RESEARCH ARTICLE

Engineering RENTA, a DNA prime-MVA boost HIV vaccine tailored for Eastern and Central Africa

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For the development of human immunodeficiency virus type 1 (HIV-1) vaccines, traditional approaches inducing virus-neutralizing antibodies have so far failed. Thus the effort is now focused on elicitation of cellular immunity. We are currently testing in clinical trials in the United Kingdom and East Africa a T-cell vaccine consisting of HIV-1 clade A Gag-derived immunogen HIVA delivered in a prime-boost regimen by a DNA plasmid and modified vaccinia virus Ankara (MVA). Here, we describe engineering and preclinical development of a second immunogen RENTA, which will be used in combination with the present vaccine in a four-component DNA/HIVA-

RENTA prime-MVA/HIVA-RENTA boost formulation. RENTA is a fusion protein derived from consensus HIV clade A sequences of Tat, reverse transcriptase, Nef and gp41. We inactivated the natural biological activities of the HIV components and confirmed immunogenicities of the pTHr.RENTA and MVA.RENTA vaccines in mice. Furthermore, we demonstrated in mice and rhesus monkeys broadening of HIVA-elicited T-cell responses by a parallel induction of HIVA- and RENTA-specific responses recognizing multiple HIV epitopes. Gene Therapy (2004) 11, 1068–1080. doi:10.1038/sj.gt.3302241; Published online 27 May 2004

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Introduction

The best hope for controlling the human immunodeficiency virus type 1 (HIV-1) epidemic is development of a safe, effective, accessible prophylactic HIV vaccine. An effective HIV vaccine may have to stimulate both neutralizing antibodies and cell-mediated immune responses and do so both systemically and at mucosal sites. While the inaccessibility and instability of neutralizing epitopes on primary HIV isolates have hampered the development of envelope-based vaccines,^{1–3} new technologies capable of inducing high levels of circulating CD8 + cytotoxic T lymphocytes (CTLs) are emerging.⁴ CTL recognize peptides that originate from both surface and inner, and structural and nonstructural HIV proteins. Unlike antibodies, CTL cannot prevent a cell-free HIV from infecting host cells. Therefore, vaccine-induced CTL will have to act quickly after initial infection to limit HIV replication, which generates HIV variants and damages the immune system. For that, CTL may have to be in sufficient numbers, which may or may not require persistent vaccine stimulation or regular revaccinations. Preferably, vaccine-induced T cells should recognize early and/or abundant HIV proteins of the transmitting virus/clade, target multiple CTL epitopes in functionally conserved protein regions to make it hard for HIV to escape and kill target cells efficiently. CTL do not act alone. Their action is supported by CD4 + T helper, dendritic, B

and other cells involved in the intricate network of interactions leading to an efficient CTL response.⁵

We have found that a successive immunization with DNA- and modified vaccinia virus Ankara (MVA)-based vaccines expressing a common immunogen is a potent way of inducing CD8 + CTL.^{6,7} Encouraged by the immunogenicity of this approach in primates,^{8–11} we have designed and constructed a DNA-MVA-based HIV vaccine candidate for clinical trials in humans.¹² With a view to proceed to efficacy trials into high-risk cohorts in central and eastern Africa, where 70% of infections are currently caused by HIV clade A, the immunogen HIVA was derived from consensus HIV clade A Gag p24/p17 sequences and a string of clade A CTL epitopes. This vaccine is the first HIV-1 clade A-derived vaccine tested in humans; it does not contain the envelope gene and focuses solely on the induction of cell-mediated immune responses. In preclinical studies, both the pTHr.HIVA DNA and MVA.HIVA vaccines were highly immunogenic in mice^{12,13} and induced multispecific T-cell responses in rhesus macaques.¹⁴ Small phase I clinical trials in healthy uninfected human volunteers run under the auspices of Medical Research Council of the United Kingdom, and the International AIDS Vaccine Initiative showed that the vaccine was safe and immunogenic.¹⁵ These results are being confirmed and extended in several trials in Europe and Africa.

Here, we describe the design and characterization of a second immunogen, designated RENTA, which is also vectored by DNA and MVA, and demonstrate the vaccine immunogenicity in mice and rhesus macaques. Providing that the RENTA vaccines are safe and immunogenic, they will be used in a combination with the HIVA vaccines in four-component DNA/HIVA-RENTA prime-MVA/HIVA-RENTA boost human trials.

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Material and methods

Protein homology search

The RENTA amino-acid sequence was compared with proteins in EMBL – Release 72.0 (09/2002), PIR-Protein – Release 71.0 (01/2002), SWISS-PROT-Protein – Release (25/09/2002), TREMBL-Protein – Release 21.0 (06/2002), and GENPEPT-Protein – Release 131.0 (09/2002) databases using the Wisconsin Package Version 10.2 (Genetics Computer Group (GCG), Madison, WI, USA) software (EXPeCt=10.000000, ALiGn=1000 and all default settings except ktup=1, that is, using a sliding window of three amino acids for the initial match).

Plasmid construction and preparation of pTHr.RENTA and pTHr.HIVA DNAs

Synthetic fragment of 2646 bp carrying the RENTA open-reading frame (ORF) has the overall structure of *HindIII*/*XmaI*-Tat/C-RT-*Bam*HI-Nef-*KpnI*-N-RT-*EcoRI*-gp41/epitopes-*XmaI*/*XbaI*. Each of the four parts flanked by restriction endonuclease sites was constructed separately from partially overlapping approximately 90-mer oligonucleotides, sequenced and corrected using a site-directed mutagenesis. The regions were then sequentially assembled in plasmid pTH (Hanke *et al*¹⁶) yielding pTH.RENTA. As the last cloning step, the β -lactamase gene was removed by collapsing the plasmid at the *Bsp*HI sites (RM Cranenburgh, Cobra Therapeutics Ltd, UK), which resulted in the final pTHr.RENTA stabilized in bacteria by the auxotrophic repressor-titration selection.¹⁷ The RENTA fragment was cut out using *XmaI* and ligated into the *XmaI* site of transfer vector pSC11 (Chakrabarti *et al*¹⁸) for the preparation of recombinant MVA.RENTA and vector pIRES2-enhanced green fluorescent protein (EGFP) (Clontech, USA) expressing EGFP, which was used in assays demonstrating inactivation of Nef functions. All recombinant DNA manipulations used standard procedures. pTHr.RENTA and pTHr.HIVA DNAs were prepared in good manufacturing practice (GMP) facilities of Cobra Therapeutics (Keele, UK).

