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Evidence for immune-mediated reduction of viral replication in *Macaca nemestrina* mucosally immunized with inactivated $SHIV_{89.6}$

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Abstract

Although most HIV-1 infections worldwide result from heterosexual transmission, most vaccine candidates have focused on induction of systemic immunity and protection. We hypothesized that combining systemic priming with mucosal boosting would induce mucosal immunity that would protect from intravaginal challenge. Macaques were primed systemically with recombinant vaccinia viruses and boosted mucosally using inactivated $SHIV_{89.6}$ plus adjuvant. Other animals received protein boosts with adjuvant alone. Priming and boosting induced antiviral IgG and IgA antibodies. Such antibodies were induced to a lesser degree in animals receiving boosts alone. Anti-SHIV T cell responses were induced only in the prime-boost animals. Immunized animals and controls were challenged intravaginally with $SHIV_{89.6}$ and significant reductions in proviral and viral RNA loads were observed in the prime-boost animals. The boost-only animals did not have significant viral load reductions. These data suggest that cellular immunity was required for protection from intravaginal challenge. This immunization regimen provides a promising lead for vaccine development.

Keywords: Macaque; SHIV; HIV; Mucosal; Immunity; Vaccine; Prime-boost; Immunization

Introduction

Mucosal transmission of HIV-1 after heterosexual contact is the primary route of infection for women throughout the world (UNAIDS/WHO, 2000). However, the majority of candidate vaccines have utilized methods to induce systemic or gastrointestinal immune responses, and they have been evaluated primarily for protection against intravenous or intrarectal challenge (reviewed in Bogers et al., 2000; Nathanson et al., 1999). Additional studies designed to test vaccine protection against intravaginal challenge are needed to explore the variables inherent in this important route of HIV infection.

Simian immunodeficiency virus (SIV) infection of macaques has been used extensively in simulating HIV-1 infection and AIDS. However, it is impractical to evaluate candidate vaccines against HIV envelope (Env) proteins for use in humans in this model due to the inadequacy of infection of and disease in macaques and chimpanzees with HIV-1. Thus, SIV/HIV chimeric viruses (SHIV) have been constructed, in which the *rev*, *tat*, and *env* genes of SIV have been replaced with those of HIV-1 (Igarashi et al., 1994; Li et al., 1992; Luciw et al., 1995; Reimann et al., 1996b).

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These chimeras replicate in macaques with relatively low pathogenicity, unless serially passaged in vivo (Joag et al., 1996; Reimann et al., 1996a; Stephens et al., 1996). Vaccine candidates targeting HIV-1 envelope can be assessed in the SHIV macaque model of infection.

While extensive research on the immune system of the genital tract of female macaques during intravaginal infection with SIV have been performed (Miller, 1994), few reports have been published showing protective effects of vaccine candidates when the animals are challenged intravaginally with SIV or SHIV (Buge et al., 1997; Israel et al., 1999; Joag et al., 1998; Johnson et al., 1999; Marthas et al., 1992; Marx et al., 1993; Mascola et al., 2000; Miller et al., 1997). These mucosal immunization protocols, as well as other HIV vaccines, can be classified into four groups; (1) subunit or nonreplicating, such as viral proteins; (2) replicating recombinant vectors, such as recombinant vaccinia virus (rW) encoding HIV or SIV genes; (3) live-attenuated SIV, HIV, or SHIV; and (4) combinations of these.

From these studies, as in the macaque vaccine literature as a whole, it is not clear which immune responses correlate with protection, especially because reports which have assayed neutralizing plasma and vaginal antibodies together with cytotoxic T lymphocytes (CTL) in the PBMC have only recently been published. Live, attenuated lentivirus infection has been shown to provide the best protective efficacy against different routes of challenge (Johnson and Desrosiers, 1998), suggesting that sustained, high-level antigen production is necessary in an effective vaccine. However, the safety of such a vaccine has been questioned after disease progression in neonatal and adult macaques vaccinated with highly attenuated SIV has been reported (Baba et al., 1995, 1999). Two studies published recently have demonstrated that human HIV neutralizing monoclonal antibodies of the IgG subclass delivered intravenously can protect against oral or vaginal challenge with SHIV (Baba et al., 2000; Mascola et al., 2000). Thus as with intravenous infection, very high titers of neutralizing antibodies can block infection. However, if HIV vaccines fail to achieve and sustain such high titers, additional cellular and humoral immunity at the mucosal surface would be beneficial.

Prime-boost vaccines, where the priming and boosting components consist of different modalities, are found to be particularly effective in inducing protective immunity. Efforts have focused on replicating vectors that could produce viral antigens inside host cells and allow epitopes to be presented in MHC class I for CTL recognition. Combining vector priming (rVV) with subunit antigen boosts (gp120 or gp160) was found to enhance protection from i.v. challenge above subunit vaccination alone (Hu et al., 1992). However, prime-boost regimens using rVV or recombinant adenovirus with subunit boosts intramuscularly or orally and intratracheally have not been successful in protection from intravaginal challenge (Buge et al., 1997; Israel et al., 1999).

Cholera toxin (CT) and heat-labile enterotoxin (LT) have been extensively explored as mucosal adjuvants in murine models of infection (reviewed in Freytag and Clements, 1999; Snider, 1995). However, these proteins are highly toxic and are not recommended for human immunizations. An LT mutant, LT(R192G), has a mutation at one amino acid, eliminating the trypsin cleavage site required for biological activation and thus greatly reducing its toxicity (Dickinson and Clements, 1995). This compound has the ability to induce mucosal and systemic antibodies and CTL against a variety of pathogens in mice (Belyakov et al., 2000; Cardenas-Freytag et al., 1999; Choi et al., 2002; Chong et al., 1998; Lee et al., 1999; Morris et al., 2000; O'Neal et al., 1998) and macaques (Belyakov et al., 2001) when administered with antigens.

Thus, we wanted to utilize LT(R192G) as an adjuvant in a prime-boost vaccination of *Macaca nemestrina*, which we hypothesize will provide potent mucosal immunity against intravaginal challenge with $SHIV_{89.6}$. In this article, we describe a novel immunization protocol of systemic priming with rVV expressing HIV-1 Env and SIV Gag-Pol along with mucosal boosting intragastrically (i.g.) and intranasally (i.n.) of LT(R192G) plus aldrithiol-2 (AT-2)-inactivated $SHIV_{89.6}$. This inactivation procedure appears to retain the functional conformation of envelope of lentiviruses (Rossio et al., 1998).

