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Multigene DNA Priming-Boosting Vaccines Protect Macaques from Acute CD4$^+$-T-Cell Depletion after Simian-Human Immunodeficiency Virus SHIV89.6P Mucosal Challenge

N. A. Doria-Rose,[1,2] C. Ohlen,[3] P. Polacino,[4] C. C. Pierce,[1]† M. T. Hensel,[1] L. Kuller,[4] T. Mulvania,[3] D. Anderson,[4] P. D. Greenberg,[3] S.-L. Hu,[2,4,5] and N. L. Haigwood[1,2,6]*

Seattle Biomedical Research Institute,[1] and Department of Pathobiology,[2] Department of Immunology,[3] Washington National Primate Research Center,[4] Department of Pharmaceutics,[5] and Department of Microbiology,[6] University of Washington, Seattle, Washington

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We evaluated four priming-boosting vaccine regimens for the highly pathogenic simian human immunodeficiency virus SHIV89.6P in *Macaca nemestrina*. Each regimen included gene gun delivery of a DNA vaccine expressing all SHIV89.6 genes plus Env gp160 of SHIV89.6P. Additional components were two recombinant vaccinia viruses, expressing SHIV89.6 Gag-Pol or Env gp160, and inactivated SHIV89.6 virus. We compared (i) DNA priming/DNA boosting, (ii) DNA priming/inactivated virus boosting, (iii) DNA priming/vaccinia virus boosting, and (iv) vaccinia virus priming/DNA boosting versus sham vaccines in groups of 6 macaques. Prechallenge antibody responses to Env and Gag were strongest in the groups that received vaccinia virus priming or boosting. Cellular immunity to SHIV89.6 peptides was measured by enzyme-linked immunospot assay; strong responses to Gag and Env were found in 9 of 12 vaccinia virus vaccinees and 1 of 6 DNA-primed/inactivated-virus-boosted animals. Vaccinated macaques were challenged intrarectally with 50 50% animal infectious doses of SHIV89.6P 3 weeks after the last immunization. All animals became infected. Five of six DNA-vaccinated and 5 of 6 DNA-primed/particle-boosted animals, as well as all 6 controls, experienced severe CD4$^+$-T-cell loss in the first 3 weeks after infection. In contrast, DNA priming/vaccinia virus boosting and vaccinia virus priming/DNA boosting vaccines both protected animals from disease: 11 of 12 macaques had no loss of CD4$^+$ T cells or moderate declines. Virus loads in plasma at the set point were significantly lower in vaccinia virus-primed/DNA-boosted animals versus controls ($P = 0.03$). We conclude that multigene vaccines delivered by a combination of vaccinia virus and gene gun-delivered DNA were effective against SHIV89.6P viral challenge in *M. nemestrina*.

An effective vaccine to reduce human immunodeficiency virus (HIV) infection and subsequent disease is not yet available. In 2002 alone, an estimated five million people became infected with HIV type 1 (HIV-1) (66). Ninety-five percent of new infections occur in developing countries. Although effective treatments are available, they have a high failure rate and their long-term effects are unknown and, more important, they are unaffordable in the countries hit hardest by the epidemic. Vaccines are likely to be the only viable long-term solution.

A wide variety of HIV vaccine candidates are being tested in small clinical trials with humans; however, to date only one has been evaluated in a phase III efficacy trial (31). As new vaccine concepts are developed, the nonhuman primate models of AIDS allow direct testing of a vaccine followed by a viral challenge. The macaque monkey can be infected with viruses related to HIV. Several strains of simian immunodeficiency virus (SIV), and chimeras of HIV and SIV called simian HIV (SHIV), infect macaques and cause a disease similar to human AIDS (43). Both SIV and SHIV have been used to evaluate protection from infection or from disease. Use of SHIV further allows testing of vaccines that contain HIV Envelope, which could be moved directly into formulations for human trials. Importantly, a number of approaches have shown promise in these models, supporting further development and testing in humans. The predictive value of nonhuman primate models will only be determined after successful human trials.

DNA vaccination is a promising strategy that has yet to be fully exploited. In principle, DNA vaccines share major advantages with live attenuated vaccines: the vaccine antigen is made by transfected host cells, will thus be folded and modified in its native conformation, and can be presented to the immune system by major histocompatibility complex (MHC) class I and II molecules. DNA vaccines are safer than live attenuated viruses because they can be made replication incompetent by carrying only single genes or genomes with large deletions, and a number of animal and human studies have demonstrated their safety. Both cellular and humoral immunity can be generated, and protection from disease has been achieved against a variety of pathogens in animal models (16, 54). Human trials of DNA vaccines for AIDS show induction of antibodies and cellular immune responses to HIV-1 (13, 31, 39), while several macaque studies have shown reduced SIV or SHIV viral load in animals vaccinated with DNA alone (10, 20, 28, 38, 42, 55). However, the relatively low levels of immunity elicited by current methods of DNA vaccine delivery have failed to provide sterilizing immunity against the primate lentiviruses. Thus, ef-

* Corresponding author. Mailing address: Seattle Biomedical Research Institute, 4 Nickerson St., Seattle, WA 98109. Phone: (206) 284-8846, ext. 338. Fax: (206) 284-0313. E-mail: Nancy.Haigwood@sbri.org.

† Present address: Hollister-Stier Laboratories, LLC, Spokane, Wash.

forts have been directed toward enhancing the low-level immune responses elicited by DNA vaccines by combining them with a different type of vaccine modality to boost the priming response (29, 42, 55). The priming-boosting concept showed early promise for SIV and HIV vaccines with recombinant vaccinia virus and subunit protein boosting (33). Several recent studies have demonstrated good protection of macaques from disease induced by SHIV89.6P after priming-boosting vaccines including a DNA priming vaccine (2, 60).

There is increasing evidence that inclusion of multiple immune targets in vaccines is more effective than using a single antigen. Many candidate AIDS vaccines have been made against Env only (reviewed in reference 64), Gag only (20, 60), or Tat only (24). While Env is the sole target of neutralizing antibody, all of the viral genes may be targets for cytotoxic T lymphocyte, and individual infected patients and animals develop cytotoxic-T-lymphocyte responses to multiple viral gene products (12, 44). Different MHC haplotypes are able to present epitopes from different genes; and even in related animals which share some MHC alleles, epitopes from different genes are targeted (23). Given the variable breadth of responses in individuals, single-antigen vaccines may not be effective in all recipients. Furthermore, HIV sequences vary greatly; several antigens in a vaccine increase the chance of providing at least one that is cross-reactive with a virus to which a vaccinee is exposed. Therefore, it is compelling to use multiple antigens in an AIDS vaccine. Indeed, recent studies suggest that multiantigen vaccines are superior to comparable Gag-only or Env-only formulations (1, 47).

We have evaluated the immunogenicity and protective efficacy of several priming-boosting vaccine regimens for SHIV89.6P, a highly virulent challenge virus that causes rapid $CD4^+$-T-cell depletion in unvaccinated *Macaca mulatta* (rhesus macaques) and in *Macaca nemestrina* (pigtailed macaques). Each regimen included a multigene DNA vaccine delivered by gene gun. We found significant protection from disease in *M. nemestrina* macaques given the combination of DNA and recombinant vaccinia virus, with DNA either as the priming vaccine or as the boosting vaccine.

MATERIALS AND METHODS

DNA vaccinations. The construction of vaccine components is described elsewhere (6, 18, 20). Plasmids for vaccination were prepared with the EndoFree Maxiprep kit (Qiagen, Valencia, Calif.). DNA vaccines were administered with the Helios gene gun (Bio-Rad, Hercules, Calif.) at 400 lb/in^2. DNA was precipitated onto 1-μm-diameter gold beads, and bullets were prepared according to the manufacturer's instructions (Bio-Rad). Mice were shaved on the abdomen and received one or three shots of 1 μg of DNA on 0.5 mg of gold. Macaques received 30 shots of gold; each shot had 2 μg of total DNA on 0.5 mg of gold. Skin was shaved on each thigh, each upper arm, and between the shoulder blades; six shots were administered at each site. The choice of the number of shots was based on a dose-response study (18).

Vaccinia virus vaccinations. Two recombinant vaccinia viruses were used: v-ELgp160(89.6P), which contains the full-length Env gene of SHIV 89.6P clone KB9 (34), and vELgag/pol(mac239)b(2)9.1, which contains *gag* and *pol* (up to but not including *integrase*) of SIVmac239. The construction and propagation of these viruses is described in reference 18. Macaques were inoculated with 10^8 PFU of each recombinant virus by skin scarification at two or three sites on the back.