Construction and preparation of MVA.RENTA

Recombinant MVA.RENTA was made as described previously.¹² Briefly, chicken embryo fibroblast (CEF) cells grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) of American origin, penicillin/streptomycin and glutamine (DMEM 10) were infected with parental MVA at a multiplicity of infection (MOI) of 1, transfected using Superfectin (Qiagen, Germany) with 3 μ g of endotoxin-free pSC11.RENTA also carrying the β -galactosidase gene, and the recombinants were identified by a blue color reaction in the presence of X-gal. Recombinants were subjected to five rounds of plaque purification, after which a master virus stock was grown, purified on a 36% sucrose cushion, titered and stored at -80°C until use. The presence of the correct RENTA ORF was confirmed by sequencing and immunofluorescent detection of the protein in MVA.RENTA-infected cells. The GMP lots of MVA.HIVA were prepared by IDT (Germany).

Immunofluorescence

Six-well plates containing sterile slides pretreated with poly-L-lysine of 70 000–150 000 relative molecular mass (Sigma) were seeded with 293T cells (2×10^5 cells per slide). After 24 h, the cell monolayers were transfected with either pTHr.RENTA or pIRES2-RENTA-EGFP using the Superfect Transfection reagent (Qiagen, Germany), or infected with MVA.RENTA at an MOI of 5. After a 24 h incubation at 37°C in 5% CO_2 , the cells were washed, their membranes were perforated, the slides were blocked with 2% FCS/phosphate-buffered saline (PBS) at 4°C for 1 h and incubated at 4°C overnight with 1:200 dilution of one of the several primary mouse monoclonal antibodies against the Pk tag (Serotec, Oxford, UK), Nef, RT or Tat (EVA352, EVA3019 and EVA3106, respectively, were kindly provided by the Centralized Facility for AIDS Reagents, UK). The following day, the cells were washed in PBS and incubated at 4°C overnight with a 1:500 dilution of an Alexa fluor 594-conjugated anti-mouse antibody (Molecular Probes, OR, USA). After washing in PBS, the slides were stained with DAPI nuclear stain Vectashield (Vector Laboratories, USA) and photographed on a Zeiss immunofluorescence microscope at $40 \times$ magnification. For the colocalization studies following the application of the secondary antibody detecting the anti-Pk monoclonal antibody (mAb), FITC-conjugated anti-GM130 or anti-CD63 antibodies were applied overnight at 4°C and the slides were washed and examined using a confocal microscope.

SDS-polyacrylamide gel (SDS-PAGE) and Western blot analysis

Human 293T cells were either transiently transfected with pTHr.RENTA or infected with MVA.RENTA and lysed 48 h later in the presence of protease inhibitors. Individual polypeptides of the cell lysates were separated on SDS-PAGE crosslinked with 15% (*N,N*-diallyl-tartardiamide (DATD) using thin (0.75 mm) minislab gels of the Bio-Rad electrophoresis system. Separated polypeptides were transferred onto a nylon filter (Amersham International) using a semidry gel electroblotter (LKB). The filters were blocked with PBS + 20% Marvel and incubated with anti-Pk mAb in PBS + 5% Marvel. Bound antibodies were detected using horse radish peroxidase-conjugated protein A (Amersham International) in PBS + 5% Marvel followed by enhanced chemiluminescence (Amersham International).

HLA class I and CD4 downregulation assay

A measure of 6 μ g of either pIRES2.RENTA-EGFP, pIRES.Nef-EGFP (NC and X-NX, unpublished) or pIRES-EGFP (Clontech) was nucleofected into 5×10^6 human peripheral blood mononuclear cells (PBMC) as per the vendor's recommendation (Amara Biosystems, Germany). At 48 h postnucleofection, cells were costained with PE-conjugated anti-CD4 (PharMingen) and APC-conjugated anti-HLA-A, -B, -C (PharMingen) mAbs, fixed and stored at 4°C until use. The percentage of downregulation was calculated using mean cell fluorescent intensities in the upper left (UL) and upper right (UR) quadrants as follows: $(\text{UL} - \text{UR}) / \text{UR} \times 100$. This calculation does not take into consideration cells with 100% downregulation, that is, cells in the lower right quadrant.

Chloramphenicol acetyl transferase (CAT) assay

CAT assay was carried out to detect the transactivation of Tat; six-well plates were seeded with 293T cells (5×10^5 cells per well) and 24 h later cotransfected with 5 μ g of DNA per each plasmid using Superfect Transfection reagent (Qiagen, Germany) following the vendor's protocol. In brief, cells were transfected with plasmid pOGS210 containing wild-type HIV-1 long terminal repeat (LTR) fused to the bacterial CAT reporter gene¹⁹ alone as a negative control, a mixture of plasmids pOGS210 and pOGS213, which expresses wild-type Tat under the control of a CMV promoter as a positive control, or a mixture of pOGS210 and pTHr.RENTA plasmids (the pOGS210 and pOGS213 plasmids were kindly provided by Dr W James, Oxford University). At 24 h post-transfection, cells were washed once with PBS, scraped from the wells, re-suspended in 2 ml of 0.25 M Tris-HCl pH 7.5 and subjected to three freeze-thaw cycles using methanol/dry-ice mixture and a 37°C waterbath. The lysates were chilled on ice and the supernatant collected by centrifugation for 5 min at 240 g at 4°C. The econofluor diffusion method²⁰ was used to assess CAT activity in 50 μ l of cell lysates.