Results

Immunization procedure

Three groups of animals were immunized: primed and boosted (Group 1, $n = 5$), boosted only (Group 2, $n = 3$); and controls (control group, $n = 3$; Table 1). Priming was done systemically using replicating rVV expressing $HIV\text{-}1_{89.6}$ Env (vT107) and SIV_{mac251} Gag-Pol (vAbT394) at weeks 0 and 4. Sham priming was performed with wildtype vaccinia virus (NYCBH) at weeks 0 and 4. Boosting was performed i.g. and i.n. at weeks 12 and 19 with AT-2-inactivated $SHIV_{89.6}$ and LT(R192G) and i.n. only at week 36. Control animals received boosts of the LT mutant only at the same time points via the same routes.

SHIV challenge

After completion of the immunization regimen, all 11 animals were inoculated intravaginally with 200 50% tissue culture infectious doses ($TCID_{50}$) $SHIV_{89.6}$ at week 40, 4 weeks after the third booster. One animal in the control group (F91286) did not become infected after challenge (Table 1, Figs. 3 and 4) and was rechallenged 14 weeks later. Four weeks prior to all challenges and the rechallenge, the animals were treated with medroxyprogesterone, which has been shown to decrease the thickness of the vaginal epithelium and increase the rate of infection (Marx et al., 1996; Sodora et al., 1998). In exploratory studies, 200 $TCID_{50}$ of the same viral stock was found to infect 2/2 *M.*

Table 1
Immunization components and coculture results

Animal	Primes[a]	Boosts[b]	Coculture results (w.p.i.)[c]										
			0	1	2	3	4	5	6	7	8	10	12
A98045	vT107+vAbT394	LT(R192G)+SHIV	−	−	+	+	+	−	+	+	+	−	−
93169[d]	vT107+vAbT394	LT(R192G)+SHIV	−	+	+								
J91354	vT107+vAbT394	LT(R192G)+SHIV	−	−	−	+	+	+	+	+	+	−	−
98063	vT107+vAbT394	LT(R192G)+SHIV	−	−	+	+	+	+	−	−	−	−	+
A98068	vT107+vAbT394	LT(R192G)+SHIV	−	+	+	+	+	+	+	+	−	−	−
F93006	NYCBH	LT(R192G)+SHIV	−	−	+	+	+	+	+	+	+	+	−
J92468	NYCBH	LT(R192G)+SHIV	−	−	−	+	+	+	+	+	+	−	−
M92077	NYCBH	LT(R192G)+SHIV	−	−	−	+	+	+	+	+	−	+	+
J93076	NYCBH	LT(R192G)	−	−	+	+	+	+	+	+	+	+	+
98129	NYCBH	LT(R192G)	−	−	−	+	+	+	+	+	+	+	+
F91286[e]	NYCBH	LT(R192G)	−	−	−	−	−	−	−	−	−	−	−

[a] Priming was done at weeks 0 and 4 by skin scarification.
[b] Boosting was done i.g. and i.n. at weeks 12 and 19 and i.n. at week 36.
[c] All animals were challenged with 200 $TCID_{60}$ $SHIV_{89.6}$ intravaginally.
[d] Animal 93169 died during anesthesia for a routine blood draw at 2 weeks postchallenge.
[e] Animal F91286 was rechallenged 14 weeks after initial challenge, results reflect cocultures after second challenge.

nemestrina inoculated intravaginally, whereas 0/2 animals that received 20 $TCID_{50}$ became infected (data not shown).

SHIV-specific antibodies in plasma

Plasma antibodies against SHIV were analyzed by ELISA (Tables 2 and 3). Anti-SHIV IgG was first detected at 1 week after the first mucosal boost (Table 2). All five Group 1 animals and one of the animals in Group 2 had detectable IgG antibodies against SHIV (1:50–1:1250) by week 21, 2 weeks after the second mucosal booster. At 3 weeks postchallenge, an antibody response could be detected only in the plasma of those five immunized animals that had detectable antibody levels prior to challenge, suggesting an anamnestic response (Table 2). At 3 weeks postinfection (w.p.i.) all but one of the remaining Group 1 animals and the Group 2 responder had increases in their IgG titers (1:1250–8800). None of the control animals had detectable IgG levels at this time point. All of the Group 1 animals had anti-SHIV IgG titers of 1:3900–7000 at 6 w.p.i. These titers were significantly higher than those of the Group 2 and control animals ($P = 0.02$ and $P = 0.001$, respectively). In addition, the two seronegative animals in Group 2 seroconverted by 6 weeks postchallenge but their responses were lower (1:250-1:3200) than those seen in the animals that were both primed and boosted. None of the three control animals had detectable anti-SHIV antibody responses at this time point. At 8 w.p.i. two of the controls developed antibodies to SHIV (1:250), whereas the remaining animal in the control group (F91286) did not seroconvert and was subsequently discovered to be uninfected by other parameters (Table 1). ELISA results were confirmed

Table 2
Anti-SHIV plasma IgG antibody titers

Animal	Immunization[c]	Titers (week)[a,b]											
		0	3	5	6	13	14	16	21	40	43	46	48
A98045	Prime+boost	<50	<50	<50	ND	<50	ND	<50	**100**	**300**	**1250**	**5000**	**4700**
93169[d]	Prime+boost	<50	<50	<50	ND	<50	ND	**100**	**100**	**300**	ND	ND	ND
J91354	Prime+boost	<50	<50	<50	ND	**250**	ND	**1250**	**1250**	**300**	**250**	**6500**	**5800**
98063	Prime+boost	<50	<50	ND	<50	**50**	ND	**50**	**50**	**100**	**4100**	**3900**	**4400**
A98068	Prime+boost	<50	<50	ND	<50	<50	ND	**50**	**250**	**100**	**8800**	**7000**	**7500**
F93006	Boost only	<50	<50	ND	<50	ND	<50	<50	**100**	**100**	**5800**	**3200**	**4200**
J92468	Boost only	<50	<50	ND	<50	ND	<50	<50	<50	<50	<50	**1250**	**250**
M92077	Boost only	<50	<50	ND	<50	<50	ND	<50	<50	<50	<50	**250**	**1250**
J93076	Control	<50	<50	ND	<50	ND	<50	<50	<50	<50	<50	<50	**250**
98129	Control	<50	<50	ND	<50	ND	<50	<50	<50	<50	<50	<50	**250**
F91286	Control	<50	<50	ND	<50	<50	ND	<50	<50	<50	<50	<50	<50

[a] Animals were challenged with $SHIV_{89.6}$ at week 40.
[b] Numbers in bold indicate positive results.
[c] Animals were primed at weeks 0 and 4 and boosted at weeks 12, 19, and 36.
[d] Animal 93169 died during anesthesia for a routine blood draw at week 42 (2 weeks postchallenge).