Inactivated virus. SHIV89.6 virus was grown in CEMx174 cells, inactivated with AT-2 as described in reference 57 and purified in sucrose gradients. Characterization of the preparation is described in reference 18. For each dose, 200 μg (by protein) of the inactivated virus was mixed with adjuvant, 0.025% alhydrogel (Cedarlane, Hornby, Ontario, Canada) in acetate buffer (pH 6.2), and 500 μg of CpG oligonucleotides. The latter were phosphorothioate at all linkages, synthesized by Genosys, Inc. (The Woodlands, Tex.), with the sequence 5′-TC GTCGCTGTTGTCGTTTCTT (32). Macaques received 1 ml of vaccine intramuscularly.

Animals. Juvenile *M. nemestrina* macaques were housed in the Washington National Primate Research Center under the care of licensed veterinarians. The University of Washington Institutional Animal Care and Use Committee approved all experimental procedures. Euthanasia was performed on the basis of the following criteria: (i) AIDS, (ii) termination of the experiment, or (iii) an unrelated cause. Euthanasia is considered to be AIDS related if the animal exhibits ≤200 $CD4^+$ cells/mm^3 in the peripheral blood at two or more consecutive time points, and two or more of the following conditions are present: wasting (loss of >15% of normal body weight), unsupportable diarrhea, opportunistic infection(s), proliferative disease(s) (e.g., lymphoma), and abnormal hematology (most commonly anemia). Mice were housed at the Seattle Biomedical Research Institute in a Food and Drug Administration-approved facility. All work was approved by the Institutional Animal Care and Use Committee.

Virus challenge. $SHIV89.6P_{MN}$ stock was derived from monkey-passaged SHIV89.P stock (gift of N. Letvin) (50, 51) by two passages in $CD8^+$-depleted peripheral blood mononuclear cells (PBMC) from *M. nemestrina* (18). The in vivo titer in *M. nemestrina* was determined to be 25 50% animal infectious doses (AID_{50})/ml (63). Vaccinated and control macaques were challenged with two doses, 1 h apart, of 1 ml of undiluted virus atraumatically in the rectum (50 AID_{50} total).

Virus load determinations. Viral loads in plasma and PBMC were determined by real-time reverse transcription (RT)-PCR and real-time PCR, respectively, as described in reference 18. The limit of detection was 100 copies/ml of plasma. Viral load assays, as well as immunophenotyping, were performed by the Virology Core of the Washington National Primate Research Center.

Antibody assays. Binding antibody responses to SHIV antigens were measured by enzyme-linked immunosorbent assay (ELISA) as described previously (18). Briefly, Immunosorp plates (Nalge Nunc, Rochester, N.Y.) were coated with 2 μg of recombinant gp120/ml. Diluted plasma was incubated for 1 h on the plates and detected with biotin-conjugated anti-human immunoglobulin G (IgG) (ICN Biomedicals, Costa Mesa, Calif.) followed by Extravidin-horseradish peroxidase. Neutralization assays were performed with the cMAGI assay. cMAGI cells (15) were maintained in Dulbecco's modified Eagle's medium (DMEM)–10% fetal bovine serum (FBS) with Geneticin (250 μg/ml), hygromycin (100 μg/ml), and puromycin (1 μg/ml). Cells were seeded in 96-well flat-bottom plates at 10^4 cells/well in 100 μl of DMEM–10% FBS medium and incubated for 24 h prior to infection. Virus was diluted in DMEM–10% FBS to give 100 to 200 infectious units per well. Heat-inactivated plasma was serially diluted twofold in DMEM–10% FBS, mixed with equal volumes of input virus, and incubated for 1 h at 37°C in 5% CO_2. DEAE-dextran was then added to a final concentration of 20 μg/ml. The virus-plasma mixture (110 μl/well) was then added to duplicate aspirated cell monolayers. Plates were incubated for 2 h, after which an additional 200 μl of medium/well was added. After 48 h, cells were fixed with 400 μl of fixing solution (1% formaldehyde, 0.2% glutaraldehyde in phosphate-buffered saline [PBS]) and stained for 50 min with 50 μl of staining solution (5 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-Gal; Sigma]/ml, 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM $MgCl_2$ in PBS) and washed thoroughly with PBS. A positive reaction for virus infection is denoted by nuclei or syncytia that are stained deep blue. Wells were counted on a Bioreader (Biosys, Heidelberg, Germany) calibrated to a manual count of at least 6 wells. The percent neutralization at a given titer is calculated by the equation $(Vo - Vn)/Vo \times 100$, where Vn is the number of infected cells in the virus-plus-antibody wells and Vo is the number of positive cells in the virus alone wells. Titers were normalized to the titer of a standard HIV-positive human serum pool that was included on each assay plate.

ELISpot assay. A gamma interferon (IFN-γ) enzyme-linked immunospot (ELISpot) assay was performed on frozen PBMC. The cells were thawed in RPMI and 10% fetal calf serum 1 day before the assay and incubated overnight at 37°C. The assay was carried out with an ELISpot system monkey IFN-γ kit (U-CyTech, Utrecht, The Netherlands) according to the manufacturer's protocol. Duplicate wells of 10^5, 5×10^4, 2.5×10^4, and 1.25×10^4 PBMC were plated in a 96-well format. Overlapping 15-mer peptides obtained from the National Institutes of Health AIDS Research and Reference Reagent Program were used at between 0.5 and 10 μg/ml (Gag peptides derived from SIVmac239; Env from SHIV89.6; Pol from SIVmac239; the accessory pool contained peptide from HIV Tat and Rev and SIVmac239 Nef, Vpr, and Vpx). Each well received the same amount of peptide. As a positive control, streptococcal enterotoxin B was added to responder cells at 1 μg/ml; as a negative control, an irrelevant peptide was

TABLE 1. Plasmids used in DNA vaccine

Plasmid	Gene product(s)	Source virus	Assay(s) showing in vitro expression[c]	Antibody response in mice[a]
pV1Jns:89.6Penv160OPTcleavable	Env (gp160)	SHIV89.6P	IFA, Western blotting, sCD4 binding	5/5 to gp160
pCIneo89.6	Env (gp160)	SHIV89.6	IFA, cell fusion, Western blotting, sCD4 binding	2/20 to gp160
pEMC*896	Env (gp120)	SHIV89.6	Western blotting, sCD4 binding	5/5 to gp160
pC3a896gpe	Gag	SHIV89.6	Western blotting, ELISA	2/5 to Gag-Pol
	Pol		Western blotting	NT
	Tat		cMAGI assay	NT
	Env (gp160)		IFA, cell fusion, sCD4 binding	2/5 to gp160
	Vif, Vpr, Vpv, Rev		NT[b]	NT
pVIR SIV OPT GAG	Gag	SHIV89.6	Western blotting, ELISA	NT
pC3a Nef	Nef	SHIV89.6	Western blotting	NT
All 6 mixed				5/5 to gp160 2/5 to Gag-Pol

[a] Number of mice responding/total number of mice.
[b] NT, not tested.
[c] IFA, immunofluorescence assay; sCD4, soluble CD4.

added. After drying, the plates were stripped and read with a Zeiss microscope by using KS ELISpot (Carl Zeiss Vision GmbH, Göttingen, Germany) software. The response to the irrelevant peptide was subtracted from the response to each antigen peptide pool, and a response to antigen was considered positive if it was greater than twice the response to irrelevant peptide and >40 per 10^6 PBMC (which is the mean plus three standard deviations of the naive animal response to antigen).

RESULTS

Construction and characterization of SHIV89.6-based DNA vaccines. The DNA vaccine consisted of six plasmids, which together allow high levels of expression of all SHIV genes under the control of the human cytomegalovirus Immediate Early-1 promoter. The plasmids pV1Jns:89.6Penv160OPT-cleavable and pVIR SIV GAG OPT have been described elsewhere (6, 20) and are immunogenic in macaques (10). The other plasmids were constructed for this study and were validated in vitro (18) and found to elicit antibodies to the expected viral gene products in DNA-immunized mice (Table 1). pV1Jns:89.6Penv160OPTcleavable contains codon-optimized *env* gp160 from the animal-passaged virus SHIV89.6P; the other five plasmids correspond to SHIV89.6, the parental construct. Additional *env* constructs were the *rev-env* construct pCIneo89.6 and pEMC*896, a gp120 expression construct in which gp120 is fused to the signal sequence of tissue plasminogen activator. pVIR SIV GAG OPT encodes codon-optimized Gag from SIVmac239, which is identical to the gene in SHIV89.6. Nef expression is provided by pC3aNef. Accessory genes *tat*, *rev*, *vif*, *vpr*, *vpx*, and *vpu*, as well as *gag*, *pol*, and *env*, are provided by pC3a896gpe, which carries nucleotides 536 to 8903 in the SHIV89.6 genome (34). We demonstrated that this plasmid can produce virus-like particles (VLP) in COS cells with processed Gag and fully functional Envelope (18), as was shown previously for an SIV plasmid that directed the expression of SIV VLP (42).

