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Construction and immunogenicity in a prime–boost regimen of a Semliki Forest virus-vectored experimental HIV clade A vaccine

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A novel, experimental subunit human immunodeficiency virus (HIV) vaccine, SFV.HIVA, was constructed. This consists of Semliki Forest virus (SFV), which is a suitable vaccine vector for use in humans, and a passenger gene encoding HIVA, which is an immunogen derived from HIV-1 clade A that is being currently tested in clinical trials of combined DNA- and modified vaccinia virus Ankara (MVA)-vectored vaccines in Oxford (UK) and Nairobi (Kenya). In the mouse, the SFV.HIVA vaccine was highly immunogenic for T cell-mediated immune responses and induced T cell memory that lasted for at least 6 months. SFV.HIVA was also compared to the vaccines currently used in the clinical trials and was shown to be as effective in T cell induction as pTHr.HIVA DNA but less immunogenic than MVA.HIVA. When tested in a prime–boost regimen, SFV.HIVA-induced responses could be boosted by MVA.HIVA. This work is a part of a long-term effort to build a panel of subunit vaccines expressing a common immunogen, which will allow both a direct comparison of various vaccine vectors and combined vaccination regimens in humans and provide more flexibility and/or a potential optimization of vaccinations for individuals based on their pre-existing anti-vector immunity.

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INTRODUCTION

Human immunodeficiency virus (HIV) infection continues to spread at an alarming rate. Education has been slow to make an impact and highly active anti-retrovirus therapy is, for the majority of people, too expensive and complex and in any case fails to clear the virus from the body. Moreover, the vast majority of HIV-infected people do not know that they are infected. Under these circumstances, development of a safe and effective prophylactic vaccine is the best hope for controlling the HIV epidemic. If successful, very similar vaccination strategies might be used in a therapeutic setting to benefit people who are already infected.

An effective HIV vaccine may have to stimulate a range of host defences, including mucosal and innate immunities, neutralizing antibodies and cell-mediated immune responses. The variability of the HIV envelope and inaccessibility of potentially neutralizing epitopes on primary isolates (Kwong *et al.*, 1998; Wyatt *et al.*, 1998) continues to hamper the development of vaccines that induce neutralizing antibodies. This shifted the focus of many vaccinologists towards the induction of $CD8^+$ cytotoxic T lymphocytes (CTLs), which have been shown to play an important role in the control of HIV infection (Borrow *et al.*, 1994, 1997; Goulder *et al.*, 1997; Haas *et al.*, 1996; Jin *et al.*, 1999; Kent *et al.*, 1997; Koenig *et al.*, 1995; Koup *et al.*, 1994; Phillips *et al.*, 1991; Price *et al.*, 1997, 1998; Rowland-Jones & McMichael, 1995; Schmitz *et al.*, 1999; Wagner *et al.*, 1998; Wilson *et al.*, 1999, 2000; Wolinsky *et al.*, 1996; Yang *et al.*, 1997; Zhang *et al.*, 1996). However, determining the level of protection that vaccine-induced CTLs can confer against HIV exposure and in already infected individuals on anti-retrovirus therapy will be possible only through development of strategies that reliably elicit strong and durable CTL responses in humans.

Conceptually, gene-based vaccines consist of an immunogen, vaccine vector and an optional immunomodulator. While the immunogen defines vaccine specificity and provides a basic level of 'intrinsic' immunogenicity, the choice of a vaccine vector determines the strength and longevity of the elicited immune responses. These can be further enhanced by particular combinations of heterologous vectors expressing a common immunogen in a prime–boost

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application (Allen *et al.*, 2000; Amara *et al.*, 2001; Hanke *et al.*, 1998, 1999; Heeney *et al.*, 2000; Kent *et al.*, 1998; Nilsson *et al.*, 2001; Osterhaus *et al.*, 1999; Robinson *et al.*, 1999; Schneider *et al.*, 1998). Our finding that a successive immunization with DNA- and modified vaccinia virus Ankara (MVA)-based vaccines is particularly immunogenic for $CD8^+$ CTLs (Hanke *et al.*, 1998; Schneider *et al.*, 1998) is being evaluated in phase I/II clinical trials in Oxford (UK) (unpublished observations) and Nairobi (Kenya). In these trials, an immunogen, which is derived from HIV-1 clade A, termed HIVA (Hanke & McMichael, 2000), is used.