Peptides and preparation of peptide pools

High-performance liquid chromatography-purified peptides were purchased from Sigma-Genosys (Cambridge, UK), with a purity of at least 80% by mass spectroscopy. Individual peptides were dissolved in dimethyl sulfoxide (Sigma-Aldrich, Irvine, UK) to yield a stock of 10 mg/ml and stored at -80°C.

For both HIVA and RENTA immunogens, 15-mers overlapping by 11 amino-acid residues across HIV proteins were employed. For HIVA, four pools of 20–23 peptides corresponding to the gag p24/p17 regions were prepared. The RENTA-derived peptides were combined into six pools of 22 (called RENTA1 and corresponding to Tat), 43 (RENTA2 – C-RT), 36 (RENTA3 – Nef), 36 (RENTA4 – N-RT), 36 (RENTA5 – N-RT) and 34 (RENTA6 – gp41) peptides. To prepare pools, 20 μ l of each peptide was combined and PBS was added to 5 ml. The pools were filtered, aliquoted, stored at 4°C and used within 1 week. For the assays, a final concentration of 4 μ g/ml of each peptide was used.

Mouse immunizations and isolation of splenocytes

Groups of 5- to 6-week-old female BALB/c mice were injected with pTHr.RENTA or pTHr.HIVA plasmid DNA or MVA.RENTA or MVA.HIVA into their anterior tibial muscles in PBS (Sigma) under general anaesthesia. The amounts of DNA and number of plaque-forming units (PFU) of MVA are indicated for each experiment in the figure legends. At 10 days after the last immunization, the animals were killed, their spleens were removed and pressed individually through a cell strainer (Falcon) using a 2-ml syringe rubber plunger. The splenocytes were washed twice and suspended in 10 ml of Lymphocyte medium (RPMI 1640 supplemented with 10% FCS penicillin/streptomycin, 20 mM HEPES and 15 mM 2-mercaptoethanol). All animal procedures and care strictly conformed to the UK Home Office Guidelines.

Bulk CTL cultures

Cell suspensions (8 ml) containing 80% of the total splenocytes were incubated with 2 μ g/ml of peptide in a humidified incubator in 5% CO₂ at 37°C for 5 days. On the day of the CTL assay, the effectors were washed 3 $\times$ with RPMI, resuspended at 10^7 per ml in R10 (RPMI 1640 supplemented with 10% FCS and penicillin/streptomycin) and used in a ⁵¹Cr-release assay as described below.

Target cells and standard ⁵¹Cr-release assay

The effector cells were diluted two-fold in U-bottom wells of a 96-well plate (Costar) to yield after addition of the target cells effector to target ratios between 200:1 and 3:1. ⁵¹Cr-labelled P815 cells (5000) in a medium with or without 2 μ g/ml of peptide was then added to the effectors and incubated at 37°C for 5 h. Spontaneous and total chromium release was estimated from wells, in which the target cells were kept in a medium alone or 5% Triton X-100, respectively. The percentage specific lysis was calculated as (sample release – spontaneous release)/(total release – spontaneous release) $\times$ 100. The spontaneous release was lower than 5%.

Production of tetrameric MHC/peptide complexes

The genes coding for the Mamu-A*01, and H-2D^d chains were kindly provided by Drs DI Watkins (Wisconsin Regional Primate Research Center, USA) and JD Altman (Emory University School of Medicine, USA). Tetrameric complexes were prepared using standard procedures.²¹ Briefly, both heavy and light chains of MHC were expressed in *E. coli* strain BL-21, purified from inclusion bodies, denatured in 8 M urea and refolded in the presence of peptide, biotinylated using the BirA enzyme (Avidity) and purified on FPLC and monoQ ion exchange columns. The formation of tetrameric complexes was induced by the addition of chromogen-conjugated streptavidin (ExtraAvidin; Sigma) to the refolded biotinylated monomers at molar ratio of MHC-peptide monomer:PE-streptavidin of 4:1. Labeled tetrameric complexes were stored in the dark at 4°C until use.

Mouse IFN- γ ELISPOT assay

The ELISPOT assay was carried out using the Mouse IFN- γ Secreting Cell Kit (BD Biosciences, UK) according to the manufacturer's instructions. In brief, 10^5 isolated splenocytes depleted of red blood cells were restimulated in duplicates in anti-IFN- γ -precoated 96-well plates with R10 alone, supplemented with concanavalin A at 4 μ g/ml or specific peptide at 2 μ g/ml for 18 h at 37°C in 5% CO₂. Following lysis of the cells by a 10 min incubation with water on ice, spots were visualized using sequential applications of a biotin-conjugated secondary anti-IFN- γ antibody, avidin-horseradish peroxidase and AEC (3-amino-9-ethyl-carbazole, Sigma, UK) and H₂O₂ (30%). Spots were counted using an ELISPOT reader (Autoimmun Diagnostika GmbH, Germany) and expressed as spot-forming units (SFU) per 10^6 splenocytes.

Mouse intracellular cytokine staining

Isolated mouse splenocytes were stimulated with appropriate peptide- or peptide pool-pulsed P815 cells in the presence of anti-CD28/antiCD49d mAbs for 90 min at

37°C in 5% CO₂. Brefeldin A was then added to inhibit cytokine secretion and the samples were incubated for additional 6 h before terminating the reaction with EDTA and the FACS fix solution. The cells were permeabilized and incubated with anti-CD8-PE (BD PharMingen) and anti-IFN- γ -FITC (BD PharMingen) monoclonal antibodies and analyzed using flow cytometry.