Table 3
Anti-SHIV plasma IgA antibody titers

Animal	Immunization[c]	Titers (week)[a,b]									
		0	3	5	6	13	14	16	21	40	43
A98045	Prime+boost	<50	<50	<50	ND	<50	ND	<50	**50**	**100**	**50**
93169[d]	Prime+boost	<50	<50	<50	ND	<50	ND	<50	**50**	**50**	ND
J91354	Prime+boost	<50	<50	<50	ND	<50	ND	<50	**100**	**100**	**50**
98063	Prime+boost	<50	<50	ND	<50	<50	ND	<50	<50	<50	<50
A98068	Prime+boost	<50	<50	ND	<50	<50	ND	<50	**50**	**100**	**450**
F93006	Boost only	<50	<50	ND	<50	ND	<50	<50	**50**	**50**	<50
J92468	Boost only	<50	<50	ND	<50	ND	<50	<50	<50	**50**	<50
M92077	Boost only	<50	<50	ND	<50	<50	ND	<50	<50	<50	<50
J93076	Control	<50	<50	ND	<50	ND	<50	<50	<50	<50	<50
98129	Control	<50	<50	ND	<50	ND	<50	<50	<50	<50	<50
F91286	Control	<50	<50	ND	<50	<50	ND	<50	<50	<50	<50

[a] Animals were challenged with $SHIV_{89.6}$ at week 40.
[b] Numbers in bold indicate positive results.
[c] Animals were primed at weeks 0 and 4 and boosted at weeks 12, 19, and 36.
[d] Animals 93169 died during anesthesia for a routine blood draw at week 42 (2 weeks postchallenge).

by Western blot analysis (data not shown). Anti-gp120 serum antibodies were not detected in any of the animals after priming or boosting (data not shown).

Low levels of anti-SHIV IgA plasma antibodies were detected by ELISA after two boosts in the primed and boosted animals (Table 3). By the time of challenge, four of the animals in Group 1 and two of the Group 2 animals had detectable IgA antibodies (1:50–1:100). There was no evidence for an anamnestic IgA response in the plasma during the short follow-up period. At 3 weeks postchallenge, all Group 1 animals and two animals in Group 2 had increased or sustained levels of vaginal IgG anti-SHIV antibodies (not shown). SHIV-specific IgA antibodies or neutralizing antibodies were not detected in any of the vaginal washes of these animals (data not shown). Anti-SHIV IgG and IgA antibodies from the rectal swabs also were not detected by ELISA assay (data not shown). Neutralizing antibodies in the plasma before and early after challenge of all 11 animals were not detected (data not shown).

$CD4^+$ T cell proliferation against SHIV

PBMC from the animals were stimulated with serial dilutions of AT-2 inactivated $SHIV_{89.6}$ in a proliferation assay (Fig. 1). Responses were detected in the two Group 1 animals that were tested after priming (98063 and A98068). After two boosts, high proliferation responses were detected only in the PBMC of the Group 1 animals, and the responses were higher for animals 98063 and A98068 than observed after priming alone. The responses in the Group 1 animals were significantly higher than those of the animals in Group 2 and the control group ($P = 0.01$ and $P = 0.03$, respectively).

SHIV-specific $CD8^+$ intracellular interferon-γ (IFNγ) responses

$CD8^+$ T cell responses against Env and Gag-Pol were determined at time points throughout the study using an intracellular cytokine assay to measure antigen-specific IFNγ production (Fig. 2). Responses against Env were not prevalent in the PBMC of the Group 1, Group 2, or control animals both before and after SHIV challenge (Fig. 2A). However, Gag-Pol-specific PBMC responses were detected in Group 1 and Group 2 animals (Fig. 2B). Responses to these antigens were seen prior to challenge in 17/43 PBMC samples from the Group 1 animals (40%), 7/24 PBMC samples from the Group 2 animals (29%), and 3/26 PBMC samples from the controls (12%). The difference in $CD8^+$ T cell responses between Group 1 and Group 3 was significant ($P = 0.003$).

Between 3 and 8 weeks postchallenge, few $CD8^+$ T cell responses were seen in any of the 11 animals for Env (Fig. 2A). However, anti-Gag-Pol responses were observed in the PBMC of animals in all three groups (Fig. 2B).

Cervical cytobrushings sampled throughout this study did not yield consistent, usable cell numbers for the intracellular IFNγ assay. Therefore, cervical T cell responses could not be evaluated.

Virus isolation and viral load

All animals were tested for circulating virus by three methods: PBMC coculture, PCR for PBMC proviral DNA, and plasma viral RNA by bDNA assay. The immune responses of the individual animals together with their level of protection are presented in Table 4. All animals except one became infected by coculture assay (Table 1). The animals in Group 1 became negative by coculture between 6 to 10 w.p.i. Two animals in Group 2 became virus negative by week 12 postinfection and one animal remained virus positive through 12 w.p.i. During the 12 weeks of follow up, we could consistently isolate virus from the PBMC of the two infected controls and one of the Group 2 animals.

The two control animals that did become infected had high proviral loads in their PBMC through 10–15 w.p.i.

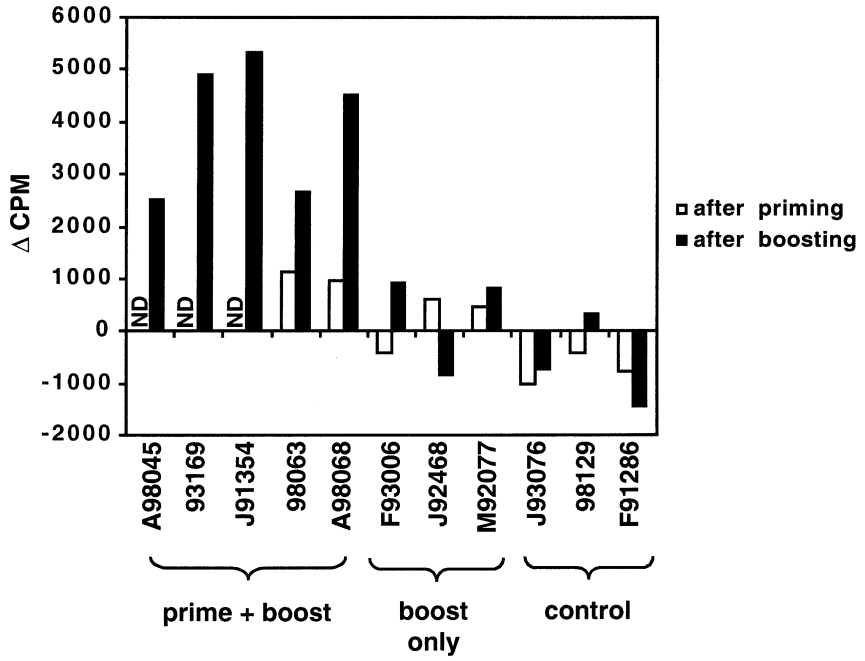

Fig. 1. PBMC proliferation responses when stimulated with AT-2-inactivated $SHIV_{89.6}$ (0.1 μg/ml) 1–2 weeks after the second priming (white bars) and 2 weeks after the second booster (black bars) of Group 1, Group 2, and control animals. Responses are represented as Δcpm; ND, not done. Five $SHIV_{89.6}$-infected animals had responses ranging 768–3370 (positive controls) and three uninfected animals had responses ranging 27–520 (negative controls).

with peaks of 4.9×10^3 and 2.5×10^4 copies/μg (Fig. 3C). Other *M. nemestrina* (97070 and 94075) inoculated intravaginally with the same dose of this $SHIV_{89.6}$ stock had peak proviral loads of $1.1–6.7 \times 10^3$ copies/μg and remained positive through 15 weeks postchallenge (Ambrose et al., 2001).

