Enhanced cellular immunity and systemic control of SHIV infection by combined parenteral and mucosal administration of a DNA prime MVA boost vaccine regimen

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The immunogenicity and protective efficacy of a DNA and recombinant modified vaccinia Ankara (MVA) vaccine administered by two different routes were investigated. DNA expressing HIV-1 IIIB *env*, *gag*, *RT*, *rev*, *tat* and *nef*, and MVA expressing HIV-1 IIIB *nef*, *tat* and *rev* and simian immunodeficiency virus (SIV) macJ5 *gag/pol* and vaccinia HIV-1 *env*, were used as immunogens. Four cynomolgus macaques received DNA intramuscularly (i.m.) at month 0 and intrarectally (i.r.) and intra-orally (i.o.) at 2 months, followed by MVA i.m. at 4 months and i.r. and i.o. at 8 months. Another group of four monkeys received the same immunogens but only i.m.. Overall, stronger cellular immune responses measured by ELISPOT and T-cell proliferation assay were detected in the group primed i.m. and boosted mucosally. Following homologous intravenous simian-human immunodeficiency virus (SHIV) challenge, one of eight vaccinated animals was completely protected. This monkey, immunized i.m. and i.r. + i.o., exhibited the highest levels of HIV Env, Nef and Tat antibodies, high HIV Tat cytotoxic T-lymphocyte activity and T-lymphocyte proliferative responses to HIV Env. Four weeks post-challenge none of the monkeys immunized i.m. and i.r. + i.o., and only two out of four animals immunized i.m., demonstrated detectable plasma viral RNA levels. In contrast, all eight control animals had demonstrable plasma viral RNA levels 4 weeks post-challenge. Thus, stronger cellular immune responses and reduction of challenge virus burden were demonstrated in animals immunized i.m. as well as mucosally, compared with animals immunized i.m. only. The breadth and magnitude of the induced immune responses correlated with protective efficacy.

INTRODUCTION

A safe and effective AIDS vaccine is urgently needed to control the HIV-1 epidemic. Many new vaccination strategies rely on DNA vaccination. Genetic vaccination has been shown to elicit durable, protective, humoral and cell-mediated immune responses in different animal models for viral, bacterial and parasitic diseases (Liu, 2003). DNA vaccines also offer many advantages over traditional live attenuated, whole killed or protein vaccines. They are safer than live vaccines, temperature-stable and simple to manufacture.

To improve the immunogenicity of DNA vaccines, different combination strategies have been used, such as inclusions of proteins, virus vectors and immunomodulator molecules. The best results have often been achieved when combining DNA vaccines with live vector-based vaccines. Poxviruses are effective expression vectors and are known to induce cellular and humoral immune responses in both animal models and humans. Modified vaccinia Ankara (MVA) is a highly attenuated vaccinia virus and has safely been used as a smallpox vaccine (Mayr & Danner, 1978) and in a pre-clinical study in immune-suppressed macaques (Stittelaar *et al.*, 2001). MVA is replication-deficient in human cells,

and has enhanced immunogenicity because of modification of certain immune evasion genes (Blanchard et al., 1998; Drexler et al., 1998). A recent study in rhesus macaques has shown that repeated rMVA immunizations alone elicited lower levels of cytotoxic T lymphocyte (CTL) but higher levels of antibodies than a DNA prime/ MVA boost regimen. However, both strategies led to similar control of a virulent mucosal simian-human immunodeficiency virus (SHIV) 89.6P challenge (Amara et al., 2001, 2002).

The ability of a multiprotein DNA/MVA vaccine, or rMVA only, to control intrarectal exposure of highly pathogenic immunodeficiency virus challenge is promising, as HIV infection is primarily sexually transmitted. Therefore, a highly desirable feature of an AIDS vaccine would be to induce mucosal immunity as a first line of defence against infection. Different routes of administration and methods of delivery of the immunogen have been shown to influence the elicited immune response. Parenteral immunizations, either by intravenous, intramuscular, subcutaneous or intradermal injections of non-replicating antigens, induce mostly systemic immune responses, whereas mucosal delivery of antigens is able to trigger both mucosal and systemic immunity. When antigens are delivered mucosally an adjuvant is generally required to generate appropriate immune responses. In rhesus monkeys mucosal immunization with an AIDS peptide vaccine formulated with an Escherichia coli toxin elicited mucosal T-cell responses and reduced virus disease after mucosal SHIV-Ku2 challenge (Belyakov et al., 2001). Besides topical application, delivery of antigen mucosally can be done simply and rapidly by high-pressure jet injectors. Intra-oral jet injection of HIV-1 DNA in solution has been shown to induce mucosal IgA in mice and activation of T cells in humans (Lundholm et al., 1999, 2002). Here, we explored the influence of different routes of delivery of a DNA prime and MVA boost regimen on inducing protective immunity against a homologous intravenous SHIV challenge. One group of monkeys was immunized by intramuscular needle injections combined with intra-oral and intrarectal jet injections, and another group of monkeys was immunized i.m. with only needle injections. Animals vaccinated mucosally as well as i.m. had better immune responses and control of the virus.

METHODS

Animals. The 16 cynomolgus macaques used in this study were housed and handled at the Primate Research Center, Swedish Institute for Infectious Disease Control, according to the guidelines of the Swedish Ethical Committee for Animal Protection. Before entering the study, animals were confirmed negative for simian immunodeficiency virus (SIV), simian T-cell lymphotropic virus and simian retrovirus type D.

Immunogens. The plasmids expressing single genes contained the HIV-1 nef (HXB3), tat, rev, RT, p37 gag (HXB2) and gp160 (BRU) genes under the control of the human cytomegalovirus (CMV) immediate early promoter, and have been described previously (Boshart et al., 1985; Hinkula et al., 1997; Isaguliants et al., 1999; Okuda et al., 1995).

Recombinant MVAs separately expressing the coding sequences for HIV regulatory proteins Tat, Rev and Nef were constructed following previously described methodology (Drexler et al., 1999). Briefly, HIV-1 LAI nef (kindly provided by Marie-Paule Kieny, Transgene, Strasbourg), tat and rev (a gift from Kai Krohn, University of Tampere) were inserted into the MVA vector plasmid pUCII LZdel P7.5 to be placed under transcriptional control of the vaccinia virus early late promoter P7.5. Transfection of these plasmids into MVA-infected chicken embryo fibroblast cells (CEF) allowed isolation of the viruses MVA-HIV-1$_{LAI}$nef, MVA-HIV-1$_{LAI}$tat and MVA-HIV-1$_{LAI}$rev, which were cloned by plaque purification on CEF screening for transient co-expression of the E. coli lacZ marker gene. Another recombinant MVA expressing the gag–pol coding sequences of SIV mac251 32H clone pJ5 (Rud et al., 1994) was previously described (Nilsson et al., 2001), and served as a vaccine for the delivery of SIV Gag-Pol antigens.

Aliquots of virus preparations contained 5×10^8 infectious units ml^{-1} and were stored at $-70\,^{\circ}$C. The vector vaccine preparations were tested in tissue culture infections for their capacity to synthesize HIV-1 LAI or SIV macJ5 antigens monitoring cell lysates for Nef, Tat, Rev or Gag–Pol proteins by Western blot analysis (data not shown). Vaccinia HIV-1 BRU gp120 65.0 was constructed by Albert Osterhaus (now at Erasmus Medical Centre, Rotterdam) and Jaap Goudsmit, (now at Crucell, Leiden), the Netherlands.

