Virology **294,** 270–281 (2002)
doi:10.1006/viro.2001.1345, available online at http://www.idealibrary.com on IDEAL®

Comparison of Vaccine Strategies Using Recombinant *env–gag–pol* MVA with or without an Oligomeric Env Protein Boost in the SHIV Rhesus Macaque Model

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Received September 18, 2001; accepted December 27, 2001

Rhesus macaques were immunized with a replication-deficient vaccinia virus (MVA) expressing human immunodeficiency virus type 1 89.6 envelope (*env*) and SIV *gagpol* (MVA/SHIV89.6) with or without a protein boost consisting of soluble 89.6 env (gp140). Immunization with MVA/SHIV89.6 alone elicited binding antibodies in all animals and neutralizing antibodies in 5 of 15 animals. Both types of antibodies were enhanced by protein boosting. In addition, CD8 cells exhibiting CM9 tetramer binding were detected in the subset of animals that were Mamu-A*01 positive. Animals were challenged intravenously with either SHIV-89.6 (Study 1) or the more pathogenic derivative SHIV-89.6P (Study 2). In Study 1, all control and vaccinated animals except one became infected. However, the levels of viremia were as follows: controls > rMVA alone > rMVA + protein. The differences were statistically significant between immunized and control groups but not between the two immunized groups. In Study 2, all animals became infected; however, the vaccinated group exhibited a 5-fold reduction in peak viremia and a 10-fold reduction in the postacute phase viremia in comparison to the controls. All of the controls required euthanasia by 10 months after challenge. A relationship between vaccine-induced antibody titers and reduction in virus burden was observed in both studies. Thus, immunization with MVA/SHIV89.6 alone or with a protein boost stimulated both arms of the immune system and resulted in significant control of viremia and delayed progression to disease after challenge with SHIV-89.6P.

Key Words: SHIV89.6; SHIV-89.6P; vaccine; recombinant modified vaccinia virus Ankara; rhesus macaque; oligomeric env protein.

INTRODUCTION

Development of a safe and effective vaccine against human immunodeficiency virus type 1 (HIV-1) is the focus of worldwide efforts. Numerous vaccine modalities have been tested in animal models, including soluble recombinant proteins (Earl *et al.,* 2001; Heeney *et al.,* 1999; Kumar *et al.,* 2000; Stott *et al.,* 1998; VanCott, 1997; Verschoor *et al.,* 1999), DNA vectors (Barouch *et al.,* 2000; Boyer *et al.,* 1996, 1997; Cherpelis *et al.,* 2001; Egan *et al.,* 2000; Lu *et al.,* 1996a; Mossman *et al.,* 1999; Shiver *et al.,* 1997; Wang *et al.,* 2000), recombinant viruses (Barouch *et al.,* 2001; Buge *et al.,* 1997; Caley *et al.,* 1997; Ourmanov *et al.,* 2000; Robert-Guroff *et al.,* 1998; Seth *et al.,* 1998), and various prime–boost combinations (Amara *et al.,* 2001; Cho *et al.,* 2001; Hirsch *et al.,* 1996; Kent *et al.,* 1998; Letvin *et al.,* 1997; Polacino *et al.,* 1999; Robinson *et al.,* 1999). In general, DNA and recombinant virus vectors stimulate cellular immunity (Amara *et al.,* 2001; Barouch *et al.,* 2000; Robinson, 1997; Seth *et al.,* 1998) while subunit proteins are more effective at inducing antibodies (Barnett *et al.,* 1997; Berman *et al.,* 1990; Clements-Mann *et al.,* 1998; Earl *et al.,* 2001; Graham *et al.,* 1993). An ideal vaccine would stimulate both arms of the immune system.

Poxviruses are attractive vaccine vectors because of their large capacity for added DNA and ability to induce cellular and humoral immune responses in both animal models and humans. Although vaccinia virus was extensively used to prevent smallpox, safety concerns have led to the testing of avian poxviruses and highly attenuated strains of vaccinia virus as vaccine vectors. Modified vaccinia virus Ankara (MVA) was attenuated for use as a safe smallpox vaccine by serial passage in chicken embryo fibroblasts (CEF). During these passages, the virus underwent numerous deletions and other mutations resulting in an inability to efficiently replicate in most mammalian cells, though it still replicates to high titer in CEF (Antoine *et al.,* 1998; Carroll and Moss, 1997; Mayr *et al.,* 1975; Meyer *et al.,* 1992). Consequently, MVA was safe when administered to humans and was aviru-

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0042-6822/02 $35.00

lent even for immunosuppressed animals (Mahnel and Mayr, 1994; Mayr *et al.,* 1975, 1978; Stittelaar *et al.,* 2001). Because the replication defect in human cells affected virus assembly, viral and recombinant gene expression remained unimpaired (Sutter and Moss, 1992). Most importantly, recombinant MVA induced protective cellular and humoral immune responses in both small animals and nonhuman primates that were at least as good as those achieved by standard replicating strains of vaccinia virus (Belyakov *et al.,* 1998; Hirsch *et al.,* 1996; Sutter *et al.,* 1994). The immunogenicity of MVA may have been enhanced by the spontaneous deletion of many immune evasion genes during the extensive passage history of MVA (Antoine *et al.,* 1998; Blanchard *et al.,* 1998).

The inability of HIV to cause disease in nonhuman primates has led to the development of the simian–human hybrid virus or SHIV (Lu *et al.,* 1996b). SHIV-89.6 was created by replacing the envelope (*env*), *tat,* and *rev* genes of SIV with those from the dual-tropic clade B primary isolate 89.6 (Reimann *et al.,* 1996b). It replicates at high levels for several weeks in macaques but then declines in magnitude without causing serious disease. A more pathogenic derivative, termed SHIV-89.6P, was isolated after serial passage of SHIV-89.6 in macaques (Reimann *et al.,* 1996a). SHIV-89.6P exhibits more robust and prolonged replication resulting in rapid CD4 T cell loss and subsequent AIDS-like disease and death. The difference between the two viruses has been attributed to 12 amino acid changes in gp120, which affect not only the replicative potential and pathogenic capacity of the virus but also the neutralization characteristics (Crawford *et al.,* 1999; Karlsson *et al.,* 1997). Thus, sera from monkeys infected with SHIV-89.6 do not readily neutralize SHIV-89.6P.

