cell line and a stock of SIVmac251 propagated in H9 cells. The neutralizing antibody titer was defined as the reciprocal of the last dilution resulting in >70% protection from killing.

Virus neutralization-inhibition assay. The virus neutralization-inhibition assay was performed exactly as was the regular CPE reduction assay (assay 2) except that prior to the addition of the virus the sera were preincubated for 2 h (37°C) in the plate with purified hybrid V2/HBsAg particles (0.486 µg/ml), V2 peptide (ANRS SP90454; 10 µg/ml), or native pre-S2/HBsAg particles (1.055 µg/ml) as a control. The V2/HBsAg

and pre-S2/HBsAg antigens were identical to those used in the vaccine.

Cytotoxicity assays. PBMC were isolated from heparinized blood by density gradient centrifugation on Ficoll metrizoate (Ficoll-Paque; Pharmacia, Les Ulis, France). The specific cytotoxic T-lymphocyte (CTL) activity was studied either in a primary assay from freshly isolated PBMC or in a secondary assay from in vitro-stimulated PBMC. Target cells were autologous B-cell lines transformed by herpes papio virus and infected by recombinant vaccinia virus expressing either the Env gp140 protein of SIVmac251 (VV TG 4177) or the signal peptide deletion mutant of this glycoprotein, VV TG 6108 (8a). For secondary assays, T-cell lines were initiated by nonspecific or by SIV-specific stimulation in RPMIc, i.e., RPMI 1640 medium with 5% inactivated human AB serum and L-glutamine. Nonspecific stimulation was performed with concanavalin A (25 µg/ml), and further expansion was obtained in RPMIc supplemented with 20 to 100 IU of recombinant interleukin 2 per ml. Specific stimulation was with purified recombinant gp140 (1 µg/ml) for 4 to 5 days and expansion in RPMIc supplemented with recombinant interleukin 2. We also used autologous irradiated (50 Gy) SIV-infected lymphoblasts (responder/stimulator ratio, 10:1) in RPMIc for 4 days and further expansion with crude T-cell growth factor for 3 days and then recombinant interleukin 2 (50 IU/ml). The CTL assays were performed from days 7 to 15 after in vitro stimulation with a conventional 4-h chromium release assay as described elsewhere (55).

Virus isolation. Macaque PBMC isolated by standard density gradient centrifugation were diluted in RPMI 1640 with penicillin, streptomycin, and glutamine from 1.25×10^6 to 3 cells per well in 24-well plates. CEMx174 cells in RPMI 1640 with 20% heat-inactivated fetal bovine serum were then added at 0.15×10^6 cells per well. Cocultures were monitored for syncytium formation and RT activity (59) in supernatants twice a week and were routinely maintained for one month. Viremia was defined as the recovery of SIV from cocultures of PBMC and was semiquantified as the number of infected PBMC per 10^6 fresh PBMC.

Antigenemia. Antigenemia was measured in macaque sera by a commercial SIV core (p27) antigen capture ELISA (Coulter SIV Core Antigen Assay; Coulter, Hialeah, Fla.). The sensitivity of this assay allows the detection of 50 pg of SIV p27 per ml.

RESULTS

Production and characterization of hybrid V2/HBsAg particles. The SIVmac251 V2 domain coding sequence was inserted into the HBsAg eukaryotic expression vector pSV2S as described in Materials and Methods. The resulting plasmid contains an SIV-HBV fusion gene in which two in-frame initiation codons allow the expression of both the major protein of the HBV envelope encoded by the S gene and a hybrid SIV/HBsAg middle protein encoded by the fusion gene. The V2 peptide amino acid sequence included in the hybrid protein was GLKRDKTKEYNETW, which exactly matched the V2 sequence of the SIVmac251 BK28 clone (23).

