Short Communication

Enhanced immunogenicity using an alphavirus replicon DNA vaccine against human immunodeficiency virus type 1

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With the human immunodeficiency virus type 1 (HIV-1) epidemic expanding at increasing speed, development of a safe and effective vaccine remains a high priority. One of the most central vaccine platforms considered is plasmid DNA. However, high doses of DNA and several immunizations are typically needed to achieve detectable T-cell responses. In this study, a Semliki Forest virus replicon DNA vaccine designed for human clinical trials, DREP.HIVA, encoding an antigen that is currently being used in human trials in the context of a conventional DNA plasmid, pTHr.HIVA, was generated. It was shown that a single immunization of DREP.HIVA stimulated HIV-1-specific T-cell responses in mice, suggesting that the poor immunogenicity of conventional DNA vaccines may be enhanced by using viral replicon-based plasmid systems. The results presented here support the evaluation of Semliki Forest virus replicon DNA vaccines in non-human primates and in clinical studies.

A prophylactic human immunodeficiency virus type 1 (HIV-1) vaccine is urgently needed in large parts of the world. To meet this need, a panel of vaccine candidates designed to stimulate T-cell responses are currently moving into human clinical trials. Several of these are based on regimens using naked plasmid DNA, either alone or in combination with viral vectors expressing a matched antigen in prime-boost regimens. However, current data suggest that plasmid DNA-based vaccines are poorly immunogenic and strategies to improve their potency are therefore under development. Such strategies include the co-delivery of genes encoding cytokines, encapsulation of the DNA into microparticles and needle-less delivery for improved tissue targeting (Barouch et al., 2000, 2003; Chen et al., 2003; McKeever et al., 2002). A common goal of all these approaches is to enhance the potency of plasmid DNA-based vaccines so that adequate immune responses may be stimulated without the need for high doses of DNA or demanding immunization regimens.

An alternative strategy to increase the immunogenicity of DNA-based vaccines is to use plasmid-encoded viral replicon systems. We previously described a DNA system in which self-replicating Semliki Forest virus (SFV) vectors (replicons) were used for immunization (Berglund et al., 1998). Replicon systems have also been developed for Sindbis virus (Dubensky et al., 1996; Hariharan et al., 1998; Kirman et al., 2003) and for Kunjin virus (Anraku et al., 2002), and collectively these studies demonstrate that replicon DNA vectors provide significant dose-sparing advantages compared with conventional DNA vaccines. The increased immunogenicity of replicon DNA vaccines may result from an ability of the replicating RNA to stimulate innate antiviral signals in the transfected target cell, providing adjuvant effects that conventional DNA vaccines are unable to stimulate (Leitner et al., 2000, 2003). Recombinant SFV particle vaccines have been extensively evaluated in pre-clinical immunogenicity studies and protective immune responses have been demonstrated in several virus challenge models (Berglund et al., 1999; Fleeton et al., 2001; Morris-Downes et al., 2001a; Mossman et al., 1996). Antigen expression from SFV particles and from SFV replicon DNA is transient and rapid clearance of the target cells from tissues after immunization has been demonstrated (Morris-Downes et al., 2001a). This aspect adds an important safety feature to replicon DNA vaccines, as cell death reduces the risk of chromosomal integration of the foreign DNA and prolonged antigen expression resulting in

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tolerance is not expected to occur. In SFV replicon DNA vectors, only the viral non-structural proteins (nsp1–4) and the foreign antigen of interest are encoded. The absence of the genes encoding the viral structural proteins completely excludes the risk that replication-proficient virus would be generated and it limits potential vector-directed immune responses.