Boosting agents. To improve the potency of the DNA vaccine, we used several different boosting agents. The first group of animals received three priming doses and two additional boosting doses of DNA and is referred to as DNA-DNA or DNA alone. The second group was boosted twice with virus particles inactivated with AT-2 (57) and is referred to as DNA priming/particle boosting (DNA-Particle on figures). AT-2 inactivation does not affect the structure of Env on the surface of the virions (57) and may therefore be a better immunogen for the elicitation of conformation-dependent and neutralizing antibodies than purified protein.

Two recombinant vaccinia viruses were constructed for this study: v-ELgp160(89.6P), which contains the full-length Env gp160 gene of SHIV89.6P, and vELgag/pol(mac239)b(2)9.1, which expresses Gag-Pol of SIVmac239 (18). The third group was boosted twice with a mixture of these two recombinant vaccinia viruses and is referred to as DNA priming/vaccinia virus boosting (DNA-Vacc on figures). Previous work by our group showed that vaccinia virus priming/protein boosting vaccines provided sterilizing immunity against SIVmne (33). Therefore, we chose to also include a fourth group in which the regimen was reversed: two priming doses of recombinant vaccinia virus followed by three boosts with DNA. This group is referred to as vaccinia virus priming/DNA boosting (Vacc-DNA on figures).

FIG. 1. Time line of vaccination and viral challenge. Arrows indicate times of vaccinations. DNA, DNA vaccination by gene gun; Particle, inactivated virus particles; Vaccinia, recombinant vaccinia virus.

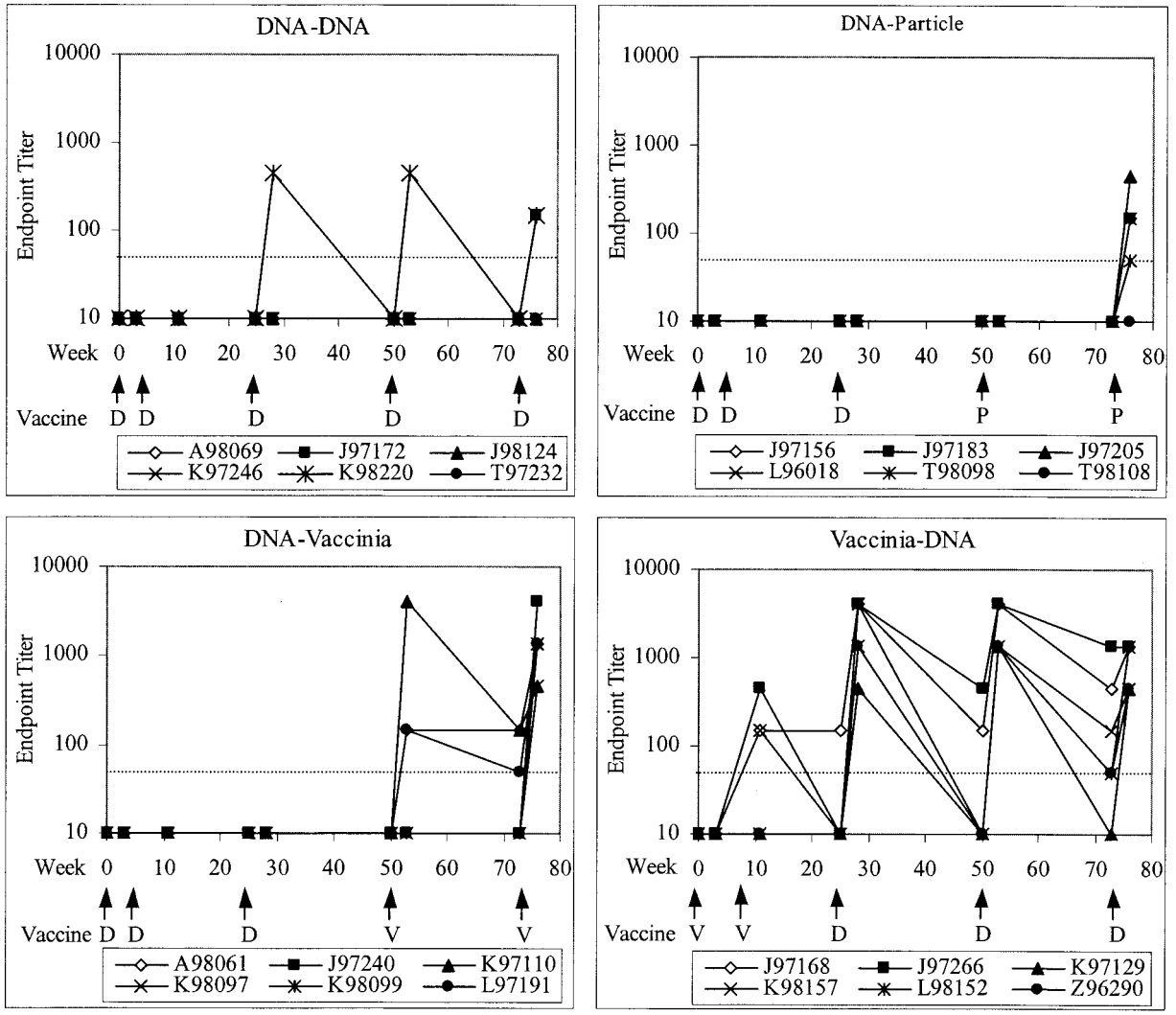

FIG. 2. Development of prechallenge anti-Env binding antibody (IgG) in vaccinated macaques. The dotted line indicates the limit of detection (1:50 dilution); values below this limit were assigned values of 10. Arrows indicate times of vaccinations. D, DNA; P, particle; V, recombinant vaccinia virus. Endpoint titer is the reciprocal of the highest dilution at which the optical density is twice that of preimmune sera at the corresponding dilution.

Vaccination of macaques and prechallenge humoral and cellular antiviral immunity. Thirty *M. nemestrina* macaques were vaccinated according to the time line shown in Fig. 1. Prior experience with vaccinia virus indicated that an 8-week interval between doses was optimal for an effective boost of the immune response (S.-L. Hu, unpublished data). Long intervals following the second and third doses were chosen to maximize the effect of boosting doses, as several groups have shown the benefits of such rest periods for both DNA (27) and protein (4) immunizations.

On the day of and 3 weeks after each immunization, blood samples were taken from the animals and tested for binding antibody to HIV-1 Env. The development of the IgG response to Env is shown in Fig. 2. Control animals had endpoint titers of <50 and were considered negative at all time points prior to challenge. DNA alone elicited very low binding antibody responses. In the group that received DNA alone (group 1), only 2 of 6 animals had detectable anti-Env IgG after five doses. Examination of all animals in the three groups primed with three doses of DNA showed that only 1 of 18 animals had detectable antibody after three doses. Boosting with inactivated virus particles greatly improved the antibody response: after the second dose, 4 of 6 animals had binding antibody against Env (Fig. 2) and all had IgG to disrupted SIV, which contains the same Gag-Pol as the vaccine (data not shown).

Recombinant vaccinia virus expressing Gag-Pol and Env was an effective boosting agent for DNA. After three doses of DNA, no animals in the DNA-Vacc group had antibodies to Env (Fig. 2); however, after one boosting dose of vaccinia, 3 of 6 animals had detectable anti-Env antibodies. After a second vaccinia dose, 6 of 6 animals had antibody to Env, with a geometric mean titer of 1:1,100. Conversely, DNA served as an effective boosting agent for vaccinia virus in the Vacc-DNA group. Animals primed with two doses of vaccinia virus had low but detectable responses, with 3 of 6 animals having antibodies to Env. After the first DNA