Following stable transfection into CHO cells, hybrid proteins were synthesized and assembled together with the HBsAg major protein into 22-nm particles, which were secreted and could be detected in cell culture supernatants by an HBsAg-specific commercial ELISA (Monolisa; Diagnostics Pasteur, Paris, France). These particles were purified on a cesium chloride gradient according to their density and were visualized by electron microscopy (Fig. 1B). Expression of V2/
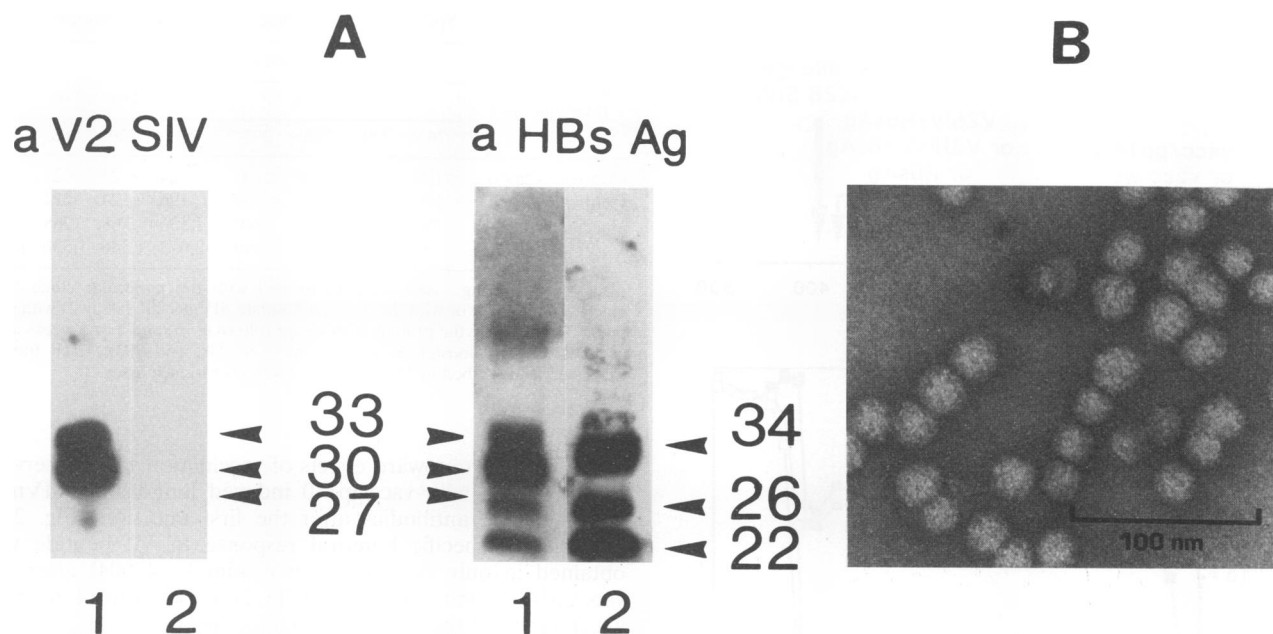
FIG. 1. Characterization of the V2/HBsAg particles. (A) Protein analysis of hybrid V2/HBsAg and native pre-S2/HBsAg particles. Particles were purified on a CsCl gradient according to their density (1.21 g/cm³), and proteins were analyzed by Western blotting. Lanes 1, 40 ng of V2/HBsAg particles; lanes 2, 40 ng of pre-S2/HBsAg particles. Proteins were revealed by MATG 2014, an anti-V2SIVmac MAb (left panel), and by H166, an anti-HBsAg MAb (right panel). Arrowheads indicate molecular masses (in kilodaltons). The 22- and 26-kDa bands are the two forms of the major polypeptide of HBsAg; the 34-kDa band corresponds to the middle polypeptide of HBsAg. The 27-, 30-, and 33-kDa bands correspond to different extents of glycosylation of the hybrid protein. (B) Electron micrograph of hybrid V2/HBsAg particles. Purified V2/HBsAg particles were adsorbed on collodium-coated grids, stained with uranyl acetate, and visualized by electron microscopy.

HBsAg in transfected cells resulted in the assembly of the SIV-HBV fusion protein with the HBV major protein into particles. The morphologies of the V2/HBsAg particles were very similar to those of the empty particles from HBV-infected human sera, sometimes with a larger diameter (Fig. 1B).

The structure of the V2/HBsAg particles was analyzed by Western blot (Fig. 1A). The hybrid protein in different glycosylated forms could be revealed by anti-SIV MAb MATG 2014 (4) as well as anti-HBsAg MAb H166 (53) as shown in Fig. 1A.

Recognition of the V2 SIV domain exposed on the hybrid particles was assessed in ELISA by several MAbs directed to the V2 domain (Table 1). V2/HBsAgs were specifically bound by MATG 2014 (4), KK13, and, to a lesser extent, to KK54 (31, 32), which mapped to the V2 epitope included in the hybrid particles. Furthermore, recognition by MATG 2014 and by KK54, which were also able to neutralize SIVmac251 infectivity in vitro (4, 32), indicates that this domain must be exposed on the hybrid particles as well as on the native SIV virus (Table

1). Particles were not fixed by MATG 2033, which mapped to an epitope on the left part of the V2 domain (4), and by KK8, which mapped to a conformational epitope on the gp140 N-terminal region (31).