For the studies described here, we immunized rhesus macaques with MVA/SHIV89.6, a recombinant MVA (rMVA) that expresses the *env, gag,* and polymerase (*pol*) genes of SHIV-89.6. Some groups of animals were boosted with soluble oligomeric 89.6 Env. Animals were challenged intravenously with SHIV-89.6 or SHIV-89.6P. Both vaccine protocols resulted in reduced replication of the challenge virus.

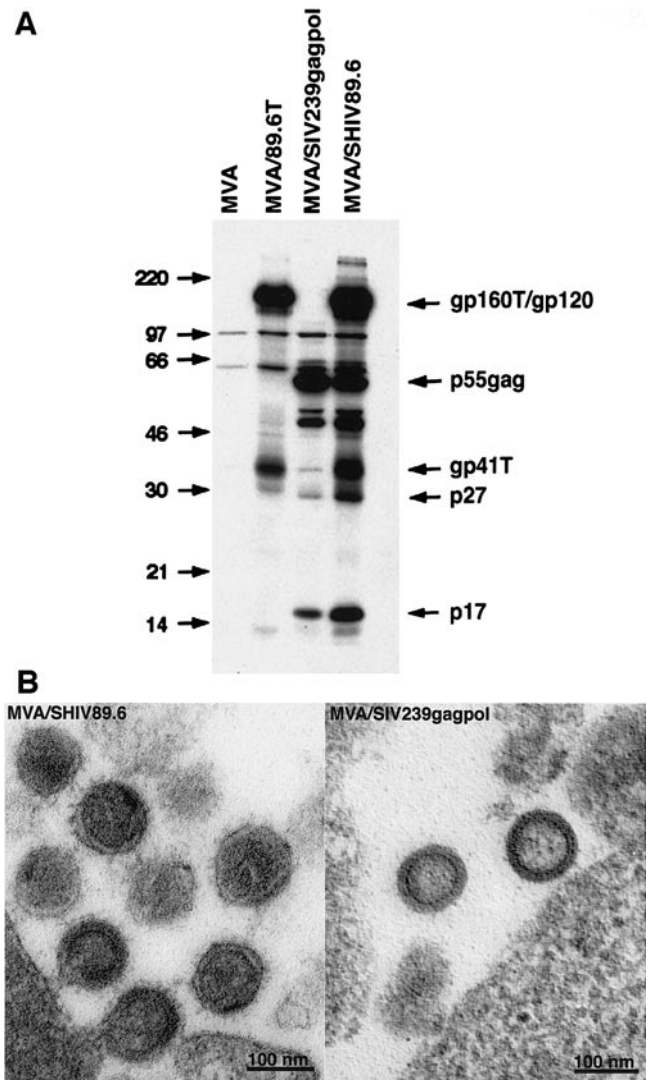

FIG. 1. Protein expression and VLP production by cells infected with MVA/SHIV89.6. (A) HIV and SIV antigens were immunoprecipitated from cells infected with single recombinant viruses (MVA/89.6T or MVA/SIV239gagpol) or double recombinant virus (MVA/SHIV89.6) using rabbit HIV-1 env serum and serum from an SIV-infected monkey. Both precursor and cleaved proteins are shown: HIV-89.6 gp160(T), gp120, and gp41(T) and SIV p55gag, p27, and p17. (B) Cells were infected with either MVA/SHIV89.6 or MVA/SIV239gagpol, fixed, embedded in plastic, and stained with uranyl acetate. Note the appearance of spikes around the edges of particles formed by infection with MVA/SHIV89.6.

RESULTS

Construction and characterization of MVA/SHIV89.6

We constructed a rMVA, named MVA/SHIV89.6, that produces Env, Gag, and Pol proteins. This virus contains a truncated 89.6 *env* (gp160T) gene and the *gagpol* gene from SIVmac239 in MVA deletions II and III, respectively. Both are regulated by the mH5 promoter which yields enhanced early expression, a factor that has been shown to be important for generation of CTL in mice (Coupar *et al.,* 1986). Further enhancement of early *env* gene expression was achieved by genetic alteration of the naturally occurring T5NT sequences within the 89.6 *env* gene without changing the amino acid sequence (Earl *et al.,* 1990).

Protein expression, induced by rMVA encoding *env* or *gagpol* or both, was analyzed by immunoprecipitation as shown in Fig. 1A. The gp160T precursor was produced and cleaved into gp41T and gp120, some of which was shed into the medium (not shown). The biological function of the expressed dual-tropic Env was demonstrated by fusion of MVA/SHIV89.6-infected cells with cells expressing CD4 and either CCR5 or CXCR4 (L. Wyatt, manuscript in preparation). Gag precursors p55 and p41

