Design and preclinical evaluation of a multigene human immunodeficiency virus type 1 subtype C DNA vaccine for clinical trial

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In this study, the design and preclinical development of a multigene human immunodeficiency virus type 1 (HIV-1) subtype C DNA vaccine are described, developed as part of the South African AIDS Vaccine Initiative (SAAVI). Genetic variation remains a major obstacle in the development of an HIV-1 vaccine and recent strategies have focused on constructing vaccines based on the subtypes dominant in the developing world, where the epidemic is most severe. The vaccine, SAAVI DNA-C, contains an equimolar mixture of two plasmids, pTHr.grttnC and pTHr.gp150CT, which express a polyprotein derived from Gag, reverse transcriptase (RT), Tat and Nef, and a truncated Env, respectively. Genes included in the vaccine were obtained from individuals within 3 months of infection and selection was based on closeness to a South African subtype C consensus sequence. All genes were codon-optimized for increased expression in humans. The genes have been modified for safety, stability and immunogenicity. Tat was inactivated through shuffling of gene fragments, whilst maintaining all potential epitopes; the active site of RT was mutated; 124 aa were removed from the cytoplasmic tail of gp160; and Nef and Gag myristylation sites were inactivated. Following vaccination of BALB/c mice, high levels of cytotoxic T lymphocytes were induced against multiple epitopes and the vaccine stimulated strong CD8+ gamma interferon responses. In addition, high titres of antibodies to gp120 were induced in guinea pigs. This vaccine is the first component of a prime–boost regimen that is scheduled for clinical trials in humans in the USA and South Africa.

INTRODUCTION

The tragic consequences of the AIDS pandemic are worst-felt in Africa, where approximately 25 million people are infected with human immunodeficiency virus (HIV) and millions more are affected by the disease (UNAIDS, 2004). Countries in the southern African region have been the worst affected. In South Africa, HIV prevalence in women attending government antenatal clinics is 29-4%, and an estimated 6-3 million South Africans are infected with HIV (Department of Health, South Africa, 2005). HIV prevalence levels of between 16 and 40% occur in antenatal-clinic attendees in neighbouring countries in the region, such as Botswana, Zambia, Mozambique and Swaziland, with no apparent signs of decline (UNAIDS, 2004).

Whilst access to treatment for those infected remains a high priority in the region, the need for a vaccine to prevent new infections is of paramount importance in bringing the epidemic under control. The vaccine development process is long and arduous, but more candidate vaccines are now entering or currently being tested in phase I and II safety trials in humans than ever before (HVTN, 2005; IAVI, 2005). Several of these candidates are DNA vaccines, which have shown great promise in inducing strong T-cell responses in non-human primates and humans when boosted with viral vectors or with matching antigens (Hanke & McMichael, 2000; Amara et al., 2001; Shiver et al., 2002). T-cell responses have been associated with control of virus
replication and delay of disease progression in HIV vaccine studies in non-human primates (Barouch et al., 2000; Amara et al., 2001; Shiver et al., 2002). A DNA-vaccine prime in a prime–boost approach elicits a more potent immune response than the response to either of the vaccine candidates separately; this has been demonstrated with recombinant modified vaccinia virus Ankara (MVA) and adenovirus boosting (Hanke et al., 1999; Robinson et al., 1999; Shiver et al., 2002).

HIV-1 subtype C accounts for over half of HIV-1 infections globally (Osmanov et al., 2002) and over 90% of infections in southern Africa (van Harmelen et al., 1999; Guevara et al., 2000; Novitsky et al., 2002; Travers et al., 2004). Ethiopia, with the second-highest population in Africa, also has a subtype C epidemic (Hussein et al., 2000) and subtype C viruses are responsible for India’s growing epidemic (Lole et al., 1999; Ramalingam et al., 2005). HIV-1 subtypes can vary by 35% in the genetic distance between the vaccine immunogen and circulating viruses. The genetic distance between the vaccine immunogen and infecting strains (Lynch et al., 2000) and subtype C viruses are responsible for India’s growing epidemic (Loele et al., 1999; Ramalingam et al., 2005). HIV-1 subtypes can vary by 35% in the genetic distance between the vaccine immunogen and infecting strains (Lynch et al., 1998; Altfeld et al., 2002). Thus, current vaccine designs take genetic diversity into consideration in order to elicit better intra-clade as well as cross-clade responses (Gao et al., 2005).

