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Immune Responses but No Protection against SHIV by Gene-Gun Delivery of HIV-1 DNA Followed by Recombinant Subunit Protein Boosts

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The efficacy of combining immunization with human immunodeficiency vitus type 1 (HIV-1) DNA and HIV-1 recombinant proteins to obtain protection from chimeric simian/human immunodeficiency virus (SHIV) was determined. Four cynomolgus monkeys received four gene-gun immunizations intraepidermally of plasmid DNA encoding HIV-1LAI *env* (gp160), *gag, tat, nef,* and *rev* proteins. Ten micrograms of DNA was used per immunization. The animals were boosted twice intramuscularly with 50 μg of HIV-1LAI Env (MicroGeneSys), Gag, Tat, Nef, and Rev recombinant proteins mixed in Ribi adjuvant. The antibody responses were amplified following the administration of the recombinant subunit boosts. One month after the final subunit immunization, the vaccinated animals together with four control animals were challenged intravenously with 10 monkey infectious doses of SHIV that expresses the *env*, *tat* and *rev* genes of HIV-1 and gag and nef from SIV. However, only low titers of neutralizing antibodies were present at the day of challenge. The consecutive HIV-1 DNA and recombinant protein immunizations induced B- and T-cell responses but not protection against SHIV replication nor reduction of the viral load.

INTRODUCTION

To design an effective vaccine to protect people from infection with human immunodeficiency virus (HIV) and to protect against AIDS are high priorities in controlling the epidemic. Preclinical vaccine studies in nonhuman primates are important for identifying which vaccine candidates should proceed to human clinical trials. Chimeric simian/human immunodeficiency viruses (SHIVs) in which the SIV *env* gene has been replaced with the *env* gene of various HIV-1 strains are becoming increasingly important for assessing the potential efficacy of candidate HIV-1 vaccines (Li *et al.,* 1992).

To date the most potent protection against simian immunodeficiency virus (SIV) has been obtained with live attenuated vaccines (for a review, see Putkonen *et al.*, 1996). However, a live attenuated HIV-1 vaccine in humans is associated with a number of safety concerns.

A novel approach to immunization is the induction of immune responses against an antigenic protein expressed *in vivo* from an introduced gene (Vogel *et al.,* 1995). This so-called DNA vaccination is attractive because it mimics the effects of live attenuated viral vaccines in that both humoral and cellular immune responses can be induced (Okuda *et al.,* 1995, Wang *et al.*, 1995a; Yasutumi *et al.,* 1996).

Immunization with DNA in various animal models has been shown to be successful in generating protective immunity against, i.e., influenza (Fynan *et al.,* 1993; Ulmer *et al.,* 1993), rabies virus (Xiang *et al.,* 1994), herpes simplex virus (Bourne *et al.,* 1996), and murine cytomegalovirus (González Armas *et al.,* 1996). In these various virus models, DNA vaccines have provided protection against disease but not against infection. Limited work has been done using DNA to raise protective immune responses against primate lentiviruses. Wang and collaborators (Wang *et al.,* 1995) have shown that immunization with HIV-1 DNA in macaques achieved some reduction in postchallenge viral load with a SHIV challenge given intravenously. Boyer *et al.* (1997) have reported protection against establishment of HIV-1 infection in two chimpanzees vaccinated with HIV-1 DNA. In a simian immunodeficiency virus vaccine trial in rhesus macaques, a DNA vaccine failed to achieve protection against disease (Lu *et al.,* 1996). In HIV-1-infected humans, HIV DNA representing regulatory genes were recently shown to induce new and increased levels of HIV-specific cytotoxicity (Calarota *et al.,* 1998).

Four reports provide indications of a synergistic relationship between genetic and recombinant subunit-based immunization (Leong *et al.,* 1995; Fuller *et al.,* 1996, 1997; Letvin *et al.,* 1997). By using a combined DNA

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TABLE 1

A. HIV-1 IgG Titers in Four Cynomolgus Monkeys after Immunizations with HIV-1 DNA and Following Recombinant Subunit Booster Immunizations

	Two weeks after the 4th DNA immunization					Four weeks after two HIV-1 protein immunizations[a]				
Monkey no.	gp160	p24	Tat	Nef	Rev	gp160	p24	Tat	Nef	Rev
C84	<50	300	450	500	300	9500	5800	1200	500	<50
C85	360	410	<50	200	200	$>10^4$	8200	$>10^4$	6000	4000
C90	300	<50	<50	125	<50	$>10^4$	1000	$>10^4$	4000	210
C91	140	<50	<50	<50	<50	$>10^4$	$>10^4$	$>10^4$	1050	<50

[a] Sera were taken 4 weeks after HIV-1 protein immunizations.