In addition, the presence of the V2 SIV domain on the surface of the HBsAg particles was demonstrated by recognition of these hybrid particles with a panel of sera of SIV-mac251-infected rhesus monkeys in an ELISA (data not shown).

Specific humoral response. As shown in Fig. 2A, four macaques (*M. mulatta*) were immunized at 0 and 2 months (days 0 and 63) with 5×10^7 and 4×10^8 PFU, respectively, of either vacc/gp140 (macaques 47004 and 47157) or wt vacc (macaques 42896 and 42915). All animals developed localized lesions within a few days of the primary inoculation that were healed by the time of the second primer injection, except animal 42896, which developed a large, necrotic lesion at the site of the primary inoculation that also healed completely.

TABLE 1. V2 SIV-specific antigenicity of native pre-S2/HBsAg and hybrid V2/HBsAg particles

MAb[a]	Peptide[b]	Neutralization[c]	Reference(s)	HBsAg[d]	V2/HBsAg[d,e]
MATG 2014	GLKRDKTKEYNET	+	4	0.098	1.900
MATG 2033	CKFTMTGLKRD	+	4	0.093	0.122
KK13	TMTGLKRDKTKEYNETWYSTC	±	14, 31, 32	0.070	2.761
KK54	TMTGLKRDKTKEYNETWYSTC	+	32	0.074	0.235
KK8	gp130 N-terminal region	−	31	0.554	0.496

[a] V2-specific MAb ascitic fluids (1:1,000) applied as coats to the solid phase.
[b] Peptides were those used to define the MAbs.
[c] +, present; −, absent; ±, equivocal.
[d] ELISA reactivities (optical densities) of captured native HBsAg or hybrid V2/HBsAg (10 ng per well) revealed by a rabbit polyclonal anti-HBsAg serum.
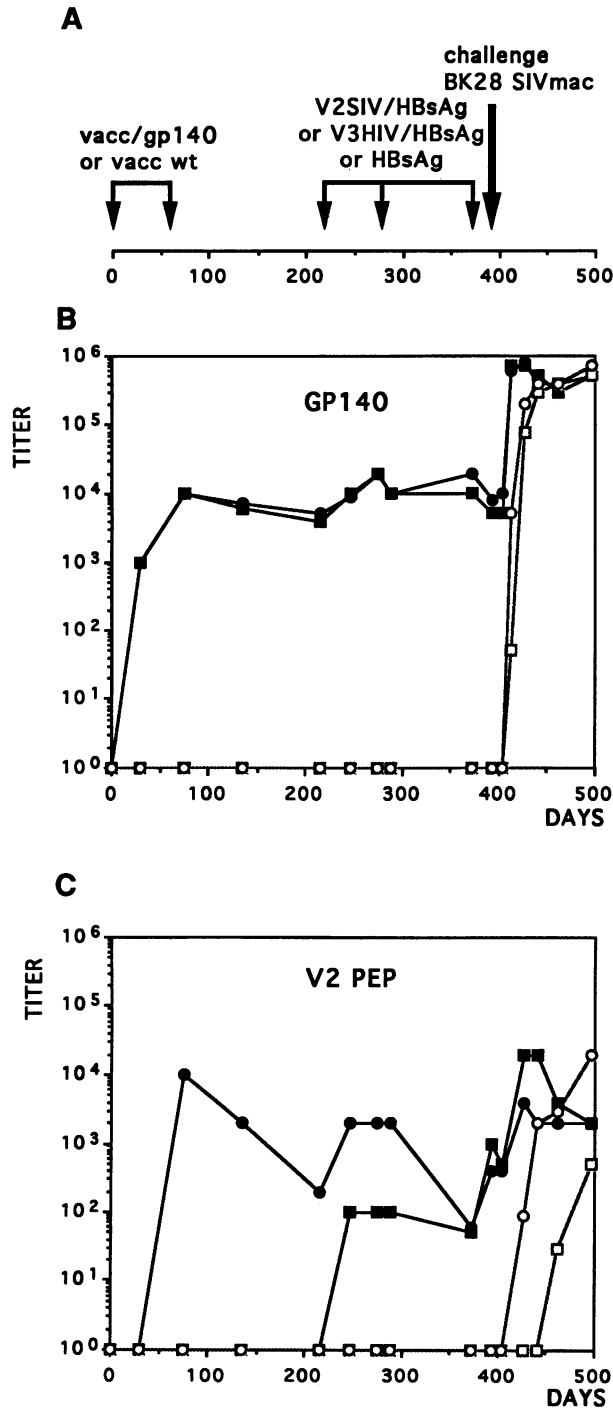[e] The V2 peptide amino acid sequence included in V2/HBsAg antigen was GLKRDKTKEYNETW.