Monkey immunizations and isolation of PBMC

Rhesus macaques (*Macaca mulatta*) positive for the Mamu-A*01 allele of MHC class I received four successive immunizations, two using the plasmid pTHr.HIVA and pTHr.RENTA DNA and two with recombinant MVA.HIVA and MVA.RENTA (see legend of Figure 8). The animals received 1 mg of each DNA in 0.5 ml of 140 mM NaCl, 0.5 mM Tris-HCl pH 7.7 and 0.05 mM EDTA intramuscularly (i.m.) and 5×10^7 PFU of each MVA in 0.1 ml of 140 mM NaCl and 10 mM Tris-HCl pH 7.7 intradermally. The HIVA vaccines were delivered into the animals' arms and the RENTA vaccines into thighs. Monkey PBMC were isolated using the Lymphoprep cushion centrifugation (Nycomed Pharma As). All immunizations and venipunctures were carried out without sedation and the animals were regularly clinically examined. All procedures and care strictly conformed to the UK Home Office Guidelines.

Monkey IFN- γ ELISPOT assay

The IFN- γ release upon a HIVA-specific restimulation was assessed in an ELISPOT assay. The procedures and reagents of the MABTECH kit (Cat. No. 3420M-2A) were used throughout. Briefly, PBMC were isolated on a Lymphoprep cushion and incubated at 37°C in 5% CO₂ for 24 h with peptides. The released IFN- γ was captured by a monoclonal antibody immobilized on the bottom of assay wells, visualized by combination of a second monoclonal antibody coupled to an enzyme and a chromogenic substrate, and the spots were counted using the AID ELISpot Reader System (Autoimmun Diagnostika GmbH).

Monkey tetramer staining

MHC/peptide tetrameric complex analysis was carried out as described previously.⁸ Briefly, heparinized blood drawn from immunized macaques was first restimulated with the appropriate peptide for 2 weeks at 37°C in 5% CO₂ with an addition of huIL-7 on day 0 and huIL-2 on day 3. On the day of the assay, the cells were reacted with PE-conjugated Mamu-A*01/peptides tetrameric complexes and mouse anti-huCD8-PerCP (BD PharMingen) monoclonal antibody and analyzed using flow cytometry.

FACS analysis

All chromogen-labelled cells were analyzed by flow cytometry using the CellQuest software (BD Biosciences, UK).

Monkey bulk CTL cultures and a standard ⁵¹Cr-release assay

Eight million of isolated PBMC were restimulated with 10 μ M peptide in 100 μ l of R20 in 5% CO₂ at 37°C for 1 h and resuspended in a total of 4 ml of R20 supplemented with 25 ng/ml of huIL-7 in two 24-well plate wells. On

day 3, Lymphocult-T (Biotest AG) was added to the final concentration of 10% (v/v). On day 8, 5×10^6 peptide-pulsed irradiated autologous B lymphoblastoid cell lines (B-LCL) was added to the cultures followed by Lymphocult-T on day 11. Cytolytic tests were carried out on day 14.

In a ⁵¹Cr-release assay, the effector cells were diluted sequentially two-fold in U-bottom wells (96-well plate; Costar) at effector to target ratios indicated in the figures. ⁵¹Cr-labelled autologous B-LCL (5000) pulsed (2 μ g/ml) or unpulsed with peptide was added to the effectors and incubated at 37°C for 6 h. The percent-specific lysis was calculated as for the mouse lysis assays. Spontaneous release was for all samples below 20% of the total counts.

Results

Design of the RENTA immunogen

An important aspect of vaccine development is finding formulations capable of inducing T-cell responses specific for multiple HIV proteins. To broaden responses induced by the existing HIVA vaccines, which deliver p24/p17 of Gag and a string of CTL epitopes recognized by human, monkey and mouse CTL, we designed a second immunogen designated RENTA and inserted its gene into the plasmid pTHr DNA and MVA vectors. RENTA is derived from the reverse transcriptase (RT), Env, Nef and Tat proteins of HIV-1 clade A sequence and is 871 amino acids long (Figure 1). It contains regulatory proteins Tat and about 69% of the CTL epitope-rich C-terminus of Nef, which are expressed abundantly and early after HIV infection. In tissue culture, CTL recognizing early proteins killed HIV-infected cells before they produced more virus virions,^{22,23} and produced chemokines that inhibit HIV replication.^{24–27} *In vivo*, vaccine-induced CTL against early proteins provided a degree of protection against pathogenic virus challenges.^{28–30} However, the protective role of Tat alone has been controversial. Despite initial successful reports,^{31,32} subsequent studies failed to demonstrate protective efficacy of Tat-specific CTL only.^{33,34} Nevertheless, simian immunodeficiency virus (SIV) Tat is an early protein against which responses impose a selective pressure on incoming virus,³⁵ and therefore it is an important component for a prophylactic vaccine. RT was included into the RENTA immunogen because it is a relatively conserved protein with a large number of identified CTL epitopes. To further increase the potential for elicitation of a broad CTL response, two CTL epitope-rich regions of the envelope transmembrane glycoprotein gp41, which may be an important CTL target and is less variable than gp120, were included. Until CTL vaccines are proven to induce broad T-cell responses in humans, it is important to match the vaccine clade to the HIV strain predominant in the target population. Therefore, similar to HIVA, all RENTA subunits were derived from the HIV-1 clade A consensus sequences.³⁶ The use of the consensus sequence is justified by the shortest distances between this 'average' and individual isolate sequences.³⁷ In addition, the HLA-A*6802-restricted CTL epitope DTVLEDINL from HIV pol was inserted, which was associated with long-term nonprogression.³⁸ Also incorporated were a *Plasmodium berghei*-derived epitope SYIPSAEKI, called pb9, presented by H-2K^d (Romero *et al*³⁹) and SIV

Figure 1 The RENTA immunogen. (a) Schematic representation of immunogen RENTA with HIVA also shown for a reference. The two proteins will be used together in clinical trials expressed from DNA and MVA vectors. (b) The amino-acid sequence of the RENTA immunogen with indicated origins of each segment. Small letters correspond to restriction endonuclease sites used to assembled to synthetic gene. Relevant epitopes are color coded.