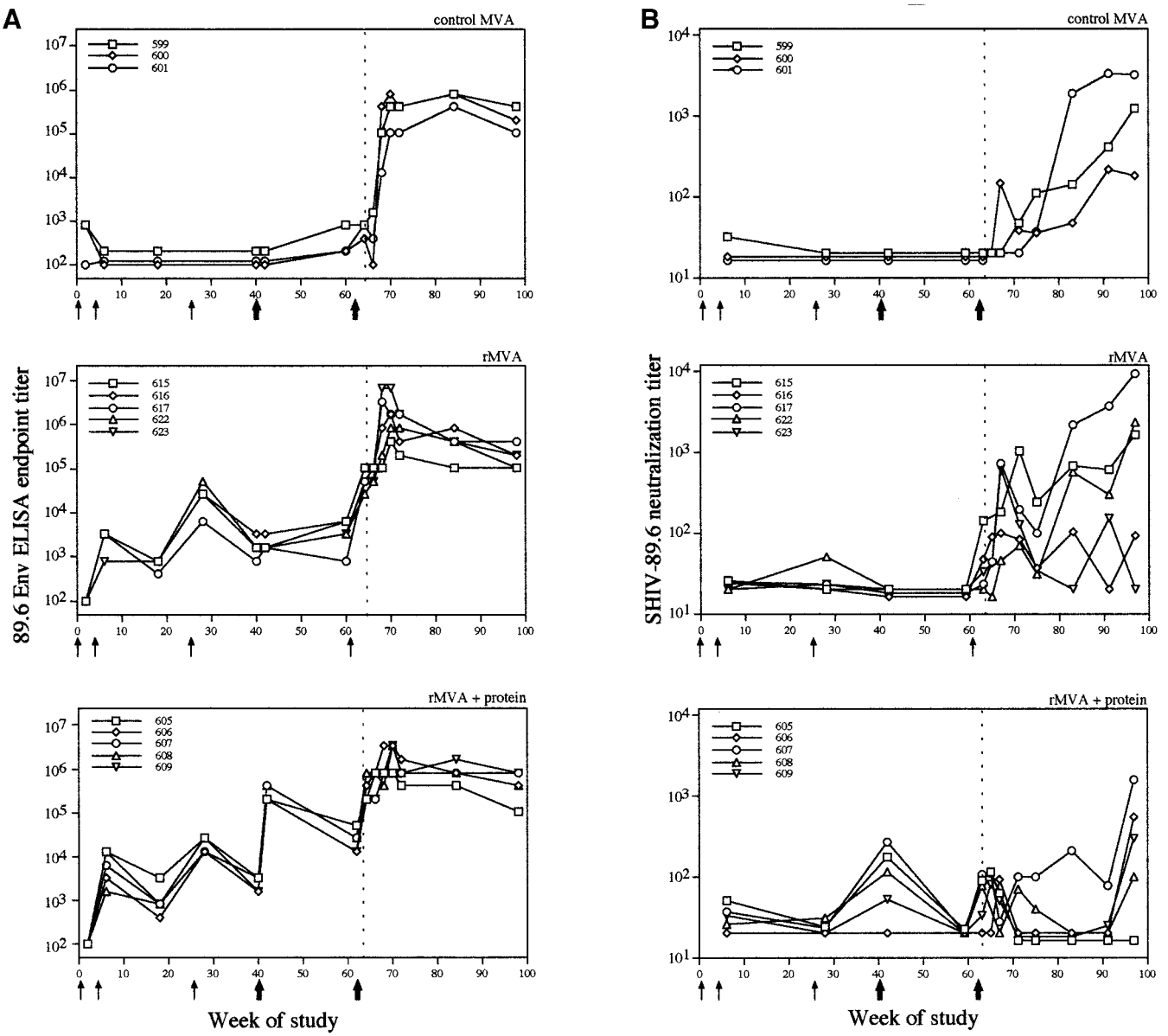

FIG. 2. Temporal antibody responses—Study 1. (A) Serum IgG end-point titers were measured by ELISA using 89.6 gp140 captured via antibody to the C-terminus of gp120. (B) SHIV-89.6 neutralizing antibody titers were determined in an MT-2 cell killing assay. Titers are the reciprocal of the dilution giving 50% neutralization. Thin and thick arrows depict times of MVA/SHIV89.6 and soluble 89.6 gp140 immunizations, respectively. The time of challenge with SHIV-89.6 is shown by the dotted vertical line.

as well as cleavage products p27 and p17 were also produced (Fig. 1A). Virus-like particles (VLP), recovered by sedimentation of the medium of infected cells through a sucrose cushion, contained Gag products p27 and p17, as well as reverse transcriptase activity (not shown).

To visualize VLP, thin sections of infected cells were analyzed by electron microscopy. Budding and released VLP were seen with both the double recombinant, MVA/SHIV89.6, and the single recombinant, MVA/SIV239gagpol (Fig. 1B). The surface of VLP formed from the double recombinant had a characteristic fuzzy appearance not seen in those formed from the single recombinant, suggestive of the presence of Env spikes. In support of this, Env was visualized on VLP produced by MVA/SHIV89.6 by immunogold labeling with Env-specific antibodies while no labeling was found in VLP produced by MVA/SIV239gagpol (not shown).

Vaccine-induced humoral responses in rhesus macaques

Studies were designed to test the immunogenicity of SHIV-89.6 proteins expressed by MVA/SHIV89.6 alone or after boosting with soluble oligomeric gp140. After two immunizations with MVA/SHIV89.6, all of the animals developed antibody to 89.6 Env as measured by enzyme-