Immunizations and SHIV infection. Table 1 shows more detailed information on the immunization regimen. Four monkeys (group A) were immunized with 500 µg of each DNA (HIV-1 nef, tat, rev, gag, RT, gp160) construct intramuscularly (i.m.) at month 0 and intrarectally (i.r.) and intra-orally (i.o.) at 2 months. These immunizations were followed by 10^8 p.f.u. of each recombinant MVA (HIV-1 nef, tat, rev and SIV gag/pol) and 500 µg of each DNA (HIV-1 rev, env) together with 250 µg DNA (HIV-1 gag) i.m. at 4 months and the same MVA recombinants and vaccinia HIV-1 env i.r. and i.o. at 8 months. Because of difficulties in obtaining DNA expressing SIV gag/pol, we used DNA expressing HIV-1 gag and RT. No recombinant MVA expressing HIV-1 env was available, therefore we used DNA HIV-1 env for the third immunization and a vaccinia HIV-1 env for the fourth immunization. Another group of four monkeys (group B) was immunized with the same antigens i.m. at 0, 2, 4 and 8 months. Granulocyte-macrophage colony-stimulating factor (GM-CSF, 75 µg; Leukomax) was given 15 min before DNA vaccination and at the same location to all macaques at 2 months. A control group of four monkeys was given a control plasmid pkCMV i.m. at month 0 and i.r. at 2 months, and non-recombinant MVA F6(28.1) i.m. at 4 and 8 months. The i.r. and i.o. immunizations, three to five 0·2 ml injections for each dose, were delivered with a SyriJet Mark II jet injector (Mizzy). The i.o. jet injections were placed bilaterally in the cheeks in the oral cavity. The i.m. injections were given with needle injections in the hind leg.

One month after the last MVA boost, all vaccinated monkeys and four naive controls were challenged intravenously with approximately 25 MID$_{50}$ (50% monkey infectious doses) of a homologous SHIV-4 expressing SIV mac239 gag, pol, vif, vpx, vpr and nef genes and HIV-1$_{LAI}$ env, tat, rev (Berglund et al., 1997).

Preparation of peripheral blood mononuclear cells (PBMC). PBMC were isolated from EDTA blood using FicollPaque (Pharmacia-Upjohn) gradient centrifugation and either used immediately or cryopreserved in liquid N$_2$.

Stimulation of PBMC for enzyme-linked immune spot (ELISPOT) assay. PBMC were either infected with recombinant poxviruses or stimulated with antigen or mitogen in tubes for 1–2 h at 37 $^{\circ}$C in 7·5% CO$_2$ prior to addition to the plates.

Table 1. Immunization schedule

i.m., Intramuscular; i.r., intrarectal; i.o., intra-oral.

Group	Immunization 1	Dose/route	Immunization 2	Dose/route	Dose/route	Immunization 3	Dose/route	Immunization 4	Dose/route	Dose/route
A (D71–74)	DNA HIV-1 *nef, tat, rev, gag, env, RT*	3 mg/i.m. 0·5 mg per plasmid	DNA HIV-1 *nef, tat, rev, gag, env, RT*	3 mg/i.r.* 0·5 mg per plasmid	3 mg/i.o.†	MVA HIV-1 *nef, tat, rev*	3×10^8 p.f.u./i.m.	MVA HIV-1 *nef, tat, rev*	$1·5 \times 10^8$ p.f.u./i.r.	$1·5 \times 10^8$ p.f.u./i.o.
						MVA SIV *gag/pol*	1×10^8 p.f.u./i.m.	MVA SIV *gag/pol*	$0·5 \times 10^8$ p.f.u./i.r.	$0·5 \times 10^8$ p.f.u./i.o.
						DNA HIV-1 *rev, gag, env*	1·25 mg/i.m.	Vac HIV-1 *env*	$0·5 \times 10^8$ p.f.u./i.r.	$0·5 \times 10^8$ p.f.u./i.o.
B (D75–78)	DNA HIV-1 *nef, tat, rev, gag, env, RT*	3 mg/i.m. 0·5 mg per plasmid	DNA HIV-1 *nef, tat, rev, gag, env, RT*	3 mg/i.m.* 0·5 mg per plasmid		MVA HIV-1 *nef, tat, rev*	3×10^8 p.f.u./i.m.	MVA HIV-1 *nef, tat, rev*	3×10^8 p.f.u./i.m.	
						MVA SIV *gag/pol*	1×10^8 p.f.u./i.m.	MVA SIV *gag/pol*	1×10^8 p.f.u./i.m.	
						DNA HIV-1 *rev, gag, env*	1·25 mg/i.m.	Vac HIV-1 *env*	1×10^8 p.f.u./i.m.	
C (D79–81)	Control plasmid	0·5 mg/i.m.	Control plasmid	0·5 mg/i.r.*		Control MVA	4×10^8 p.f.u./i.m.	Control MVA	4×10^8 p.f.u./i.m.	
						Control plasmid	0·5 mg/i.m.			
D (D82–86)	–		–			–		–		

*GM-CSF was given to all macaques 15 min before and at the same location as the following DNA immunization.

†i.o. immunization given 2 days after i.r. immunization.

The following poxviruses were used for infection: MVA expressing HIV-1 (LAI) *tat* or *nef*, ALVAC HIV-1 encoding gp120 (MN); transmembrane anchor regions of gp41 (IIIB); Gag and protease (IIIB); Nef CTL domains; pol CTL epitopes (IIIB) (vCP 300), ALVAC SIV encoding *gag/pol* (mac142) (vCP172) at m.o.i. 5, 2 and 5, respectively. MVA wild-type and parental ALVAC were used at m.o.i. 1–5 to determine reactivity against the vector.

T-helper cell responses were measured using the following antigens: HIV-1 IIIB and HIV-2$_{6669}$ whole virus lysate (Biberfeld *et al.*, 1988) at a concentration of 5 µg ml^{-1} and recombinant gp120 (LAV) (Protein Sciences) at a concentration of 0·5 µg ml^{-1}. Because of cross-reactivity with SIV, HIV-2 viral lysate was used in this study.

For peptide stimulation, PBMC were pulsed directly with the peptide pools in the plate at a final concentration of 2·5–5 µg ml^{-1} per peptide. The HIV-1 LAI nef (1–24), tat (1–12), env 1 (1–40), env 2 (41–80), env 3 (81–122) peptide pools consisted of 15 mers overlapping by 8 aa (aa sequences derived from pKCMVnef, pKCMVtat and pKCMVgp160 B plasmids; Kjerrstrom *et al.*, 2001; Ljungberg *et al.*, 2002). The synthetic peptides above were purchased from Thermo Hybaid. The SIV gag (ARP 714.1–22, EVA 775.1–13, EVA 776.1–14) and SIV nef (EVA 777.1–25) pools consisted of 20 mers overlapping by 10 aa (kindly provided by Harvey Holmes, Programme EVA). Peptides were solubilized in DMSO at 10 mg ml^{-1} and stored in small aliquots at −70 °C. Medium with DMSO corresponding to the highest concentration in the peptide solution was used as a mock control. Depletion of CD8$^+$ T cells was accomplished by incubating the PBMC with magnetic beads covered with anti-CD8mAb (Dynabeads). Two cell fractions were obtained after CD8-depletion, one depleted of CD8$^+$ T cells and the other containing CD8$^+$ T cells + beads (enriched CD8$^+$ T cells).

IFN-γ ELISPOT assay. ELISPOT was used to determine the frequency of antigen-specific IFN-γ-producing cells as described previously (Makitalo *et al.*, 2002). Responses were considered to be positive if the number of spot-forming cells (SFC) was at least twice that of the control, and >50 SFC per 10^6 PBMC.