B. T-cell Proliferation against HIV-1 Antigens and Regulatory Protein Peptides

	Frequency of responses after DNA immunizations and protein boost					Frequency of responses after SHIV challenge immunizations[a]				
Monkey no.	gp160	p24	Tat + P[b]	Nef + P	Rev + P	gp160	p24	Tat + P	Nef + P	Rev + P
Vaccine										
C84	2/9	2/9	4/9	5/9	6/9	4/7	3/7	6/7	7/7	3/7
C85	0/9	2/9	4/9	5/9	5/9	4/7	3/7	4/7	5/7	5/7
C90	3/9	3/9	4/9	7/9	2/9	2/7	2/7	2/7	6/7	1/7
C91	1/9	2/9	3/9	4/9	5/9	4/7	4/7	3/7	5/7	3/7
Control										
C23	0/9	1/9	1/9	5/9	1/9	0/7	0/7	1/7	5/7	0/7
C28	1/9	0/9	1/9	5/9	2/9	0/7	0/7	2/7	2/7	1/7
C88	0/9	0/9	3/9	3/9	1/9	0/7	1/7	2/7	1/7	1/7
C89	0/9	0/9	1/9	0/9	1/9	2/7	3/7	3/7	7/7	4/7

[a] Number of positive SI of number of tests.
[b] P, synthetic peptide.

plus protein immunization regime, enhanced immune responses were induced. Improved responses were seen even when the DNA was only weakly immunogenic. The present study addresses whether immunization with HIV-1 DNA followed by subunit boosts confers immune responses and protective benefit against a SHIV challenge.

RESULTS

Immune responses in vaccinated monkeys

Serum IgG responses were low to moderate after the DNA immunizations but rapidly increased after the protein boosts (Table 1A, Fig. 1). After the protein boosts, the four vaccinees had high antibody responses to structural as well as to regulatory proteins, but anti-rev IgG remained low in two animals. Development of HIV-1 virus neutralizing antibodies was detected only after protein immunizations and was seen in all the vaccinees. The four vaccinated monkeys developed neutralizing antibodies to HIV-1_{LAI} with IC_{50} titers ranging from 40 to 220 (Table 2). Two animals showed IC_{90} titers of 40 in one animal (C85) and 70 in another (C91). Neutralizing antibodies against the primary clade B isolate 6794 was detected in Monkey C85 only. SHIV-neutralizing antibodies were seen in three vaccinees (C85, C90, and C91).

It was not possible to detect any specific CTL against HIV-1 Env, HIV-1 Nef, nor against SIV Nef before SHIV challenge.

T cell proliferation to all protein antigens and synthetic peptides representing the regulatory proteins were performed once before and nine times during immunizations and seven times after SHIV challenge (Table 1B). The highest responses were seen during the DNA immunizations and after SHIV challenge (Fig. 2). Both structural and regulatory antigens contributed to the anti-HIV-specific reactivities. Macaques C90 and C91 had the broadest reactivities, while Macaque C85 had high stimulation indexes. Among control animals an unspecific proliferative reaction was seen with the Nef antigen (Table 1B).

Local reactions were observed as a skin rash in two of the vaccinees (C90 and C91) This reaction was localized in the groin of the site of DNA-immunization with a size of approximately 1.5 × 1.5 cm. These reactions increased in size to 3 × 5 cm following the protein immunizations, which were given intramuscularly. A skin biopsy contained inflammatory cells with no atypia. None of the two other vaccinated monkeys nor the four control DNA im-