TABLE 2. Specificity of SIVmac-neutralizing antibodies

Source of serum (macaque no.)	Titer[a] on indicated day with:							
	SIVmacBK28				SIVmac251			
	76	229	288	393	76	229	288	393
Control (42896)	<100	<100	<100	<100	<24	<24	<24	48
Control (42915)	<100	<100	<100	<100	96	24	<24	24
Vaccinee (47004)	100	<100	100	100	1,536	768	768	1,536
Vaccinee (47157)	100	<100	100	100	>3,072	1,536	1,536	3,072

[a] SIV-neutralizing assays were performed with the molecular clone SIV-macBK28 (assay 1) or with the biological isolate SIVmac251 (assay 2) with sera taken 2 weeks after the priming with vacc/gp140 (day 76) and 2 or 3 weeks after each V2/HBsAg booster injection (days 229, 288, and 393). Each titer is expressed as described in Materials and Methods for each assay.

Otherwise, no untoward effects of vaccination were observed. Immunization with vacc/gp140 induced long-lasting SIVmac gp140-specific antibodies after the first injection (Fig. 2B). However, a specific humoral response to V2 peptide was obtained in only one of the two animals (47004) after the vacc/gp140 priming injections (Fig. 2C). The control animals, which received the wt vacc, remained negative.

At months 7, 9, and 12 (days 215, 274, and 372), animals received booster immunizations with either V2/HBsAg (macaques 47004 and 47157), native HBsAg (macaque 42896), or an SIV-nonrelated hybrid HBsAg particle, the V3 HIV-1/HBsAg (macaque 42915) (Fig. 2A). As shown in Fig. 2B, the V2/HBsAg injections slightly increased the anti-gp140 antibody titers whereas the control animals remained negative. Specific antibodies to V2 peptide were boosted by the first two V2/HBsAg injections in macaque 47004. These injections also allowed animal 47157 to develop low titers of antibodies to V2 peptide that were dramatically increased after the third booster injection in adjuvant (Fig. 2C). It should be noted that titers to V2 peptide equaled or exceeded titers observed in experimentally infected macaques (5, 57a).

The humoral response to native HBsAg was also evaluated. Control animals have previously developed consistent HBsAg-specific antibodies (titers from 10^5 to 10^6) which were not affected by the wt vacc immunizations but were increased 10-fold after the three booster injections of either native HBsAg or V3 HIV-1/HBsAg. These last injections also induced an anti-HBsAg response in the vacc/gp140-vaccinated animals that was of the same order as that in the hybrid V3 HIV-1/HBsAg-immunized control (data not shown).

Sera from vaccinees neutralized the challenge virus BK28 and the biological isolate SIVmac251 in vitro. Neutralization assays were done with sera collected 2 to 3 weeks after the priming with vacc/gp140 and after each V2/HBsAg booster injection (Table 2). A first assay for neutralizing antibodies (assay 1) was performed with BK28 SIVmac, a molecular clone derived from SIVmac251 which was also used as the homologous challenge virus. Titers were expressed as the dilution that gave >50% inhibition of RT activity in comparison with preimmune sera. A reference serum from an asymptomatic rhesus monkey infected with SIVmac251 was also monitored in each experiment as a positive control. Neutralizing antibodies were obtained after the second injection of vacc/gp140 in the sera of the two immunized animals (Table 2). At the time of

FIG. 2. SIV-specific antibody responses in immunized macaques. (A) Immunization and challenge protocol. Arrows indicate the times of primer injection with vacc/gp140 (macaques 47004 and 47157) or wt vacc (macaques 42896 and 42915) at days 0 and 63; of booster injections with V2 SIV/HBsAg (macaques 47004 and 47157) or V3 HIV/HBsAg (macaque 42915) or native HBsAg (macaque 42896) at days 215, 274, and 372; and of challenge at day 393. (B and C) Data for SIV-immunized macaques (●, 47004; ■, 47157) and control macaques (□, 42896; ○, 42915) are shown. (B) SIV gp140 ELISA. Recombinant SIV gp140 (Repligen) was applied as a coat at 0.5 μg/ml on the solid phase. Dilutions of macaque sera collected at the indicated times were added and then revealed by a sheep anti-human immunoglobulin peroxidase conjugate (Amersham). (C) SIV V2 peptide ELISA. V2

peptide (ANRS SP90454, KFTMTGLKRDKTKEYNETWY) was applied as a coat at 5 μg/ml on the solid phase.