DNA PCR revealed significant levels of provirus in the PBMC of only one of four surviving Group 1 animals (A98045), despite the fact that all four were productively infected (see below). This animal had a peak proviral load of 6.8×10^3 copies/μg DNA (Fig. 3A) and became provirus-negative at 12 weeks postinfection. Two other animals in this group were consistently negative and one animal (J91354) was slightly positive above the limit of detection (100 copies/μg DNA) at weeks 4 and 10 postinfection only. One Group 2 animal (F93006) had multiple positive PCRs for viral DNA in the PBMC with a peak of 1.3×10^3 copies/μg and becoming negative at 12 weeks p.i., and the other two animals were negative throughout the study (Fig. 3B). The proviral peaks of the Group 1 animals only were significantly lower than the levels of the control animals ($P = 0.05$).

Viral RNA was detected in all coculture-positive animals and varied in magnitude in each group (Fig. 4). The four surviving Group 1 animals had viremia peaks at 3–4 weeks postchallenge (Fig. 4A). One of these animals had a high viremic peak (A98045, 3.1×10^6 copies/ml) and had undetectable levels by 8 w.p.i., whereas the average viral RNA peak of the other three was 1.6×10^4 copies/ml and they became negative by 4–6 weeks postchallenge. The Group 2 animals had an average viral RNA peak of 1.0×10^5 and became negative between 4–8 weeks after challenge (Fig. 4B). The two infected controls had peaks of 3.5×10^5 and 7.2×10^6 copies/ml and they were still positive at 10–15 weeks postinfection (Fig. 4C). Two other unimmunized control animals had viral RNA peaks of $1.1–8.7 \times 10^5$ (Ambrose et al., 2001). Plasma viral RNA peaks of the Group 1 animals were significantly lower than those of the control animals ($P = 0.05$) but not of the Group 2 animals ($P = 0.4$). The viremia peaks of the Group 2 animals showed trends toward significance as compared to the peaks of the control animals ($P = 0.09$).

Discussion

We have described a novel method of immunization, which combines systemic priming with mucosal boosting, using inactivated, conformationally intact SHIV virions. This immunization regimen induced significant antiviral systemic and mucosal immune responses in the limited number of animals used in this study. Suppression of viral replication in animals that were immunized using this protocol suggests partial protection mediated through these immune responses, presumably through a reduction in early viral replication. Alternatively, the observed partial protection could be the result of blocking of target cell infection at the vaginal mucosa. We detected only low levels of nonneutralizing vaginal IgG antibodies against $SHIV_{89.6}$ and consider the latter alternative therefore unlikely.