Proliferation assay. Freshly isolated PBMC ($1·5 \times 10^5$ cells per well) were cultured in triplicate with or without antigen or phytohaemagglutinin (PHA) in complete medium in 96-well flat-bottom plates at 37 °C in 7·5% CO$_2$ for 6 days, and thereafter pulsed with 1 µCi (37 kBq) per well of [^{3}H]thymidine for 6 h. The antigens used were whole virus lysate of HIV-1 IIIB and HIV-2$_{6669}$ and recombinant HIV-1 gp160 (Protein Sciences) at final concentrations of 5 and 0·5 µg ml^{-1}, respectively. Cultures were harvested and the thymidine incorporation was measured with a 1450 MicroBeta PLUS counter (Wallac Sverige AB). Stimulation index (SI) was calculated by dividing mean [^{3}H]thymidine incorporation in antigen-stimulated wells by mean incorporation in control wells. SI = 2·0 was considered positive when proliferation against HIV-2 whole viral lysate was measured. For HIV-1 IIIB whole viral lysate and HIV-1 gp160, responses were defined as positive when SI exceeded 3·0 and 5·0, respectively (Berglund *et al.*, 1997).

Effector cells. For stimulation of effector cells, autologous B-lymphoblastoid cell lines (BLCL) were infected separately with each recombinant poxvirus, MVA encoding HIV-1 *tat* and HIV-1 *nef*,

ALVAC (vCP 300) and SIV *gag/pol* (vCP172) at m.o.i. 5, 2 and 5, respectively. After infection the cells were washed and incubated overnight at 37 °C. The next day the infected BLCL were pooled, washed and fixed with 1·5% (w/v) paraformaldehyde in PBS for 15 min and thereafter treated with 0·2 M glycine in PBS for 15 min. In one experiment, effector cells were stimulated with BLCL infected with ALVAC vCP300 only. Stimulators were washed three times and added to monkey PBMC at a responder:stimulator cell ratio of 15:1. After 2–4 days recombinant human interleukin 2 (rhIL2, 40 U ml⁻¹) was added to the cells. On day 7 the cultures were restimulated by addition of fixed autologous stimulator cells as above.

When peptide-sensitized PBMC were used, PBMC (5×10^6) were prepulsed with an HIV-1 Tat peptide pool (EVA 770CS) for 60–90 min in 100 µl complete medium, then diluted up to $2·5 \times 10^6$ cells ml⁻¹ in complete medium (10 µg final peptide concentration ml⁻¹) supplemented with 25 ng rhIL7 ml⁻¹ (R&D Systems). After 2–4 days rhIL2, 40 U ml⁻¹, was added to the cells. Fresh medium containing rhIL2, 20 U ml⁻¹, was added twice weekly to all cell cultures and CTL activity was measured after 12–16 days.

⁵¹Cr release cytotoxicity assay. Recombinant poxvirus-infected or peptide-sensitized autologous BLCL were used as target cells. For peptide-sensitized targets, BLCL were enumerated and pulsed with peptide pools as described above. As a mock target, BLCL were pulsed with medium containing 0·2% DMSO. A standard chromium release assay was used with different effector:target cell ratios (12:1–25:1). Percentage-specific lysis was calculated as [(sample release−spontaneous release)/(total release−spontaneous release)] × 100. In assays where MVA-infected targets were used, a 40-fold excess of unlabelled MVA (0·1 m.o.i.) infected targets (cold targets) was added to the cultures to reduce vaccinia virus-specific cytolysis present in the effectors, as previously described (Nilsson *et al.*, 2001). Antigens used were the same as described above for stimulation of effector cells with the addition of MVA wild-type and parental ALVAC for control of vector reactivity.

Antibody assays. A commercial microparticle enzyme immuno-assay (IMx HIV-1/HIV-2 III Plus; Abbott) was used for detection of antibodies to HIV-envelope (gp41 and gp120) in serum according to the manufacturer's instructions. Serum samples were tested for IgG responses to the HIV-1 regulatory proteins Nef and Tat (kindly provided by Jonathan Karn, Medical Research Council) using ELISA as described elsewhere (Hinkula *et al.*, 1997; Putkonen *et al.*, 1998).

Collection of rectal washes and detection of HIV-1 IIIB, gp120- and SIV p27-specific IgG and IgA by ELISA was performed as described previously (Nilsson *et al.*, 2001). Native *Galanthus nivalis* agglutinin purified HIV-1 IIIB gp120 (Gilljam, 1993) and recombinant SIV mac p27 (kindly provided by I. Jones through the NIBSC's centralised facility for AIDS) were used at 1 and 0·5 µg ml⁻¹, respectively, for coating. Saliva samples were collected using two different collection devices according to the manufacturers' instructions (Omnisal and OraSure). To enhance detection of virus-specific IgA, IgG was removed by use of protein A Sepharose (Pharmacia–Amersham) prior to analysis. A buffer containing a protease inhibitor cocktail (Sigma) was used and all purification steps were performed on ice.

Assays for detection of virus and virus load determination. For virus isolation, monkey PBMC were co-cultivated with PHA-stimulated human PBMC as previously described (Nilsson *et al.*, 1995). Primers in the long terminal repeat and gag region of HIV-2 and in the env region of HIV-1 were used for the nested PCR (Walther-Jallow *et al.*, 1999). Levels of viral RNA in plasma samples were measured using a quantitative competitive RT-PCR assay with a lower detection limit of 40 RNA equivalents ml⁻¹ plasma (ten Haaft *et al.*, 1998).

Statistical analysis. Plasma virus loads taken 2, 4 and 8 weeks post-challenge were compared between groups by Mann–Whitney U-test for non-parametric observations. A value of $P < 0·05$ was considered significant.

RESULTS

Vaccine-induced antigen-specific T-cell responses to proteins

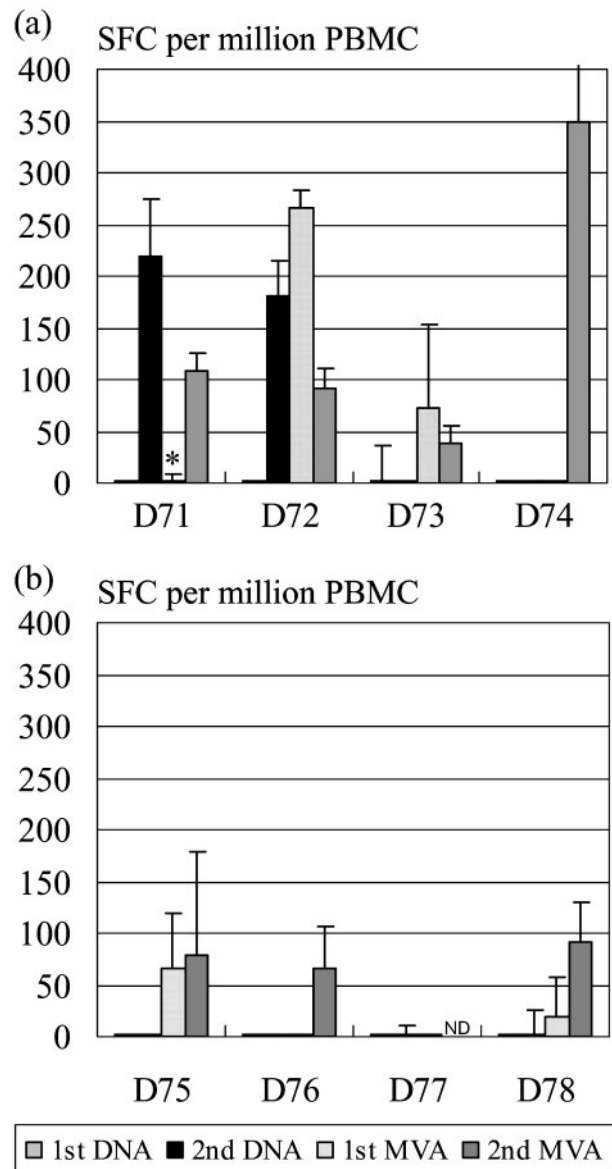T-cell-mediated IFN-γ responses to HIV-1 and HIV-2 viral lysate and rgp120 were assessed by an ELISPOT