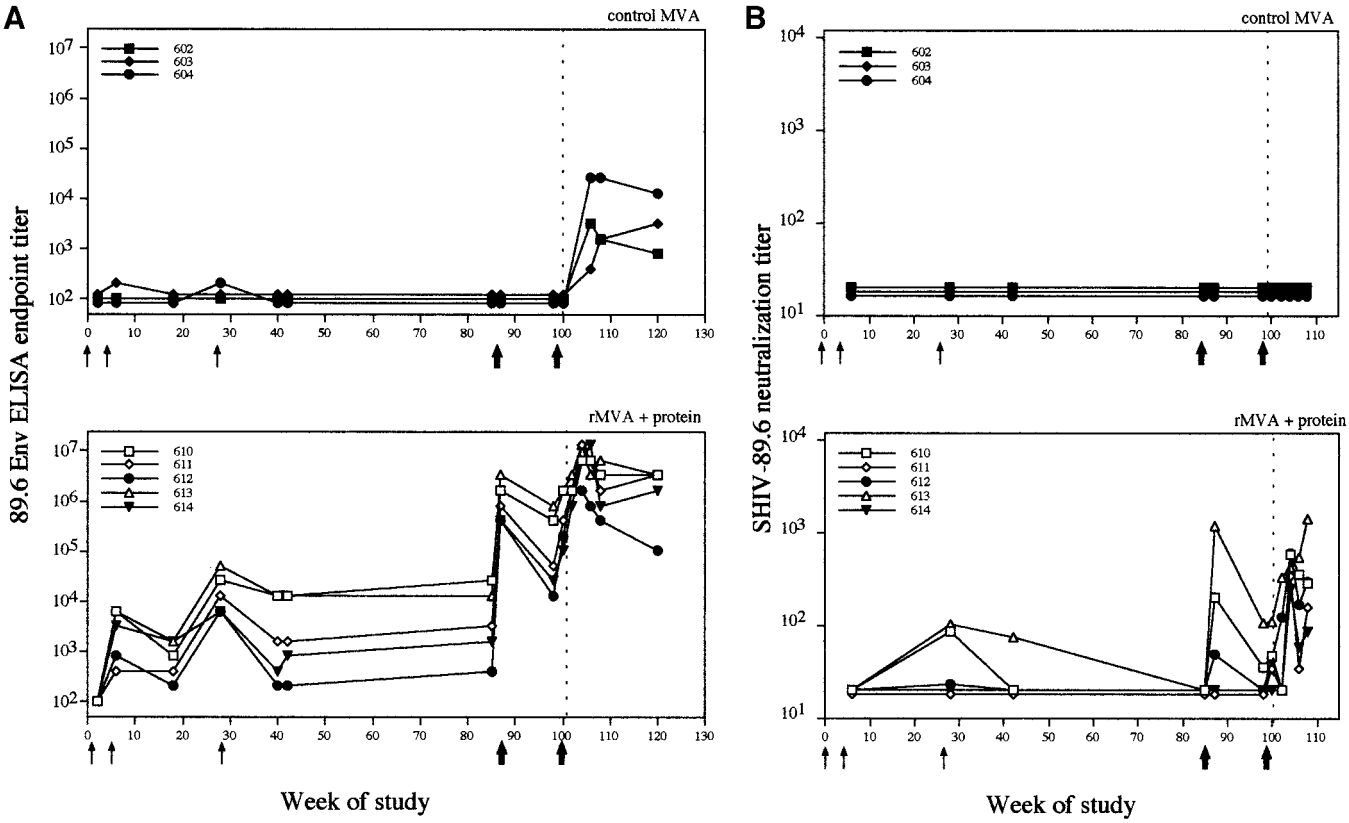

FIG. 3. Temporal antibody responses—Study 2. (A) Serum IgG end-point titers were measured by ELISA using 89.6 gp140 captured via antibody to the C-terminus of gp120. (B) SHIV-89.6 neutralizing antibody titers were determined in an MT-2 cell killing assay. Titers are the reciprocal of the dilution giving 50% neutralization. Thin and thick arrows depict times of MVA/SHIV89.6 and soluble 89.6 gp140 immunizations, respectively. The time of challenge with SHIV-89.6P is shown by the dotted vertical line. Open and closed symbols represent animals that are alive or dead 17 months after challenge, respectively.

linked immunosorbent assay (ELISA) (Figs. 2A and 3A). Titers were enhanced after both the third and fourth MVA/SHIV89.6 immunizations with geometric mean titers of 2.1×10^4 and 6.8×10^4, respectively. Protein boosting increased ELISA titers (geometric mean) to 3.1×10^5 and 4.7×10^5 in Studies 1 and 2, respectively (Figs. 2A and 3A).

Neutralizing antibodies to SHIV-89.6 developed after three or four immunizations with MVA/SHIV89.6 (Figs. 2B and 3B). After three immunizations, 3 of the 15 animals had reciprocal titers between 51 and 102. The fourth MVA/SHIV89.6 immunization (Study 1) boosted the titers in 3 of the 5 animals, with reciprocal titers ranging from 33 to 139. Boosting with gp140 enhanced the neutralizing titers in 4 of 5 animals in Study 1 and 3 of 5 animals in Study 2, with reciprocal titers ranging from 49 to 1195. In addition, 4 of the 10 animals that received the protein boost developed neutralizing antibody against the heterologous SHIV-89.6P (1:14 to 1:37) (see Table 1).

Vaccine-induced CTL responses in rhesus macaques

CTL responses for the SIV Gag epitope CM9 bound by Mamu-A*01 (Altman *et al.,* 1996) were assessed in the five Mamu-A*01-positive vaccines at week 60 or 62 of the study, at which time the monkeys had received either four immunizations with MVA/SHIV89.6 or three immunizations with MVA/SHIV89.6 and two protein boosts. Tetramer binding was detected in fresh peripheral blood mononuclear cells (PBMC) from four of the five animals, ranging from 0.14 to 0.54% of the $CD3^+CD8^+$ cells (Table 1). After *in vitro* stimulation, cells from all animals stained with Mamu-A*01/CM9 tetramer and exhibited specific lysis in a functional chromium release assay (Table 1).

Virus loads after challenge with nonpathogenic SHIV-89.6 (Study 1)

The 13 animals (10 vaccinated with MVA/SHIV89.6 and 3 with control MVA) in Study 1 were challenged intravenously with 10 animal infectious doses of SHIV-89.6 4 weeks after the fourth MVA/SHIV89.6 immunization or 2 weeks after the second protein boost. The final boosting was staggered to allow all animals to be challenged on the same day. Plasma viral RNA loads were determined by a quantitative NASBA assay (Fig. 4). Viral RNA was undetectable in animal 605 at all times after challenge. The total viral RNA load (geometric mean) in the control

TABLE 1

Summary

Immunogen	Animal no.	Peak neutralizing antibody titer (reciprocal dilution)[a] SHIV-89.6	Peak neutralizing antibody titer (reciprocal dilution)[a] SHIV-89.6P	Mamu-A*01/CM9 tetramer % CD8$^+$ T cells[a] Fresh PBMC	Mamu-A*01/CM9 tetramer % CD8$^+$ T cells[a] Stimulated PBMC	^{51}Cr release E:T (25:1) % specific lysis[a]	Challenge virus	Peak RNA (copies/ml)
MVA	599	<20	<5				SHIV-89.6	5.6×10^6
	600	<20	<5					1.4×10^6
	601	<20	<5					1.6×10^6
								Average: 2.9×10^6
MVA/SHIV-89.6	615	139	19				SHIV-89.6	$<5.0 \times 10^4$
	616[b]	47	<5	0.20	26	37.9		5.1×10^5
	617	23	<5					1.2×10^5
	622[b]	<20	<5	0.15	22	28.3		$<5.0 \times 10^4$
	623	33	9					6.7×10^5
								Average: 2.8×10^5
MVA/SHIV-89.6 + gp140	605[b]	171	25	0.09	10	66.0	SHIV-89.6	$<5.0 \times 10^3$
	606	<20	<5					1.2×10^6
	607[b]	261	26	0.14	17	61.5		1.2×10^5
	608	112	37	0.03	0.18	−1.7		$<5.0 \times 10^4$
	609	52	<5					8.5×10^4
								Average: 2.9×10^5
MVA	602	<20	<5				SHIV-89.6P	1.1×10^8
	603	<20	<5					1.9×10^8
	604	<20	<5					4.5×10^7
								Average: 1.2×10^8
MVA/SHIV-89.6 + gp140	610	210	5				SHIV-89.6P	2.9×10^7
	611[b]	34	<5	0.54	68	67.7		1.6×10^7
	612	49	<5					1.4×10^8
	613	1195	14					4.8×10^5
	614	<20	5					3.1×10^7
								Average: 4.3×10^7

[a] Prechallenge values.
[b] Mamu-A*01 positive.

group of animals was 1 log higher than that in either vaccinated group ($P = 0.0335$ and 0.0125 using the Fisher test; $P = 0.05$ and 0.016 using Shaeffer's modification of the Bonferonni method). Although the RNA levels of the protein boost group were lower than the rMVA alone group, the difference was not considered statistically significant.