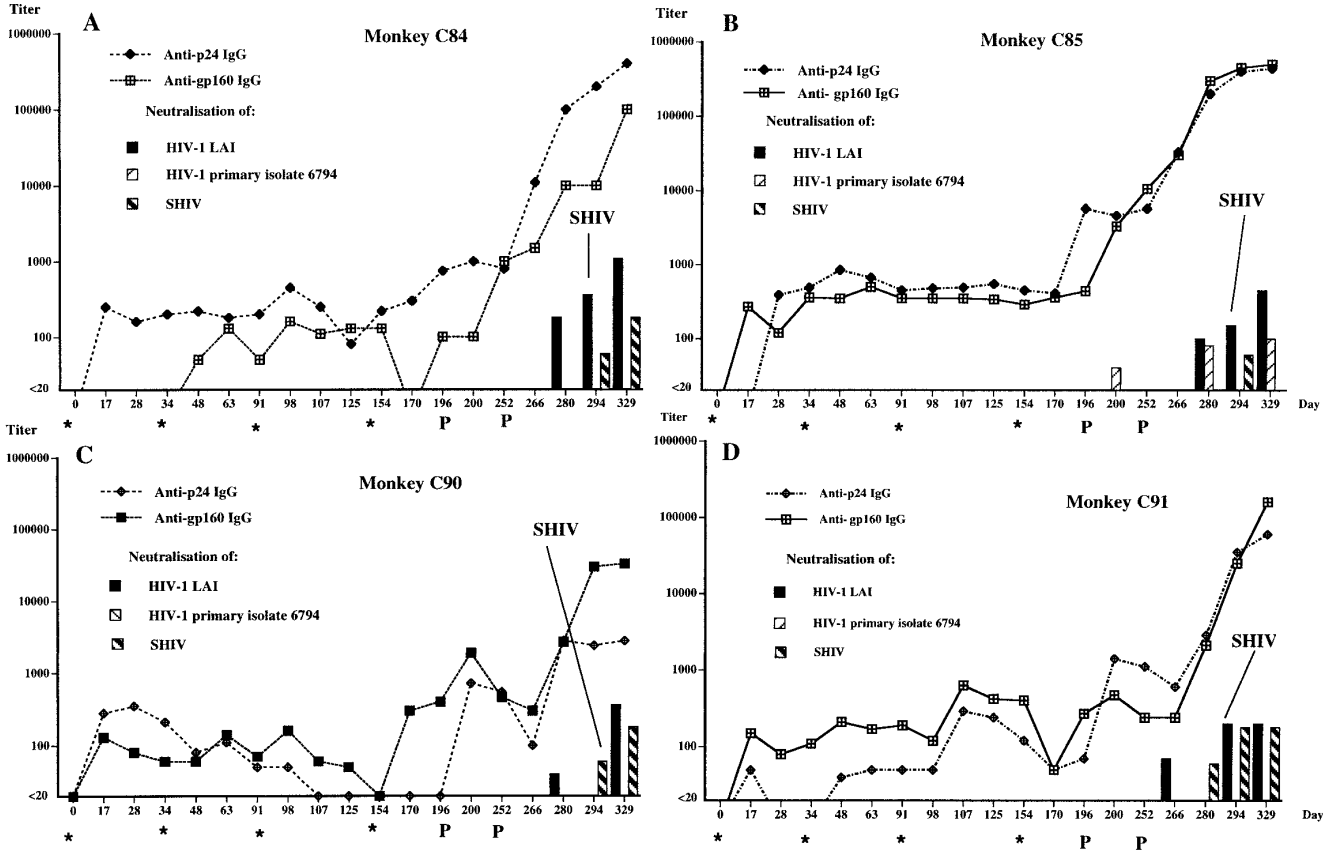

FIG. 1 HIV-1 antigen specific serological responses in Monkey C84 (A), Monkey C85 (B), Monkey C90 (C), and Monkey C91 (D), before and during immunizations and postchallenge. Serum IgG to HIV-1 gp160 and p24 as well as neutralizing antibody titers to HIV-1LAI (50 $TCID_{50}$), primary isolate 6794 (40 $TCID_{50}$) and SHIV (10 $TCID_{50}$) are shown. DNA immunization time is indicated by * and protein boosts by P. SHIV challenge intravenously was performed at Day 294 as indicated.

munized monkeys developed skin reactions at the sites of immunization.

HIV-1-specific B-cell IgG synthesis *in vitro*

Two DNA plasmid immunizations were required before HIV-1-specific IgG were produced by lymphoid cells collected from blood. All four immunized macaques responded by developing B cells secreting IgG specifically reactive with HIV-1 recombinant antigens. The highest levels were directed against Nef and Tat, with the main reactivity directed to the C-terminal region of Nef (aa 165–210) and to the central region of Tat (aa 31–65). Following the HIV-1 protein immunizations, all vaccinated animals developed B cells secreting HIV-1-specific

TABLE 2

HIV-1 Neutralization Titers of Serum from Four Cynomolgus Monkeys after Immunizations with HIV-1 DNA, Recombinant Subunit Boosters and Post-SHIV Challenge