the first V2/HBsAg booster injection, neutralizing antibodies were not detectable and were not boosted by this injection. The second V2/HBsAg boost induced persistent neutralizing antibodies for several weeks in one animal and a transient neutralizing activity in the other one (data not shown). These neutralizing antibodies probably differed from the ones obtained after the vacc/gp140 immunizations, since these were no longer detectable after the first V2/HBsAg injection. No further increase of the neutralizing-antibody titers to the molecular clone BK28 SIVmac was obtained after the third V2/HBsAg booster injection (i.e., at the time of challenge).

Neutralizing specificity of plasma samples was also assayed against the biological isolate SIVmac251 in a second assay based on CPE reduction in CEMx174 cells (assay 2). Both vacc/gp140 vaccinees developed neutralizing antibodies to SIVmac251 after the two primer injections (day 76) (Table 2). After several months, these titers fell slightly. After the third V2/HBsAg booster with SAF-1 adjuvant, there was an increase of the SIVmac251-neutralizing titers. An experimentally infected animal was used as positive control and was able to neutralize SIVmac251 at a titer of >5,760 (data not shown). The variation of neutralizing titers obtained against SIVmac251 compared with those against SIVmacBK28 could be related to differences in the methods used in the two assays.

To further evaluate the specificity of these neutralizing antibodies, competition with hybrid V2/HBsAg or native HBsAg particles was assessed in an SIVmac251 neutralization-inhibition assay. On the day of challenge (day 393), no evidence of reduction of neutralization titers was observed in the presence of either V2/HBsAg or HBsAg in samples from vaccinees (data not shown). Because the concentration of the V2/HBsAg particles may have been too low or because the steric hindrance of the V2/HBsAg particles could have impaired a possible competition in this neutralization-inhibition assay, V2 peptide was used in further experiments (Fig. 3). On the day of challenge (day 393), neutralizing antibodies that were present in the sera of the two immunized macaques, 47004 and 47157, were partially inhibited by the V2 peptide (Fig. 3A and C). This demonstrates that at least a part of the SIV-neutralizing activity present at the time of challenge was directed to the V2 region. Furthermore, after the vacc/gp140 primer injections (day 76), neutralizing activity was also partially inhibited by V2 peptide in the sample taken from macaque 47004, which was the only vaccinee that developed antibodies directed to the V2 peptide at this time of the immunization protocol (Fig. 3B). This shows that the V2 domain was presented efficiently enough by the recombinant vacc/gp140 to induce V2-specific neutralizing antibodies, at least in one animal.

Cellular cytotoxic response. Primary cytotoxic activity from fresh PBMC was not detectable when measured at days 76 and 134. To evaluate secondary CTL, T-cell lines were derived from specific or nonspecific stimulated PBMC collected at days 76, 134, 173, 229, 274, 288, 295, 334, and 393 of the immunization protocol, as described in Materials and Methods. No envelope-specific CTL activity was detected from these T-cell lines tested against autologous transformed B-cell lines infected with recombinant vaccinia virus expressing the envelope of SIVmac251 (data not shown).