This paper describes a multigene HIV-1 subtype C DNA vaccine, SAAVI DNA-C, which was developed as part of the South African AIDS Vaccine Initiative (SAAVI). To minimize the impact of genetic variability on vaccine effectiveness, the genes incorporated in the vaccine were derived from two primary HIV-1 subtype C isolates, Du151 and Du422, which were selected based on their amino acid similarity to a derived South African consensus sequence (Williamson et al., 2003). This approach minimizes the genetic distance between the vaccine immunogen and circulating viruses. The gag, pol and env genes utilized in this study had 98.7, 98.9 and 95.0% similarity, respectively, to the South African consensus sequence. In addition, the env gene was obtained from an individual within 2 months of infection and was shown to be R5-tropic (Williamson et al., 2003). Here, we report on the vaccine design, the modification of genes for safety considerations and the characterization of integrity, potency and function of the expressed proteins. In addition, the cellular immune responses in BALB/c mice and humoral responses in guinea pigs are described. This vaccine is the first component of a prime–boost combination planned for clinical trials in the USA and South Africa, and represents one of several candidate subtype C vaccines targeted for clinical trial (IAVI, 2005).

### METHODS

**Modification of genes and construction of plasmids.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Length (bp)</th>
<th>Origin</th>
<th>Modification</th>
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<tr>
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<td>1326</td>
<td>Du422</td>
<td>Myristylation site removed by mutated MGA to MAA (aa 2); codon-optimized</td>
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<tr>
<td>RT</td>
<td>1354</td>
<td>Du151</td>
<td>RT site inactivated by mutating YMDDL to YMAAL (aa 337); codon-optimized</td>
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<td>365</td>
<td>Consensus Du422/151</td>
<td>Inactivation of tat by shuffling; codon-optimized</td>
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<tr>
<td>nef</td>
<td>592</td>
<td>Du151</td>
<td>Inactivation of nef by deletion of 30 bp (5’ end); codon-optimized</td>
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<tr>
<td>env</td>
<td>2208</td>
<td>Du151</td>
<td>Deletion of 372 bases at 3’ end. HIV-1 BALB/c mouse env V3 sequence (10 aa) inserted; codon-optimized</td>
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</tbody>
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This paper describes a multigene HIV-1 subtype C DNA vaccine, SAAVI DNA-C, which was developed as part of the South African AIDS Vaccine Initiative (SAAVI). To minimize the impact of genetic variability on vaccine effectiveness, the genes incorporated in the vaccine were derived from two primary HIV-1 subtype C isolates, Du151 and Du422, which were selected based on their amino acid similarity to a derived South African consensus sequence (Williamson et al., 2003). This approach minimizes the genetic distance between the vaccine immunogen and circulating viruses. The gag, pol and env genes utilized in this study had 98.7, 98.9 and 95.0% similarity, respectively, to the South African consensus sequence. In addition, the env gene was obtained from an individual within 2 months of infection and was shown to be R5-tropic (Williamson et al., 2003). Here, we report on the vaccine design, the modification of genes for safety considerations and the characterization of integrity, potency and function of the expressed proteins. In addition, the cellular immune responses in BALB/c mice and humoral responses in guinea pigs are described. This vaccine is the first component of a prime–boost combination planned for clinical trials in the USA and South Africa, and represents one of several candidate subtype C vaccines targeted for clinical trial (IAVI, 2005).
then ligated into this site, introducing the 10 aa HIV-1 V3 sequence and a stop codon (Fig. 1a). These plasmids were modified for manufacturing and clinical trials by removal of the β-lactamase gene at BspHI sites (Cobra Biomanufacturing) to yield plasmids pTH.grtttnC and pTHr.gp150CT, which use an antibiotic-free bacterial repression titration selection system (Williams et al., 1998; Hanke & McMichael, 2000; Cranenburgh et al., 2001).

For the Tat transactivation assay, wild-type codon versions of shuffled or wild-type tat genes were cloned in-frame with green fluorescent protein (GFP) into the EcoRI and BamHI sites in pcDNA3GFP (kindly supplied by T. Kouzarides, University of Cambridge, UK), giving pWTatGFP and pMutTatGFP. For immunocytochemical assays, the gag gene containing a myristylation site was cloned into pcDNA3 (Invitrogen), giving pcDNA3.HMgag. All constructs were sequenced and determined to be error-free.

Preparation of DNA plasmids. pTHgrtttnC, pTHgp150CT and pTH plasmids were manufactured by Aldevron or prepared by using endotoxin-free Maxi/Giga kits (Qiagen). The vaccine constructs for clinical trial making up SAAVI DNA-C, pTH.grtttnC and pTHr.gp150CT were manufactured under Good Manufacturing Practice (GMP) by Cobra Biomanufacturing without the use of any antibiotics for plasmid maintenance (Williams et al., 1998; Cranenburgh et al., 2001). Plasmids in 10 mM Tris/HCl, 1 mM EDTA, 0–9 % NaCl buffer were provided individually or mixed in an equimolar ratio. Mixed plasmids are referred to as SAAVI DNA-C.