Fig. 1. IFN-γ ELISPOT responses to HIV-1 whole viral lysate. PBMC were obtained 2 weeks after each immunization from monkeys immunized intramuscularly and mucosally (a) and monkeys immunized intramuscularly only (b). *, Poor phyto-haemagglutinin stimulation; ND, not done.

assay using cryopreserved PBMC from vaccinated animals 14 days after each immunization. In addition, T-cell proliferative responses were analysed 14 days before challenge against HIV-1 and HIV-2 whole viral lysate (containing the structural proteins env, gag and pol) and rgp160.

Group A monkeys, immunized both i.m. and mucosally, mounted earlier and higher responses to HIV-1 whole viral lysate than the animals in group B, immunized i.m. only. After only two DNA immunizations positive IFN-γ responses were detected in two animals (D71 and D72), and after the second MVA boost also in a third animal (D74). In group B animals, positive responses were detected in PBMC against HIV-1 lysate in one monkey (D75) following the first MVA boost and in two monkeys (D76 and D78) after the second MVA boost (Fig. 1). No IFN-γ response to HIV-2 lysate could be detected at any time point in animals in group A, and only one monkey (D76) in group B showed a weak HIV-2-specific IFN-γ response (72 SFC per 10^6 PBMC) after the second MVA boost (data not shown). IFN-γ secretion in response to rgp120 was detected in PBMC from only two monkeys (D71 and D72) (272 and 240 SFC per 10^6 PBMC) in group A, after the second DNA immunization and also after MVA boost (112 and 176 SFC per 10^6 PBMC) (data not shown).

When measuring HIV-1-specific lymphocyte proliferation to HIV-1 lysate and rgp160, positive responses were detected in three monkeys (D71, D72 and D74) in group A. Two monkeys (D77 and D78) in group B had proliferative responses to HIV-1 whole viral lysate, and one of these animals (D78) also responded to rgp160. The proliferative responses to rgp160 were analysed at two different laboratories at this time point and the positive results were concordant (Table 2). No proliferative responses to HIV-2 viral lysate could be detected in either group of monkeys (data not shown).

Vaccine induction of CD8+ T-cell responses

CD8+ T-cell activity in vaccinated animals was assessed by a functional chromium-release assay and a poxvirus vector-based ELISPOT assay before and 2 weeks after each immunization.

CTL assay. CTL responses against MVA HIV-1 Tat, ALVAC HIV-1 gp120; Gag/Pol; Nef (vCP300) and ALVAC SIV Gag/Pol (vCP172) were measured using cryopreserved PBMC stimulated with formaldehyde-fixed autologous B-cell lines infected with corresponding poxvirus constructs.

Only one animal (D71) in group A, immunized i.m. and mucosally, showed an HIV-1 Tat-specific CTL activity after MVA boost (Fig. 2a). In animals immunized i.m. only, two

Table 2. Immune responses 2 weeks prior to challenge and plasma virus load 2 weeks post-challenge

i.m., Intramuscular; i.r., intrarectal; i.o., intra-oral; positive reactions in bold type; ND, not done.

			i.m. × 2 and (i.o. + i.r.) × 2				i.m. × 4			
			D71	D72	D73	D74	D75	D76	D77	D78
T-helper cell responses	Proliferation	HIV-1 IIIB viral lysate (SI)	**8·6**	**63**	1·8	**4·4**	1·7	1·6	**7·8**	**32·9**
		rgp160 (SI)	**23·9**	**17·2**	2·1	**6·3**	1·0	1·0	1·3	**13·5**
	IFN-γ ELISPOT	HIV-1 IIIB viral lysate (SFC per 10^6 PMBC)	**108**	**92**	36	**416**	80†	**64**	ND	**92**
		rgp120 (SFC per 10^6 PBMC)	**112**	12	24	0	0	36	ND	52†
CD8+ T-cell responses*	IFN-γ ELISPOT CTL	vCP 300 (SFC per 10^6 PBMC)	12	**2523**	**144**	**84**	48	4	8	0
		HIV-1 Tat (% lysis)	**22·5**	8·4	8·7	5·5	**17·6**	**26·4‡**	15·8§	8·3
		Tat peptides (% lysis)	**43·4**	0·1	ND	1·0	8·1	ND	ND	ND
Humoral responses	Serum IgG	gp41, gp120 (signal per cut off)	**14·28**	<1	<1	<1	<1	<1	<1	<1
		rNef (titre)	**1250**	**110**	**220**	**750**	**110**	**70**	**125**	**200**
		rTat (titre)	**700**	**120**	<50	**110**	<50	**70**	**110**	**210**
Viral load	Plasma RNA	Equivalents per ml	<40	<40	$1·2 \times 10^3$	$2·0 \times 10^2$	$2·9 \times 10^3$	$5·3 \times 10^3$	$5·0 \times 10^4$	$8·8 \times 10^3$

*Mainly CD8+ T-cell responses.
†Not positive due to high medium control.
‡PBMC from day of challenge.
§Not positive due to high zero point value.

monkeys had CTL activity against Tat, D76 appearing after the first DNA inoculation and D75 appearing after the MVA boost (Fig. 2b). As the sensitivity of the CTL assay

(a) specific lysis (%)

(b) specific lysis (%)

☐ day 0 ▦ 1st DNA ■ 2nd DNA ▤ 1st MVA ▨ 2nd MVA

(c) specific lysis (%)

■ medium
☐ Tat peptide pool

may have been reduced by the use of MVA vectors and cold targets, a pool of Tat peptides was used to assess cytotoxic activity in PBMC from four monkeys 14 days before challenge. One animal (D71) showed high specific lysis to Tat peptides at that time point and another animal (D75) showed weak reactivity (although high background), while the other two monkeys (D72 and D74) showed no specific reactivity (Fig. 2c). This was consistent with the earlier findings in these animals of CTL reactivity against target cells infected with MVA HIV-1 Tat (Fig. 2a, b).

No cytolytic T-cell activity was detected in PBMC from vaccinated animals against HIV-1 (vCP300) or SIV (vCP172). To exclude the possibility that stimulation of effector cells with a pool of BLCL infected with different poxviruses was less efficient than stimulation with a single vector, an additional CTL assay with effector cells stimulated with only ALVAC vCP300 was performed. No CTL activity against vCP300 could be detected at this time point (data not shown).

IFN-γ ELISPOT. PBMC infected with poxvirus vectors ALVAC HIV-1 (vCP300), SIV (vCP172) and MVA HIV-1 Tat were also assessed for their ability to secrete IFN-γ in an ELISPOT assay. Only animals immunized both i.m. and mucosally showed IFN-γ production in response to vCP300 (Fig. 3a, b). Positive responses were shown in one animal (D74) after the first DNA inoculation, and in two animals (D71 and D72) after the second DNA inoculation. Following the third and fourth immunizations, three animals (D72, D73 and D74) in group A continuously produced IFN-γ in response to vCP300. Depletion of CD8+ T cells demonstrated that the IFN-γ response detected in the poxvirus-based ELISPOT assay was CD8+ T-cell-dependent (data not shown).