Challenge of rhesus macaques with the nonpathogenic BK28 SIVmac251. Vaccine efficacy was tested by intravenous challenge with live cell-free SIV 3 weeks after the third V2/HBsAg booster injection (day 393). The molecular clone BK28 of SIVmac, which was considered to be a homologous challenge virus stock, was diluted, and 10 50% macaque infectious doses were injected. The proper dilution factor was
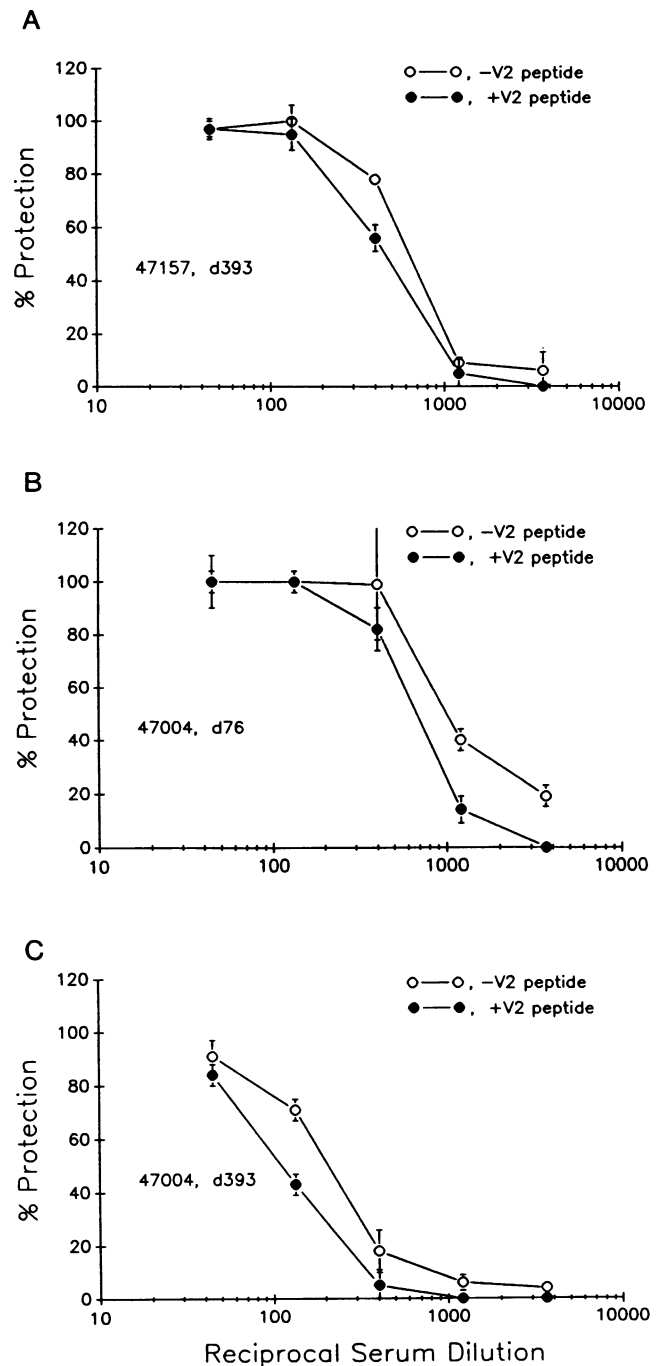

FIG. 3. SIVmac251 neutralization-blocking experiments with V2 peptide. Neutralization-blocking experiments were performed only when antibodies specific to the V2 peptide were present in ELISA, at day 393 (d393) for the two vaccinated macaques (47004 [C] and 47157 [A]) and at day 76 for macaque 47004 (B). Assay 2 was used for neutralization with the biological isolate SIVmac251. The day 76 sample was taken 2 weeks after the priming with vacc/gp140. The day 393 samples were taken 3 weeks after the third V2/HBsAg booster injection, i.e., at the time of the challenge.

TABLE 3. Virus isolation after challenge

Macaque no.	No. of infected PBMC/10^6 fresh PBMC[a] at indicated time[b]									
	393 (0)	404 (0)	413 (0)	426 (1)	440 (1)	461 (2)	497 (3)	517 (3)	566 (5)	637 (8)
47004	<2	250	100	20	4	4	4	100	20	100
47157	<2	1,250	100	100	100	100	20	20	100	100
42896	<2	250	500	100	<2	2	<2	<2	<2	<2
42915	<2	1,250	100	100	4	ND[c]	<2	4	<2	4

[a] Virus isolation was attempted from PBMC of SIV-immunized macaques (47004 and 47157) and from controls (42896 and 42915) at the time of (day 393) and after challenge by cocultivation with CEMx174 cells. SIV replication was quantified in positive cocultures after 2 weeks of cocultivation.
[b] Number of days of immunization (number of months after challenge).
[c] ND, not done.

calculated for the stock on the basis of previous in vivo 50% macaque infectious dose determinations.

Biological parameters of vaccinated and control macaques. Immunized and control macaques were monitored for hematologic parameters and T-cell subsets throughout the study. Hematologic parameters were not significantly different between vaccinees and controls (data not shown). Mean absolute numbers of circulating $CD4^+$ cells and $CD8^+$ cells remained stable in SIV-vaccinated and in control animals throughout the 7-month period after challenge (data not shown). It should be noted that BK28 SIVmac infection of rhesus macaques does not cause a detectable acute disease syndrome with a rapid decline of $CD4^+$ cell numbers, as has been observed with the biological SIVmac251 isolate (12). However, in a different study, Rivière et al. (54a) recently observed a decline in CD4 numbers at 15 months after the BK28 challenge in one rhesus macaque out of eight.