Semliki Forest virus (SFV) as a vaccine vector has a number of selling features. First of all it is very safe. While even the highly pathogenic strains in mice are non-pathogenic in humans, the experimental SFV vaccines are derived from strains that are, in mice, highly attenuated (Atkins *et al.*, 1999). SFV replicates in the cytoplasm through amplification of its RNA genome, i.e. resulting in a high copy number of mRNA, the translation of which is not limited by processing. Cytoplasmic replication also removes the risk of chromosomal integration. In addition, SFV induces apoptosis of infected cells; therefore, the virus genome does not persist in the tissue. Recombinant SFV (rSFV) vaccines can be delivered in three forms: RNA, DNA or virus particles. For the stock production of particles, three mRNAs are co-transfected into packaging cells to reduce the possibility of recombination, which could reconstitute replication-competent particles: one mRNA with the packaging signal encoding the SFV polymerase and an immunogen, and two other mRNAs supplying the capsid and envelope proteins *in trans* (Smerdou & Liljeström, 1999b). As a vaccine, rSFV induced better protective responses than a plasmid DNA in mice (Fleeton *et al.*, 2000) and was immunogenic in primates alone (Berglund *et al.*, 1997) and in a combined immunization protocol (Mossman *et al.*, 1996; Heeney, 2000; Nilsson *et al.*, 2001). In addition, most people do not have a pre-existing immunity to SFV. These properties make SFV a suitable and potentially very attractive vector for human subunit vaccines.

Here, we describe the construction of rSFV particles expressing the HIVA protein and assess their immunogenicity in mice on their own and in a combined SFV.HIVA prime–MVA.HIVA boost regimens.

METHODS

Recombinant DNA. All enzymes used for recombinant DNA work were purchased from New England Biolabs or Boehringer Mannheim and used under the reaction conditions recommended by the vendors.

Cell lines. Baby hamster kidney (BHK)-21 cells were maintained in complete BHK medium supplemented with 5 % foetal calf serum, 10 % tryptose phosphate broth, 2 mM glutamine, 20 mM HEPES and antibiotics (10 μg streptomycin ml^{-1} and 100 IU penicillin ml^{-1}).

Preparation of stock SFV.HIVA particles. The sequence encoding HIVA was isolated from pTHr.HIVA (Hanke & McMichael, 2000) as a *Hin*dIII–*Not*I fragment and ligated into the pET-43 vector (Novagen). A *Pml*I–*Sma*I fragment containing the HIVA open reading frame (ORF) was then inserted into the *Sma*I site of pSFVb12a, which attached a 34 aa enhancer sequence of the capsid and the foot-and-mouth disease virus 2a cleavage site to the HIVA gene (Smerdou & Liljeström, 2000). Packaging of recombinant RNA encoding HIVA into rSFV particles was done using a two-helper RNA system (Smerdou & Liljeström, 1999a). In brief, BHK cells were co-transfected with the recombinant and two additional helper mRNAs, one of which coded for the SFV capsid and the other for the envelope proteins. After 48 h of incubation, medium containing recombinant virus stock was harvested and purified (Fleeton *et al.*, 1999). Indirect immunofluorescence of infected BHK cells was performed to determine the titre of the recombinant virus stocks (Liljeström & Garoff, 1994).

Analysis of expression of HIVA antigen from rSFV and rMVA particles. Metabolic labelling of SFV.HIVA- or MVA.HIVA-infected cells with [^{35}S]methionine has been described previously (Liljeström & Garoff, 1994). Briefly, BHK cells were infected with SFV.HIVA or MVA.HIVA at an m.o.i. of 5. After 15 h, growth medium was replaced with methionine-free minimum essential medium for 30 min prior to the addition of fresh medium containing 75 μCi (2·7 MBq) [^{35}S] methionine ml^{-1}. After a 15 min labelling period, the cells were incubated further for various times in medium containing unlabelled methionine. Supernatants were collected and the cells lysed with Nonidet P-40 buffer containing 100 mM iodoacetamide.

Protein sample preparation and analysis. Cell lysates were analysed by immunoprecipitation followed by SDS-PAGE, as described previously (Liljeström & Garoff, 1994). Cell lysates were immunoprecipitated with protein A–Sepharose and an anti-Pk-tag monoclonal antibody (mAb) (Serotec) overnight at 4 °C. Cell pellets were washed, resuspended in SDS sample buffer and heated at 95 °C for 5 min prior to SDS-PAGE on a 10 % acrylamide reducing gel.