The infection status of the animals was determined with two additional assays. First, PBMC from the monkeys were cocultured with CEM cells and p27 was quantified from the supernatant. Cells from all animals except 605 were positive for at least one time point after challenge (data not shown). Second, seroconversion to p27 was assayed by ELISA. All animals except 605 strongly seroconverted to p27 in this assay (data not shown).

Humoral responses following challenge with SHIV-89.6

ELISA binding titers to Env increased after challenge in all control and all vaccinated animals except 605 (Fig. 2A). Geometric mean titers increased from 6.7×10^4 (MVA/SHIV89.6) and 3.1×10^5 (MVA/SHIV89.6 + gp140) prior to challenge to 1.4×10^6 (both groups) by 6 weeks postchallenge, suggesting a secondary response induced by replication of SHIV-89.6 in vaccinated animals. At this time the challenged control animals had a geometric mean titer of only 3.3×10^5.

Neutralizing antibody responses to SHIV-89.6 were observed in all control animals by 12 weeks postchallenge (1:35–1:110). In 3 of the 10 immunized animals (Nos. 615, 617, and 623), a spike in titer (up to 1:619–1:1031) was seen within 4 to 8 weeks after challenge. Animal 605, which had the second highest prechallenge neutralizing antibody titer (1:171), showed a drop in both binding and neutralizing antibodies postchallenge, consistent with its apparent resistance to infection as measured by plasma RNA and virus isolation. SHIV-89.6 infection typically results in development and maintenance of high neutralizing antibody titers. In the MVA/SHIV89.6 + gp140 group, neutralizing antibody titers

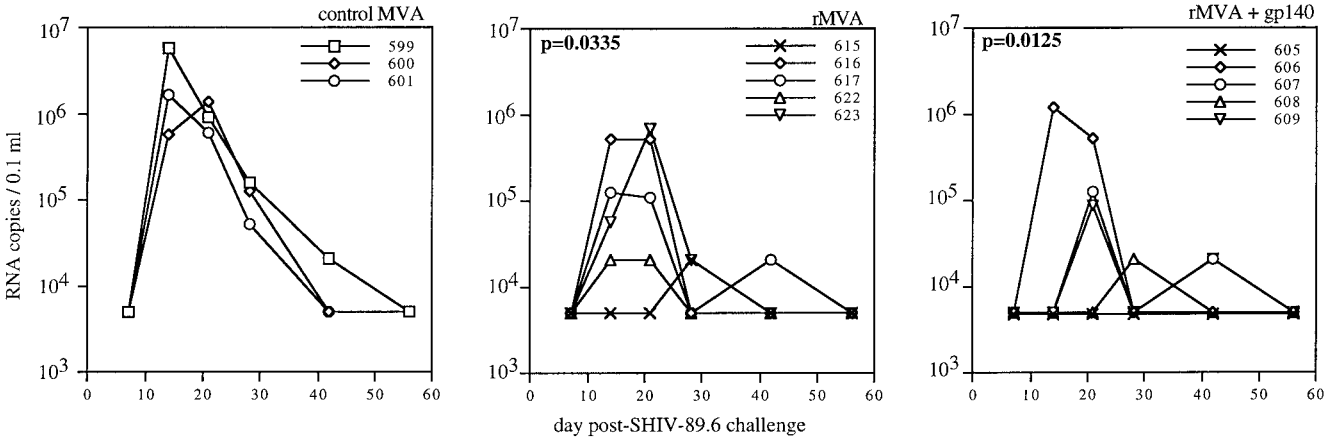

FIG. 4. Temporal viral RNA loads after challenge with SHIV-89.6—Study 1. Plasma viral RNA (copies/ml) was determined by NASBA. Data from individual animals are plotted. The statistical significance of comparison between each vaccinated group and control group is given.

were low to undetectable for several months (see Fig. 2B, weeks 71–91) following challenge in 3 of the 4 infected animals, suggesting that the animals were able to temporarily control viral replication.

Virus loads after challenge with SHIV-89.6P

Approximately 1 year after the third immunization with MVA/SHIV89.6 one unchallenged group of animals was boosted with Env protein (Study 2). Following intravenous challenge with 10 animal infectious doses of the pathogenic SHIV-89.6P, all of the animals became infected (Fig. 5A). In the three control animals, viral RNA peaked at 2 weeks postchallenge with a geometric mean of 9.7×10^7 copies/ml. RNA was then maintained at levels of 10^5 to 10^6 copies/ml. In the five vaccinated animals, viral RNA loads varied considerably. At the peak, the geometric mean was 1.7×10^7 RNA copies/ml, fivefold lower than in the controls (Fig. 5A). However, RNA values quickly dropped to low levels in two animals and declined more slowly in two others. The profile from animal