Evaluation of SIV replication after challenge. Virus isolation from all four challenged macaques was attempted at the time of and after challenge by cocultivation with CEMx174 cells (Table 3). Cocultures were monitored for SIV replication by measurement of RT activity in supernatants. The numbers of infected PBMC per 10^6 PBMC at 2 weeks from each coculture are shown in Table 3. At day 404 (11 days after challenge), SIV was isolated from the PBMC of the two controls and the two vacc/gp140- and V2/HBsAg-immunized animals. Although SIV-vaccinated macaque 47004 had lower viral loads for the first month of the study, this was not the case afterwards, as the two control animals strongly decreased their virus load and one control animal became negative for virus isolation 3 months after challenge. Quantitative variations observed throughout the duration of the study (8 months) may reflect individual variation. To further clarify the variation in viral load that may be due to control of viral replication by $CD8^+$ cells, coculture at day 517 (3.5 months after challenge) was monitored on PBMC with or without $CD8^+$ cells. $CD8^+$ cells were depleted from the PBMC with an anti-T8 MAb fixed on beads (IOT8; Immunotech) according to the manufacturer's protocol, and the depletion was verified by FACScan as described in Materials and Methods. Cocultures with or without $CD8^+$ cells were monitored in parallel. Depletion in $CD8^+$ cells did not change viral loads for control animal 42896 and vaccinated animal 47004 (data not shown). In immunized macaque 47157, $CD8^+$ cells appeared to have an effect on SIV replication, as 20 infected cells per 10^6 cells were found in the presence of $CD8^+$ cells, versus 100 infected cells per 10^6 cells in the absence of $CD8^+$ cells. However, we could not rule out the possibility that this was due to internal variation, as control animal 42915 showed the opposite result.

Antigenemia measured in sera from all four macaques was at the limit of detection (data not shown), as observed previ-

ously in macaques infected by BK28 SIVmac251 during in vivo titration experiments with this virus dose (27a).

SIV-specific antibody responses after challenge. Control and vaccinated macaques seroconverted to SIV by 3 weeks following challenge (Fig. 2). Macaques that received the vacc/gp140 and V2/HBsAg injections steadily increased anti-gp140 and anti-V2 peptide antibody titers at day 413 (day 20 after challenge), presumably due to an anamnestic response associated with the replication of the challenge virus. Control animals also developed a gp140-specific response at day 413, and this response reached the same titers as those of the vaccinated animals at day 440. Later, the anti-gp140 titers plateaued to a level of 5×10^5. One of the control animals also had specific V2 peptide antibodies at day 426 (i.e., 1 month after challenge), whereas the other one developed this response only at day 461, 2 months after challenge. This shows that there may be individual variation in the induction of V2 peptide-specific antibodies following viral infection similar to that seen after immunization with vacc/gp140.

Neutralizing antibodies were measured after challenge by assay 1. One month after challenge, neutralizing antibodies were stable at a titer of 1:100 in the SIV vaccinees and in control animals. Two weeks later (day 440), neutralizing antibodies had fallen in all four challenged macaques to undetectable levels 2 months after challenge. This may be due to the fact that neutralizing antibodies can form complexes with the replicating virus. However, 3 months after challenge (day 497), neutralizing antibodies at 1:100 were again observed in SIV-vaccinated macaque 47157, unlike the other vaccinee, 47004, and control animals. This may be related to this monkey's viral load, which was higher and more persistent than those in the other animals, or it could reflect an escape of the virus by mutation under the immune pressure, which therefore liberated antibodies from complexes.

DISCUSSION

Efforts to develop a safe and effective AIDS vaccine have been facilitated by the use of several animal models. In the chimpanzee model, protection from HIV-1 experimental infection by vaccination has correlated with induction of HIV-1-neutralizing antibodies directed to the V3 region of the envelope protein. In contrast, protection of macaques from SIV infection has not been yet correlated with any component of the immune response. Here, two macaques were primed twice with vacc/gp140 and received booster injections with the V2 presented by a highly immunogenic carrier, the HBsAg (44, 58), whereas the two control animals received wt vacc for primer injections and either native HBsAg or V3 HIV-1/HBsAg for booster injections. After priming with vacc/gp140, a specific humoral response to gp140 was induced in the two