Immunofluorescence for the detection of HIVA expression. Indirect immunofluorescence of SFV.HIVA- or MVA.HIVA-infected BHK cells was carried out to detect the expression of the HIVA protein. BHK cells were infected with SFV.HIVA or MVA.HIVA at an m.o.i. of 5. After a 15 h growth period, cells were fixed in methanol and protein expression was detected by incubation of the cells with anti-Pk-tag mAb at a concentration of 0·1 μg ml^{-1} followed by anti-mouse IgG conjugated to FITC (Sigma).

Vaccines and immunizations. Groups of four 5- to 6-week-old female BALB/c mice were immunized at weeks 0, 2 or both with pTHr.HIVA DNA, SFV.HIVA or MVA.HIVA alone or in combinations (Table 1). For pTHr.HIVA and MVA.HIVA, clinical batches of vaccines produced by COBRA Therapeutics (Keele, UK) and Impfstoffwerk Dessau-Tornau (IDT, Germany), respectively, were used. Either a total of 50 μg pTHr.HIVA DNA in 0·1 ml 140 mM NaCl, 0·05 mM EDTA and 0·5 mM Tris/HCl, pH 7·7, solution or 10^6 p.f.u. MVA.HIVA in 0·1 ml 140 mM NaCl and 10 mM Tris/HCl, pH 7·7, solution was administered using needle injections of the tibial muscles of hind legs. All intramuscular (i.m.) injections were carried out under general anaesthesia. SFV.HIVA particles were administered subcutaneously (s.c.) at a dose of 10^6 IU. All procedures and care strictly conformed to the UK Home Office guidelines.

Preparation of MHC–peptide tetrameric complexes. The MHC–peptide tetrameric complexes were prepared as described before (Altman *et al.*, 1996).The gene encoding the *H-2D^d* heavy chain was modified so that the expressed protein missed the transmembrane and cytosolic tail segments and contained a BirA biotinylation site at its C terminus. For the human β_2-microglobulin gene, the fragment encoding the leader sequence was deleted (Garboczi *et al.*, 1992). Both chains were expressed in *Escherichia coli* strain

Table 1. Immunization schedules

Group	Designation	Vaccination schedule (day)			
		0	14	24	180
Experiment 1					
1	SFV–	SFV.HIVA	–	Sacrifice	NA
2	–SFV	–	SFV.HIVA	Sacrifice	NA
3	2SFV	SFV.HIVA	SFV.HIVA	Sacrifice	NA
4	DNA–	pTHr.HIVA	–	Sacrifice	NA
5	–DNA	–	pTHr.HIVA	Sacrifice	NA
6	2DNA	pTHr.HIVA	pTHr.HIVA	Sacrifice	NA
7	MVA–	MVA.HIVA	–	Sacrifice	NA
8	–MVA	–	MVA.HIVA	Sacrifice	NA
9	2MVA	MVA.HIVA	MVA.HIVA	Sacrifice	NA
10	DNAMVA	pTHr.HIVA	MVA.HIVA	Sacrifice	NA
11	SFVMVA	SFV.HIVA	MVA.HIVA	Sacrifice	NA
Experiment 2					
12	SFV	SFV.HIVA	–	–	Sacrifice
13	2SFV	SFV.HIVA	SFV.HIVA	–	Sacrifice
14	DNA	pTHr.HIVA	–	–	Sacrifice
15	2DNA	pTHr.HIVA	pTHr.HIVA	–	Sacrifice
16	MVA	MVA.HIVA	–	–	Sacrifice
17	2MVA	MVA.HIVA	MVA.HIVA	–	Sacrifice
18	DNAMVA	pTHr.HIVA	MVA.HIVA	–	Sacrifice
19	SFVMVA	SFV.HIVA	MVA.HIVA	–	Sacrifice

NA, Not applicable.

BL-21 (DE3) pLysS (Novagen) as inclusion bodies, which were purified, refolded by dilution in the presence of the peptide RGPGRAFVTI and biotinylated. The 45 kDa refolded complex was purified on FPLC and ion-exchange columns. An ELISA using alkaline phosphatase-conjugated streptavidin (Sigma) followed by a colorimetric reagent was used to measure the concentration of the biotinylated MHC–peptide complex monomer. To induce the formation of tetrameric complexes, Streptavidin–phycoerythrin conjugate (ExtrAvidin, Sigma) was added to the refolded MHC–peptide complex monomer solution in a 1 : 4 molar ratio.