TABLE 2. Cellular immunity measured by IFN-γ ELISpot assay

Group	Animal	Result[a] for viral antigen:															
		Gag				Env				Pol				Accessory peptide			
		Prime[b]	Wk 0[c]	Wk 2	Wk 4	Prime	Wk 0	Wk 2	Wk 4	Prime	Wk 0	Wk 2	Wk 4	Prime	Wk 0	Wk 2	Wk 4
Control	J96258	—[d]	2	ND[e]	50	—	0	ND	90	—	2	ND	0	—	2	ND	0
	J97167	—	5	10	25	—	5	0	40	—	5	35	5	—	15	0	25
	J97200	—	0	0	5	—	15	5	5	—	5	0	0	—	0	0	0
	J98071	—	0	5	10	—	0	10	0	—	0	0	10	—	0	0	0
	K96166	—	20	**120**[f]	**70**	—	0	30	0	—	0	0	5	—	0	5	**150**
	K97107	—	0	**100**	55	—	5	0	10	—	0	5	10	—	0	20	0
DNA-DNA	A98069	0	5	**320**	**160**	10	5	0	60	0	0	ND	0	5	10	ND	30
	J97172	0	0	25	50	0	5	**75**	90	0	5	**430**	0	0	5	**145**	0
	J98124	0	0	**75**	**100**	0	0	**560**	0	0	0	0	20	5	0	5	5
	K97246	5	0	**225**	**90**	15	5	**890**	**75**	0	0	35	0	0	0	**55**	**100**
	K98220	0	0	**60**	0	5	0	**345**	25	0	5	25	0	0	0	**630**	0
	T97232	0	0	**300**	0	0	20	15	10	0	0	0	0	0	0	35	0
DNA-particle	J97156	0	10	ND	0	0	0	ND	0	0	0	ND	0	0	0	ND	0
	J97183	0	10	5	5	0	10	**70**	0	0	5	0	0	0	5	25	0
	J97205	0	15	**595**	**120**	0	0	**180**	0	0	0	0	0	0	5	35	0
	L96018	10	**160**	**60**	**595**	15	30	**345**	**45**	0	35	25	**70**	10	10	**630**	30
	T98098	**5**	0	ND	30	0	0	ND	10	10	0	ND	10	5	0	ND	15
	T98108	0	0	55	0	0	0	0	0	0	0	15	10	0	0	305	20
DNA-vaccinia virus	A98061	5	**398**	**1,090**	**230**	20	**362**	**500**	**220**	15	0	**1,300**	0	0	0	15	70
	J97240	0	**90**	**910**	**180**	0	**300**	**130**	**1,485**	0	0	10	15	0	0	0	0
	K97110	0	**1,018**	0	20	0	**1,315**	**110**	**45**	0	0	0	0	0	0	**170**	**235**
	K98097	0	10	**705**	**445**	0	15	**670**	**520**	0	0	0	0	0	15	0	0
	K98099	40	40	ND	10	**65**	**145**	ND	**1,595**	10	0	ND	0	**55**	0	ND	10
	L97191	10	**325**	**875**	30	0	**255**	**70**	**50**	0	0	0	0	0	0	**205**	**35**
Vaccinia virus-DNA	J97168	0	25	**100**	**145**	10	15	0	25	10	0	0	25	10	10	5	65
	J97266	0	20	**440**	**235**	**55**	**440**	**205**	**230**	0	0	0	0	5	0	0	0
	K97129	10	**210**	**430**	**185**	**10**	**635**	5	**925**	0	22	0	5	0	10	0	**110**
	K98157	0	10	**230**	**150**	10	15	**1,425**	10	0	0	15	10	0	0	**95**	0
	L98152	**215**	**85**	**815**	**280**	35	35	**455**	10	5	5	15	0	0	0	10	10
	Z96290	**310**	**90**	**70**	5	**210**	**575**	**470**	15	**90**	0	0	5	**135**	0	5	5

[a] Spot-forming cells/10^6 PBMC measured by IFN-γ ELISpot with pools of overlapping peptides.
[b] Prime, samples taken after 3 doses of DNA or 2 doses of recombinant vaccinia virus.
[c] Wk 0, day of challenge (3 weeks after the last boosting dose).
[d] —, Not applicable.
[e] ND, not determined.
[f] Numbers in boldface type are >2 times the background for that animal and >40 spot-forming cells/10^6 PBMC.

boosting dose, 6 of 6 animals responded, with a geometric mean titer of 1:1,900.

We measured cellular immunity to SHIV89.6 by ELISpot assay and compared samples after priming and after full vaccination (Table 2). PBMC isolated after the last priming dose (dose 2 or 3) (Fig. 1) or the last boosting dose (day of challenge) were stimulated with one of four pools of peptides, corresponding to Gag, Pol, Env, or accessory proteins of SHIV89.6. Responses ranged from 70 to 1,595 spot-forming units/million PBMC and were found in 9 of 24 vaccinated animals and none of the controls on the day of challenge. After three DNA priming immunizations, 17 of the 18 DNA-vaccinated animals were negative for cellular responses while 1 animal was very weakly positive for the Env and accessory gene peptides. In contrast, after two priming doses of recombinant vaccinia virus, 2 of 6 animals were positive by ELISpot assay for Gag and Env peptides. After the boosting immunizations, 1 of 6 DNA priming/particle boosting animals was positive for Gag while 5 of 6 DNA priming/vaccinia virus boosting animals and 4 of 6 vaccinia virus priming/DNA boosting animals were positive for Gag, Env, or both. Three macaques had low-level prechallenge responses to Pol or accessory proteins that were above the background; one vaccinia virus Gag and Env-primed macaque (Z96290) showed an unexpected response to Pol and accessory genes that did not persist with boosting. These data indicate that, as seen for antibody, DNA was an effective priming or boosting agent for cellular immune responses elicited by the vaccinia virus recombinants.

Lymphocyte depletion following viral challenge. Three weeks after the fifth immunization, all animals were challenged intrarectally with 50 AID_{50} of SHIV89.6P. We chose the mucosal route to model sexual transmission, the dominant form of HIV transmission worldwide. Lymphocyte subsets in the blood of all macaques were measured over the course of the experiment for 42 weeks after challenge. As shown in Fig. 3, naive animals experienced dramatic and rapid $CD4^+$-T-cell loss in the first 4 weeks postchallenge, similar to that documented for *M. mulatta*. One animal died of simian AIDS at week 10, 2 animals gradually recovered some $CD4^+$ T cells, and the remaining 3 control animals had counts of less than 500 cells/μl for 42 weeks. The animals vaccinated with DNA alone or DNA priming/particle boosting were not protected from this pathological effect; in each group, 5 of 6 animals experienced dramatic loss and sustained low levels of $CD4^+$ T cells, and 1 of 6 animals showed slow $CD4^+$-T-cell decline over several months.

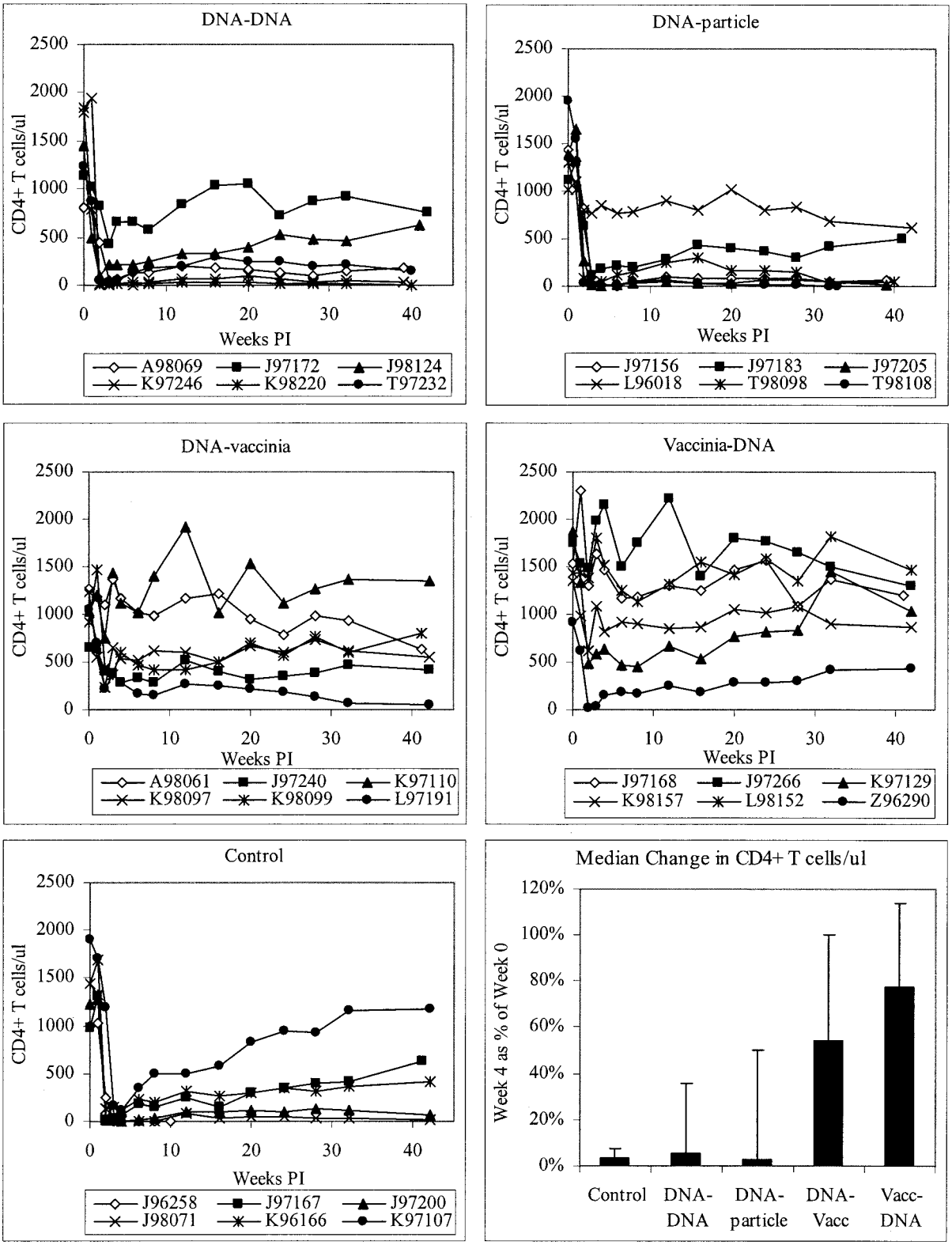

FIG. 3. CD4$^+$-T-cell counts of infected macaques. The bottom right panel shows the median for each group of each animal's CD4$^+$-T-cell count at week 4 compared to week 0. Error bars indicate interquartile ranges. Vacc, vaccinia virus; PI, postinfection.