vaccinated animals and this response was also specific to the V2 domain in macaque 47004. These antibodies were able to neutralize the molecular clone BK28 of SIVmac251, which was used for challenge, as well as the biological isolate SIVmac251, which contains high genetic diversity. In competition-neutralization experiments, the SIVmac251-neutralizing activity was inhibited in part by V2 peptide in animal 47004, which had developed the V2-specific antibodies. Booster injections with V2/HBsAg permitted the two vaccinees to develop or to increase the V2-specific response. Especially, the third injection of V2/HBsAg, which was given in Syntex adjuvant, increased the SIVmac251-neutralizing-antibody titers in the two vaccinated macaques. This neutralizing activity was also partially inhibited in V2 peptide competition experiments, showing that at least a proportion of neutralizing antibodies were directed to the V2 domain. Because induction of neutralizing antibodies in monkeys with SIV peptides has not been easy, our results could certainly be ascribed to the antigen presentation system used in this study. However, despite having consistent SIV-neutralizing-antibody titers that nearly reached the levels found in infected macaques, animals were not protected against homologous challenge with SIVmac BK28. No SIV Env-specific cellular cytotoxic response was detectable throughout the immunization protocol, suggesting that neutralizing antibodies directed to SIV envelope gp140 and especially to the V2 domain were unable on their own to protect against SIV challenge. These data support other studies in which the induction of neutralizing antibodies by recombinant vaccinia virus priming followed by SIV particle boosting was unable to protect macaques, whereas a *nef*-deleted, live, attenuated SIV vaccine afforded protection despite inducing lower neutralizing-antibody titers (11). In contrast, another research group has shown that partial protection obtained after vaccination with *nef*-deleted live SIV may have correlated with neutralizing antibody titers (33). Results obtained by Stott et al. (64), in which macaques inoculated with attenuated SIV vaccine were protected against challenge with a chimeric SIV with an HIV-1 envelope (39), are puzzling. Experiments with passive transfer of antibody, in which infusion of pooled anti-SIVsm serum to cynomologus monkeys was able to protect them from SIVsm challenge, are informative, but SIV-neutralizing titers of the serum pool were only at 1:80 (54). Furthermore, Gardner et al. (18) have shown that injection of inactivated plasma or purified immunoglobulin with high SIV-neutralizing antibodies and no anti-human cell antibody might have enhanced infection and disease of passively immunized monkeys.

Indeed, in our study, the viral loads of infected vaccinees were similar to those of infected controls during the first month following challenge. Afterwards, the control animals seemed to have lower viral loads than vaccinees. Despite the small number of monkeys used in this study, this result raises the question of enhancement of infection by the humoral response induced by vaccination. Antibodies that enhance HIV-1 and SIV infection in vitro have been observed in sera from infected and vaccinated humans and macaques, respectively (48). Infection enhancement has been associated with vaccine failures in a number of other lentivirus infections, including visna virus, caprine arthritis-encephalitis virus, and equine infectious anemia virus, which has raised concerns regarding AIDS vaccines (40). Several hypotheses relating to our data can be invoked. (i) Enhancing antibodies may have developed, even if the V2 region has never been shown before to induce them. (ii) It is possible that in vitro-measured SIV-neutralizing antibodies are not neutralizing in vivo, as has been shown for neutralizing antibodies directed to murine hepatitis virus 4 by Talbot et al.

(66). If this is the case, the fact that three macaques remained virus positive by coculture could have been due to random variation. (iii) Immune pressure induced in vaccinees may lead to faster mutation of the challenge virus. It is widely believed that the host neutralizing-antibody response is one of the selective forces driving antigenic variation in the lentivirus envelope. Indeed, emergence of neutralization-resistant HIV-1 mutants has been observed in HIV-1-infected humans (67). Furthermore, neutralization-resistant HIV-1 mutated in the V3 domain was generated in vitro (41) and in HIV-1-infected chimpanzees (51). Besides the V3 domain, it is becoming clear that the HIV-1 V2 region contains neutralization epitopes (17, 22, 25, 43, 49, 65) and is involved in soluble CD4 neutralization (34), subunit association (65), replication and tropism (34, 61, 68, 69), and syncytium formation (2, 21, 65). Again, in vitro generation of neutralization-resistant HIV-1 has been recently obtained with MAbs directed to the V2 domain (70).

Concerning SIV, mutants resistant to serum neutralization have been shown to arise during the course of persistent infection of rhesus monkeys (8). In our case, it is possible that the neutralizing antibodies induced by vaccination and directed to the SIV envelope, and perhaps to the V2 region, have selected viral escape mutants. This mechanism is certainly worthy of intensive investigation and raises some concern for SIV envelope-targeted immunization.

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