Tat-derived epitope STPESANL presented by Mamu-A*01 (Allen *et al*³⁵) to facilitate the measurement of vaccine-induced immune responses in mice and rhesus macaques, respectively. Finally, mAb epitope Pk (Hanke *et al*⁴⁰) was added at the C-terminus for convenient detection of the RENTA protein.

Amino-acid sequence of the RENTA polypeptide was compared with proteins in the available databases. With the exception of sequences derived from exo- and endogenous retroviruses, no homologies to human proteins greater than 27% over 175 amino acids (hepatocyte nuclear factor 3- β HNF-3B) were found. Therefore, immunization with the RENTA vaccines is not likely to trigger an autoimmune reaction.

Design of the RENTA gene, and the recombinant DNA and MVA vectors

The RENTA gene was designed to maximize the expression of the immunogen. The whole gene fragment of 2.6 kbp was made synthetically, which made it possible to use consensus sequence and humanize amino-acid codons, which increase the stability of heteronuclear RNA and possibly the efficiency of translation.⁴¹ The ORF was preceded by a consensus

Table 1 Genetic stability of MVA.RENTA

	Blind passage 0	Blind passage 7
Experiment 1		
Neutral red	132 ^a	60
X-gal	146	66
Experiment 2 (in duplicate)		
Neutral red	175	104
	186	86
X-gal	165	90
	199	95
Total experiment 1+2		
Neutral red	493	250
X-gal	510	251

^aData are expressed as numbers of plaques per well.

Kozak sequence to -12 nucleotides, which increases the usage of the first methionine as the translation initiation codon.⁴²

In the pThr vector, the RENTA transcription is controlled by an efficient enhancer/promoter/intron A cassette derived from the human cytomegalovirus strain

AD169 (Whittle *et al*)⁴³ and a bovine growth hormone polyadenylation site.⁴⁴ The pTHr.RENTA plasmid uses a repressor-titration system for bacterial selection and does not carry any antibiotic-resistance gene.¹⁷ This lowers the total amount of delivered DNA and increases the safety of the plasmid DNA, especially should the vaccine be delivered mucosally, that is, at sites potentially colonized by microorganisms.

MVA is an attenuated smallpox vaccine that fails to replicate in most mammalian cells.⁴⁵ The fragment coding for RENTA was inserted into the thymidine kinase locus of the virus genome under the P7.5 early/late promoter using plasmid pSC11, which codelivered a β -galactosidase gene facilitating the screening, titration and stability studies of the recombinant MVA.RENTA.¹⁸ This marker enzyme is commonly expressed by human enteric bacteria and has been safely used in several clinical trials including healthy HIV-uninfected volunteers vaccinated with MVA.HIVA.¹⁵

Expression in human cells

The RENTA protein expression was characterized in detail. First, we assessed the expression of the RENTA protein in human 293T cells transiently transfected with pTHr.RENTA. Using immunofluorescence and mAb recognizing HIV Tat, RT, Nef and the C-terminal epitope Pk, clear signals were obtained in the plasmid-transfected cells (Figure 2a–d). Combinations of anti-Pk and anti-CD63 (lysosomal marker; Figure 2e and g) or anti-GM130 (Golgi matrix marker; Figure 2f and h) mAbs

were used to approximate the subcellular localization of RENTA, or strictly speaking its C-terminal Pk tag. While the anti-Pk mAb did not colocalize significantly with the lysosome/late endosome marker, RENTA seemed to accumulate largely in the Golgi apparatus (Figure 2g and h). The fact that on a Western blot, the anti-Pk mAb detected primarily a full-size protein of a predicted relative molecular mass of 99.4 kDa only in the presence of proteasome inhibitor lactacystin suggests that the majority of the RENTA is normally degraded (Figure 2j). The expression of RENTA in MVA-infected cells was also confirmed using the anti-Pk mAb (Figure 2i), but no protein was detected on a Western blot (Figure 2j). This was similar to our previous experience with unstable recombinant proteins expressed from recombinant MVAs.^{12,46} As CTL recognize peptide fragments derived from degraded HIV proteins, this is not a concern for the vaccine immunogenicity (see below).

Genetic stability of MVA.RENTA

The genetic stability of the inserted RENTA and β -galactosidase genes was confirmed by seven blind sequential passages of the MVA.RENTA in CEF. The original (passage 0) and final (passage 7) virus stocks were used to infect duplicate wells, of which one well was stained with neutral red to detect any MVA plaques (both the no-insert parental MVA and MVA.RENTA) and the other with X-gal to detect the inserted β -gal gene (Table 1). Comparison of the two titers suggested that MVA.RENTA was stable. In addition, immunofluores-

Figure 2 Expression of RENTA. The expression of the RENTA chimeric protein in human 293T cells from pTHr.RENTA (a–d, g and h) and MVA.RENTA (i) was detected using immunofluorescence and mAb to the indicated subdomains. For, (a–d), the nuclei are shown in blue, Tat, RT and Pk in red and Nef in green. Colocalization of a related fusion protein containing unmutated Tat (red anti-Pk; e and f) and RENTA (red anti-Pk; g and h) with lysosomal/late endosomal marker (green; e and g) and the Golgi matrix protein (green; f and h) in transfected 293T cells. The arrows indicate the presence of a recombinant protein in the nucleus consistent with the NLS of unmutated Tat. (j) shows a Western blot analysis of polypeptides from DNA-transfected and MVA-infected cells using the anti-Pk mAb. Only in DNA-transfected cells, a 99.4 kDa band was detected. Relative molecular masses of protein markers are indicated.

cence analysis of CEF cells infected with viral stocks from passages 0 and 7 indicated that the numbers of cells expressing RENTA were comparable (not shown).