Potency assay. A regulatory requirement for DNA vaccines entering phase 1 clinical trials is that the potency be determined (docket no. 96N-0400, Food and Drug Administration). This may be done by showing expression of proteins by Western blot. The potency of SAAVI DNA-C was compared with that of the research batch of pTHgrtttnC/pTHgp150CT mixed in equimolar ratios (pTH DNA-C) in a Good Laboratory Practice (GLP)-certified laboratory (UCTVRG, IIDMM, UCT, South Africa). 293 cells (2 × 10^6; ATCC) were transfected with SAAVI DNA-C or pTH DNA-C diluted serially from 2 to 0–25 µg or with 2 µg pTH empty vector by using FuGENE6 transfection reagent according to the manufacturer’s instructions (Roche). To determine transfection efficiency, reactions were spiked with 0–0125 µg pcDNA3.1CAT + reporter plasmid (Invitrogen). Following harvesting and lysis 48 h later, protein concentration was determined and a CAT ELISA (Roche) was performed. Samples positive for CAT expression were used for Western blot analysis. A 20 µg aliquot of each cell lysate was separated by electrophoresis on 7–5% SDS-PAGE gels and blotted onto PVDF membranes (Bio-Rad) by standard procedures. Precision Plus molecular mass standards (Bio-Rad) were included on the gels. Membranes were probed with anti-RT (NIBSC Centralised Facility for AIDS Reagents, MRC, UK) and anti-gp120 (Biogenesis), followed by anti-sheep IgG conjugated to alkaline phosphatase (Sigma). Detection was performed by using NBT/BCIP (Roche). Assays were repeated six times for repeatability and reproducibility.

Protein expression from single versus mixed plasmids. 293 cells were transfected with 20 µg DNA (either single plasmids, SAAVI DNA-C or pTH vector backbone) as described above. Following harvesting and lysis 48 h later, 50 µg cell lysate was analysed by Western blot with anti-RT and anti-gp120 antibodies as described.

Immunocytochemistry. HeLa cells were grown on coverslips to 60% confluence in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum (FCS) at 37 °C, 5% CO₂. Cells were transfected with pTHgrtttnC or pcDNA3.HMgag by using FuGENE6 (Roche). Cells were processed for immunocytochemistry 48 h post-transfection by
using standard techniques (Hasson et al., 1997). The following antibodies were used: anti-p24 (Aalto BioReagents) and anti-Nef [National Institutes of Health (NIH) AIDS Research and Reference Reagent Program], followed by anti-goat–fluorescein isothiocyanate (FITC) (Dako) and anti-rabbit–Alexa 488 (Molecular Probes). Stained cells were visualized under a Zeiss microscope.

Chloramphenicol acetyltransferase (CAT) assay. HLCD4-CAT cells (NIH AIDS Research and Reference Reagent Program) were maintained in RPMI (Gibco) with 10% FCS at 37°C, 5% CO₂. This cell line harbours integrated copies of an HIV-1 long terminal repeat (LTR) that drives expression of the CAT gene. Cells were transfected with 0, 0.5, 5 and 20 µg pWtTatGFP, pMutTatGFP or SAAVI DNA-C, harvested 24 h later and 60 µg cell lysate was tested for the presence of the reporter protein by using a CAT ELISA kit (Roche) according to the manufacturer’s instructions. Expression of Tat–GFP constructs was confirmed by fluorescence microscopy, and expression of SAAVI DNA-C mix in HLCD4-CAT cells was confirmed by Western blot analysis with anti-RT as described above.

RT assay. A Reverse Transcriptase Assay colorimetric kit (Roche) was used to determine RT activity in 293 cell lysates transfected with 20 µg SAAVI DNA-C. Cell lysate (3 µg) was measured in the assay and RNase inhibitor (Promega) was included. Cell lysate transfected with the subtype C Pol plasmid p96ZM651pol-opt (NIH AIDS Research and Reference Reagent Program) was included as a positive control. Expression levels of Pol and GttnC protein were determined by Western blot analysis.