Isolation of mouse peripheral blood mononuclear cells (PBMCs). Approximately 100 μl blood was taken from individual mice by a venepuncture on the day of sacrifice. Blood was prevented from coagulation by the addition of 200 μl blood buffer (PBS, 10 mM EDTA and 100 U heparin ml^{-1}). Red blood cells (RBCs) were lysed by the addition of 1·5 ml RBC lysis buffer (Puregene) followed by centrifugation at 3500 r.p.m. for 5 min. PBMCs were then washed once with R0 (RPMI 1640 supplemented with penicillin/streptomycin).

Flow cytometry. About 10^6 mouse PBMCs were washed once with PBA (PBS, 1 % BSA and 0·1 % sodium azide) and incubated on ice with 1 μg MHC–peptide tetrameric complex for 20 min and for a further 20 min after the addition of an anti-mouse CD8 mAb conjugated to Tricolor (Caltag). Cells were then washed three times with PBA prior to a formaldehyde fixation (PBS, 2 % formaldehyde and 1 % BSA) and analysed on a Becton Dickinson FACScalibur flow cytometer using the CELLQUEST software (Becton Dickinson).

Isolation of splenocytes. At 10 days or 6 months after the last immunization, spleens were removed and pressed individually through a cell strainer (Falcon) using the rubber plunger of a 2 ml syringe. Splenocytes were washed twice with R0 and suspended in 10 ml lymphocyte medium [RPMI 1640 supplemented with 10 % foetal bovine serum (FBS), penicillin/streptomycin, 20 mM HEPES and 15 mM 2-mercaptoethanol]. A 2 ml sample of splenocyte suspension was used for the interferon (IFN)-γ ELISPOT assay and the rest was used for a bulk CTL culture.

Enumeration of IFN-γ-secreting splenocytes by ELISPOT assay. The ELISPOT assay was carried out using the Mouse IFN-γ-Secreting Cell kit (U-Cytech), according to the manufacturer's instructions. In brief, splenocytes isolated 10 days or 6 months following the last immunization of BALB/c mice were restimulated in 48-well plates at 8×10^6 cells per well in R10 (RPMI 1640 supplemented with 10 % FBS and penicillin/streptomycin) alone, supplemented with 4 μg concanavalin A ml^{-1} or specific peptide RGPGRAFVTI derived from HIV-1 and restricted by *H-2D*^{d} (Takahashi *et al.*, 1988) at 4 μg ml^{-1} for 15 h at 37 °C in 5 % CO_2. The cells were then removed from the wells by careful washing in R0, set up in anti-IFN-γ, pre-coated 96-well plates with the same stimulation as before at concentrations of 6, 3 or $1·5\times10^5$ cells per well in triplicates and incubated for a further 5 h at 37 °C. Following lysis of the cells by a 10 min incubation with water on ice, spots were visualized using a biotin-conjugated anti-IFN-γ antibody and an enhancement system followed employing a dual activator system. Spots were counted using an ELISPOT reader (Autoimmun Diagnostika) and expressed as spot-forming units (s.f.u.) per 10^6 cells.

Bulk CTL cultures. An 8 ml sample of cell suspension containing 8/10 of the total number of splenocytes was incubated with 2 μg peptide ml^{-1} in an humidified incubator in 5 % CO_2 at 37 °C for 5 days. On the day of the CTL assay, effector cells were washed three times with RPMI, resuspended at 10^7 cells ml^{-1} in R10 medium and used in a ^{51}Cr-release assay, as described below.

Target cells and standard ^{51}Cr-release assay. Effector cells were diluted twofold in a 96-well, U-bottom plate (Costar) to yield after addition of the target cells 50 : 1, 25 : 1, 12 : 1 and 6 : 1 effector to target ratios. A total of 5000 ^{51}Cr-labelled P815 cells in a medium containing 10^{-7} M peptide was then added to the effector cells and incubated at 37 °C for 4 h. Spontaneous and total chromium releases were estimated from the wells, in which the target cells were kept in a medium alone or 5 % Triton X-100, respectively. Percentage specific lysis was calculated as [(sample release−spontaneous release)/(total release−spontaneous release)]×100. Spontaneous release was lower than 5 % of the total c.p.m.