Neither group A nor group B macaques showed responses to SIV Gag/Pol before challenge (data not shown). Antigen-specific IFN-γ secretion was also not detectable in response to MVA HIV-1 Tat because of the high background induced by the MVA vector.

Overlapping 15–20 mer peptide pools of HIV-1 Tat, Nef and Env and SIV Gag and Nef were used to measure IFN-γ secretion in PBMC 2 months after the third immunization. The number of monkeys responding to the peptide pools were: group A, 3/4 to HIV-1 Tat; 4/4 to HIV-1 Nef; 4/4 to any of the HIV-1 Env pools; 1/4 to SIV Gag and 1/4 to SIV Nef; group B, 1/4 to HIV-1 Tat; 3/4 to HIV-1 Nef; 1/4 to any of the HIV-1 Env pools; 3/4 to SIV Gag; 2/4 to SIV Nef.

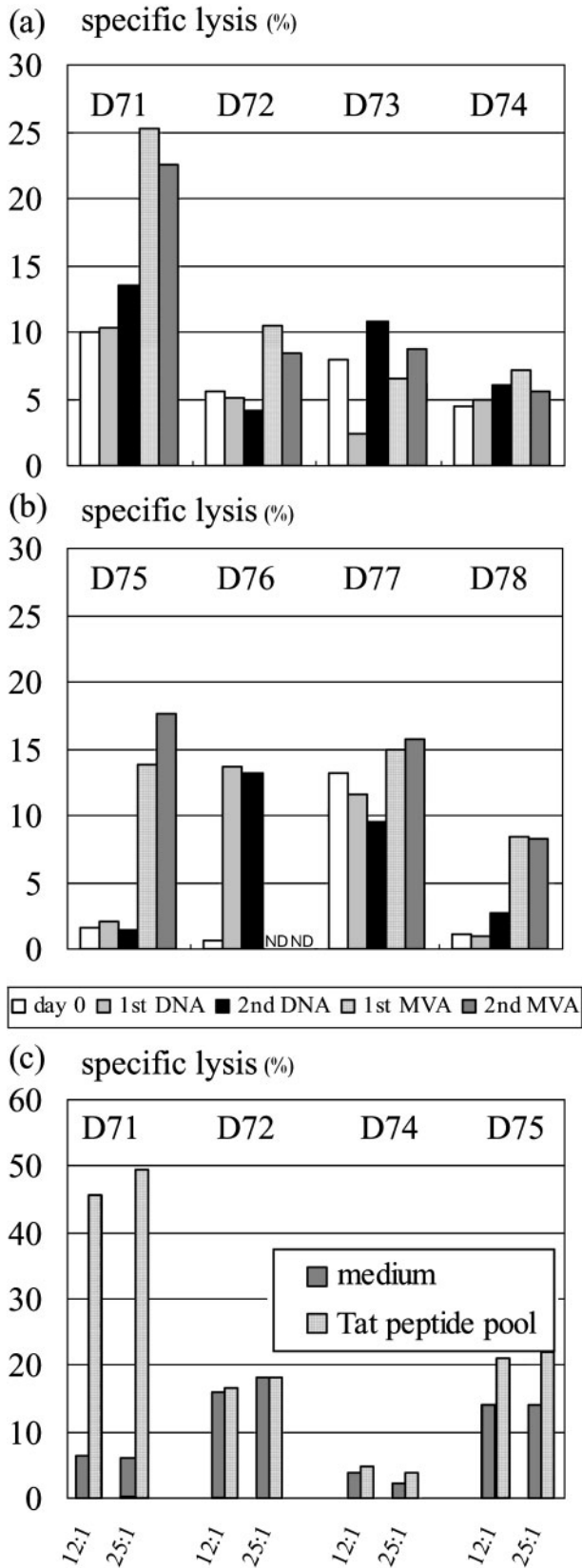All four monkeys in group A responded to at least three

Fig. 2. HIV-1 Tat-specific CTL responses measured before immunization and 2 weeks after each immunization in monkeys immunized intramuscularly and mucosally (a) and intramuscularly only (b). Effector : target cell ratio 25 : 1. (c) CTL responses against an HIV-1 Tat (EVA 770CS) peptide pool in four monkeys 2 weeks before challenge. ND, Not done.
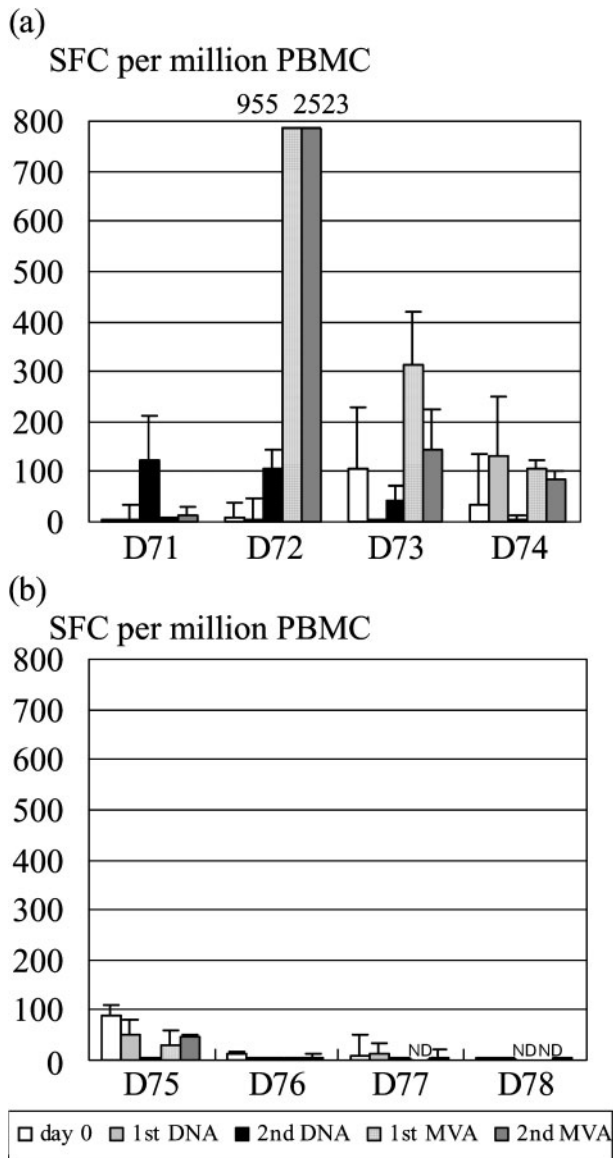
(a)

SFC per million PBMC

(b)

SFC per million PBMC

☐ day 0   ☐ 1st DNA   ■ 2nd DNA   ☐ 1st MVA   ■ 2nd MVA

Fig. 3. IFN-γ ELISPOT responses to ALVAC vCP300. PBMC were obtained before immunization and 2 weeks after each immunization from monkeys immunized intramuscularly and mucosally (a) and intramuscularly only (b). ND, Not done.

out of seven peptide pools with a range of 57–697 SFC per 10^6 PBMC (Fig. 4). Three monkeys in group B showed responses of a lower magnitude ranging from 52–155 SFC per 10^6 PBMC.

CD8$^+$ T-cell dependence of the IFN-γ response was determined in six animals, three in group A and three in group B, by CD8$^+$ T-cell depletion. Removal of CD8$^+$ T cells reduced the number of IFN-γ-secreting cells. Only monkey D72 still exhibited some IFN-γ production in response to Nef after CD8$^+$ T-cell depletion. Interestingly, when enriched CD8$^+$ T cells were used responses not detected with whole PBMC were observed. Thus three monkeys

(D71, D77 and D78) demonstrated CD8$^+$ T-cell reactivity against Tat when enriched CD8$^+$ T cells were used. Two of these monkeys (D77 and D78) also showed some HIV-1 Env reactivity in the CD8$^+$ T-cell-enriched fractions.