DNA immunizations. Groups of ten female BALB/c mice (6–8 weeks old) were inoculated on days 0 and 28 with 100 µl of either the single-plasmid vaccines or SAAVI DNA-C by injecting 50 µl into the tibialis anterior muscle of each hind leg. Single plasmids were prepared at a concentration of 1 mg ml⁻¹, whilst SAAVI DNA-C was at a concentration of 2 mg ml⁻¹. Outbred Dunkin–Hartley guinea pigs (6 weeks old) were immunized intramuscularly with 500 µg SAAVI DNA-C (at 2 mg ml⁻¹) in a volume of 250 µl. Six animals were given two inoculations 4 weeks apart, and two immunized animals were included as controls. Animals were housed at UCT and all procedures were performed in accordance with guidelines and approval of the UCT Research Ethics Committee.

In vitro generation of antigen-specific cytotoxic T-lymphocyte (CTL) effector cells. Ten days post-immunization, mice were sacrificed and spleens were harvested. Spleenocyte pools were prepared and cultured (10⁶ cells ml⁻¹) in RPMI and 10% FCS (Gibco) supplemented with 15 mM β-mercaptoethanol, 100 µU penicillin ml⁻¹ and 100 µg streptomycin for 5–6 days with the RT peptide VYDPSKDLIA in Pol (Casimiro et al., 2002) or V3 peptide RGPGRAVT (Wild et al., 2004) for responses to Env, or irrelevant peptide TSYTVASSL. At the end of the culture period, lymphocytes were harvested by using Lympholyte-M (Cedarlane), washed three times and resuspended in splenocyte-culture medium.

⁹¹Chromium-release assays. A standard ⁵¹Cr-release assay containing ⁵¹Cr-labelled P815 target cells and effector cells at ratios of 200:1–1:1 in the presence or absence of the peptide (2 µg ml⁻¹) that was used to generate the effector cells was set up to evaluate the CTL activity of effector cells. After a 4 h incubation period in 5% CO₂ at 37°C, the supernatant was harvested and assayed for ⁵¹Cr release. The percentage of specific release of ⁵¹Cr was calculated as 100 × (experimental c.p.m.–spontaneous c.p.m.)/(total c.p.m.–spontaneous c.p.m.).

Intracellular gamma interferon (IFN-γ) staining and fluorescence-associated cell-sorting (FACS) analysis. Intracellular IFN-γ production by CD8⁺ cells within the effector population in response to a further stimulation with the peptide that was used to generate the effectors was evaluated. For this, effectors were incubated with P815 cells at a 1:1 ratio in the presence and absence of the relevant peptide (2 µg ml⁻¹) and 10 µg brefeldin A ml⁻¹ (4 h, 5% CO₂ at 37°C). Cells were washed with FACS buffer (PBS supplemented with 1% FCS and NaN₃) and stained with FITC-labelled anti-CD8 (Pharminingen). Intracellular IFN-γ was detected by using anti-IFN-γ (Pharminingen) after the cells were fixed and permeabilized by using a Cytoperm/Cytofix kit (Pharminingen) according to the manufacturer’s instructions. Analysis was done on a Becton Dickinson FACScan with CellQuest software.

Antibody-binding assay. Anti-HIV-1 gp120 antibodies were detected in a standard ELISA. HIV-1 subtype C gp120 (TV1 strain, p11) and an ELISA protocol were kindly provided by Indresh Srivastava, Ying Lian and Susan Barnett, Chiron Corporation, USA. Briefly, gp120 protein was coated at 0.1 µg per well in 96-well ELISA plates (Nunc Immunoplate Maxisorp) in PBS overnight. Sera were serially diluted twofold in blocking buffer (PBS containing 0.05% Tween 20 and 1% fat-free milk powder) and incubated for 2 h at 37°C. After washing in PBS with 0.05% Tween 20, bound antibodies were detected by using horseradish peroxidase-conjugated rabbit anti-guinea pig IgG (Dako) and TMB substrate (KPL). Absorbance was read on a microplate reader (Molecular Devices). End-point titres were defined as the reciprocal of the highest dilution whose absorbance value was threefold over that of the background pre-immunization sera at the lowest dilution.

RESULTS

Vaccine design and construction

Two plasmids were constructed, one expressing a poly-protein of 1224 aa comprising Gag–RT–Tat–Nef and a second plasmid expressing a truncated Env (Fig. 1a). These genes were included based on closeness to a South African consensus sequence (Williamson et al., 2003). The vaccine-vector backbone used in the construction of the plasmids, pPTH, has been used in trials in humans and is safe and well-tolerated (Mwau et al., 2004).