RESULTS AND DISCUSSION

Construction of rSFV particles expressing the HIVA immunogen

The HIVA ORF was inserted into the pSFV vector and rSFV particles were produced as described in Methods. Using the C-terminal mAb epitope Pk, the expression of the HIVA protein in SFV.HIVA-infected BKH cells was confirmed by indirect immunofluorescence (Fig. 1A). When compared with MVA.HIVA-infected cells, similar expression levels of the HIVA antigen were observed. Production of the HIVA antigen was also demonstrated by [^{35}S]methionine pulse–chase experiments followed by immunoprecipitation (Fig. 1B). All of the radioactively labelled HIVA protein was found in the cell lysate and a majority of it was degraded within 3 h after synthesis. No HIVA was detected in the tissue culture supernatant.

SFV.HIVA particles induced HIV-specific immune responses that lasted for at least 6 months

The induction of HIV-specific T cell responses by SFV.HIVA particles was assessed and compared to the pTHr.HIVA DNA and MVA.HIVA vaccines (Hanke & McMichael, 2000) in the mouse. A sensitive readout was facilitated by employing epitope RGPGRAFVTI (Takahashi *et al.*, 1988), which is included in the multi-CTL epitope string of HIVA. Thus, groups of BALB/c mice were immunized either with 10^6 IU SFV.HIVA particles s.c., 50 μg pTHr.HIVA DNA i.m. or 10^6 p.f.u. MVA.HIVA i.m. once or twice at a 2 week interval. Mice were sacrificed for immunological analysis 10 days after the last immunization (Table 1, experiment 1). Both the ^{51}Cr-release and ELISPOT assays carried out on splenocytes from individual mice showed that a single delivery of SFV.HIVA particles induced in all animals CTL responses similar to those elicited by pTHr.HIVA DNA but lower compared to the more complex and immunogenic MVA.HIVA vaccine (Fig. 2A and Table 2). T cell induction was also confirmed by detection of PBMCs reactive with the *H-2D^d*–RGPGRAFVTI tetrameric complexes, although this assay seemed to be, in this case, less informative (Table 3).

To assess the longevity of the induced immune responses and also as a more stringent comparison of immunogenicities of these three vaccine vectors, CTLs were analysed

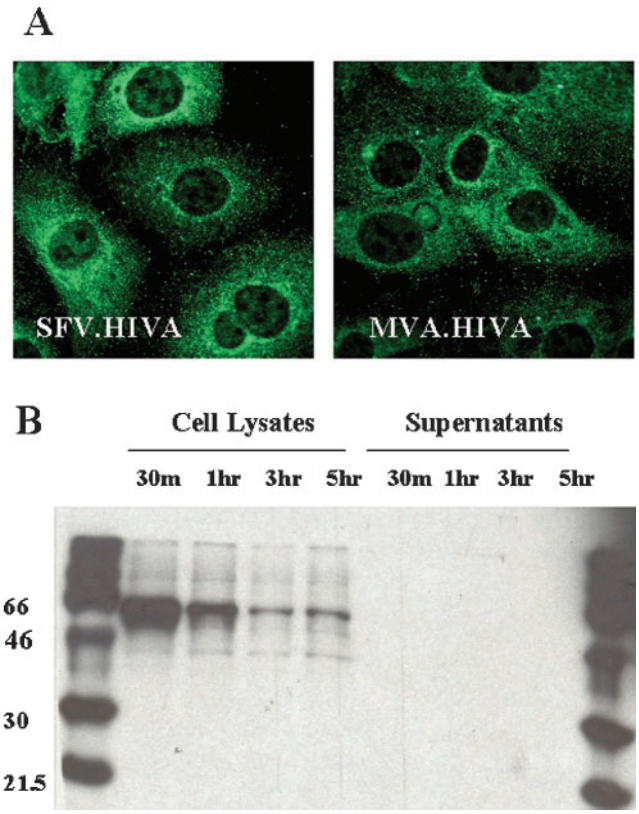

Fig. 1. Expression of HIVA. (A) Immunofluorescence of cells infected with either SFV.HIVA (left) or MVA.HIVA (right) using the C-terminal mAb epitope Pk for detection of the HIVA protein. (B) Analysis of HIVA antigen expressed by rSFV in transfected BHK-21 cells. After labelling with [^{35}S]methionine, cells were chased with cold methionine for the indicated times. Both the cell lysates and supernatants were analysed by immunoprecipitation, 10 % SDS-PAGE and autoradiography.

6 months after immunization (Table 1, experiment 2). The ^{51}Cr-release assay involving a 5 day *in vitro* peptide restimulation readily detected persisting CTL precursors (Fig. 3A). These lytic activities were again similar between the SFV.HIVA and pTHr.HIVA vaccines and stronger for MVA.HIVA. The frequencies of IFN-γ-releasing cells over 6 months approximately halved (Table 4).