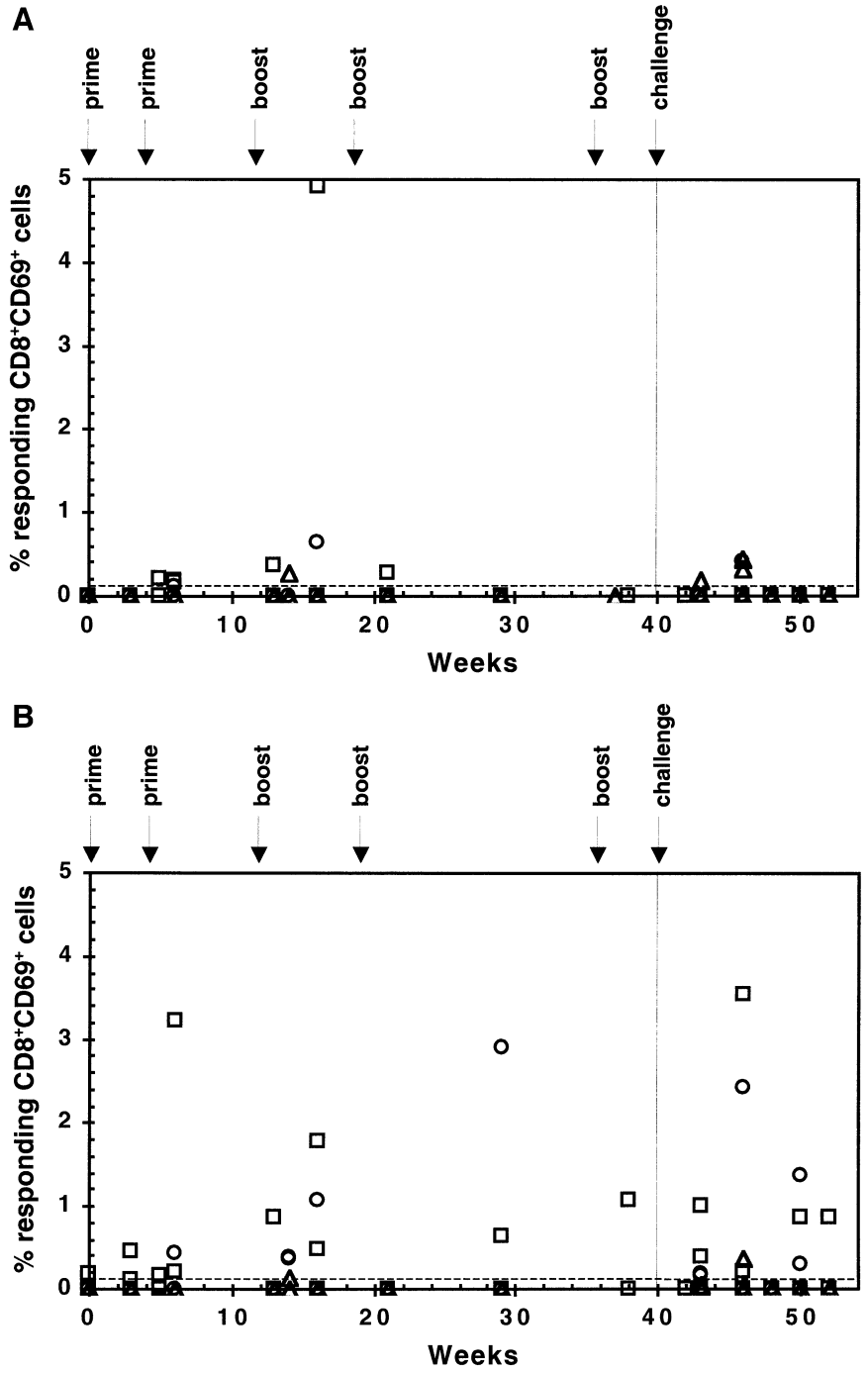

Fig. 2. Intracellular IFNγ staining of $CD8^+CD69^+$ PBMC in response to Env (A) and Gag-Pol (B) stimulation. Responses from all individual animals are pooled by group: Group 1 (□), Group 2 (○), and controls (△). The percentages of antigen-specific IFNγ-positive cells were plotted after subtraction of spontaneous IFNγ-producing cells with wild-type W stimulation. The limit of detection was 0.1% of the $CD8^+CD69^+$ cells (dashed line).

Previous studies have demonstrated possible roles for both neutralizing antibodies and components of the cellular immune response in suppressing viral replication (Barouch et al., 2002; Wyand et al., 1999). We did not detect any virus-neutralizing antibodies in either plasma or mucosal secretions of the immunized animals. This lack of induction of neutralizing antibodies could be a function of the viral preparations used for the mucosal immunizations: AT-2 inactivated SHIV retains the native Env glycoprotein conformation (Rossio et al., 1998), but recent data suggest that some lentiviral preparations may rapidly lose gp120 from the virion surface (Chertova et al., 2002). Although not specifically investigated for the $SHIV_{89.6}$ preparation used here, such loss of glycoproteins could be a potential cause for the observed lack of induction of virus-neutralizing antibodies. The absence of neutralizing antibodies after

Table 4
Summary of immune responses detected during immunization protocol and the viral outcomes postchallenge

Group	Animal	Plasma IgG[a]	Plasma IgA[a]	Vaginal IgG[b]	$CD4^+$ prolif[c]	$CD8^+$ IFNγ[d]	Plasma RNA[e]	PBMC DNA[f]	Coculture[g]
	A98045	300	100	++	2.0	4.90	3.1×10^6	6820	++
Prime-boost	93169	300	50	−	1.8	0.86	NA	NA	NA
	J91354	1250	100	++	3.3	1.79	1.3×10^4	129	++
	98063	100	<50	+	2.1	0.47	1.9×10^4	<101	+
	A98068	250	100	+	1.7	3.21	1.5×10^4	<101	++
	M93006	100	50	++	1.1	2.89	1.7×10^5	1340	+++
Boost	F92468	<50	50	+/−	0.7	1.07	3.1×10^4	114	++
	M92077	<50	<50	−	1.3	0	1.0×10^5	<101	++
	J92076	<50	<50	−	0.7	0	7.2×10^5	25400	+++
Control	98129	<50	<50	−	1.1	0.28	3.5×10^5	4930	+++
	F91286	<50	<50	−	0.8	0	<500	<101	−

NA, not available; ND, not done.

[a] Titers of anti-SHIV IgG or IgA determined by ELISA.

[b] +, detectable antibodies after challenge before controls became positive; ++, detectable antibodies before challenge; +/−, borderline responses detected.

[c] Proliferation response against 0.1 μg/ml SHIV after two boosts, expressed as stimulation indices (SI).

[d] Highest $CD8^+CD69^+$ cell response against Env or Gag-Pol, expressed as % responding $CD8^+CD69^+$ cells.

[e] Peak plasma RNA load, expressed as SIV copies/ml plasma.

[f] Peak PBMC DNA load, expressed as SIV copies/μg DNA.

[g] +, 1–4 positive cocultures; ++, 5–7 positive cocultures; +++, 8–10 positive cocultures.

challenge is puzzling; it is possible that viral challenge primarily enhanced the preexisting immune response induced by immunization. Efficient induction of antibody responses to new epitopes may require infection with a higher replication rate than the one we used for these studies (Rasmussen et al., 2001).

Comparison of the two vaccination regimens demonstrates that priming and boosting is more efficient in inducing antiviral immunity than boosting alone, in accordance with earlier studies (Hu et al., 1992). The Group 1 animals had higher levels of antibodies than the Group 2 animals. In addition, T cell proliferation was only observed in the PBMC from the animals that had been primed and boosted, but not in those of the animals that received the mucosal booster vaccinations after sham priming. Interestingly, there was no clear difference in the levels or frequencies of $CD8^+$ T cell responses to viral antigens between the two groups. Other studies have suggested that the LT(R192G) adjuvant directs the immune response toward a Th1 bias (Chong et al., 1998), and our data, albeit limited, support that suggestion.

Parameters for viral replication that were studied in our animals after challenge include the reisolation of virus from peripheral blood cells, the quantitation of PBMC proviral loads by DNA PCR, and the determination of plasma viral loads in plasma by bDNA assay. Each of these assays suggest reduced levels of virus replication in the immunized animals compared to the controls. The most striking reduction of viral burden is found in the fully immunized animals: those that received both the recombinant vaccinia priming and the protein boost with adjuvant. Conversion to a "virus negative" status by coculture occurs relatively early in these animals, and both quantitative assays for viral nucleic acids show significant differences compared to the control animals. When comparing numerical values for these measurements, there appears to be some reduction of viral replication as well in the animals that were not primed (Group 2), but the numbers in this group and the control group are small and the differences between these two groups are not statistically significant. A finding that the mucosal immunization regimen by itself, without systemic priming, can potentially induce protective systemic and mucosal responses is of great interest as such preparations are relatively easy to administer. Although we used intragastric immunization as a part of our regimen in this study, this method can conceivably be replaced by oral administration in the presence of acid-neutralizing substances to achieve the same effect. Because parenteral immunization is not necessary for induction of mucosal antiviral immunity, an oral and/or intranasal HIV-1 vaccine would greatly increase the potential acceptance in developing countries, allowing more widespread vaccination.

If true differences in the levels of viral replication between the Group 1 and Group 2 animals exist, a more comprehensive analysis of the immune responses between greater numbers of animals in these groups could elucidate which components are required for protection. The most striking difference between the two groups is the absence of T cell proliferation to SHIV antigens in PBMC from the Group 2 animals. We have not analyzed mucosal sites for helper T cell or CTL responses, and a possibility exists that such responses were induced locally but not systemically. Such analyses will be included in future studies.