For improved levels of expression, HIV-1 gene codons were humanized and inhibitory sequence (INS) sites were removed. During synthesis, additional mutations and modifications were introduced for safety and increased immunogenicity (Table 1): the myristylation site in Gag was mutated and the RT protein was inactivated at the catalytic site (Chao et al., 1995). Tat was shuffled by dividing the gene into three fragments at regions known to be important for Tat function and rearranging these fragments. The important functional regions were the cysteine-rich domain (aa 22–36), responsible for enhancing virus replication and stimulating monocyte dysfunction (Boykins et al., 1999), and the transactivation response basic lysine- and arginine-rich region (TAR; aa 49–56), which is responsible for TAR RNA binding and mediating uptake of exogenous Tat by cells (Betti et al., 2001; Park et al., 2002) (Fig. 1b). In order to prevent any potential T-cell epitopes from being lost, fragments of tat were extended by 10 aa to overlap with neighbouring fragments prior to shuffling. To avoid possible recombination, the nucleotide sequences of the overlapping regions were designed to be heterologous, whilst
maintaining amino acid sequence. To inactivate Nef, the 10 amino-terminal residues, including the myristylation site, were deleted. These residues are responsible for directing Nef to the cell membrane and are essential for Nef function, including its ability to downregulate the CD4 receptor and major histocompatibility complex (MHC) class I molecules (Aiken et al., 1994; Schwartz et al., 1996).

Env was modified by removing 124 aa residues from the carboxy-terminal cytoplasmic domain of gp41, to yield gp150 (Fig. 1a). There is the potential for a neutralizing-antibody response, as there is evidence that truncation of the cytoplasmic tail may result in a ‘partially triggered’ conformation of the Env protein, which may expose neutralizing-antibody epitopes (Edwards et al., 2002) and also result in increased surface expression of gp120, leading to higher antibody titres (Vzorov et al., 1999). The HIV-1 V3 sequence RGPGRAFVTI, an H-2d-restricted epitope in BALB/c mice, was inserted at the carboxy terminus of gp150 in order to assess immunogenicity of the vaccine in mice. This sequence is not naturally present in this subtype C Env.

pTHgrttnC and pTHgp150CT were mixed in equimolar ratios, giving pTH DNA-C. For clinical-trial manufacture, the antibiotic-resistance gene in the plasmids was removed and an equimolar mixture of the resulting constructs, pTHr.grttnC and pTHr.gp150CT, is referred to as SAAVI DNA-C.

Expression of proteins

Western blot analysis of SAAVI DNA-C-transfected 293 cell lysates revealed expression of the full-length GrttnC polyprotein at approximately 150 kDa with anti-RT antibodies [Fig. 2a(i), lane 2]; and Gp150CT at the expected 150 kDa size with anti-gp120 antibodies [Fig. 2a(ii), lane 2]. These bands were specific, as no expression was evident in the case of empty vector-transfected lysates [Fig. 2a(i) and (ii), lane 1]. No decrease in expression of GrttnC and Gp150CT proteins was observed when the cells were transfected with the plasmid mixture (SAAVI DNA-C) compared with single-plasmid transfections [Fig. 2a(i) and (ii), lane 3].

Potency of SAAVI DNA-C vaccine for clinical trial

To ensure that the vaccine manufactured for clinical trials was equivalent in potency to the research lot, a dose-response study was performed and expression of pTH DNA-C research plasmids was compared with that of SAAVI DNA-C. No difference in levels of expression was observed in six repeated experiments using decreasing doses of SAAVI DNA-C [Fig. 2b(i) and (ii), lanes 3–6] or pTH DNA-C [Fig. 2b(i) and (ii), lanes 7–10], as detected with anti-RT or anti-gp120 antibodies. There was thus no loss in vaccine potency upon removal of the β-lactamase gene and manufacturing of SAAVI DNA-C vaccine plasmids, indicating that SAAVI DNA-C had acceptable potency for use in clinical trials.

Vaccine-modified genes are inactive

The safety of products used in clinical trial volunteers is of paramount importance and is investigated extensively prior to phase I clinical trials. In this study, we demonstrated that we had inactivated the functions of the genes included in the vaccine. No RT activity above background (empty-vector negative control) was detected in SAAVI DNA-C-transfected lysates, whilst RT activity was detected in lysates expressing wild-type Pol (positive control) (Fig. 3a). This
indicates that RT in the GrttnC polyprotein is functionally inactive.