TABLE 3. Analysis of $CD4^+$-T-cell counts in infected macaques

Group	Median $CD4^+$-T-cell count as % of wk 0 for wk:		*P* value for wk[a]:	
	4	24	4	24
Control	3.2	23.7		
DNA-DNA	5.7	19.0	0.240	0.931
DNA-particle	2.6	9.1	0.485	0.662
DNA-vaccinia virus	53.9	57.3	**0.002**[b]	0.052
Vaccinia virus-DNA	77.9	88.2	**0.002**	**0.030**

[a] *P* values from Mann-Whitney test of each vaccine group compared to controls.
[b] Boldface type indicates $P < 0.05$.

Animals vaccinated with DNA and vaccinia virus, in either regimen, fared much better. DNA priming/vaccinia virus boosting animals experienced slow or moderate declines in $CD4^+$-T-cell counts; 1 of 6 animals experienced no immunodepletion at all (Fig. 3). Macaques given vaccinia virus priming/DNA boosting were also partially protected from disease. Three of 6 animals showed no effect of infection on the level of $CD4^+$ T cells. Two of 6 animals had slow, moderate declines but maintained $CD4^+$-T-cell counts of over 500 for the 42 weeks of the study, and 1 animal initially had a severe loss but gradually recovered to 50% of its baseline $CD4^+$-T-cell level.

Since the prechallenge $CD4^+$-T-cell counts were quite variable from animal to animal, we analyzed $CD4^+$-T-cell loss by comparing each animal's counts at weeks 0 and 4. This parameter is shown in Fig. 3, lower right panel. The median loss was much more severe in control animals and the DNA-only and DNA/particle groups than in the DNA/vaccinia virus and vaccinia virus/DNA groups. $CD4^+$-T-cell loss is significantly greater in controls than in the last two groups ($P = 0.002$ for each group compared to controls) (Table 3). Additionally, we looked at the levels of $CD4^+$ T cells in the chronic phase of infection. Again, the vaccinia virus/DNA group showed significantly better maintenance of $CD4^+$ T cells ($P = 0.03$); however, the DNA/vaccinia virus group did not achieve statistically significant maintenance of $CD4^+$ T cells in the chronic phase of infection.

Virus loads in vaccinated and control macaques. Virus loads were measured by real-time RT-PCR of viral RNA in plasma. This analysis showed that all animals in the study were infected, and this was confirmed by PCR of PBMC DNA and lymph node DNA (data not shown). As shown in Fig. 4, controls had consistently high peak viremia, up to 10^8 copies/ml. All vaccine groups except DNA priming/particle boosting had small but significant reductions in peak viremia (Table 4). Set point viremia was highly peak variable in controls and in animals vaccinated with DNA alone or primed with DNA and boosted with particles. One control animal (J96258) sustained very high viremia, progressed rapidly to AIDS, and was euthanized at week 10. In concordance with the effects on $CD4^+$ T cells, we found that animals receiving DNA and recombinant vaccinia virus, in either regimen, had consistently lower viral loads in the chronic phase of infection. In macaques that were primed with DNA and boosted with vaccinia virus, 5 of 6 animals controlled viremia to less than 10^4 copies/ml by week 6 postinfection, and these low levels were stable through week 42. Among animals primed with vaccinia virus and boosted with DNA, all 6 animals controlled viremia below 10^4 copies/ml, with 5 of 6 having virus at or below the limit of detection (100 copies/ml) at multiple time points. The animals vaccinated with vaccinia virus priming/DNA boosting had significantly lower viremia at week 24 than the controls ($P = 0.03$); this was the only group that reached a level statistically different from the controls.

Quantification of postchallenge cellular immunity. Cellular immunity was measured at weeks 2 and 4 postchallenge by IFN-γ ELISpot assay (Table 2). All 12 of the animals in the DNA-vaccinia virus and vaccinia virus-DNA groups mounted robust cellular responses to Gag and/or Env by week 2, and 11 of 12 had sustained responses through week 4. All 6 animals vaccinated with DNA alone had responses at week 2, and 4 animals sustained the responses at week 4. In contrast, only 2 of 6 controls and 2 of 6 DNA-primed/particle-boosted animals mounted cellular responses. Additionally, at least 1 animal in each group developed responses to additional gene products, as shown by positive ELISpot assays for Pol and accessory gene peptides, including 3 of 6 animals in the DNA-only group. The level of cellular immunity on the day of challenge correlated strongly with protection from disease (see below). The level of response at weeks 2 and 4, however, did not correlate with the set point viral load or protection from $CD4^+$-T-cell decline. ELISpot responses at these early time points were higher and more frequent in vaccinees relative to controls.

Quantification of postchallenge antiviral humoral responses. Postchallenge antibody responses were measured for binding to Envelope gp120 (Fig. 5) and neutralizing activity to SHIV89.6 and SHIV89.6P (Fig. 6). Binding antibody increased rapidly in vaccinees that received both vaccinia virus and DNA and peaked by weeks 4 to 6 postinfection, remaining at a high level throughout the study. In contrast, while controls and animals in the first two vaccine groups generated similarly high titers of antibodies, the development was slower, plateauing at weeks 8 to 12. This observation indicates a strong memory response in the DNA priming/vaccinia virus boosting and vaccinia virus priming/DNA boosting groups.

Neutralizing antibody against the challenge strain was measured by cMAGI assay (15). No neutralizing activity was detected on the day of challenge. However, most animals developed neutralizing antibodies in the first 12 weeks postinfection. The development of neutralizing antibody differed among groups. As shown in Fig. 6, animals immunized with DNA/vaccinia virus or vaccinia virus/DNA rapidly developed neutralizing antibody, with detectable neutralization in 11 of 12 animals and a geometric mean titer of 1:53 (limit of detection, 1:10) at week 4. In contrast, control animals and those immunized with DNA alone or DNA priming/particle boosting had lower and less consistent responses, with only 12 of 18 responding and a geometric mean titer of 1:13 (limit of detection, 1:10) at week 4. The same pattern was seen for neutralization of the vaccine strain SHIV89.6, assayed in GHOST-CCR5 cells (14) at 8 weeks postchallenge (data not shown). These data indicate a memory response or faster maturation of the antibody response in animals immunized with DNA/vaccinia virus or vaccinia virus/DNA.

Correlation of immune responses and outcome of infection. We analyzed the relationship of preexisting immune responses