Inactivation of RENTA's biological activities

To increase the vaccine safety, several of the biological activities of the individual RENTA components were inactivated. While a degree of inactivation was likely to occur through the assembly of individual domains into

Figure 3 Inactivation of transcriptional transactivation from HIV LTR. Human 293T cells were transiently transfected with LTR-CAT plasmid alone (white); LTR-CAT and CMV-Tat plasmids (grey) or LTR-CAT and pThr.RENTA plasmids (black), and the amount of the induced CAT enzyme activity in cell lysates was estimated from the production of [3 H]acetylchloramphenicol. The figure shows the mean $\pm$ s.d. of two independent experiments, each carried out in duplicate.

an artificial fusion protein, some activities were specifically targeted by amino-acid substitutions or deletions and the inactivation of the RENTA protein was confirmed formally in appropriate assays. Thus, for the Tat protein, the nuclear localization signal (NLS; RKRRRQRRR) was deleted. While related immunogen RTNA containing unmutated Tat (TH, unpublished) was readily found in the nuclei (Figure 2e and f), the RENTA protein was never detected in this compartment (Figure 2G and H). Two amino acids implicated in the Tat-induced transcriptional transactivation, Cys22 and Lys41 (Ruben *et al*⁴⁷), were substituted with Gly, and the lack of transactivation from the HIV LTR promoter was confirmed using CAT reporter gene and a standard CAT assay (Figure 3). The integrin interaction domain of Tat (RGD domain) is not present in RENTA. As the Nef protein in RENTA is missing its N-terminal region, it cannot be myristylated and exert its biological functions. To demonstrate this formally, the RENTA gene was subcloned into a bicistronic plasmid expressing green fluorescent protein (GFP) and the RENTA protein production from this plasmid was confirmed by immunofluorescence (not shown). Upon transfection of human PBMC, the cells expressing GFP and therefore RENTA did not downregulate the surface expression of HLA class I and CD4 molecules, while the wild-type Nef-expressing cells did (Figure 4). HIV RT was inactivated by splitting the enzyme just downstream of the active site and separating its N- and C-terminal regions (Figure 1a). This inactivation was chosen because there are conserved CTL epitopes across the enzyme active site.

Immunogenicity in the BALB/c mouse

The immunogenicities of the pThr.RENTA and MVA.RENTA vaccines were assessed in mice using pb9

Figure 4 Inactivation of Nef activities. Human PBMC were transfected with plasmids expressing either GFP alone, wild-type Nef and GFP or RENTA and GFP. The top and bottom panels show cells from the same transfection stained for their surface expressions of MHC class I (top) and CD4 (bottom). The percentage downregulation is indicated in each graph. The method used to calculate the percentage downregulation underestimates the CD4 downregulation by Nef as it does not take into account cells with 100% downregulation, that is, cells in the lower right quadrant. The experiment was carried out three times with two different Nefs as positive controls and gave similar results for the effects of RENTA.

epitope SYIPSAEKI incorporated towards the protein's C-terminus (Figure 1a). Groups of mice were immunized once with either pThr.RENTA DNA or MVA.RENTA using doses and routes indicated in the legend of Figure 5 and killed 10 days later. Vaccine-elicited CTL responses were measured for individual mice in a ^{51}Cr -release assay after a 5-day *in vitro* peptide restimulation. The assay showed that all animals responded to the immunization and relatively high levels of lytic activities were detected (Figure 5a).

The RENTA vaccines will be used ultimately in a DNA prime-MVA boost protocol together with the currently clinically tested HIVA vaccines. Therefore, it is important to demonstrate the immunization efficiencies of the RENTA vaccines in a prime-boost protocol alone and in combination with the HIVA vaccines. Elicited immune responses to both RENTA and HIVA immunogens were monitored using epitopes pb9 (in RENTA) and P18-I10 (in HIVA) in ^{51}Cr -release and IFN- γ ELISPOT assays. Whether the vaccines were applied alone or in a combination, both epitopes were approximately equally immunogenic, and in the bulk peptide-restimulated cultures induced similar lytic activities (Figure 5b) and comparable numbers of SFU producing IFN- γ upon peptide stimulation (Figure 5c). Thus, no decrease in the induction of these effector functions was observed following the HIVA and RENTA coimmunization (Figure 5b and c).

To optimize the combined vaccine delivery, the effect of delivering the HIVA and RENTA vaccines into the same or separate hind legs was assessed. A DNA prime-MVA boost regimens were used, whereby the HIVA constructs were injected into the left hind legs and the RENTA constructs into the right hind legs, that is, separate DNA prime-separate MVA boost (SS), the HIVA and RENTA DNAs were injected into separate legs while the MVAs were mixed and applied into both legs (SM), the HIVA and RENTA DNAs were mixed and MVAs injected into separate legs (MS), or both hind legs were injected with mixed HIVA and RENTA DNAs and mixed MVAs (MM). The vaccine dosing is described in the legend of Figure 6. At 10 days after the second immunization, elicited immune responses were assessed first in an intracellular IFN- γ staining assay employing the P18-I10, pb9, RT1 and RT2 epitopes, (Figure 1) and pools of 15-mer peptides overlapping by 11 amino acids across the entire RENTA immunogen. The observed frequencies of INF- γ -producing cells upon peptide restimulation *in vitro* suggested that mixing provides a certain advantage over separate vaccine deliveries (Figure 6a). This hierarchy was also seen analyzing the H-2D^a/P18-I10 tetramer reactivities (Figure 6B); a similar trend was suggested by *ex vivo* IFN- γ ELISPOT assay (Figure 6c), but could not be seen in the ^{51}Cr -release assay, which, however, *in vitro* expands the memory cells for 5 days and might obscure initial cell number differences (Figure 6d). Examples of the intracellular cytokine and tetramer staining of representative mice are shown in Figure 7a and b, respectively. Thus, mice immunized using the combined DNA-MVA/HIVA-RENTA responses to at least five distinct T-cell epitopes: P18-I10 of HIVA, and pb9 and three peptide pools of RENTA (Figure 7a).