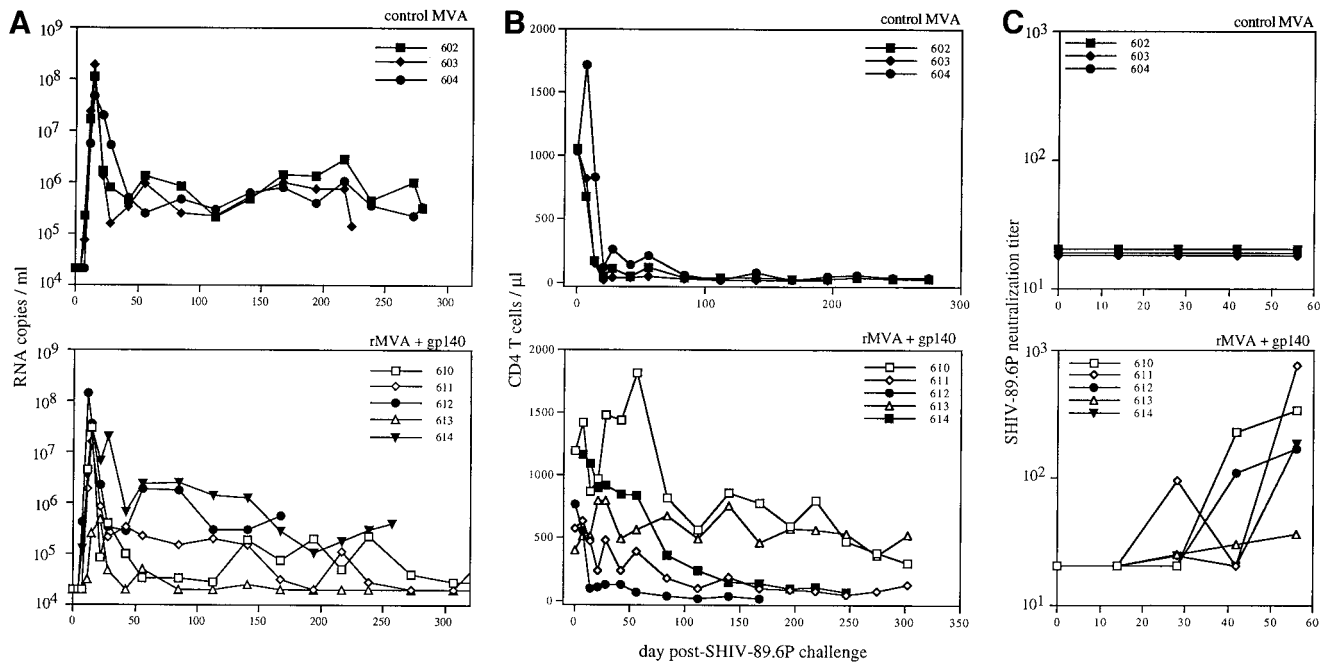

FIG. 5. Temporal viral RNA loads, CD4 cell counts, and neutralizing antibody titers against SHIV-89.6P—Study 2. (A) Plasma viral RNA (copies/ml) was determined by NASBA. Plot lines terminate at the time of death; three animals (Nos. 610, 611, and 613) are still alive. (B) CD4 T cell counts were measured by FACS. (C) SHIV-89.6P neutralizing antibody titers were determined in an MT-2 cell killing assay. Titers are the reciprocal of the dilution giving 50% neutralization. Data from individual animals are plotted. Open and closed symbols represent animals that are alive or dead 20 months after challenge, respectively.

612, however, was similar to that of the controls. A statistically significant difference between the log RNA values in the control and vaccinated groups was reached in the postacute phase of infection ($P = 0.032$).

Humoral responses following challenge with SHIV-89.6P

After challenge with SHIV-89.6P, Env ELISA titers increased rapidly in all five vaccinated monkeys (Fig. 3A) as expected, since the 89.6 and 89.6P Env proteins are very similar. The geometric mean titers increased from 4.7×10^5 on the day of challenge to 6.6×10^6 4 weeks later, suggestive of an anamnestic response that had been primed by vaccination. By comparison, titers to 89.6 Env were at least two orders of magnitude lower in the control animals. In addition, enhanced neutralization of SHIV-89.6 (Fig. 3B) and SHIV-89.6P (Fig. 5C) was seen in all vaccinated animals following the challenge. The challenged control animals developed only low levels of binding antibodies and no neutralizing antibodies against either the vaccine or the challenge virus (Fig. 5C). Thus, immunization with MVA/SHIV89.6 primed for a secondary neutralizing antibody response to SHIV-89.6 as well as SHIV-89.6P.

CD4 T cell counts and clinical outcome in animals challenged with SHIV-89.6P

All control monkeys exhibited a rapid and dramatic loss of CD4 T cells within 3 weeks after challenge (Fig. 5B). A similar pattern was seen in one vaccinated monkey (No. 612). Two others (Nos. 611 and 614) also had severe loss of CD4 cells but the decline occurred more slowly than in the controls, reaching similar levels only at week 21 after challenge. Animals 610 and 613 have maintained CD4 cell levels at >500 cells/μl for 20 months.

The animals were followed for clinical signs of disease after challenge. To date, all three control and two vaccinated animals (Nos. 612 and 614) have been euthanized due to severe AIDS-like symptoms including chronic diarrhea, weight loss, poor appetite, and unresponsiveness to therapy. The remaining three vaccinated monkeys remain in good health with no evident signs of disease. We found, as have others, that high RNA levels and CD4 cell decline were associated with more rapid disease progression and death.

DISCUSSION

Induction of both humoral and cellular immunity are goals in development of an effective HIV vaccine. To this end we designed a preclinical immunization protocol aimed at stimulating both arms of the immune system. We chose the SHIV model that incorporates the *env* gene from the primary HIV-1 isolate 89.6 and constructed a rMVA virus, MVA/SHIV89.6, that expresses functional 89.6 env and SIVmac239 gagpol. While production of correctly folded and functional proteins is not necessary for eliciting CTL, it is likely to be important for generation of potent neutralizing antibodies. As a means of enhancing antibody production, we included a protein boost, consisting of oligomeric 89.6 gp140, in some of the study groups.

Our studies were designed to answer several questions. First, could we generate both humoral and cellular immunity with the rMVA alone? Second, does boosting with soluble Env enhance immunogenicity? Third, can either immunization protocol effectively lower viral loads after challenge with a homologous SHIV? And finally, using the pathogenic SHIV challenge, does immunization limit not only virus replication but also virus-induced CD4 T cell loss and progression to AIDS-like disease?

Immunization with MVA/SHIV89.6 elicited both binding and neutralizing antibodies, with the former appearing earlier and more consistently in all animals. In contrast, Barouch *et al.* (2001) found no evidence of neutralizing antibodies after three successive immunizations of macaques with a mixture of the two single rMVA viruses that were used to generate our MVA/SHIV89.6. Perhaps under those conditions most cells were not simultaneously infected with the two viruses so that small amounts of VLP containing Env were formed. In addition, Amara *et al.* (2001) did not detect neutralizing antibodies after two DNA immunizations followed by a single boost with MVA/SHIV89.6. We found substantially enhanced antibody titers after a protein boost. However, they were not as high as those obtained in animals immunized 4–6 times with 89.6 Env alone (P. Earl, unpublished results). In addition, neutralization of SHIV-89.6P was found in 4 of the 10 animals that received protein boosting. Thus, although enhancement of the humoral response was achieved by administration of soluble Env, a more effective immunogen is needed to further increase both the magnitude and the breadth of the antibody response.

We also detected a CTL response to Gag in Mamu-A*01-positive animals by CM9 tetramer staining of PBMC. Peak tetramer staining was previously reported to occur at 1–2 weeks following a second or third rMVA immunization (Barouch *et al.,* 2001; Seth *et al.,* 1998, 2000). Other groups have reported a correlation between peak tetramer staining and reduction in virus load (Barouch *et al.,* 2000, 2001). Since our study was initiated before the tetramer technology was widely available, we performed the assay at only a single time point, just prior to challenge, when the T cell pools were in memory phase. Because of this as well as the limitation in number of Mamu-A*01 animals in the study, no correlations with virus load were possible.