To assay for Tat activity of the shuffled Tat, two fusion constructs were made consisting of the shuffled Tat fused to GFP and a wild-type Tat–GFP fusion (positive control). Fluorescent microscopy of transfected cells demonstrated expression of both constructs (data not shown). To determine whether Tat was functional, HLCD4-CAT indicator cells, where CAT expression is under the control of an HIV-1 LTR and is expressed in the presence of a functional Tat protein, were used. No biological activity above background was apparent for the shuffled Tat–GFP construct (Fig. 3b); however, a dose-dependent increase in CAT activity was demonstrated for increasing amounts (0–20 μg) of the wild-type Tat–GFP constructs. No transactivation activity was observed when cells were transfected with 20 μg SAAVI DNA-C. Thus, the shuffling of the Tat protein resulted in inactivation of Tat transactivation activity.

Localization studies were performed, demonstrating that removal of myristylation sites in Gag and Nef abrogated localization of the GrttnC polyprotein to the membrane. In the case of cells transfected with plasmid expressing myristylated Gag (pcDNA3.HMgag), Gag was localized to the cell membrane [Fig. 2c(i–iii)]. In cells transfected with pTHgrttnC

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**Fig. 3.** Inactivation of the biological activities of vaccine-expressed proteins. (a) Reverse transcriptase assay demonstrating inactivation of vaccine-modified RT. Cells were transfected with SAAVI DNA-C or p96ZM651pol-opt (pPol), expressing HIV-1 subtype C Pol, and lysates were tested for RT activity. Results shown are data from three independent experiments ± SD. (b) CAT assay demonstrating inactivation of the transactivation activity of vaccine-modified Tat. Increasing concentrations (0, 0.5, 5 and 20 μg) of wild-type and shuffled Tat–GFP and 20 μg SAAVI DNA-C were tested for transactivation activity. Results shown are data from three independent experiments ± SD. (c) Immunocytochemical localization of GrttnC polyprotein. HeLa cells were transfected with a plasmid expressing myristylated Gag (pcDNA3.HMgag) and probed with anti-p24 antibody (i) or with pTHgrttnC and probed with anti-Nef antibody (iv). The DAPI-stained images (ii and v) were merged to produce panels (iii) and (vi). Bars, 20 μm.
and probed with anti-Nef antibodies, the polyprotein was localized to the cytoplasm [Fig. 2c(iv–vi)]. This result was confirmed with anti-p24 antibodies (data not shown).

The individual vaccines and SAAVI DNA-C induce potent CTL responses in mice
A strong, peptide-specific CTL response was detected after two inoculations of pTHr.grttnC, pTHr.gp150CT or SAAVI DNA-C (Fig. 4). No CTL activity was detected if an irrelevant peptide was used in the 51Cr-release assay. At an effector:target ratio of 50:1, the mean net RT peptide-specific lysis was 51±24% for an inoculation with pTHr.grttnC (Fig. 4a) and 59±13% for an inoculation with SAAVI DNA-C (Fig. 4c), after the background lysis in the absence of peptide was subtracted. Similarly, mean net V3 peptide-specific CTL activity of splenocytes from mice vaccinated with either pTHr.gp150CT or SAAVI DNA-C was 55±17 and 59±15±6%, respectively (Fig. 4b, d). Thus, no significant difference was observed between the responses to individually administered plasmids and the plasmids administered in an equimolar mixture to mice. Furthermore, the CTL activity generated by the plasmids without the ampicillin-resistance gene was no different from that generated by the experimental plasmids that contained this gene (data not shown).

SAAVI DNA-C elicits high frequencies of IFN-γ-producing CD8+ T cells
Mice immunized with pTHr.grttnC and pTHr.gp150CT elicited high frequencies of CD8+ T cells producing IFN-γ in response to RT and V3 peptides. The percentage of peptide-specific CD8+/IFN-γ+ cells was considered positive if it was greater than twice the background (the absence of peptide stimulation). IFN-γ was not produced by CD8+ cells when stimulated with an irrelevant peptide (data not shown). Responses were at least twice the background number of IFN-γ-producing cells in the absence of peptide. Inoculation of the plasmids in combination (SAAVI DNA-C) did not alter the response to the individual plasmids (Table 2). Furthermore, these results were the same as the results obtained with the experimental vaccines, which contained the ampicillin-resistance gene (data not shown).