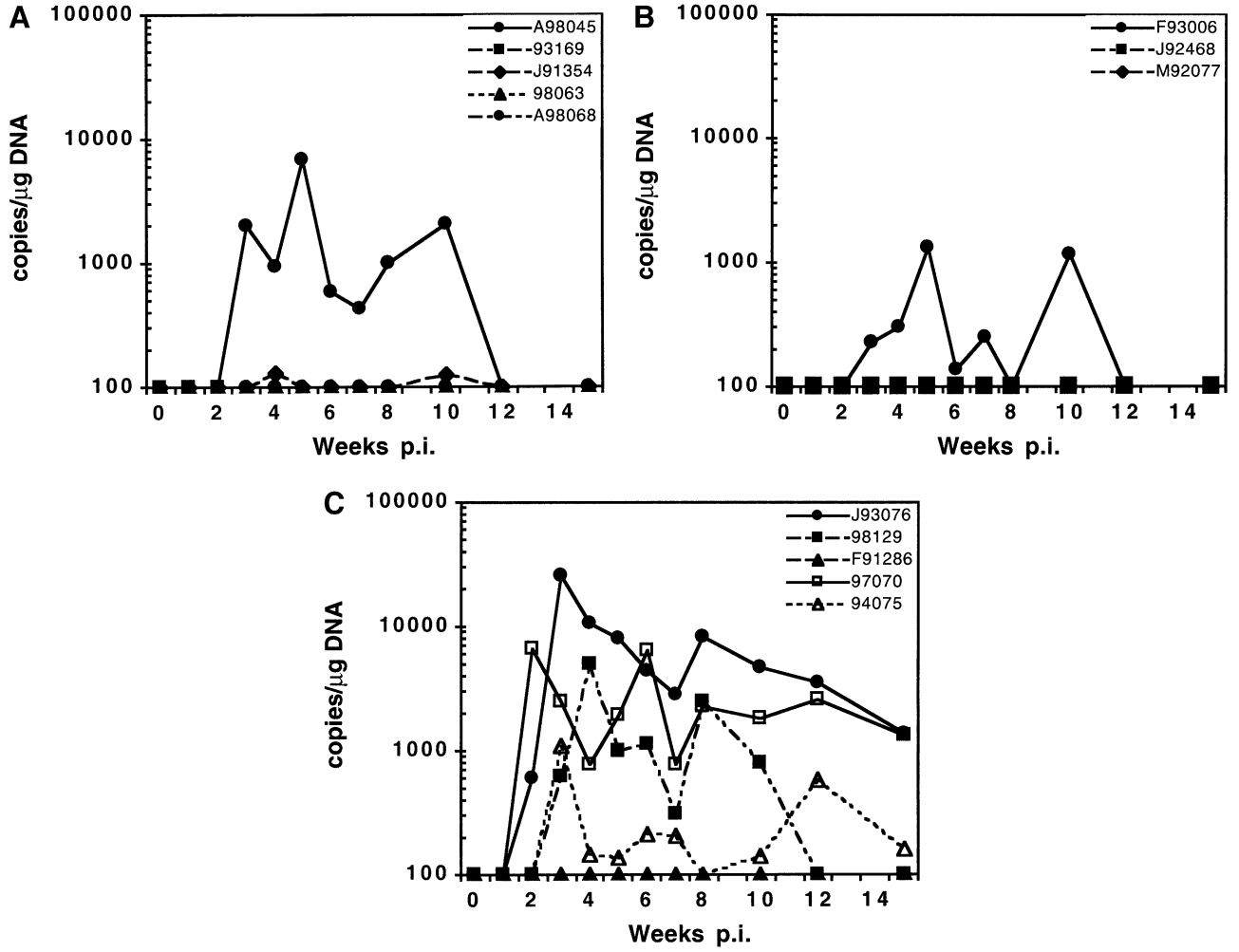

Fig. 3. Viral DNA levels in the PBMC of the Group 1 (A), Group 2 (B), and control (C) animals after intravaginal challenge with $SHIV_{89.6}$. Viral DNA is represented as SIV copies per microgram of PBMC DNA as determined by PCR. Animals 97070 and 94075 are unimmunized controls infected intravaginally with the same challenge dose (see text).

Materials and methods

Animal care

Eleven adult female pigtailed macaques (*M. nemestrina*) which were negative for SIV, SRV-2, and STLV-1 were used. These animals were between 4 and 7 years old and mensing. The animals were housed at the Washington Regional Primate Research Center. All macaque procedures were carried out with the approval of the Animal Care and Use Committee at the University of Washington. During all procedures, the animals were anesthetized with an intramuscular injection of 10–15 mg ketamine-HCl/kg of body weight.

Cell-culture medium

In vitro cultures were performed in RPMI 1640 (Gibco-BRL, Gaithersburg, MD) containing 25 mM HEPES, 10% fetal calf serum (FCS; Hyclone, Logan, UT), 100 U each of penicillin and streptomycin (Sigma, St. Louis, MO), and 50 μm 2-mercaptoethanol (JT Baker, Phillipsburg, NJ).

SHIV stock and inactivation

The virus used in this study was $SHIV_{89.6}$ (a kind gift from Y. Lu, Avant Immunotherapeutics, Needham, MA), which has been described previously (Reimann et al., 1996b). The stock used was passaged once in vivo in a rhesus macaque and briefly cultured on rhesus macaque PBMC. The viral concentration of the stock was determined to be 10^3 $TCID_{50}$/ml by culture on CEM $\times$ 174 cells and p27 production (SIV-1 p27 Antigen ELISA kit; ZeptoMetrix, Buffalo, NY).

Aldrithiol-2 (AT-2) inactivation was performed as previously reported with modifications (Rossio et al., 1998). Briefly, bulk cultures of AA2 cells were infected with the virus stock at 37°C until cytopathic effects (CPE) were