Figure 5 Immunogenicity of the RENTA vaccines in the BABL/c mouse. (a) Immunogenicities of the individual pThr.RENTA (25 μg i.m.) and MVA.RENTA (10^6 PFU i.m.) vaccine components alone are shown. Splenocytes from individual animals were restimulated for 5 days in culture with the pb9 peptide and tested in a ^{51}Cr -release assay on peptide-pulsed (full) or -unpulsed (open) target cells. The establishment of relative immunogenicities is complicated by the fact that these experiments were not carried out in parallel on the same day. (b) The DNA prime/MVA boost regimens using the same total doses and routes as in 'a' of HIVA (top left) or RENTA (top right) alone or a mixed HIVA-RENTA immunization (bottom) were assessed for the elicitation of T-cell responses against the RGPGRFVTL epitope of HIVA (diamonds) and SYIPSAEKI epitope of RENTA (circles). A 5-day *in vitro* restimulated effectors were assayed on peptide-pulsed (full) or unpulsed (open) targets. (c) Splenocytes from the same mice as in (b) (immunizations indicated below) were also tested *ex vivo* for production of IFN- γ in an ELISPOT assay using the RENTA (hatched) or HIVA (open) epitopes. In all panels, the results are shown as arithmetic mean $\pm$ s.d.

Figure 6 Optimizing delivery of combined HIVA + RENTA vaccines. Groups of mice were immunized with pTHr.HIVA DNA and MVA.HIVA into their left hind legs and pTHr.RENTA DNA and MVA.RENTA into their right hind legs (SS – thin stripes bottom left to top right), DNAs into separate hind legs and mixed MVAs into both hind legs (SM – thick stripes top left bottom right) mixed DNAs into both legs and MVA into separate legs (MS – thick stripes bottom left to top right) or mixed DNAs and mixed MVAs into both legs (MM – thin stripes top left to bottom right). Plasmid DNA (25 μ g) i.m. and 5×10^4 PFU of each MVA i.m. were used. Naïve mice served as a control (empty). Several T-cell assays were employed to characterize the elicited immune responses: (a) Intracellular IFN- γ staining of splenocytes following *ex vivo* restimulation with peptides indicated below the graph; (b) H-2D^b/P18-I10 tetramer staining; (c) IFN- γ ELISPOT assay using peptides indicated below and (d) ⁵¹Cr-release assay using regimes SS (gray circles), SM (gray squares) MS (black circles) and MM (black squares) and target P815 cells unpulsed (open) or pulsed (full) with peptides indicated at the top of the graphs. For all assays, splenocytes from individual mice were treated separately and the results are expressed as an arithmetic mean $\pm$ s.d. of a particular treatment group.

Immunogenicity in non-human primates

Vaccine immunogenicity in the mouse does not always transfer into primates. Here, we demonstrated that the new RENTA vaccines and the HIVA vaccines currently used in humans can be delivered together and induce multispecific immune responses in rhesus macaques. Mamu-A*01 + animals were immunized twice with the DNA and twice with the MVA vaccines at short or long intervals between the DNA and MVA copying two schedules of human phase II trials (unpublished; see Figure 8 legend). Taking advantage of the Gag CTPY-DINQM and Tat STPESANL Mamu-A*01-restricted epitopes in the HIVA and RENTA immunogens, respectively, immune responses were analyzed using corresponding tetrameric MHC Mamu-A*01/peptide complexes. Although tetramer reactivities of *ex vivo* PBMC were near to the sensitivity of this technique (not shown), tetramer-reactive CD8 + cells were readily detected after DNA immunization only following peptide-specific expansion of PBMC *in vitro* (Figure 8a). Similar results were obtained in animals immunized by the DNA prime-MVA boost regimen (Figure 8b). Using

both the Mamu-A*01-restricted and overlapping peptides derived from the HIVA and RENTA immunogens, multispecific responses were detected in both vaccines in an IFN- γ ELISPOT assay *ex vivo* (Figure 8c) and in a standard ⁵¹Cr-release assay following a 2-week peptide restimulation in culture (Figure 8d). Note that the p11C C-M epitope, which is immunodominant during SIV infection of Mamu-A*01 + animals, is not immunodominant in this setting.¹⁴ Thus, the majority of these animals responded to at least seven different CTL epitopes: CTPYDINQM and two peptide pools of HIVA, and STPESANL and three peptide pools of RENTA (Figure 8d).

Discussion

Novel candidate prophylactic HIV-1 vaccines pTHr.RENTA and MVA.RENTA were engineered. These are vectored by plasmid DNA and MVA (an attenuated small pox vaccine) delivered in a sequential prime-boost regimen. Both vaccine components express a common

Figure 7 Representative examples of intracellular cytokine and H-2D^d/P18-I10 tetramer staining of mouse splenocytes. (a) shows IFN- γ production by splenocytes isolated from a mouse immunized using mixed HIVA and RENTA vaccines in a DNA prime and MVA boost regimen, and a naive mouse as a control. The breadth of vaccine-elicited immune responses was assessed by using individual epitope peptides or overlapping peptide pools across RENTA indicated above. Inserted numbers indicate IFN- γ -producing cells as a percentage of CD8⁺ splenocytes. (b) Effect of separate or mixed deliveries of the HIVA and RENTA vaccines on immunogenicity (see text for the immunization protocols). Inserted numbers give the percentage of CD3⁺ CD8⁺ splenocytes reactive with the tetramer.

immunogen RENTA and were shown to induce broad T-cell responses in mice and rhesus macaques as measured by several different functional T-cell assays.