SAAVI DNA-C induces high-titre antibody responses to gp120
In order to investigate whether SAAVI DNA-C elicited an antibody response, sera from outbred guinea pigs immunized twice with DNA were tested for the presence of antibodies to HIV-1 subtype C gp120 by ELISA. As shown in Fig. 5, binding antibodies to gp120 were detectable in all six animals, with end-point titres ranging from 500 to 81,920.
Table 2. Frequency of CD8+/IFN-γ+ cells detected by flow cytometry

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<tr>
<th>Inoculum Peptide</th>
<th>Mean CD8+/IFN-γ+ cells* (%)</th>
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<tr>
<td></td>
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<tr>
<td>pTHR.grttnC RT (VYYDPKSDLIA)</td>
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</tr>
<tr>
<td>pTHR.gp150CT V3 (RGPGRAFVTI)</td>
<td>4.0 ± 1.3</td>
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<tr>
<td>SAAVI DNA-C RT (VYYDPKSDLIA)</td>
<td>7.2 ± 1.3</td>
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*Data values are means of four experiments ± SD.

at 8 weeks after the second immunization. No antibody responses were detectable in the two unimmunized animals.

DISCUSSION

Although there are currently numerous candidate HIV-1 vaccines entering the clinical-trial pipeline, those based on subtype B have predominated and are at the most advanced stages of clinical testing (IAVI, 2005), despite the burden of new infections being due to subtype C (Osmanov et al., 2002). Many of the early HIV-vaccine strategies were proof-of-concept vaccines, where only single genes were expressed as immunogens. More recently, new subtype C candidate vaccines, containing multiple HIV-1 genes, have entered clinical trials (IAVI, 2005). Here, we describe the development and preclinical testing of the first HIV-1 subtype C multigene vaccine developed in Africa, SAAVI DNA-C.

The two plasmids making up SAAVI DNA-C expressed full-length GtrtnC (Gag, RT, Tat and Nef polyprotein) and Gp150CT (Env) proteins. The immunogens are expected to be safe, with the function of Tat and RT shown to be abrogated. We have used a novel way to shuffle Tat to inactivate the biological activity of the protein, but still preserve T-cell epitopes. Furthermore, SAAVI DNA-C was found to elicit high frequencies of T cells specific for multiple HIV-1 genes in BALB/c mice, and these were capable of killing target cells and producing high levels of IFN-γ. In addition, high titres of binding antibodies to gp120 were elicited in guinea pigs.

There are numerous obstacles to developing an effective vaccine against HIV-1. We have sought to overcome these in various ways. To overcome inefficient expression of the proteins, all genes in SAAVI DNA-C were codon-optimized and placed under the control of a potent CMV promoter, enhancer and intron A cassette. Low expression levels of artificial proteins, possibly due to the shuffling of proteins, have been reported previously (Nkolo et al., 2004), as these may be targeted for degradation more rapidly. However, the GtrtnC polyprotein was expressed stably in human cells. There was also no evidence of interference in expression or immunogenicity due to the mixture of two plasmids being used, a concern raised in previous studies (Kjerstrøm et al., 2001; Muthumani et al., 2002). A similar DNA vaccine has been developed by the Vaccine Research Centre (NIH, USA) and is currently in phase I clinical trial (HVTN, 2005). This vaccine consists of four plasmids: one encoding a fusion protein (encoding Gag, Pol and Nef from subtype B) in combination with three plasmids encoding Env (from subtypes A, B and C) (Kong et al., 2003; Seaman et al., 2005). The Vaccine Research Center has developed a second-generation DNA vaccine that appears to be more immunogenic. This vaccine contains six plasmids, with the gag, pol and nef genes contained on separate plasmids (Barouch et al., 2005). This concept is also in phase I trial and is scheduled to be tested in a phase II clinical trial in combination with a multiclade recombinant adenoviral-vector vaccine boost (HVTN, 2005).

HIV-1 diversity remains a major challenge in vaccine development and, in order to reduce the impact of diversity, the genes included in the vaccine were selected from recently transmitted viruses that were most similar to a consensus sequence derived from viruses circulating within South Africa (Williamson et al., 2003). The Du422 gag gene has been included in a number of vaccines currently in clinical trial, including the VEE replicon (AlphaVax) and adenovirus-associated virus vaccine (Targeted Genetics; IAVI, 2005).

Fig. 5. Antibody responses to SAAVI DNA-C. Six guinea pigs (761–766) were immunized twice with 500 µg SAAVI DNA-C and antibody responses to HIV-1 subtype C gp120 were evaluated by ELISA. DNA immunizations are indicated by arrows. Data values represent end-point serum titres. The open squares (□) represent control unimmunized animals (792, 760).
and comparative studies could be performed using the same immunogen in different vector backbones in order to compare delivery strategies.