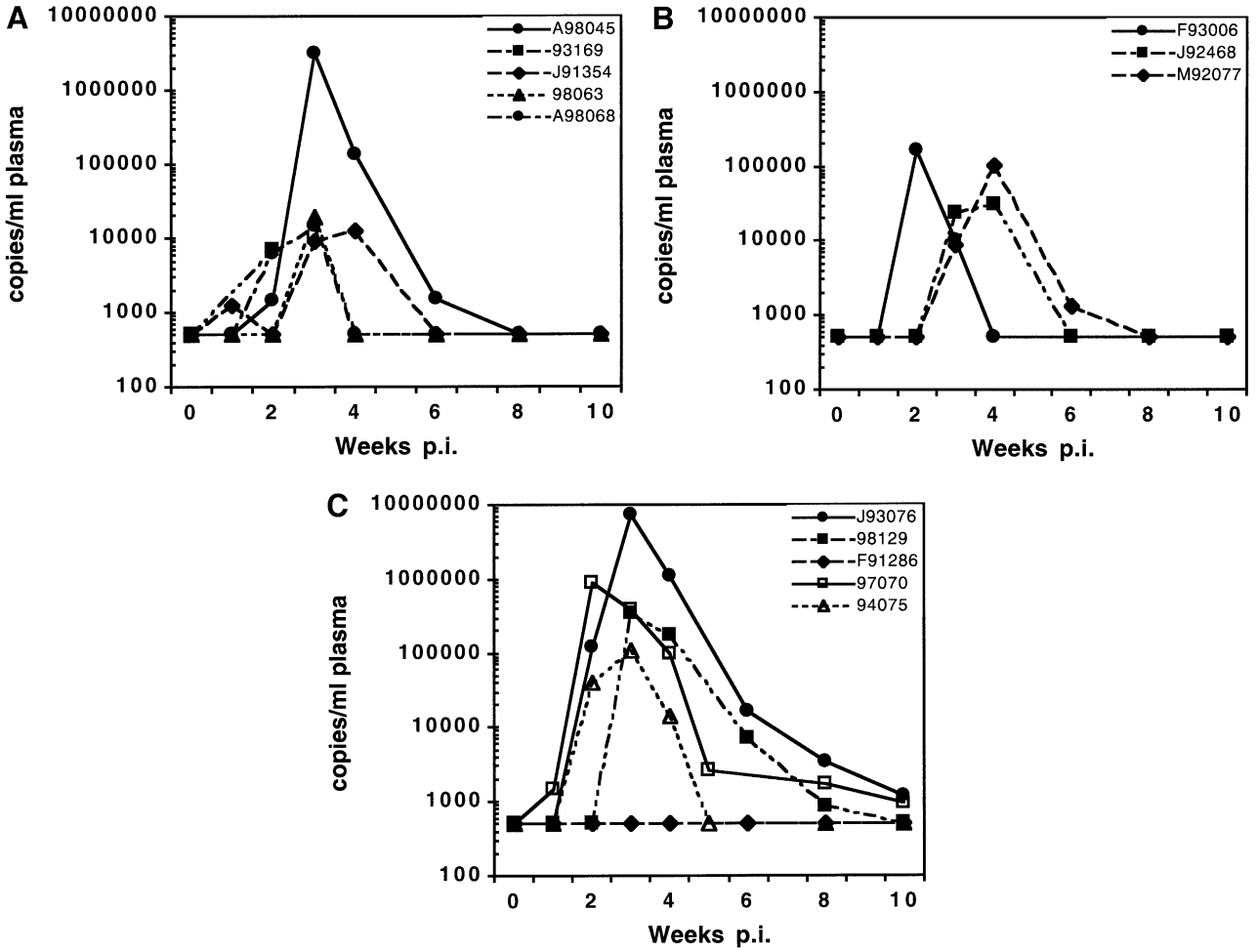

Fig. 4. Viral RNA levels in the plasma of the Group 1 (A), Group 2 (B), and control (C) animals after intravaginal challenge with $SHIV_{89.6}$. Viral RNA is represented as SIV^{+} copies per milliliter of plasma as determined by bDNA assay. Animals 97070 and 94075 are unimmunized controls infected intravaginally with the same challenge dose (see text).

significant (approximately 2 days). Cell supernatant was harvested and stored at 4°C. Pelleted cells were resuspended in fresh medium and incubated for two more days. Supernatant was harvested and combined with the previous harvest. The supernatant volume was concentrated fivefold in a tangential flow concentrator (Millipore, Bedford, MA) using a molecular weight cutoff of 100 kDa. AT-2 was added to the virus at a final concentration of 400 μM and the mixture was incubated for 1 h in a 37°C water bath. Inactivated virus was pelleted through 20% glycerol in PBS at 19K for 1.5 h at 4°C. The pellet was gently resuspended in PBS and layered over a cold 30%/45% sucrose step gradient and centrifuged at 45K for 1.5 h at 4°C. The virus was collected from the interface and mixed 2.1 with PBS and overlaid on 20% glycerol/PBS at 45K for 1 h at 4°C. The virus pellet was gently resuspended in PBS and frozen at −70°C in 1 ml aliquots. Total protein concentration was determined to be 850 μl/ml (BCA assay; Pierce, Rockford, IL).

Inactivation was verified by culturing 10 μg of the AT-2 inactivated virus on 2×10^7 AA2 cells for 2 weeks. No cytopathic effects were observed.

Immunization

Animals were inoculated by dermal scarification on the back at weeks 0 and 4: five animals received 1×10^8 PFU vT107 ($HIV\text{-}1_{89.6}$ Env) and 1×10^8 PFU vAbT394 (SIV_{mac251} Gag-Pol) and six animals received 2×10^8 PFU NYCBH (wild-type; Table 1). At weeks 12 and 19, eight animals received (1) an i.n. inoculation in one nostril of 85 μg AT-2 inactivated $SHIV_{89.6}$ and 50 μg LT(R192G) and (2) 85 μg AT-2-inactivated $SHIV_{89.6}$ and 50 μg LT(R192G), following 25 ml 0.2 M sodium bicarbonate via an intragastric feeding tube placed through the other nostril into the stomach. Three animals received the same mucosal boosts with the omission of AT-2 inactivated $SHIV_{89.6}$, which was replaced with the same volume of RP-10. At week 36, i.n. inoculations were repeated in all 11 animals.

SHIV challenge

Four weeks prior to and 1 week following challenge, each animal was given 30 mg medroxyprogestrone acetate

i.m. (Depo-Provera; Pharmacia & Upjohn, Kalamazoo, MI). Inoculation of all animals was given atraumatically through a small tube inserted into the vagina with 200 $TCID_{50}$ of $SHIV_{89.6}$ within 1 h after thawing. Animal J91286 was rechallenged in the same manner 14 weeks after the first challenge.

Blood and mucosal samples

Blood was taken at multiple time points throughout the study in vacutainers containing preservative-free heparin (immunological assays) or EDTA (virological assays). PBMC were separated over a 95% lymphocyte separation medium (immunological assays; Organon Teknika, Durham, NC) or 95% lymphoprep medium (virological assays; Life Technologies, Rockville, MD) gradient and used fresh or frozen at −80°C or in liquid nitrogen. Plasma was also separated by centrifugation and frozen at −20°C. Vaginal washes were obtained by instilling and removing 1 ml of sterile PBS into the vagina, which was frozen at −20°C. Vaginal washes were discarded if they contained visible blood contamination. Rectal swabs were collected by wiping the rectal mucosal surface with a cotton swab, which was placed in 1 ml of sterile PBS and frozen at −20°C.