Augmentation of immune responses by SFV.HIVA prime–MVA.HIVA boost regimen

Data are emerging that combined heterologous prime–boost vaccinations are superior to repeated immunizations with a single vaccine modality (Hanke, 2001). To test the suitability of SFV for priming of CTLs, mice were immunized with SFV.HIVA and boosted with MVA.HIVA 2 weeks later. As a reference, a sequential immunization with pTHr.HIVA and MVA.HIVA currently tested in the clinic was used. For both the DNA and SFV vaccinations, MVA increased the lytic activities of restimulated splenocyte

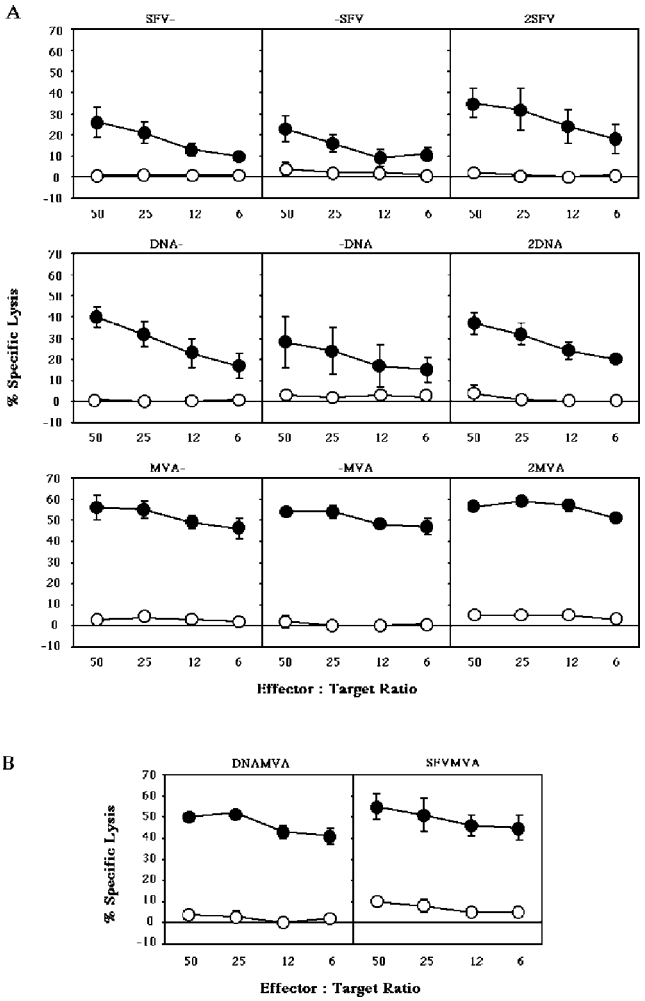

Fig. 2. Short-term analysis of CTL precursors. The immunogenicities of three vectors expressing HIVA alone (A) or in a heterologous prime–boost regimen (B) were assessed by employing an epitope recognized by mouse CTLs included into the polyepitope string. Mice were sacrificed 10 or 28 days after the last immunization (see Table 1), splenocytes from individual mice were isolated, separately peptide-restimulated *in vitro* for 5 days and tested in a ^{51}Cr-release assay against peptide pulsed (full) or unpulsed (open) targets. Lines represent an average lysis ± SD of each immunization group.

cultures compared to the two single vaccine applications, although these responses were not significantly higher than MVA.HIVA alone (Fig. 1B and 2B). Similarly, for the IFN-γ ELISPOT assay carried on *ex vivo* splenocytes, the heterologous immunizations were more potent than two sequential doses of SFV.HIVA or pTHr.HIVA (Table 2). Although the tetramer reactivities on PBMCs were not significantly different (Table 3), the first two assays indicated that the immune responses induced by SFV.HIVA could be augmented by MVA.HIVA. Good memory levels of CTLs were detected 6 months after the last immunization, indicating that the responses were relatively long lasting (Fig. 3 and Table 4).

Table 2. IFN-γ ELISPOT assay on *ex vivo* splenocytes 10 or 28 days after the last immunization

Freshly isolated splenocytes were restimulated with peptide for 16 h and the number of IFN-γ-producing cells was determined (s.f.u. per 10^6 splenocytes). Values for individual animals in each group are shown.