In human vaccine trials, safety is the prime concern. This applies to HIV vaccines given as a prophylaxis to general HIV-uninfected population including children/neonates and as an immunotherapy for HIV infected, potentially immunocompromised individuals. Each HIV protein has its biological function(s) involved in the virus life cycle and virus–host interactions. As many of these biological activities contribute to the pathology of HIV infection, the use of these proteins even as subunits in vaccine formulations raises safety concerns. This is particularly true for the early multifunctional regulatory proteins of HIV. To minimize the chances of adverse effects caused by the overexpressed virus-derived immunogens, we inhibited a number of activities of the RENTA subdomains and confirmed formally the lack of these activities in appropriate assays, although the protein instability might have also contributed. We believe that this may facilitate the approval of RENTA clinical trials by the regulatory authorities.

We are currently running clinical trials involving immunogen HIVA.¹² To increase the likelihood that vaccines will benefit from the CTL approach, we will use a second immunogen RENTA together with HIVA to broaden the elicited immune responses to multiple HIV proteins and thus make it harder for HIV to escape. There is a theoretical advantage in using several smaller immunogens delivered individually by separate vaccine vectors compared to one large multiprotein expressed

from a single vector, because the former may reach separate antigen-presenting cells and induce at least two immunodominant responses, one to HIVA and one to RENTA.⁴⁸ Unless crosspriming plays a central role, no matter how many viral components a multiprotein will express, they will be all produced by one cell and open to a competition with each other. However, a balance has to be struck between the breadth of elicited immune responses, and practicalities and cost of vaccine development and production, the former increasing and the latter decreasing the number of vaccine components.

We found immune responses to be stronger by at least two functional assays when the HIVA and RENTA vaccines were coinjected into the same sites (Figure 6). This was unexpected, but could be explained by increased migration of T cells of different specificities into the same areas because of the broader available antigenic spectrum, by increased adjuvancy coming from doubling the total local doses of vaccines or by doubling the number of involved antigen-presenting cells by injecting each vaccine into two separate sites for the mixed prime-mixed boost delivery compared to one site for the separate deliveries. These possibilities are being addressed in further experiments with a particular attention to the effect of the number of injection sites on the overall immunogenicity and the detailed analysis of the immune responses to the HIVA and RENTA immunogens and their interplay in the BALB/c mouse (E-J Im, unpublished).

Both the HIVA and RENTA immunogens are derived from HIV-1 sequences, thus there is no relevant challenge

Figure 8 Immunogenicity of the combined DNA-MVA/HIVA-RENTA immunization in non-human primates. Mamu-A*01 + rhesus monkeys were vaccinated twice using 1 mg of each pThr.HIVA and pThr.RENTA plasmid DNA on weeks 0 and 4 followed by 5×10^7 PFU of each MVA.HIVA and MVA.RENTA on weeks 20 and 24 (monkeys 1–3) or weeks 8 and 12 (monkeys 4 and 5). MHC/peptide tetramer reactivities after pThr.RENTA DNA alone in PBMC (week 16; a) and after a prime-boost vaccination (week 22; b) following a 2-week peptide expansion in culture are shown and percentages of CD8+ PBMC reactive with tetramers are indicated. Examples from two monkeys only are presented for the sake of space. c IFN- γ ELISPOT assay was carried out on freshly isolated PBMC (week 22) using both the Mamu-A*01-restricted epitope peptides (G for Gag and T for Tat) and overlapping pools of peptides across the HIVA and RENTA proteins (numbers below). Only one animal is shown because of high no-peptide backgrounds (b) in the other monkeys' PBMC. (d) Standard ^{51}Cr -release assay after a 2-week peptide restimulation *in vitro* of PBMC (week 26). Dark blue – Tat peptide; light blue – Gag peptide; orange and red – HIVA peptide pools 1 + 2 and 3 + 4, respectively; and dark green, light green and purple – RENTA peptide pools 1 + 3, 4 + 5 and 2 + 6, respectively. B-LCL of monkey 1 did not grow, thus monkey 1 was not tested in this assay.

virus to assess the efficacy of this approach in an animal model. For the efficacy experiment, SIV-derived HIVA- and RENTA-equivalent vaccines would need to be constructed. Although without a doubt, the induction of HIV-neutralizing antibodies by a prophylactic vaccine would be highly desirable, the DNA-MVA/HIVA-RENTA vaccines focus solely on the induction of T-cell memory. This is because a reliable method for induction of such antibody specificities by active immunization is still awaited. Therefore, leaving a 'gap' for envelope in our vaccination approach will enable us to assess the role in the protection against HIV infection and/or disease of cell-mediated responses alone and add an envelope-derived component in the future when it becomes available.

Providing no toxicity is observed, we will aim to test the four-component DNA-MVA/HIVA-RENTA vaccines in efficacy clinical trials in central and eastern Africa. It is not known what level and/or kind of memory CD8 T-cell response will be needed to justify an efficacy trial in humans. This will be informed partly by the levels and qualitative aspects of these responses in HIV-resistant

sex workers,⁴⁹ partly by macaque studies,⁵⁰ and partly by the vaccines ability to induce immune responses capable of imposing a selective pressure on breakthrough HIV infections in phase IIB clinical trials in high-risk volunteers in Africa.

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