	Pre-SHIV challenge at time for challenge[a]					Four weeks post-SHIV challenge				
	HIV-1LAI		6794	SHIV		HIV-1LAI		6794	SHIV	
Monkey no.	IC_{90}	IC_{50}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{50}	IC_{90}	IC_{50}
C84	<20	180	<20	<20	<20	540	1080	<20	60	360
C85	40	100	70	20	60	180	450	80	60	540
C90	<20	40	<20	<20	20	80	360	<20	20	180
C91	70	220	<20	20	60	200	750	<20	20	60

[a] Sera were taken 4 weeks after HIV-1 protein immunizations; this also was the time of challenge with SHIV. Inhibiting serum virus neutralisation concentrations (IC) IC_{50} and IC_{90} titers are shown.

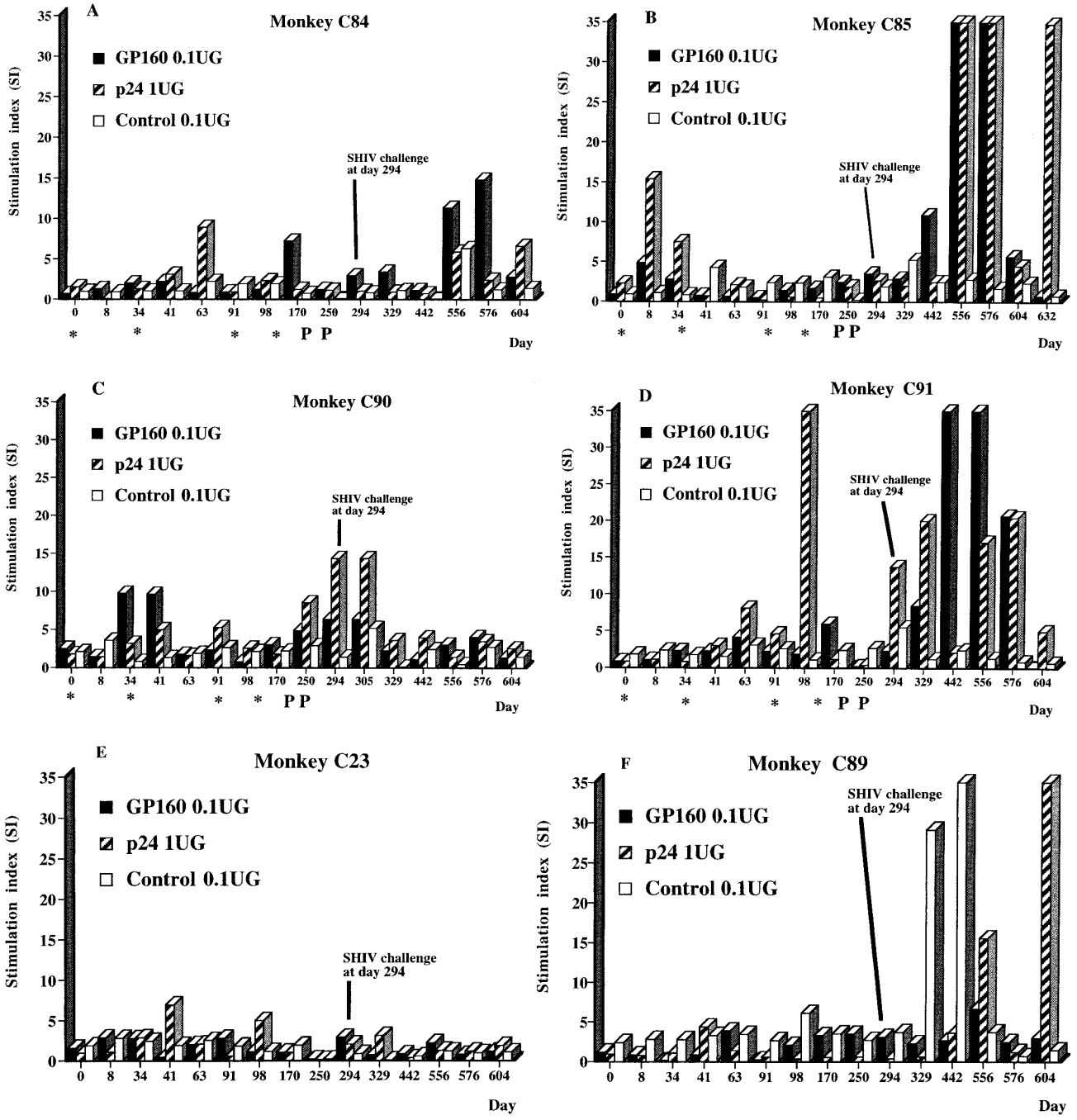

FIG 2. (A–D) Kinetics of the HIV-1 and baculovirus control antigen specific T-cell proliferation *in vitro* with peripheral blood cells from vaccinated Monkeys C84, C85, C90, and C91 during immunizations and after challenge. (E and F) T-cell proliferation *in vitro* with peripheral blood cells from two representative control monkeys, C23 and C89, before and after challenge. DNA immunization is indicated by * and protein boosts by P. Control monkeys received DNA plasmid without any inserted gene and Ribi adjuvant boosts without protein. SHIV challenge intravenously was performed at Day 294 as indicated.

IgG to all HIV-1 antigens except the Rev protein. Lymphoid cells taken from the control monkeys remained negative.

Intravenous challenge with SHIV

After SHIV challenge, virus was isolated from PBMCs of all vaccinated monkeys as well as from control DNA immunized controls. One of the vaccinated animals (C85) had the lowest post challenge levels of infected PBMCs of any challenged animal (Fig. 3). His infection was detectable by virus isolation at Day 14 and thereafter only by PCR. At 14 days postchallenge, the concentration of plasma p26 antigen was low in three of four vaccinated animals and in two of four in the control group (Fig. 4).

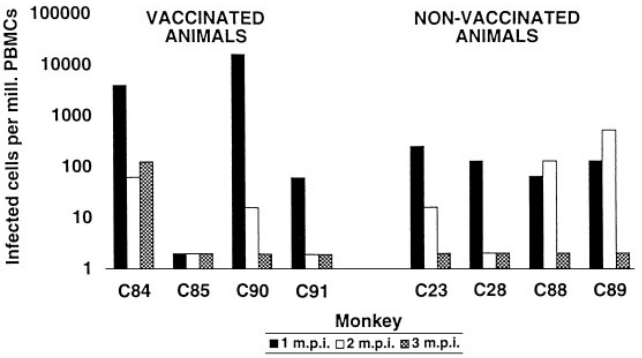

FIG. 3. Cell-associated viral loads at 1, 2, and 3 months postinfection (m.p.i) of cynomolgus monkeys following SHIV challenge.

Despite the early reductions in viral load, the vaccinated animals did not clear their infections. The trial was terminated 8 months postchallenge.

Immune responses to SHIV challenge