		IFN-γ-producing cells (s.f.u. per 10^6 splenocytes)				
Group	Vaccination	1	2	3	4	Average ± SD
1	SFV–	91*	78	187	67	106 ± 48
2	–SFV	130	54	81	244	127 ± 73
3	2SFV	268	286	404	367	331 ± 56
4	DNA–	340	287	229	547	351 ± 120
5	–DNA	56	107	414	213	198 ± 137
6	2DNA	337	276	267	293	293 ± 27
7	MVA–	393	253	453	520	405 ± 98
8	–MVA	660	409	547	410	507 ± 105
9	2MVA	501	1046	512	1149	802 ± 298
10	DNAMVA	1017	889	1153	1033	1023 ± 94
11	SFVMVA	887	1084	807	1009	947 ± 107

*Numbers of s.f.u. are shown after subtracting the no-peptide background.

This paper reports on the construction of a novel experimental subunit HIVA vaccine, SFV.HIVA. Using three different mutually complementing T cell assays, the relatively high and long-lasting immunogenicity of the vaccine in

Table 3. Reactivities of MHC–peptide tetrameric complexes with PBMCs 10 or 28 days after the last immunization

Blood was drawn from the immunized mice on the day of sacrifice and PBMCs were tested for their reactivities (% $CD8^+$ PBMCs) with the RGPGRAFVTI/*H-2D*^{d} tetramer. Values for individual animals in each group are shown.

		Reactivity (% $CD8^+$ PBMCs)				
Group	Vaccination	1	2	3	4	Average ± SD
1	SFV–	4	12	12	6	9 ± 4
2	–SFV	16	16	16	16	16 ± 0
3	2SFV	7	31	32	34	26 ± 11
4	DNA–	18	13	33	16	20 ± 8
5	–DNA	23	4	12	4	11 ± 8
6	2DNA	15	11	12	6	11 ± 3
7	MVA–	66	29	12	20	32 ± 21
8	–MVA	19	15	30	19	21 ± 6
9	2MVA	20	27	14	28	22 ± 6
10	DNAMVA	29	67	22	19	34 ± 19
11	SFVMVA	40	24	24	25	28 ± 7

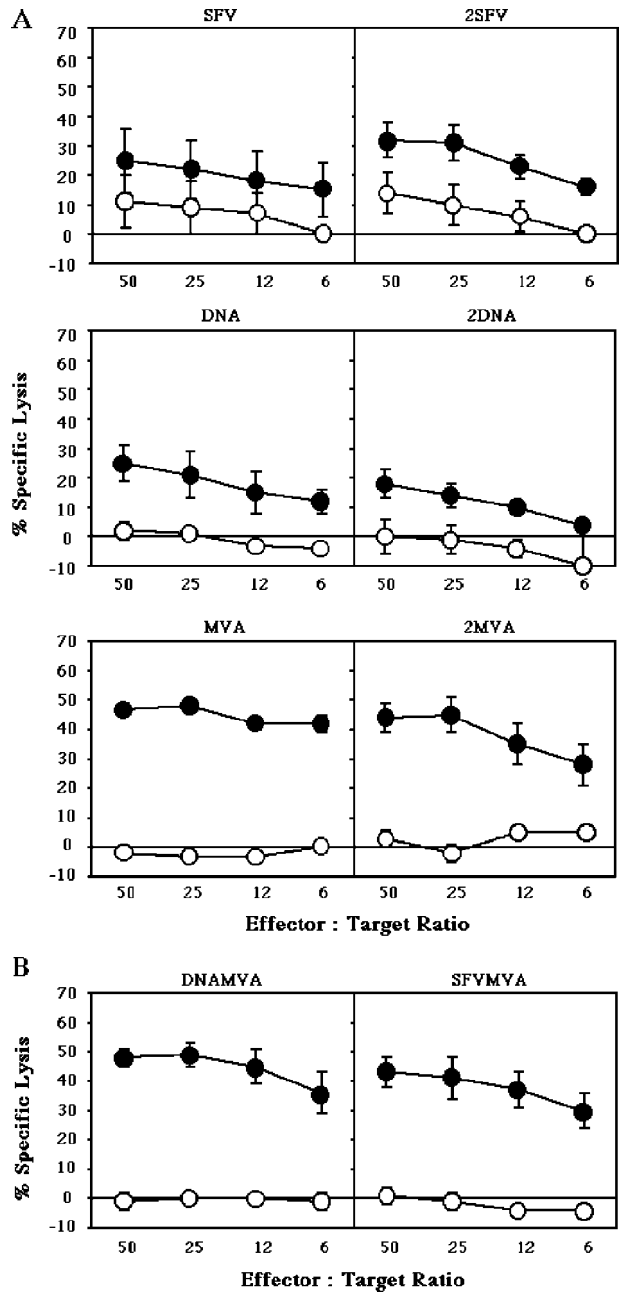

Fig. 3. Analysis of CTL precursors 6 months after immunization. The immunogenicities of three vaccines expressing HIVA alone (A) or in a heterologous prime–boost regimen (B) were immunized (Table 1) and assessed 6 months after immunizations, as described in the legend of Fig. 2. Lines represent an average lysis ± SD of each immunization group of either peptide pulsed (full) or unpulsed (open) targets.