Humoral immune responses

HIV-1 Env-specific serum antibodies were measured by a commercial microparticle enzyme immunoassay prior to and 2 weeks after each immunization. Monkey D71 was the only animal that developed antibodies to HIV-1 envelope proteins, which appeared after the second DNA immunization (signal/cut off = 3·30) and were further boosted after the following immunizations (signal/cut off = 7·92 and 14·28, respectively).

Serum antibodies to HIV-1 Nef and Tat were investigated by ELISA at 1 and 2 months after the first MVA and 2 weeks after the second MVA immunization. Weak systemic IgG responses against the regulatory HIV-1 proteins Nef and Tat were seen in sera from all vaccinated monkeys after the first MVA. In two animals (D71 and D74), a moderate increase of antibodies to Nef was seen after the second MVA immunization (Fig. 5). The presence of specific IgG and IgA antibody responses to HIV-1 gp120 and SIV rp27, in saliva and rectal washes collected 14 days after each immunization, was determined by ELISA. No antigen-specific IgG or IgA antibodies of mucosal origin were observed in the immunized animals.

Outcome of homologous SHIV challenge in relation to immune responses prior to challenge

The animals were challenged intravenously with SHIV-4, 1 month after the last MVA immunization. Infection was monitored by virus isolation, quantification of plasma viral RNA and DNA PCR for detection of provirus from PBMC 2 weeks, 1 month and 2 months after challenge. One monkey (D71) in group A, vaccinated i.m. and mucosally, was consistently negative in all three assays. By 2 weeks post-challenge, two out of four monkeys in group A had RNA levels below the detection limit, whereas all naive control monkeys had almost 1 log higher RNA virus load than any of the vaccinated animals (Fig. 6). Statistical analysis demonstrated that monkeys immunized i.m. and i.o. + i.r. had significantly ($P = 0.0209$) lower virus load at 2 weeks post-challenge than monkeys immunized i.m. only and sham-vaccinated controls ($P = 0.043$) (Fig. 6c).

One month after challenge, all four animals in group A (Fig. 6a) and two of four animals in group B (Fig. 6b) had RNA levels below the detection limit (< 40 RNA equivalents ml^{-1}), whereas all eight control animals had demonstrable plasma viral RNA levels (Fig. 6). Both group A and B animals had significantly lower virus load at 2 and 4 weeks post-infection than naive controls ($P = 0.0209$) (Fig. 6d). No significant difference in virus load was noted between vaccinated controls and naive controls.

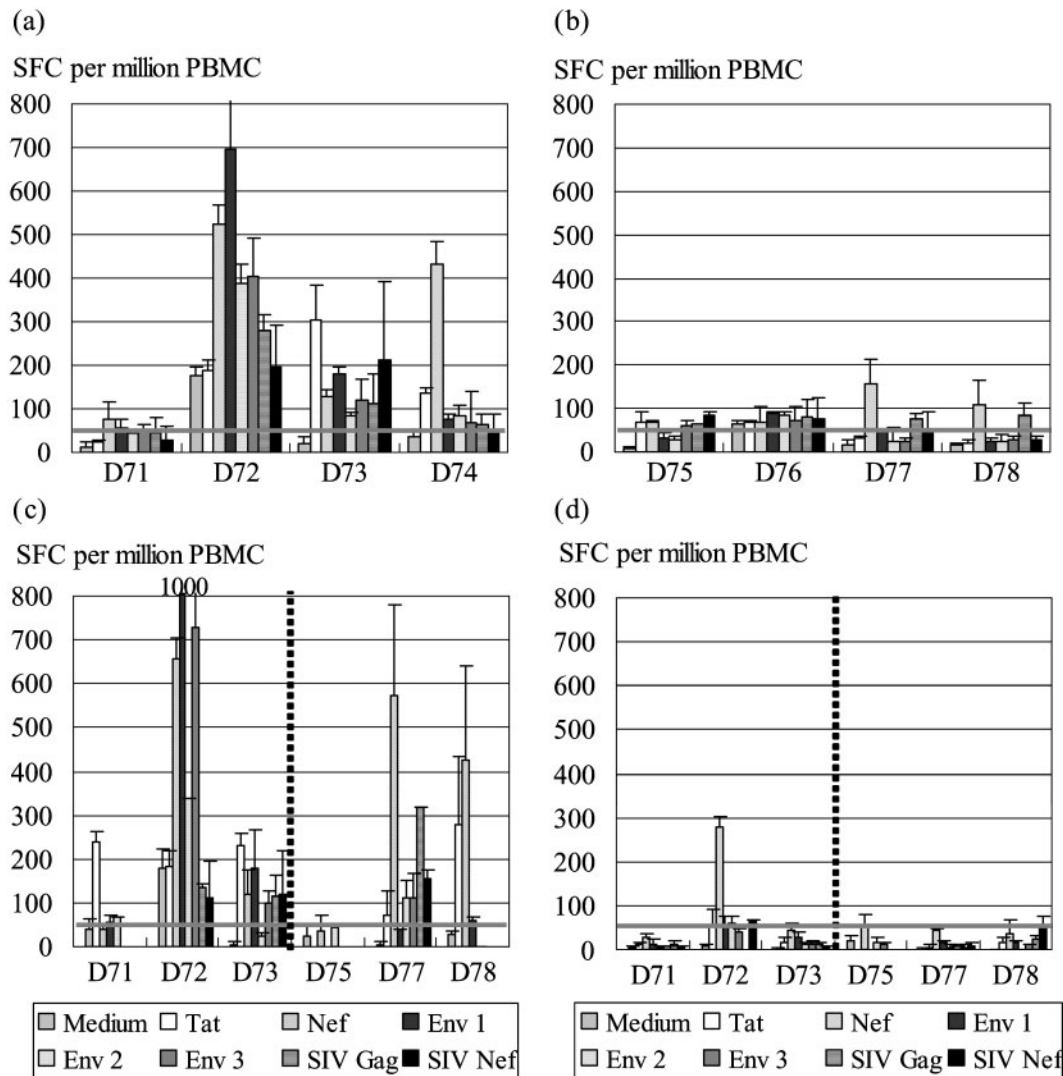Virus was isolated from PBMC 1 month post-challenge in

Fig. 4. Breadth of DNA/MVA-induced IFN-γ responses. Monkey PBMC collected 2 months after the first MVA were cultured in the presence of peptide pools in an ELISPOT assay. Monkeys immunized intramuscularly and mucosally (a) and intramuscularly only (b). PBMC from six animals were depleted of CD8⁺ T cells; the fraction with CD8⁺ T cells (c) and the fraction depleted of CD8⁺ T cells (d) were assayed in an ELISPOT against the same peptide pools.

2/4 monkeys in group A and 3/4 monkeys in group B. At 2 months all vaccinated monkeys were isolation-negative, whereas 5/8 controls were still virus isolation-positive. Proviral DNA was demonstrable at 6 months post-challenge in all animals except in the only protected monkey (D71). The low replication capacity of this non-pathogenic SHIV-4 stock has been described previously (Quesada-Rolander et al., 1996).