The humoral and cellular immune responses were characterized also after challenge. Here responses were measured to both HIV-1 and HIV-2 antigens because SIV, SHIV, and HIV-2 are closely related. In the vaccine group, anamnestic humoral and cellular immune responses appeared to both HIV-1 and HIV-2 after SHIV inoculation (Table 3). After SHIV challenge all four control monkeys made primary anti-SHIV antibodies by 12 weeks.

The four vaccinated animals showed an increase of neutralizing antibody titers to HIV-1$_{LAI}$ from 40–220 to 360–1080 due to the SHIV infection (Fig. 1). A similar effect was also seen in neutralizing titers against SHIV. In all control animals, neutralizing antibodies remained undetectable until 2 months post-SHIV challenge, when all four developed low titers against HIV-1 or against SHIV. At 1 month postchallenge, CTL activity was not detected in the control group, but low levels of $CD8^+$-dependent HIV-1env and SIVnef specific CTL responses were identified in three immunized monkeys (C85, C90, and C91).

DISCUSSION

A low dose of epidermally given DNA vaccine in combination with recombinant subunit proteins had a poor impact on SHIV replication. There was no clear difference after challenge in the virus burden of the immunized monkeys compared with the controls. Both the protein boosts and particularly the SHIV challenge had significant effects in increasing anamnestic humoral immune responses.

Our inability to induce sterilizing immunity was disappointing. The slight reduction of viral recovery in one vaccinated monkey and the weak immune responses after the DNA immunizations were probably due to the small doses of DNA used (10 μg per immunization).

Our study should be compared with those using higher doses of DNA. For example, good antibody titers have been observed in chimpanzees injected with 2 mg of plasmid DNA encoding the major hepatitis B envelope proteins (Davis *et al.,* 1996). Letvin and collaborators (Letvin *et al.,* 1997) have demonstrated that milligram doses of HIV-1 DNA combined with HIV envelope boosts protected monkeys from intravenous challenge with a nonpathogenic SHIV. Chimpanzees injected intramuscularly six times with 100 μg and twice with 1 mg of HIV-1 env and gag/pol DNA showed low-level env-specific neutralizing antibodies, and low levels of Env and gag/pol specific cytotoxicity. They were protected from HIV-1 infection (Boyer *et al.,* 1997). In HIV-infected humans, repeated 100 μg doses of HIV-1 DNA have been demonstrated to increase the HIV-specific cytotoxicity (Calarota *et al.,* 1998).