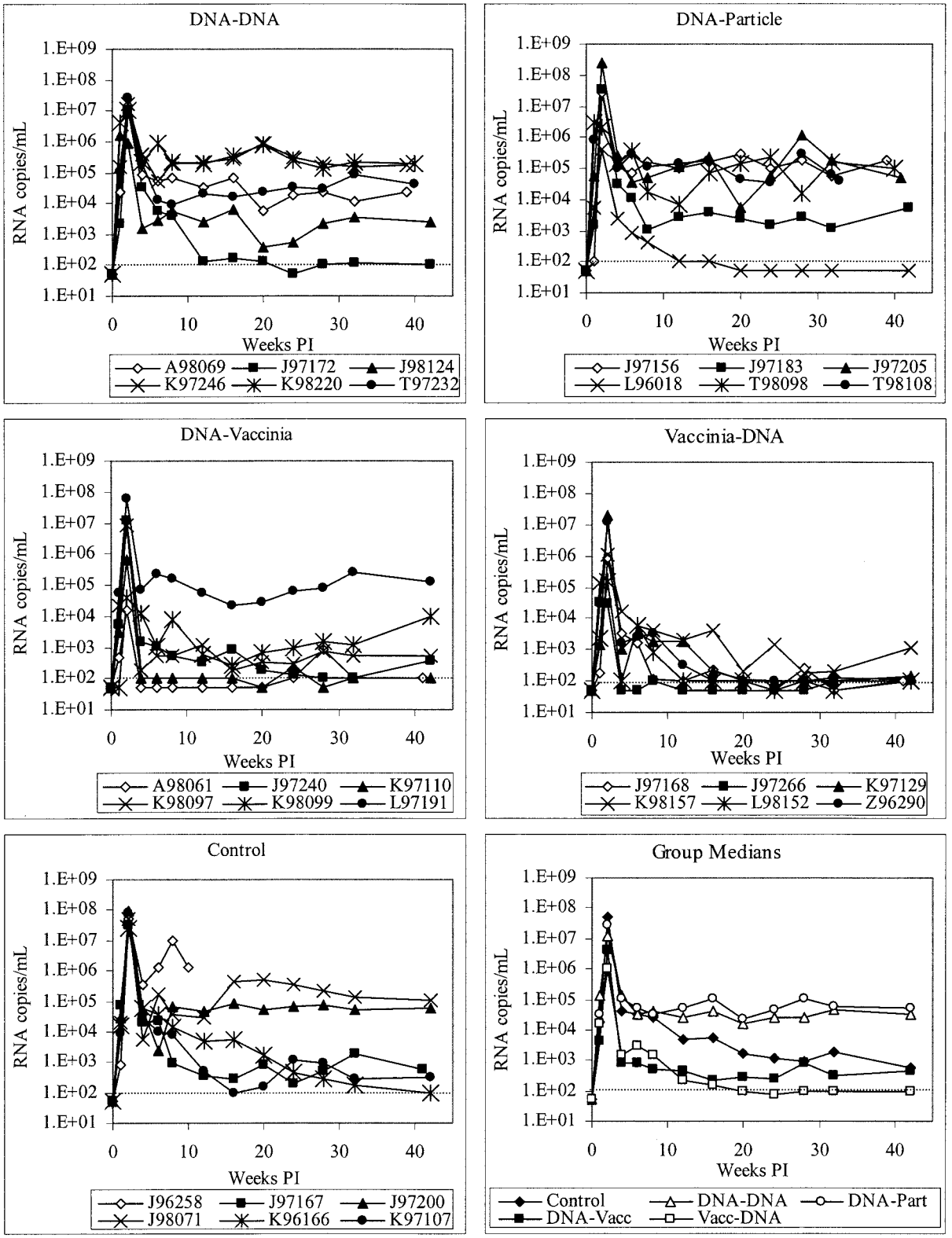

FIG. 4. Virus load in plasma in infected macaques. Viremia was quantified by real-time RT-PCR. The dotted line indicates the limit of detection (100 copy equivalents/ml); values below this limit were assigned values of 50 copies/ml. Vacc, vaccinia virus; Part, particle; PI, postinfection.

TABLE 4. Analysis of plasma viral load in infected macaques

Group	Median viral load (RNA copies/ml of plasma) for wk:		*P* value for wk[a]:	
	4	24	4	24
Control	5.2×10^7	1.2×10^3		
DNA-DNA	1.2×10^7	2.6×10^4	**0.004**[b]	0.931
DNA-particle	2.9×10^7	4.5×10^4	0.310	0.792
DNA-vaccinia virus	4.3×10^6	2.6×10^2	**0.026**	0.178
Vaccinia virus-DNA	9.8×10^5	1.0×10^2	**0.002**	**0.030**

[a] *P* values from Mann-Whitney test of each vaccine group compared to controls.
[b] Boldface type indicates *P* value of <0.05.

to the outcome of viral infection. Protection from rapid immunodepletion (defined as reduction of $CD4^+$ T cells to $<20\%$ of baseline in the first 4 weeks) correlated with detection of anti-Env IgG on the day of challenge ($P = 0.003$, Fisher's exact test) and detection of cellular immunity to Gag or Env by IFN-γ ELISpot assay on the day of challenge ($P = 0.004$). The magnitude of the immune response also correlated with protection (*P* values for the Spearman rank correlation test are shown in Table 5). The total ELISpot response on the day of challenge, the anti-Env IgG titer on the day of challenge, and the neutralizing antibody titers at week 4 postinfection all correlated positively with the $CD4^+$-T-cell count (as a percentage of the levels on the day of challenge) at weeks 4 and 24 postinfection and correlated negatively with viral load at week 24.

Clinical outcome in challenged macaques. One animal in the control group progressed rapidly to AIDS and was euthanized at week 10 postchallenge. This animal did not seroconvert. One animal in the DNA priming/particle boosting group was euthanized at week 33. All other animals were still alive at the conclusion of the study (weeks 39 to 42), despite the fact that many had extremely low $CD4^+$-T-cell counts for >9 months. Only minor illnesses, such as diarrhea which resolved, were noted.

DISCUSSION

In this study, we found that priming vaccinations with gene gun-delivered DNA followed by boosting with vaccinia virus, or vaccinia virus priming/DNA boosting, provided significant protection from disease caused by SHIV89.6P in *M. nemestrina*. Neither DNA alone nor DNA boosted with inactivated virus particles provided protection from disease. While these two groups, as well as controls, experienced severe and rapid loss of $CD4^+$ T cells, most animals that received DNA priming/vaccinia virus boosting or vaccinia virus priming/DNA boosting remained healthy for more than 39 weeks. Statistically significant differences were seen for these two groups versus controls in the first 4 weeks after infection, and long-term maintenance of $CD4^+$ T cells was significant for the vaccinia virus priming/DNA boosting group. These two regimens also resulted in reduced viral load in the chronic phase of infection. Set point viremia is predictive of disease progression in HIV-1-infected humans (41) and in macaque models with SIV (61, 69). In the highly pathogenic SHIV89.6P model, the relationship is less clear; however, lower viremia generally correlates with preservation of $CD4^+$ T cells (22, 52, 65). We found a statistically significant reduction of set point viremia in the animals primed with vaccinia virus and boosted with DNA relative to controls. The animals that received DNA priming/vaccinia virus boosting also had reduced viral loads, but the reduction was not statistically significant. The median viral load for controls was lower than that in the groups vaccinated with DNA or DNA with particles; this may be due to the early death (week 10) of the control animal with the highest viral load (J96258).

In most previous vaccine studies which used DNA with a viral vector, DNA was used as priming agent but not as a boosting agent (reviewed in references 17 and 48). In a landmark study of malaria in mice, DNA priming/modified vaccinia virus Ankara (MVA) boosting provided sterilizing immunity while MVA priming/DNA boosting was ineffective (58). It has been suggested that DNA can only be effective as a priming agent and that its success may be due to low-dose priming of high-affinity T cells which can be expanded by a viral vector boosting agent (21). Conversely, several groups have suggested that poxviruses are only effective as boosting agents (21, 59) because they prime low-avidity T cells. However, other studies imply that this order may not be required, at least for lentiviruses. A study of SIV in macaques with DNA and vaccinia virus did not show clear differences when the order of priming and boosting was reversed (29). Several experiments by our group show complete (33) or partial (46) protection from SIV challenge after priming with vaccinia virus and boosting with recombinant protein; thus, we expected that vaccinia virus would be effective in priming a DNA vaccine.

Here we present two lines of evidence to show that a SHIV DNA vaccine is effective as a boosting agent for recombinant vaccinia virus. First, the animals immunized with recombinant vaccinia virus priming/DNA boosting had the best outcomes of any group in this study. Second, the time course of development of cellular and humoral immunity reveals a boosting effect of DNA. A comparison of the antibody titers (Fig. 2) illustrates this point. The responses in the vaccinia virus priming/DNA boosting group increase from 3 of 6 responders after vaccinia virus alone to 6 of 6 responders after the DNA inoculations; thus, DNA works as a boosting agent. Furthermore, all 6 macaques primed with DNA and boosted with vaccinia virus developed antibody after the boost, with higher titers than the animals given vaccinia virus first. Thus, DNA primes the antibody responses elicited by the vaccinia virus, as expected. Likewise, ELISpot assays for cellular immunity show that, while vaccinia virus alone elicited strong responses in 2 of 6 macaques, boosting these animals with DNA increased the number of responders to 4 of 6, and although DNA alone did not elicit cellular responses, the animals primed with DNA and boosted with vaccinia virus had the highest responses, with 5 of 6 animals responding (Table 2).