In Study 1, following intravenous challenge with SHIV-89.6, all but one vaccinated animal and all control animals became infected. Nevertheless, both groups of im-

munized animals exhibited a statistically significant reduction in viral load in comparison to the control group. The viral RNA loads were lower in the protein boost group than in the group that received only recombinant MVA. Interestingly, the animal that apparently resisted infection (No. 605) was one that received the protein boost and had one of the highest vaccine-induced neutralizing antibody titers, but the lowest tetramer staining. Using data from both groups of immunized animals and excluding controls, we found an inverse correlation between peak prechallenge neutralizing antibody titer and log total viral RNA. Although the *P* value (0.089, determined by either Kendall or Spearman rank correlation) did not reach statistical significance, it is suggestive that neutralizing antibodies contributed to the control of viremia. In another study, we found that repeated immunization of monkeys with 89.6 Env alone induced neutralizing antibodies in all animals. In addition, those animals with the highest titers (>1:2000 at peak) were fully protected against SHIV-89.6 challenge, indicating that with sufficiently high neutralizing antibodies, protection can be achieved in the absence of CTL (P. Earl, unpublished results). Our observations that antibody production is enhanced after boosting with protein and that there is a relationship between higher neutralizing antibody titers and lower viral burden have implications for upcoming efficacy trials in which inclusion of a protein boost following canarypox immunization is under consideration.

The consequences of infection with SHIV-89.6P differ dramatically from those of SHIV-89.6 in that replication is persistent, CD4 cells undergo dramatic depletion, and animals develop AIDS-like disease in most cases (Reimann *et al.,* 1996a). In addition, the neutralizing specificities of the 89.6 Env in the vaccine and the 89.6P Env in the challenge virus differ. This challenge virus thus offers the opportunity to evaluate protection from immunologic suppression and disease in circumstances in which there is some Env heterogeneity. Although all of the animals became infected following intravenous challenge with SHIV-89.6P, the control animals exhibited uniformly high viral RNA loads, dramatic loss of CD4 cells, and onset of AIDS-like disease typical of infection with SHIV-89.6P. Although there was wide variation in the responses of the immunized animals, as a group they exhibited a considerable reduction in plasma viremia, CD4 cell loss, and progression to disease. With the exception of animal 612, which behaved like the unimmunized controls, the kinetics of CD4 loss were much slower in the immunized group. Currently, 20 months following challenge, three of the five immunized monkeys (Nos. 610, 611, and 613) are alive and healthy.

Prechallenge neutralizing antibodies to SHIV-89.6 were detected in four of the five immunized animals in Study 2 (Table 1). Animals 613 and 610, which had the highest neutralizing titers against SHIV-89.6 (1:1195 and 1:200, respectively), exhibited the lowest viral RNA loads and the most consistent preservation of CD4 T cells, supporting the role of neutralizing antibodies in control of infection. Although the only Mamu-A*01 animal in this group had a relatively high percentage of tetramer-positive $CD8^+$ T cells, it showed a progressive loss of CD4 cells.

Further evidence for the protective effects of immunization came from the postchallenge neutralizing antibody titers. Secondary responses to SHIV-89.6 were observed within 2–4 weeks of challenge in all of the immunized animals in Study 2. In addition, these animals developed neutralizing antibodies to the challenge strain, SHIV-89.6P, within 4 to 6 weeks of challenge. These latter antibodies could reflect either an anamnestic response by a small subset of B cells that were primed by vaccination or a *de novo* response to the challenge virus that was made possible due to CD4 cell preservation. In contrast, none of the control animals seroconverted to either SHIV strain because of the rapid loss of immune function.

In summary, our studies showed that immunization with rMVA induces both antibodies and CTL, although the former were enhanced by addition of a protein boost. Significant reduction of viremia was achieved against either a completely or partially homologous challenge strain. With the pathogenic virus, this translated into CD4 cell preservation and prolonged survival. In addition, our data suggested that neutralizing antibodies played a role in controlling viremia, although cell-mediated immunity could also have made a significant contribution to protection.

MATERIALS AND METHODS

Construction of rMVAs

A recombinant virus expressing the SIVmac239 *gagpol* gene was constructed by insertion of the entire open reading frame from plasmid p239SpSp5′ into a plasmid transfer vector, pLW-9 (Wyatt *et al.,* 1996). The rMVA, MVA/SIV239gagpol, was selected by immunostaining with serum from an SIV-infected macaque. For construction of the double recombinant virus, CEF were incubated simultaneously with 5 infectious units each of MVA/SIV239gagpol and MVA/89.6T (L. Wyatt, manuscript in preparation). Cells infected with MVA/SIV239gagpol have a unique rounded-up appearance unlike that seen with nonrecombinant MVA or *env*-expressing recombinant MVA. Thus, we picked foci composed of rounded cells that stained positively for Env. Approximately 20% of the foci had this characteristic. The double recombinant virus was clonally purified and amplified.

Immunoprecipitation of viral antigens from MVA-infected cells

Infection, metabolic labeling, and immunoprecipitation were performed as previously described (Sugiura *et al.,*

1999). Briefly, BS-C-1 cells were infected with 5 to 10 infectious units/cell. Five hours postinfection, cell monolayers were washed once and overlaid with methionine-free EMEM containing 5% dialyzed FBS and 100 μCi of [^{35}S]methionine (New England Nuclear, Boston, MA) per milliliter. After 20 h, cells were lysed in buffer containing 100 mM Tris–HCl, pH 8.0, 100 mM NaCl, and 0.5% Triton X-100. Cell lysates were clarified by centrifugation for 10 min at top speed in a microfuge and subjected to immunoprecipitation with rabbit HIV-Env serum 2144 and monkey serum p211. Proteins were separated by 10% polyacrylamide SDS electrophoresis and visualized by autoradiography.

Electron microscopy of VLP

BS-C-1 cells were infected at a multiplicity of infection of 10. After 24 h, the cells were prepared for conventional transmission electron microscopy as previously described (da Fonseca *et al.,* 2000).