During the DNA immunization, the antibody titers in serum were low in three of the four vaccinees. However, all animals in the vaccine group that were seronegative had B cells that were capable of secreting specific antibodies *in vitro* upon antigen stimulation. This indicates that B-cell memory was induced in all vaccinated animals. The gene-gun delivery of DNA was effective in priming for the induction of high-titered antibody responses when followed by subunit booster immunizations. Neutralizing antibodies were induced, but the levels were low before challenge. In Vaccinee C85 the neutralizing antibody titer was not better than in the other animals, but when tested against a primary isolate, this was the only animal serum capable of neutralizing a primary isolate (Fig. 1B, Table 2).

The reason for failure of inducing detectable CTLs after vaccination in the present study is not known but may be due to the low dose of DNA used or the mode of administration. Even in high-dose DNA immunized animals it seems difficult to obtain $>$10% of specific target cell lysis by CTL (Boyer *et al.,* 1997). T-cell proliferations were high following the DNA immunizations. Recall T-cell

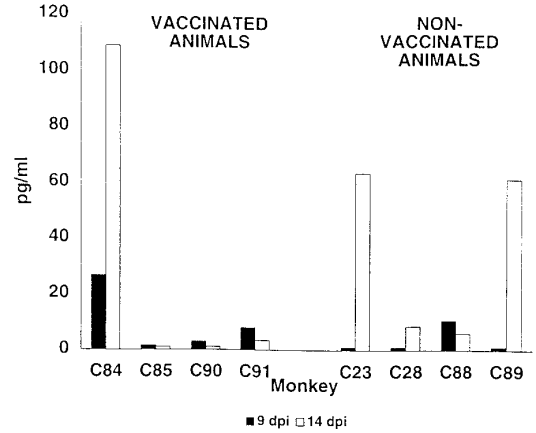

FIG. 4. Concentration of plasma p26 viral antigen at 9 and 14 days following SHIV challenge.

TABLE 3

Antibody Responses after SHIV Challenge

Group	ELISA	Antibody titers at SHIV challenge	2 weeks post challenge	1 month post challenge	3 months post challenge
Vaccine					
C84	HIV-1LAI gp120	500	500	12,500	62,500
	HIV-2 virions	500	2,500	62,500	12,500
C85	HIV-1LAI gp120	2,500	2,500	12,500	12,500
	HIV-2 virions	2,500	2,500	12,500	2,500
C90	HIV-1LAI gp120	2,500	62,500	62,500	62,500
	HIV-2 virions	2,500	12,500	62,500	12,500
C91	HIV-1LAI gp120	12,500	62,500	312,500	62,500
	HIV-2 virions	2,500	12,500	62,500	12,500
Controls					
C23	HIV-1LAI gp120	<100	<100	500	62,500
	HIV-2 virions	<100	<100	<100	2,500
C28	HIV-1LAI gp120	<100	<100	<100	12,500
	HIV-2 virions	<100	<100	2,500	2,500
C88	HIV-1LAI gp120	<100	<100	<100	12,500
	HIV-2 virions	<100	<100	<100	2,500
C89	HIV-1LAI gp120	<100	<100	<100	32,500
	HIV-2 virions	<100	<100	<100	500

proliferation responses were seen after SHIV challenge, indicating that T-cell memory was induced by the DNA immunizations. This could explain the local skin delayed type hypersensitivity-like reaction noticed in two of four vaccinees.

The present study failed to demonstrate protection against infection when the challenge inoculum was administered intravenously. Protection may be more easily obtained against mucosal than systemic challenge (Quesada-Rolander *et al.,* 1996; Putkonen *et al.,* 1997). The major mode of HIV transmission worldwide is through mucosal transmission, and we have recently demonstrated efficacy of mucosal DNA administration (Asakura *et al.,* 1997). Studies to improve the efficacy of DNA immunization by optimizing humoral and cell-mediated immune responses particularly at mucosal surfaces are therefore warranted.