The presence of preexisting antibody or cellular immunity on the day of challenge correlated with protection. Overall, a positive measurement by ELISpot or binding antibody assay on the day of challenge correlated with protection from $CD4^+$-T-cell loss, and the magnitude of the immune response correlated with higher $CD4^+$-T-cell counts and lower viremia. The two regimens that were protective were those that elicited measurable antibody and cellular responses in most animals prior to challenge. Even within the groups that fared poorly

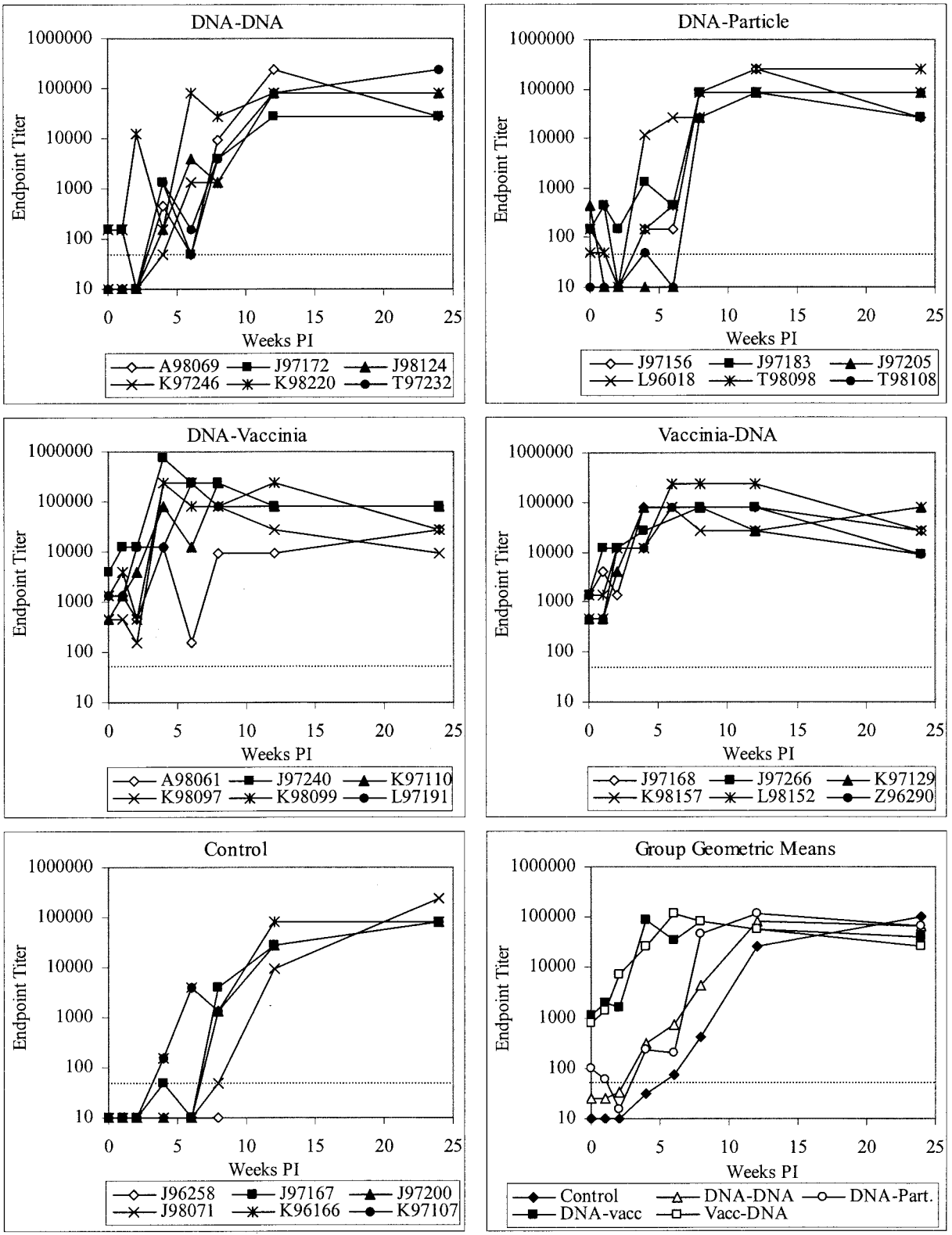

FIG. 5. Development of postchallenge anti-Env binding antibody (IgG) in vaccinated and control macaques. The dotted line indicates the limit of detection (1:50 dilution); values below this limit were assigned values of 10. Vacc, vaccinia virus; Part, particle; PI, postinfection.

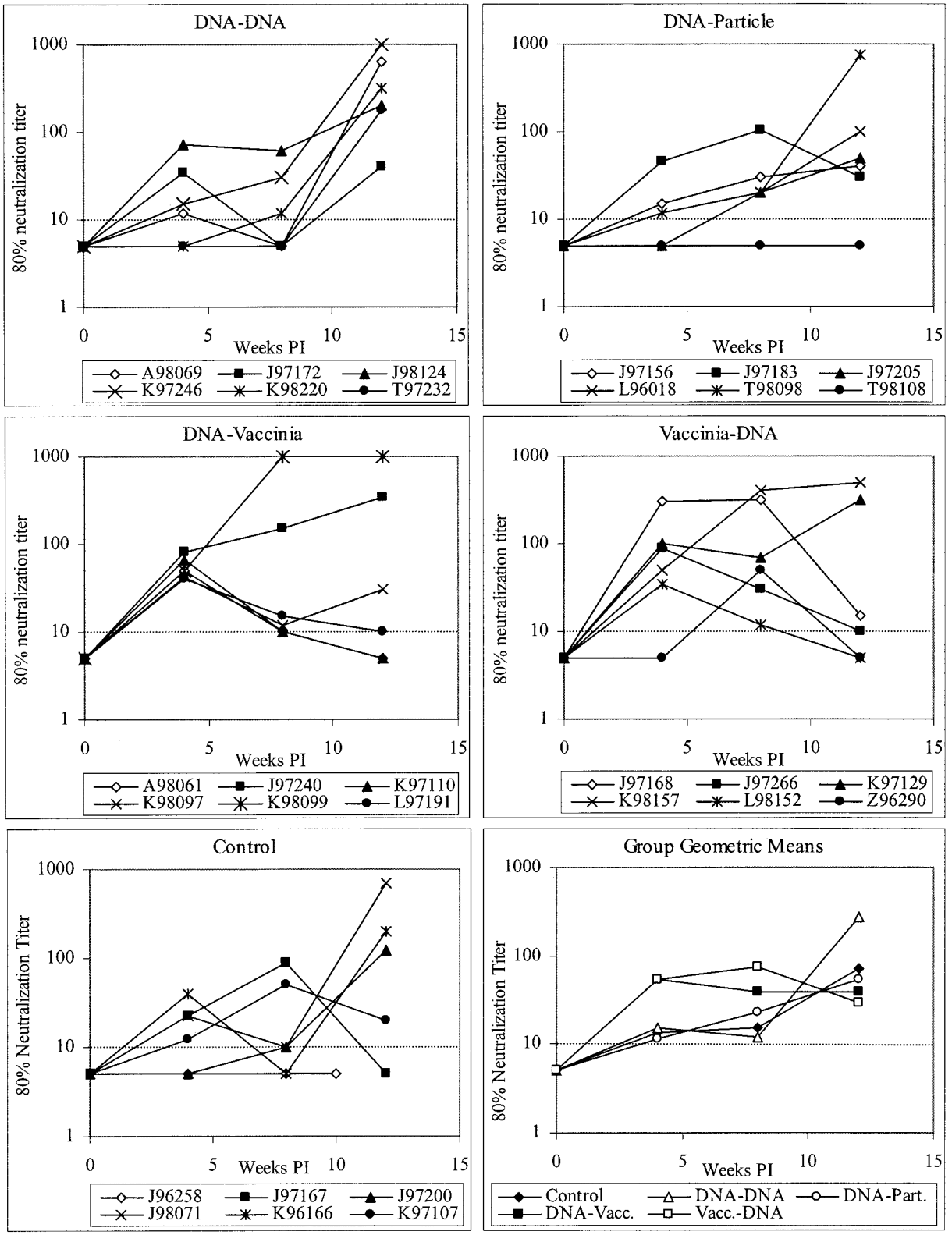

FIG. 6. Neutralizing antibody against SHIV89.6P. Neutralization was measured by the cMAGI assay. The dotted line indicates the limit of detection (1:10 dilution). Values below this limit were assigned a value of 5. Vacc, vaccinia virus; Part, particle; PI, postinfection.