Comparison of the immune responses at challenge with the plasma RNA levels 2 weeks post-challenge demonstrated a trend of correlation between type of immune responses and protection (Table 2; Fig. 6). The completely protected monkey (D71) immunized i.m. + i.r. + i.o. showed a broad cellular immune response including lymphocyte proliferation, IFN-γ production and CTL, and it also had antibody

responses. Another monkey (D72) in the same group with the highest levels of CD8⁺ T-cell-dependent IFN-γ responses and strong lymphocyte proliferative responses displayed no plasma viral RNA at any time point. In contrast, monkey (D77) (i.m.) with the highest virus load of all vaccines had only a lymphocyte proliferative response prior to challenge.

DISCUSSION

We have shown previously that a Semliki Forest virus prime and MVA boost vaccine regimen induces stronger SIV-specific immune responses than MVA alone (Nilsson et al., 2001). In this study, we evaluated the immunogenicity and protective efficacy of another prime boost
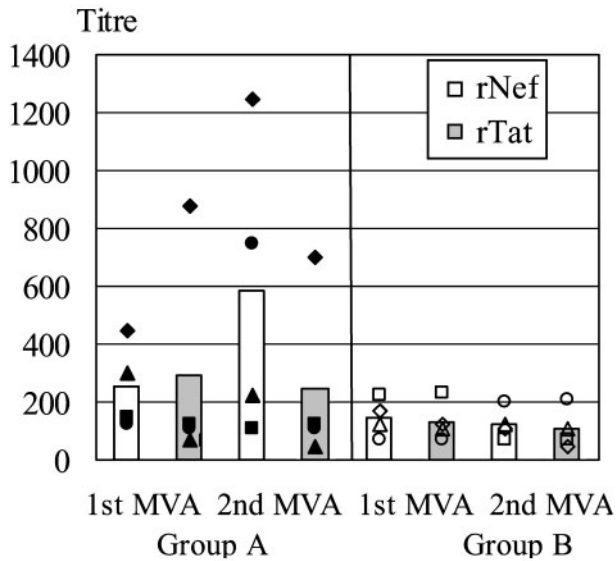
Fig. 5. Serum antibody responses to rNef and rTat in vaccinated animals as determined by ELISA, 1 month after first MVA and at 14 days after second MVA. Hatched bars, mean values for each group.

strategy using multiprotein DNA and MVA-based HIV/SIV vaccines by combining administration via parenteral (i.m.) and mucosal (i.o. + i.r.) routes.

The stronger immune responses and the more efficient virus control in animals immunized both parenterally and mucosally, in contrast to animals immunized mucosally only, may be a function of improved delivery of the plasmid and recombinant poxvirus antigens. Besides i.m. needle injection, we used a high-pressure jet injector (Syrijet) to immunize monkeys i.o. and i.r. The jet injection allows more cells to be targeted with DNA plasmids, and the mucosa provides a target tissue which contains large amounts of antigen-presenting cells and immunocompetent cells. In a previous study, mucosal jet injection of plasmid DNA in HIV-1-infected individuals triggered a Th-1-like immune response both locally and systemically (Lundholm *et al.*, 2002). Delivery of DNA i.m. with a similar jet-injection device (Biojector) was more effective in inducing immune responses and protection against a malaria vaccine than i.m. needle injection (Rogers *et al.*, 2001; Wang *et al.*, 2001). In another study with a multiprotein DNA/MVA vaccine, the Biojector was used to deliver DNA i.m. and intradermally in rhesus macaques and induction of high levels of virus-specific T cell responses was demonstrated (Amara *et al.*, 2001). Although no complete protection was seen after a mucosal challenge, the elicited immune responses in those monkeys controlled the highly pathogenic SHIV-89.6P.

The monkeys in our study immunized both i.o. and i.r. received 6 mg DNA, 3 mg at each location, while the i.m. immunized monkeys received 3 mg. The higher dose may

have increased the immune responses in this group of monkeys, although we believe that the route and method of administration may be of equal importance. In the study by Amara *et al.* (2001) two different doses of DNA were used and, while the ten times higher dose increased both the cellular and humoral immune responses, no significant effect on protection was observed.

All the immunogens used in our study appeared to be immunogenic, although the use of HIV-1 Gag and RT in the DNA immunization and SIV Gag/Pol in the MVA may explain the limited responses against these antigens.

In our study, no mucosal antibodies could be detected in either group of monkeys and mucosal CTL activity was not investigated. An intravenous challenge was used as no SHIV stock was available for mucosal challenge. Thus, future studies will address the ability of this immunization strategy to induce mucosal T-cell responses and protection against a mucosal challenge.

The importance of cellular immunity for containing virus replication in humans and monkeys has been demonstrated in several studies. Emergence of virus-specific CTL has been demonstrated to contain HIV-1 and SIV replication during primary viraemia (Koup *et al.*, 1994; Kuroda *et al.*, 1999). CTL escape mutants have been detected in primary HIV-1 infection (Borrow *et al.*, 1997). Furthermore, monkeys temporally depleted of CD8^{+} T cells were unable to control virus replication both during primary and chronic SIV infection, and during primary SHIV infection (Jin *et al.*, 1999; Matano *et al.*, 1998; Schmitz *et al.*, 1999). Strong HIV-1-specific CD4^{+} T-helper responses are associated with control of viraemia in HIV-1-infected patients (Rosenberg *et al.*, 1997, 2000). CD4^{+} T-helper cells are also necessary for the maintenance of functional virus-specific CD8^{+} T-cell responses (Heeney, 2002).

The steady-state virus load is usually predictive of subsequent clinical course and outcome in both SIV-infected monkeys and HIV-1-infected humans (Mellors *et al.*, 1996; ten Haaft *et al.*, 1998; Watson *et al.*, 1997). Others have shown a relation between the peak virus load during primary infection and the steady-state virus load, which has been demonstrated in both monkeys and humans (Lifson *et al.*, 1997; Lindback *et al.*, 2000). Because of the relatively rapid clearance of viraemia in our study, we used the plasma virus load at 2 weeks post-challenge near the peak of viraemia for analyses of correlation of protection.