mice was demonstrated and compared to two other vaccines currently tested in humans, pTHr.HIVA DNA and MVA.HIVA.

An MVA.HIVA p.f.u. of 10^6 per dose was used. Because of the high MVA immunogenicity, the MVA.HIVA results stand on their own in this work. In all three assays, one MVA.HIVA vaccination was as good as two MVA.HIVA and the heterologous prime–boost immunizations (Figs 1 and 2, and Tables 3 and 4). Perhaps, the benefit of the heterologous prime–boost protocol over the one or two MVA.HIVA schedule might be better seen at lower doses of MVA.HIVA or in more stringent immunizations of non-human primates (Hanke *et al.*, 1999; Allen *et al.*, 2000; Heeney *et al.*, 2000) and man (unpublished observations).

Our long-term aim is to build a panel of vaccine vectors expressing a common immunogen. The rationale is at least fourfold: first, to directly compare these vectors for their effectiveness in induction of both $CD8^+$ and $CD4^+$ T cell responses in animal models and humans; second, to assess the immunogenicities of various combined regimes using sequential immunizations; third, to evaluate the effect of a parallel use of different vectors on the breadth of induced T cell responses; and fourth, to generate a means of overcoming both pre-existing and vaccine-induced anti-vector immunities, which can negatively affect the immunogenicity of the passenger immunogen. For these types of studies, the HIVA immunogen is particularly suitable because it contains well-characterized CTL epitopes recognized by murine, rhesus macaque and human CTLs. Furthermore, HIVA has a growing safety record in humans, the species in which the ultimate immunogenicity evaluation of HIV vaccines has to be carried out and which no animal model can substitute.

HIV-1 is a highly variable virus, which is classified into M, N and O major groups. The M group has spread around the world and is further diversified into clades A to K in different geographical regions. We have argued previously that a candidate vaccine should match the appropriate local clades (McMichael & Hanke, 2002). Because the HIVA immunogen uses consensus clade A HIV sequences, it is designed specifically for areas with high prevalence of clade A infections, such as subSaharan Africa, Thailand and Russia (Neilson *et al.*, 1999), all of which are in a desperate need of an HIV vaccine. Therefore the addition of SFV.HIVA onto the list of clade A vaccines acceptable for use in humans might increase the chances that in the future, an effective T cell vaccination for these regions becomes available.

In conclusion, it cannot be stressed enough that only clinical trials aimed to optimize the elicitation of T cell responses in humans will provide a basis for the eventual proof or disproof of the at-present-frequently pursued hypothesis that CTLs can prevent establishment of an HIV infection and/or significantly delay the onset of AIDS in individuals who have become infected. This work represents one little step towards this goal.

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Table 4. IFN-γ ELISPOT assay on *ex vivo* splenocytes 6 months after the last immunization

Freshly isolated splenocytes were restimulated with peptide for 16 h and the number of IFN-γ-producing cells was determined (s.f.u. per 10^6 splenocytes). Values for individual animals in each group are shown.

		IFN-γ-producing cells (s.f.u. per 10^6 splenocytes)								
Group	Vaccination	1	2	3	4	5	6	7	8	Average ± SD
12	SFV	18*	51	49	0	–	–	–	–	30 ± 21
13	2SFV	56	15	17	101	–	–	–	–	47 ± 35
14	DNA	28	75	57	44	–	–	–	–	51 ± 17
15	2DNA	137	88	41	–	–	–	–	–	89 ± 39
16	MVA	229	250	228	682	–	–	–	–	347 ± 193
17	2MVA	292	527	464	409	–	–	–	–	322 ± 202
18	DNAMVA	528	125	346	630	192	205	550	375	369 ± 175
19	SFVMVA	670	542	395	472	526	520	137	486	469 ± 145

*Numbers of s.f.u. are shown after subtracting the no-peptide background.

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