MATERIALS AND METHODS

Vaccine DNAs

Plasmids carrying HIV-1LAI regulatory genes *rev, tat,* and *nef* and structural genes *p37 (gag)* and *gp 160 (env)* controlled by the human immediate early cytomegalovirus promoter were used (Wahren *et al.,* 1995; Hinkula *et al.,* 1997). The HIV-1*gag*-encoding plasmid produces a 37-kDa protein consisting of p17gag and p24gag.

Recombinant proteins and synthetic peptides

The recombinant proteins were baculovirus expressed HIV-1LAI gp160 and p24 and control antigen (MicroGeneSys Inc., Meriden, CT), Rev, Tat, and Nef (Wahren *et al.*, 1995, 1996; Hinkula *et al.,* 1997). Recombinant Nef was kindly donated by Drs. Birgit Kohleisen and Volker Erfle (GSF, Forschungscentrum Neuherberg, Oberschlossheim, Germany) and Tat by Dr. J. Karn (Medical Research Council, UK). Synthetic peptides were derived from the three regulatory proteins, Rev, Tat, and Nef of the HIV-1LAI strain, and all peptides were 20-mers with a 5-aa overlap (Hinkula *et al.,* 1997).

Viruses

Chimeric SIVmac 239, also called SHIV-4, expressing the *env, rev,* and *tat* of HIV-1IIIB (LAI strain HXBc2) (Li *et al.,* 1992) was a kind gift from Drs. J. Li and J. Sodroski (Dana-Farber Cancer Institute, Boston, MA). This SHIV chimera was grown on cynomolgus monkey peripheral blood mononuclear cells, and a cell-free virus stock was subsequently prepared. The infectious dose of the SHIV stock was determined by *in vivo* titration in cynomolgus monkeys (Berglund *et al.,* 1997). The stock contained approximately 10^4 100% monkey infectious doses (MID_{100}) of SHIV/ml. Stocks of HIV-1LAI and HIV-1SF2 were grown in human peripheral blood mononuclear cells (PBMC).

Animals, immunization, and virus challenge

Two groups of four cynomolgus monkeys (*Macaca fascicularis*) were used. Four monkeys (C84, C85, C90, and C91) received DNA immunizations by using a gene gun (Accell, Geniva, Middleton, WI) to deliver DNA-

coated gold beads to the epidermis (Pertmer *et al.,* 1995). Four doses of plasmid DNA encoding HIV-1LAI Env (gp160), Gag, Rev, Tat, and Nef proteins were given at 0, 1, 3, and 5 months. Ten micrograms of DNA was used per immunization. By the 7th and the 9th month, the animals were boosted intramuscularly with 50 μg HIV-1LAI gp160, p24, Rev, Tat, and Nef recombinant proteins mixed with Ribi adjuvant (Ribi, Immuno Chem Research Inc., Hamilton, MO). Four control monkeys (C23, C28, C88, and C89) received plasmid DNAs without inserts followed by Ribi adjuvant but no proteins. One month following the final subunit immunization, the vaccinated animals together with the four control animals were challenged intravenously with 10 MID_{100} of SHIV.

Detection of humoral immune response *in vivo*

Antibody titers to native HIV-1LAI gp120 were determined by enzyme-linked immunosorbent assays (ELISA) as previously described for HIV-2 gp125 (Nilsson *et al.,* 1995). Antibodies to the HIV-1LAI recombinant proteins and synthetic peptides representing the antigens used for immunization were also determined by ELISA. Microtiter plates (Nunc, Aarhus, Denmark) were coated with the recombinant proteins at a concentration of 0.5 μg/ml or with the synthetic peptides at 10 μg/ml (in 0.05 M carbonate buffer, pH 9.6). Coated plates were kept at room temperature overnight before use. The plates were blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 60 min and washed twice in saline with 0.05% Tween 20 before serial dilutions of animal sera were added, 100 μl/well. Plates were incubated for 2 h at 37°C, washed, and alkaline phosphatase labeled goat anti-human IgG (Sigma, St. Louis, MA) was added as tracer, 100 μl/well. One hundred microliters per well of *p*-nitrophenylenediamine was used as substrate for 30 min before the color development was measured at OD 410 nm. The color reaction was considered positive when the absorbance value was above the mean of the negative controls +2 SD. Preimmunization sera from each animal were used as negative controls.