The animals with the lowest virus loads in our study demonstrated broad and strong immune responses, while animals displaying the highest virus load showed more restricted and lower immune responses. These results suggests that: (i) broad immune responses including CD8^{+} and CD4^{+} T cells as well as antibodies are more efficient in controlling virus replication than cellular immune responses alone; (ii) a combination of CD8^{+} and CD4^{+} T-cell responses is more efficient than either CD8^{+} or CD4^{+}
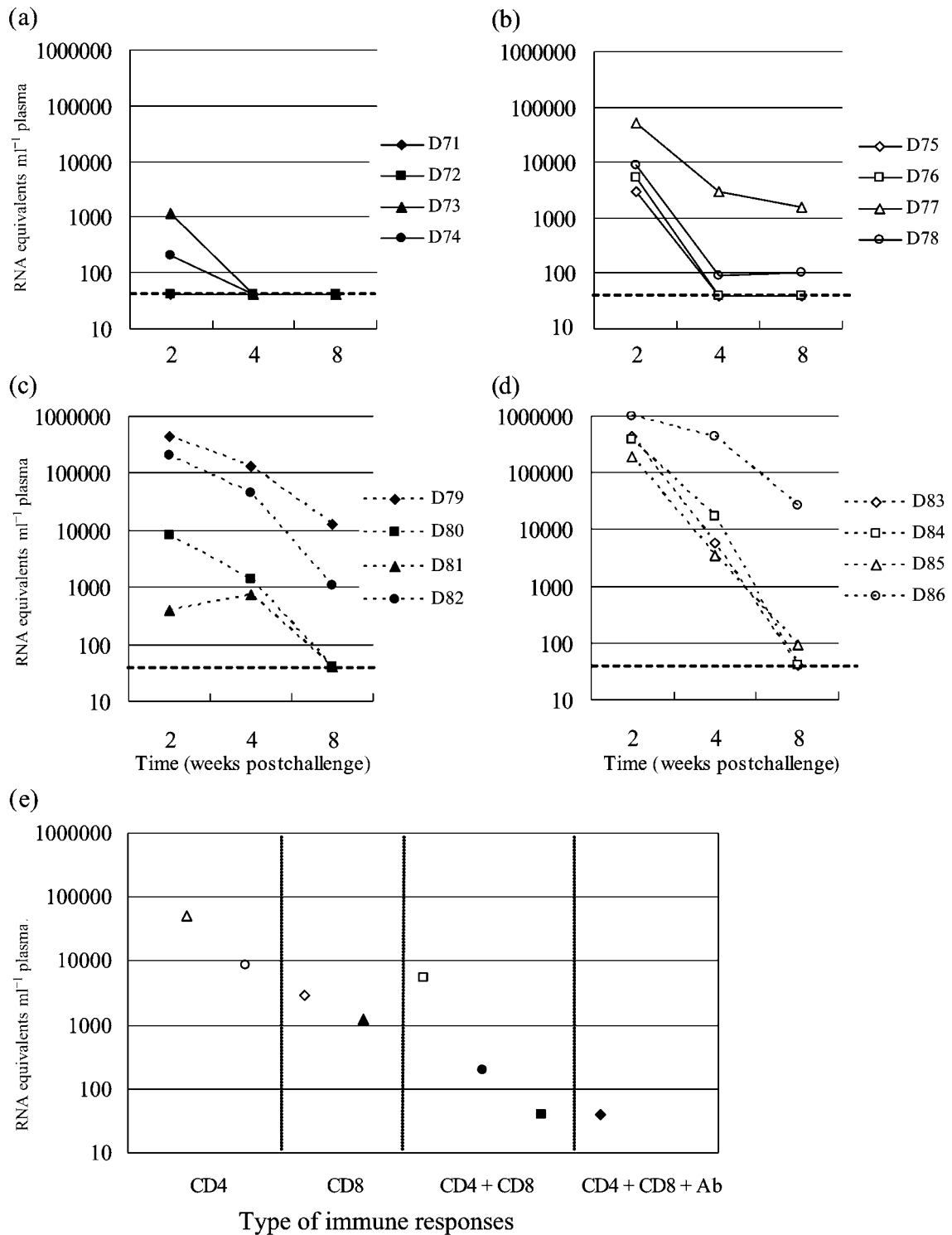
Fig. 6. Plasma virus load in cynomolgus monkeys vaccinated with a combination of DNA- and MVA-based multicomponent HIV/SIV vaccine after intravenous SHIV challenge. Monkeys immunized intramuscularly and mucosally (a), intramuscularly only (b), vaccine control monkeys (c), naive controls (d). Comparison of virus load at 14 days post-challenge and type of immune responses prior to challenge in vaccinated monkeys (e). Symbols as in (a–d).

T cells alone; (iii) CD8$^+$ T-cell responses are more efficient than CD4$^+$ T-cell responses (CD8$^+$ CD4$^+$ antibodies >CD8$^+$ CD4 >CD8 >CD4). Similar broad immune responses, consisting of both functional CD8$^+$ and CD4$^+$ T cells and antibodies, have been demonstrated to correlate positively with protective efficacy to HIV or SIV infection as well as disease (Hel *et al.*, 2002; Mooij & Heeney, 2001).

Sterilizing immunity can be obtained by administration of envelope antibodies, and it has been demonstrated that passively transferred anti-HIV-1 neutralizing monoclonal antibodies completely protected macaques against systemic as well as mucosal SHIV-89.6P challenge (Baba *et al.* 2000; Mascola *et al.*, 1999, 2000). In a recent study, it was shown that HIV-1-specific T-cell responses induced by a DNA gag/env vaccine were unable to assist humoral immunity to prevent the establishment of initial infection, although they could reduce virus replication (Mascola *et al.*, 2003). Current AIDS vaccines mainly eliciting cellular immunity do not, in general, induce sterilizing immunity, but do reduce virus loads. The limitations of these vaccines have been indicated in recent studies. Barouch *et al.* (2002, 2003) showed a breakthrough of virus replication after mutations in immunodominant CTL epitopes in monkeys with a previous effective vaccine-elicited immune control of SHIV-89.6P challenge virus replication. These data suggest that an effective AIDS vaccine should induce neutralizing antibodies as well as functional CD4$^+$ T cells and CD8$^+$ T cells. It is likely that a vaccine able to elicit broader immune responses against many virus proteins will exert more effective control of virus replication than a vaccine inducing a narrow immune response.

The protected monkey (D71) together with two monkeys immunized i.m. (D75 and D76) had CTL against Tat, and they appeared to have an earlier control of virus replication than the other monkeys in that group. CTL activity against proteins expressed early in HIV-1 replication cycle (Tat, Rev and Nef) may be more beneficial than CTL against late structural proteins in controlling infection (Calarota *et al.*, 2001; Gruters *et al.*, 2002). In studies in humans and monkeys, virus specific CTL against Rev and/or Tat has been shown to correlate with control of virus replication (Cafaro *et al.*, 2001; Osterhaus *et al.*, 1999; van Baalen *et al.*, 1997). However, data published recently suggest that immunization with only Rev and Tat vaccines is not sufficient to achieve solid protection (Allen *et al.*, 2002; Verrier *et al.*, 2002).

A peptide-based DNA/MVA HIV vaccine designed to elicit mainly CD8$^+$ T-cell responses has already entered clinical trials in Oxford and Nairobi. This vaccine has been shown to induce T-cell responses in rhesus macaques (Wee *et al.*, 2002), and preliminary studies show induction of T-cell responses in a majority of human volunteers by either vaccine component alone (Hanke *et al.*, 2002). In contrast to our vaccine, the vaccine used by Hanke and McMichael consists of a consensus HIV clade A p24/p17 and a string of CTL epitopes (Hanke & McMichael, 2000).

To increase the efficacy of DNA-based vaccines, different cytokine adjuvants have been tried. In mouse models, co-immunization of DNA vaccines with GM-CSF plasmids has been reported to enhance immunogenicity and protective efficacy of malaria DNA vaccines (Weiss *et al.*, 1998). Both groups of monkeys in our study received GM-CSF protein just before the second DNA immunization. Thus, no conclusions about the effect of GM-CSF can be drawn from this experiment. In one recent paper, the use of plasmids expressing IL12/GM-CSF in addition to an SIV DNA prime/virus-like particle protein-boost strategy enhanced protection against mucosal SIVsmE660 challenge in monkeys (O'Neill *et al.*, 2002). However, in another study of monkeys genetically immunized with an SIV library vaccine and challenged intravenously with SIV mac251, the use of plasmids encoding IL12 and GM-CSF resulted in lower survival rates (Sykes *et al.*, 2002). These results show that more work is needed to determine the effect of GM-CSF as a cytokine adjuvant.

Our results demonstrate that cellular immune responses were elicited by immunization of macaques with a multi-component DNA/MVA vaccine. The administration of the vaccine by mucosal injection both i.o. and i.r. in combination with the i.m. route was shown to be more effective in inducing cellular immune responses and a better control of challenge virus replication than i.m. injection alone. In addition, the breadth of the elicited immune response correlated with control of infection in the vaccinated monkeys. Based on this concept of a DNA prime-MVA boost strategy, a multiprotein DNA/MVA HIV vaccine will enter clinical phase I trials in the near future.

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