Immunization and challenge of rhesus macaques

A total of 21 rhesus monkeys (*Macaca mulatta*) were used in two studies (13 in Study 1 and 8 in Study 2). Experiments were conducted in an AAALAC-International accredited facility and in accordance with guidelines set forth in the National Research Council Guide for the Care and Use of Laboratory Animals (1996). The studies were conducted under Contract N01-AI65314 by the Division of AIDS, NIAID, NIH. Vaccinated ($n = 5$) and control ($n = 3$) groups were immunized intramuscularly with 10^8 infectious units of either MVA/SHIV89.6 or nonrecombinant MVA at 0, 4, and 26 weeks. Study 1 consisted of two experimental groups: one received an additional MVA/SHIV89.6 immunization at week 60, whereas the other received two immunizations with 300 μg of soluble, oligomeric 89.6 gp140 at weeks 40 and 62. The 89.6 gp140 was produced from BS-C-1 cells infected with recombinant vaccinia virus vBD1 and purified by lentil lectin and Superdex 200 chromatography (P. Earl, unpublished results). The 89.6 gp140 boosts were administered in QS-21 adjuvant (Aquila Biopharmaceuticals, Inc., Framingham, MA). Study 2 consisted of one experimental group that received three immunizations with MVA/SHIV89.6 followed by boosting with 89.6 gp140 at weeks 85 and 98. Monkeys in Study 1 were challenged intravenously with 10 animal infectious doses of the nonpathogenic SHIV-89.6 (obtained from Yichen Lu, Virus Research Institute, Cambridge, MA) 4 weeks after the fourth MVA immunization or 2 weeks after the second protein immunization. The final immunizations were staggered to allow simultaneous challenge of all monkeys. Monkeys in Study 2 were challenged intravenously with 10 animal infectious doses of the pathogenic SHIV-89.6P (obtained from Norman Letvin, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA) 2 weeks after the second protein boost.

89.6 Env ELISA

For the ELISA, Immulon-2 (Dynex Technologies, Chantilly, VA) 96-well U-bottomed plates were coated overnight at 4°C with sheep antibody to the C-terminus of gp120 (International Enzymes, Inc., Fallbrook, CA) at 0.5 μg/ml in bicarbonate buffer (Roche Molecular Biochemicals, Indianapolis, IN). Purified 89.6 gp140 was captured (0.6 μg/ml in PBS) for 2 h at room temperature. Plates were blocked with 5% nonfat dry milk in PBS. Then twofold serial dilutions of monkey sera were incubated for 2 h at room temperature followed by horseradish peroxidase-conjugated anti-monkey IgG (Accurate Chemical and Scientific Corp, Westbury, NY) for 1 h and then BM Blue substrate (Roche Molecular Biochemicals) for 30 min. Absorbency was measured at 370 and 492 nm.

Neutralization of SHIV-89.6 and SHIV-89.6P

Neutralization was measured in an MT-2 cell assay as described previously (Crawford *et al.,* 1999; Montefiori *et al.,* 1988). Virus stocks were produced in human PBMC. Titers of neutralizing antibodies were presented as the reciprocal of the serum dilution that reduced virus-induced cell killing by 50% as measured by neutral red dye uptake.

CTL assays

^{51}Cr release assays were performed on *in vitro* stimulated PBMC (Allen *et al.,* 2000).

MHC typing

Rhesus macaques were typed for the rhesus MHC class I molecule Mamu-A*01 by PCR sequence-specific priming and direct sequencing as previously described (Knapp *et al.,* 1997).

Tetramer staining

A total of 1×10^6 fresh, unstimulated PBMC or 2×10^5 lymphocytes from 2-week peptide-stimulated cultures were washed three times in FACS buffer (PBS from Gibco with 2% FCS from BioCell) in a 96-well U-bottom plate. Cells (100 μl) were incubated for 40 min at room temperature in the dark with Mamu-A*01/CM9-tetramer labeled with either phycoerythrin or allophycocyanin (0.5 μg/100 μl for fresh PBMC, 0.1 μg/100 μl for *in vitro* cultures) together with 10 μl anti-rhesus fluorescein isothiocyanate-labeled CD3 monoclonal antibody (BioSource, San Jose, CA) and 6 μl anti-CD8α-PerCP monoclonal antibody (clone SK1, Becton Dickinson) in a 100-μl volume of FACS buffer. The Mamu-A*01 molecule was refolded with the GagCM9 peptide and the tetramers

were made as described previously (Allen *et al.,* 1998; Altman *et al.,* 1996). Plates were then washed four times with FACS buffer. Finally, 450 μl of 2% paraformaldehyde in PBS was added to fix the stained cells. A CTL clone specific for these tetramers was stained in parallel with isotype controls (mouse IgG1-FITC, Biosource; mouse IgG2a-PE, Immunotech; mouse IgG1-PerCP, BD; mouse IgG1-APC, Immunotech), anti-CD3-FITC, anti-CD8β-PE (Immunotech), anti-CD8α-PerCP, or CD8α-APC (Immunotech) to establish compensation parameters. Samples were stored in the dark at 4°C and acquisition of 100,000–200,000 lymphocyte-gated events was performed on a Becton Dickinson FACSCalibur instrument and analyzed using CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA). Background tetramer staining of fresh, unstimulated PBMC from naive Mamu-A*01-positive animals was routinely less than 0.09% and less than 0.2 for in vitro stimulated cultures (data not shown).

Plasma SHIV RNA copy number

The concentration of SHIV RNA in plasma was determined by quantitative nucleic acid sequence-based amplification (NASBA) assays (Romano *et al.,* 2000).

$CD4^+$ T cell counts

Flow cytometric assays were performed on PBMC by FAST Systems, Inc. (Gaithersburg, MD).

Statistical methods

Analysis of variance (ANOVA) was used to test for differences in RNA loads between study groups using log-transformed data. Calculations were performed with StatView (SAS Institute, Inc., Cary, N.C.). In addition, we applied Shaffer's improved method for multiple, pairwise comparisons among a set of tests (Shaffer, 1986). This procedure, an extension of earlier work by Holm (1979), is a sequentially rejective method that can significantly improve on the Bonferroni method, while still protecting the family-wise error rate.

ACKNOWLEDGMENTS

We thank Chelsi Cacciatore and Lynn Frampton for ELISAs and viruses, Norman Cooper for cells, and Andrea Weisberg for electron microscopy. Mamu-A*01/CM9 tetramer was provided by John Altman. QS-21 adjuvant was provided by Charlotte Kensil. Plasmid p239SpSp5′ containing the SIVmac239 *gagpol* gene was obtained from Ronald Desrosiers through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. VBD1 was provided by Robert Doms. This work was supported in part by extramural NIH funding (AI85343).

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