Selected serum samples were tested for their ability to neutralize HIV-1 infectivity *in vitro.* For the neutralization assay, preimmune and postvaccination serum samples were heat inactivated at 56°C for 30 min, and serial twofold dilutions were prepared. Sera were diluted in RPMI 1640 medium supplemented with 4 mM L-glutamine, 50 IU/ml penicillin, 50 mg/ml streptomycin, and 10% inactivated fetal calf serum. Serum dilutions were incubated with 40–60 tissue culture infectious doses $(TCID)_{50}$ of two HIV-1 strains LAI and 6794 or with 5 $TCID_{50}$ of the SHIV strain used for challenge. Serum and virus mixtures were kept at 37°C for 60 min before target cells (human PHA-activated PBMC for the HIV-1 strains and human C8166 lymphoid cells for the SHIV isolate) were added, 100,000 cells/well. After 60 min at 37°C, the cells were washed twice with 200 μl RPMI 1640 medium (200 μl/well). The cells were cultured at 37°C in 5% CO_2 for 3 days when 50% of the medium was exchanged. At Day 6–7, the presence of HIV-1 p24 or SIV p26 antigen in the culture fluid was measured by capture ELISA (Sundqvist *et al.,* 1989; Thorstensson *et al.,* 1991). The neutralizing titer was defined as the reciprocal of the highest dilution resulting in 50 or 90% reduction (IC_{50-90}) of absorbance in comparison with serum samples collected prior to immunization.

Detection of HIV-1 antibodies after *in vitro* stimulation

HIV-1 DNA-vaccinated monkeys without detectable antibodies after immunization were tested for circulating B cells capable of secreting HIV-1-specific antibodies when stimulated *in vitro* with the antigens used for immunization. PBMCs (100,000/well in 200 μl RPMI 1640 supplemented with 5% inactivated fetal calf serum serum) were cultured for 72 h in triplicate wells at 37°C in 96-well, flat-bottomed cell culture plates (Nunclon, Nunc) with the antigens at concentrations of 1 μg/well. After washing, the bound Ig was assayed by the ELISAs described above.

Cellular immune response

T-cell stimulation. Peripheral blood cells (150,000 cells/well) were cultured for 6–7 days in RPMI 1640 supplemented with 4 mM L-glutamine, 50 IU/ml penicilline, 50 μg/ml streptomycin, and 10% human CMV negative AB+ serum in the presence of 0.1–1 μg antigen in triplicate wells. Medium alone was used as negative control, and phytohemagglutinin (PHA) was used as mitogen to measure unspecific cell stimulation. Fifty microliters/well of tritium-labeled thymidine was added (1 μCi) and cells were incubated for 5–6 h before the cells were harvested and thymidine incorporation was measured in a β counter. The stimulation index (SI) was calculated by dividing the mean cpm values in triplicate wells of the antigen stimulated wells with the mean cpm of medium control wells. A more than fivefold SI with whole proteins and a more than threefold SI with synthetic peptides was considered positive (Hinkula *et al.,* 1997; Calarota *et al.,* 1998).

The cytotoxic T lymphocyte (CTL) response was measured in a standard chromium release assay (Andersson *et al.,* 1996; Putkonen *et al.,* 1997). Target cells were autologous B-lymphoblastoid cell lines infected with 10 plaque forming units of recombinant vaccina virus expressing Env or Nef protein of HIV-1 or Nef protein of SIVmac32(pJ5) (Rud *et al.,* 1994). As a control, $CD8^+$ T cells were depleted by use of antibodies on magnetic Dynabeads (Dynal, Oslo, Norway).

Postchallenge tests for infection

SHIV isolation was performed by cocultivation of monkey PBMCs with PHA-stimulated human PBMCs (Nilsson *et al.,* 1995). The viral load following SHIV challenge in peripheral blood was determined by a limiting dilution assay. Serial twofold dilutions of monkey PBMCs (starting with 5×10^5 cells) were cocultivated with C8166 as indicator cells (3×10^5 cells/well) in complete RPMI medium in 24-well plates (Falcon, Becton Dickinson, NJ). Medium was changed twice a week, and culture media were assayed regularly for the presence of SIV proteins by antigen capture enzyme-linked immunosorbent assay (Thorstensson *et al.,* 1991). Detection and quantification of plasma SIV p27 antigen levels following SHIV challenge were performed by Innotest HIV antigen (Innogenetics N.V., Belgium), modified according to an assay procedure for HIV-1 p24 detection (Lyamura *et al.,* 1996). Before detection of SIV p27 antigen, plasma was acid and heat treated to dissociate immune complexes of SIV antigen and antibodies. Polymerase chain reaction (PCR) was performed in sera (Quesada-Rolander *et al.,* 1996).

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