It is likely that first-generation T cell-generating vaccines for HIV-1 will protect from disease rather than infection, so viral escape from vaccine-induced responses is likely to be a further challenge determining the success of these vaccines (Allen et al., 2000; Barouch et al., 2002; Barouch & Letvin, 2004). Inclusion of multiple genes in vaccines may result in broad immune responses, decreasing the likelihood of immune escape. The first evidence for this comes from a study in which macaques were given a vaccine expressing immune escape. The first evidence for this comes from a study in which macaques were given a vaccine expressing Gag, Pol and Env. Four years after challenge with virulent SHIV 89.6P, 22 of 23 animals controlled their viraemia and resisted progression to disease (Sadagopal et al., 2005). We have thus designed SAAVI DNA-C to express five viral proteins. All five proteins have been shown to contain epitopes recognized by HIV-1 subtype C-infected individuals, with Nef, Gag and Pol being the most commonly recognized (Novitsky et al., 2001, 2002; Masemola et al., 2004). Gag and Nef are known to be frequently targeted by both CD4\(^+\) and CD8\(^+\) T cells in both subtype C and subtype B infections (Betts et al., 2001; Addo et al., 2003; Kaufmann et al., 2004). Additionally, some studies show that cellular immune responses to Gag correlate with better control of virus replication in HIV-infected individuals (Connick et al., 2001; Buseyne et al., 2002; Masemola et al., 2004). Strong CD8\(^+\) T-cell responses to Nef are detected during primary infection (Lichterfeld et al., 2004) and inclusion of Nef as well as Tat, expressed early in the virus life cycle, may be important in a vaccine for early clearance of virus-infected cells.

Cellular responses to epitopes contained in the SAAVI DNA-C vaccine have recently been reported in early HIV-1 subtype C infection in southern Africa, summarized in Fig. 6 (adapted from Masemola et al., 2004). Overall, 87 % of infected individuals responded to one or more of the peptides matching the vaccine constructs in this study: 87 % recognizing Nef, 83 % recognizing Gag, 74 % RT, 63 % Env and 17 % Tat. These data demonstrate that the constructs described here are good candidates for a prophylactic vaccine. In addition, inclusion of multiple proteins with high sequence conservation, such as Gag, Nef and Pol, increases the probability of obtaining cross-clade immune responses (Coplan et al., 2005).

There has been limited success thus far with generating neutralizing antibodies against HIV-1 by vaccination. Whilst a truncated gp160 was included in SAAVI DNA-C, it is unlikely that high-titre and broadly cross-neutralizing antibodies will be elicited by the vaccine. SAAVI DNA-C did, however, elicit binding antibodies to gp120. There is evidence that macaques vaccinated with a DNA prime and recombinant MVA boost vaccine expressing Gag–Pol–Env were able to control virus replication better after challenge than those vaccinated with Gag–Pol alone (Amara et al., 2002). This may be due to binding antibodies to gp120, which protect uninfected bystander CD4\(^+\) T cells from gp120-mediated apoptosis.

DNA vaccines have been shown to elicit fairly weak immune responses when administered in clinical trials alone (McConkey et al., 2003; Moorothy et al., 2003, 2004; Mwau et al., 2004). However, much-improved responses have been elicited when DNA vaccines are used as a prime for a boost, such as recombinant MVA or adenovirus (Hanke et al., 1999; Letvin et al., 2004; Smith et al., 2004). In macaques given a DNA prime–MVA boost vaccine followed by challenge, low-level T-cell responses were detectable ex vivo 4 years after SHIV-89.6P challenge (Sadagopal et al., 2005). In humans, a DNA prime–MVA boost regimen has also been shown to result in a long-term memory response lasting at least 6 months post-vaccination (Vuola et al., 2005). SAAVI DNA-C has thus been constructed as the prime for a matching MVA containing identical genes, which is currently in production.

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**Fig. 6. IFN-\(\gamma\) ELISPOT responses in HIV-infected individuals to peptides contained in SAAVI DNA-C, demonstrating that the proteins expressed by the vaccine are frequently targeted in HIV-infected southern Africans.**

(a) Cumulative spot-forming cells (s.f.c.) per 10\(^6\) peripheral blood mononuclear cells (PBMCs) responses to peptide pools represented in SAAVI DNA-C. (b) Frequencies of individuals recognizing each of the peptide pools. Adapted from Masemola et al. (2004).
In summary, we have designed SAAVI DNA-C, a multigene DNA vaccine based on circulating subtype C strains from South Africa. The vaccine is strongly immunogenic in BALB/c mice and expresses genes that are recognized at a high frequency by HIV-infected individuals from southern Africa. This candidate vaccine is expected to enter phase I clinical trials as part of a prime–boost approach with a recombinant MVA vector expressing matching HIV-1 genes in South Africa and the USA (HVTN, 2005).

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