Binding and neutralizing antibodies

Maxisorp plates (Nunc, Naperville, IL) were coated overnight with 1 μg/ml (plasma ELISA) or 3 μg/ml (vaginal wash ELISA) of AT-2 inactivated $SHIV_{89.6}$ lysate or gp120 (AIDS Reagent and Reference Program, Rockville, MD) in carbonate buffer. Plates were washed four times with 0.1% Triton X-100 in PBS (wash buffer) and then blocked with 5% nonfat milk in PBS for 1 h at 37°C. Plasma was diluted to 1:50 in 1% Triton X-100 buffer and titrated in duplicate. Vaginal washes and rectal swabs were diluted 2:1 in 1% Triton X-100 and tested in duplicate. Samples were incubated for 1 h at 37°C. After washing, rabbit-anti-human IgG-HRP or rabbit-anti-human IgA-HRP (1:5000, Dako, Carpinteria, CA) in 1% Triton X-100 was added and incubated for 1 h at 37°C. Turbo TMB (Pierce) was added to the plates and allowed to develop and stopped with 1 M sulfuric acid. Plates were read at 450 nm on a THERMOmax ELISA plate reader with SOFTmax software (Molecular Devices, Sunnyvale, CA). Plasma titers were expressed as endpoint dilutions, defined as twofold above background of prebleeds, and vaginal washes were expressed as OD/100 μl of diluted wash.

Neutralization assays were performed on plasma at 0, 40, and 43 weeks and sterile-filtered vaginal washes at weeks 43 and 48 as described previously (Crawford et al., 1999). The $SHIV_{89.6}$ stock used in this assay was produced in human PBMC and titered in an MT-2 killing assay. The dilutions used for this assay were 1:20 for plasma and 1:5 for vaginal washes.

Western blot analysis

Western blots of SIV antigens were made as previously described (Kuller et al., 1998). The blots were incubated with plasma samples diluted 1:100 in 5% milk and 0.05% Tween 20 in PBS for 1 h at room temperature, followed by incubation with sheep anti-human IgG-HRP (Amersham Pharmacia, Piscataway, NJ). The blots were then incubated in a chemiluminescent reagent (ECL; Amersham Pharmacia) and visualized on X-ray film.

Proliferation assay

PBMC were plated at 1×10^5/well in round-bottom 96-well plates in triplicate. AT-2 inactivated $SHIV_{89.6}$ was added to each well in dilutions. Negative control wells received medium without antigen and positive control wells received PHA (1:100; Boehringer Mannheim, Mannheim, Germany). After 3 days in culture at 37°C, 5% CO_2, 1 μCi/well [^{3}H]thymidine (ICN, Costa Mesa, CA) was added. The cells were cultured for an additional 18 h and then harvested on filters (Skatron Instruments, Sterling, VA). Filters were removed and placed in 3 ml liquid scintillation fluid (Packard, Meriden, CT) and counted on a β-scintillation counter (Beckman, Fullerton, CA). Responses were plotted as the change in counts per minute (cpm) of the average of stimulated triplicate wells minus the spontaneous cpm of the cells cultured in medium only, or Δcpm.

Intracellular cytokine staining

The intracellular cytokine staining assay was described previously (Ambrose et al., 2001). In brief, monocyte target cells from PBMC in 48-well plates were infected with WT vaccinia (NYCBH) or rW (vT107 or vAbT394) at an m.o.i. of 10 for 4 h. Targets were washed with PBS. Fresh autologous effector cells were added to the targets from monocyte-depleted PBMC at a ratio of 1:1. The cells were incubated for 12 h; the last 4 h were in the presence of GolgiPlug (PharMingen, San Diego, CA). Effector cells were collected, washed in RP-10, and stained on the surface with antibodies against CD8 (biotin; Becton-Dickinson, Mountain View, CA), streptavidin-TC (Caltag, South San Francisco, CA), and CD69 (PE; Becton Dickinson). The cells were permeabilized with the Cytofix/Cytoperm kit (PharMingen) and stained intracellularly for IFNγ (FITC; PharMingen). Cells were evaluated by FACScan and CellQuest software (Becton-Dickinson).

Responses are expressed as the percentage of $CD8^+$ $CD69^+$ cells that produce IFNγ after stimulation with rW

above that of the wild-type W. Responses were not included if the percentage of IFNγ-producing cells when stimulated with WT vaccinia was above 1%. The limit of detection was 0.1% of $CD8^+$ $CD69^+$ cells.

Virus isolation and viral load assays

Infection of the animals was measured by three methods. First, fresh PBMC (5×10^6) from each animal were cocultured with CEM×174 cells (5×10^8) at 37°C for 1 week. Coculture supernatant was analyzed for viral p27 antigen (ZeptoMetrix). After sampling, half of the culture medium was replaced and additional CEM×174 cells were added if necessary. Sampling and medium replacement was repeated for 6 weeks. After two consecutive positive assays, the coculture was considered positive and discarded.

Second, quantification of SIV DNA in PBMC was determined by QC-PCR as previously described (Watson et al., 1997). Briefly, DNA samples were mixed with competitor DNA of pCon-1 plasmid containing an internally deleted SIV gag insert. Primers used for amplification were: 5′, AAAGCCTGTTGGAGAACAAAGAAG, and 3′, AATTTTACCCAGGCATTTA. The thermocycling conditions used for PCR were 96°C for 11 min, followed by 41 cycles of 96°C for 15 s, 55°C for 1 min, and 72°C for 30 s, followed by an extension at 72°C for 9 min. Amplified products were separated on 3% agarose gels and visualized on a Gel-Doc 2000 documentation system (Bio-Rad) to determine band intensities. The limit of detection was 100 copies/μg DNA.

Plasma SIV bDNA assays were performed at Bayer as previously described (Dailey et al., 1995). The limit of detection was 500 copies/ml plasma.

Statistical analysis

Statistics comparing the three groups of animals to each other for antibodies and proliferation assays were performed using a two-tailed unpaired *t* test with 95% confidence interval (CI) using Prism software (GraphPad, San Diego, CA). Comparisons of the $CD8^+$ T cell intracellular IFNγ responses of animals in the three groups were performed using generalized estimating equations with a log link and exchangeable correlation structure (courtesy of B. Richardson, Fred Hutchinson Cancer Research Center, Seattle, WA). Comparisons of viral DNA or RNA levels were done using the log values in a one-sided Chi-square test with 95% CI (log cutoffs of 3.0 and 5.0, respectively; Prism). Two unimmunized control animals that were inoculated with the same amount of virus were included in the viral load or virus isolation analyses only. Undetectable immune responses or viremia were represented as the midpoint value between 0 and the level of detection.

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