TABLE 5. Spearman rank correlation test for immune responses compared to outcomes

Immune response	*P* value for comparison to:		
	Viral load, wk 24	$CD4^+$ T cells, wk 4	$CD4^+$ T cells, wk 24
$SFC^b/10^6$ PBMC, wk 0	**<0.0001**[a]	**0.0003**	**0.0001**
Anti-Env IgG titer, wk 0	**<0.0001**	**0.001**	**0.005**
Neutralizing titer, wk 4	**0.0043**	**<0.0001**	**0.0001**

[a] Boldface type indicates *P* value of <0.05.
[b] SFC, spot-forming cells.

overall, the few animals that responded to the vaccines were more likely to remain healthy. For example, the one animal in the DNA/particle group (L96018) that had cellular immunity, as measured by ELISpot, on the day of challenge was the only animal in its group that maintained a normal $CD4^+$-T-cell count. Likewise, the animal in the DNA-only group (J97172) that had the lowest set point viral load and highest $CD4^+$-T-cell count was one of the two animals that had detectable binding antibody to Env on the day of challenge. Furthermore, strong memory antibody responses were noted in the protected groups; these indicate the presence of memory B cells and are likely also related to the maintenance of $CD4^+$ T cells which were able to provide help for a vigorous memory response. Although neutralizing antibody was not detected on the day of challenge in any animals, those immunized with both DNA and recombinant vaccinia virus rapidly developed modest neutralizing antibody titers after infection. At week 4, neutralizing antibody titers were threefold higher in the DNA/vaccinia virus or vaccinia virus/DNA groups than in the other animals. Since most animals developed titers of at least 1:40 by weeks 8 to 12, even those with severe immunodepletion, we infer that the earlier response in animals receiving DNA and vaccinia virus was not due solely to the preservation of $CD4^+$ T cells in those animals. The earlier development of a neutralizing response may have helped protect those animals from $CD4^+$-T-cell depletion. Priming of a rapid neutralizing response, in the absence of detectable neutralizing activity on the day of challenge, has been seen in other studies with SHIV89.6P (1, 10). In addition, binding antibody that was present on the day of challenge may itself have played a role in protection. Binding antibody may have protective effects that are not measured by our neutralization assay, such as antibody-dependent cell-mediated cytotoxicity (5); it has also been speculated that binding of shed gp120 glycoprotein may reduce a proapoptotic effect of gp120 on uninfected cells (1).

Several recent vaccine studies have used the highly pathogenic virus SHIV89.6P and shown protection from $CD4^+$-T-cell loss and disease. The levels of protection from SHIV89.6P provided by DNA/vaccinia virus and vaccinia virus/DNA in this study are similar to published results of vaccination of rhesus macaques *M. mulatta* with recombinant MVA (rMVA) (9), recombinant vesicular stomatitis virus (56), DNA with recombinant Semliki Forest virus (40), low doses of DNA given intramuscularly and boosted with rMVA (2), and DNA vaccines given intramuscularly (10). The latter study used two of the same plasmids used here (pV1Jns:89.6Penv160OPTcleavable and pVIR SIV GAG OPT). A few studies performed with *M. mulatta* have shown better protection from SHIV89.6P-induced disease, notably by using recombinant adenovirus (60), rMVA primed with a high dose of DNA (2), or DNA with interleukin-2/Ig (10); however, none have elicited sterilizing immunity. The major difference between these studies and the one reported here is our use of *M. nemestrina*. We found that *M. nemestrina* macaques are susceptible to SHIV89.6P infection and that naive animals have levels of peak and set point viremia similar to those of *M. mulatta* macaques (50). Furthermore, the hallmark of SHIV89.6P infection in naive rhesus monkeys is the very rapid and severe depletion of circulating $CD4^+$ T cells, and we observed this effect in naive *M. nemestrina*. Therefore, we conclude that *M. nemestrina* is an appropriate species for evaluating vaccines against this virus and that comparisons to other studies are valid.

We noted several differences in the long-term outcome of infection, notably the survival (>9 months) of animals with extremely low $CD4^+$-T-cell counts. This contrasts with several studies of SHIV89.6P in rhesus macaques in which high mortality was noted in nonvaccinated animals (10, 51). However, more recent data indicate that survival among infected rhesus macaques is highly variable, with better survival in Mamu-$A01^+$ animals (35, 72) and some naive animals partially recovering $CD4^+$ T cells (60). *M. nemestrina* macaques may be better able to survive with very low levels of $CD4^+$ T cells than *M. mulatta*. Indeed, there is precedent for this in the HIV-2-287 model, where rapid irreversible loss of $CD4^+$ T cells by *M. nemestrina* macaques is uniformly observed, and macaques can survive for long periods following HIV-2-287-induced $CD4^+$-T-cell depletion (68).

Several recent studies have compared viral vectors alone to the same vectors primed with a DNA vaccine. Macaques immunized with rMVA, or primed with DNA and boosted with rMVA, all had good control of a pathogenic SHIV challenge; however, the regimens elicited different responses (3). The combination elicited higher levels of IFN-γ-producing T cells but lower antibody titers relative to rMVA alone. Similarly, an adenovirus-based vaccine alone or as a boost after DNA vaccination protected macaques from the same virus, but the latter regimen elicited a higher cellular response (60). These studies utilized needle injection of DNA. The promise of gene gun delivery to provide equivalent immunity with much lower doses of DNA than needle injection (30) has been called into question by studies that indicate poor protection from virus challenge. No protection was seen in a study of a gene gun-delivered gp120 DNA vaccine with an SHIVsf13 challenge (67) or gene gun delivery of *gag*, *pol*, *env*, and *nef* and challenge with SHIV-IIIb (55). However, in the SIV model, protection from disease at a level similar to that indicated by the data presented here has been obtained by using the gene gun to protect against SIVdeltaB670 challenge (28). This study is the first example of gene gun-based DNA delivery protecting against disease caused by any SHIV, including the highly virulent SHIV89.6P. Although our DNA vaccine alone was not effective in eliciting strong immunity, the DNA clearly improved the responses elicited by recombinant vaccinia virus. It is possible that the effectiveness of the DNA as a priming or boosting agent was due in part to the ability of one of the plasmids to direct expression of VLP in vivo. Additionally, these gene gun-delivered vaccines primed and boosted cellular immune responses. Some previous studies have found that gene gun

delivery elicited a primarily Th2 response (26), although others have shown a balanced Th1/Th2 response (11, 45) and cellular immunity (55).

Boosting with inactivated virus particles was not as effective as we had anticipated, based on the prior experience of our group (73) with autologous macaque dendritic cells for delivery of inactivated SIV, and others which used virus particles as the sole antigen (37) or as a boosting agent for vaccinia virus (70). We used alum plus CpG oligonucleotides as adjuvants in an effort to maintain the overall virion architecture and the Envelope conformation present in the original preparation. It is possible that the low immunogenicity of the virions can be overcome by the use of more effective adjuvants or higher doses of particles.

The vaccines tested here included multiple viral genes: the inactivated virus particles and the recombinant vaccinia viruses provided Gag, Pol, and Env, while the DNA vaccine provided these plus all accessory genes. This design was chosen based on the hypothesis that a multiantigen vaccine would improve the outcome of the challenge, and it differs from the many studies which have used single antigens (7, 20, 24, 60, 64). We measured prechallenge antibody and cellular responses to multiple gene products. Some animals responded to both Gag and Env while others responded only to one or the other. Thus, including more than one antigen successfully increased the total number of animals responding to the vaccine. We were disappointed to find only limited responses to Pol or accessory genes prior to challenge. Sporadic cellular responses to Pol and Env also were noted after challenge; however, these did not correlate with any one vaccine regimen.

The utility of the SHIV89.6P model has recently come into question (25). The very rapid loss of $CD4^+$ T cells is unlike the course of HIV disease progression in humans. Furthermore, while the parental HIV89.6 is dualtropic, SHIV89.6P uses CXCR4 as a coreceptor (71), again differing from HIV infection, in which most transmitted viruses are CCR5 users. However, there are advantages to this virus as well (53). SHIVs are invaluable for testing vaccine strategies aimed at eliciting immune responses to Env, particularly neutralizing antibody, because they contain HIV-1 Env. Furthermore, while many SHIV strains are highly attenuated, the pathogenicity of SHIV89.6P allows assessment of protection from both infection and disease. In addition, a large number of recent vaccine studies have used SHIV89.6P as a challenge virus (1, 2, 10, 19, 36, 40, 49, 56); thus, these studies can all be easily compared to each other, as noted above.

Because low viral load is associated with slow disease progression, control of viral load rather than sterilizing immunity may be considered as an endpoint for vaccine-induced protection (31, 62). Most of the encouraging recent vaccine studies with macaques have used this endpoint (2, 60). Recently, however, vaccinated *M. mulatta* macaques were shown to advance to disease despite very low virus loads early after challenge with SHIV89.6P (8) or SIV (7), casting doubt on the durability of vaccine-induced virus control. It is thus desirable to design vaccines that are even more effective in limiting virus load for the long term. To most effectively improve upon current approaches, it is valuable to understand what types of immunity each vaccine component provides, alone and in combination with other components. We have found that comparative studies can provide information on which regimen(s) are most effective in preventing disease. In conclusion, we show that multigene vaccines in priming-boosting regimens comprised of gene gun-delivered DNA and recombinant vaccinia virus, in either order, are effective in preventing SHIV89.6P disease. Further modifications to the vaccines, possibly including recombinant protein to boost neutralizing antibody responses, will be needed to provide complete protection from disease or sterilizing immunity.

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