In this communication, we describe an SFV replicon DNA vaccine, DREP.HIVA designed for clinical evaluation as a preventative HIV-1 vaccine. HIVA is a well-described antigen currently under evaluation in the clinic in the context of a conventional DNA plasmid, pTHr.HIVA (Hanke & McMichael, 2000; Mwau et al., 2004). The HIVA gene encodes a scrambled HIV-1 clade A Gag protein fused to a string of HIV-1 class I epitopes recognized by human, murine and rhesus macaque cytotoxic T-lymphocytes (Hanke et al., 1998). To generate DREP.HIVA, we made a series of modifications to the previously published pBK-SFV-E vector (Berglund et al., 1998) to improve its immunopotency and to meet the demands of regulatory agencies and large-scale Good Manufacturing Practice (GMP) production. The modifications included the insertion of an 84 bp self-cleaving ribozyme (RZ) sequence from the hepatitis D virus (HDV) (Been et al., 1992; Perrotta & Been, 1991) downstream of the virus-encoded 3′ sequences to obtain enhanced vector function and the removal of redundant vector sequences to obtain a vaccine product that did not carry unnecessary sequence information. Representative expression and immunogenicity results from the development of DREP.HIVA are presented in this report.

Previous studies using Sindbis virus-based replicon DNA vectors in transient transfection experiments have shown that when the RZ element is placed immediately downstream of the viral poly(A) sequence, the expression of a reporter gene is enhanced (Dubensky et al., 1996; Yamanaka & Xanthopoulos, 2004). The HDV RZ cleaves at its own 5′ end, and thus its presence downstream of the viral poly(A) sequence facilitates the generation of viral RNA molecules with authentic SFV 3′ termini. This increases the chance of successful RNA replication initiation in transfected cells, yielding higher expression levels in the cell population as a whole. To investigate whether the RZ element enhanced the immunogenic profile of the SFV replicon DNA vector, we inserted the HDV RZ into pBK-SFV-E-LacZ to yield pBK-SFV-E-LacZ-RZ (Fig. 1a) and we immunized 6–8-week-old BALB/c female mice intramuscularly (i.m.) with 0–10 μg of the replicon DNA plasmids in 100 μl (0.9% saline) divided between both anterior tibialis muscles. Antibody titres in sera were measured at 10 days post-immunization using a standard ELISA and reciprocal end-point titres were calculated as the final positive dilution above the background mean +2SD (Fig. 1b). In animals immunized with 1 μg pBK-SFV-E-LacZ and pBK-SFV-E-LacZ-RZ, antibody responses were observed in all mice in both groups, while in animals immunized with 0.1 μg replicon DNA, a greater number of animals responded in the pBK-SFV-E-LacZ-RZ-immunized group (6/7 animals) compared with the pBK-SFV-E-LacZ-immunized group (3/7 animals). Similarly enhanced immunogenicity of

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**Fig. 1.** Insertion of the RZ into the SFV replicon DNA vector. (a) Schematic representation of the position into which the RZ was inserted to generate pBK-SFV-E-LacZ-RZ. CMV, cytomegalovirus promoter; UTR, untranslated region; REP, SFV replicase (nsp 1–4); E, SFV translational enhancer; pA, poly(A) signal. (b) ELISA for total anti-β-galactosidase IgG antibody responses in sera from mice immunized with pBK-SFV-E-LacZ and pBK-SFV-E-LacZ-RZ. Antibody titres were measured 14 days after a single DNA immunization. Data are plotted as reciprocal end-point titres and shown for each individual mouse. Values below the dashed line represent animals with reciprocal IgG titres below the detection limit (<50) in this assay. The number of responding mice out of the total number of mice in the group is indicated in parentheses above each group.
pBK-SFV-E-LacZ-RZ over pBK-SFV-E-LacZ was observed in mice that had been immunized twice, with 1 month between each immunization (data not shown). In this experiment, the positive contribution of the RZ was only apparent in mice immunized with low amounts of DNA. This may be because the uptake of plasmid DNA across cellular membranes is less efficient at lower doses of DNA and optimal function of the vector may consequently be more important at these doses.

Based on these results, the pBK-SFV-E-RZ vector was used to generate a vaccine that was suitable as a clinical product. Undesired sequences in the vector were excised, including a redundant region in the virus-encoded 3′ end, retained to facilitate prior construction work, and elements in the plasmid backbone, including the f1 origin of replication and the SV40 promoter located upstream of the kanamycin-resistance gene. Manipulations were performed by inserting endonuclease restrictions sites at appropriate positions in the plasmid using the QuikChange (Stratagene) protocol and excising the undesired regions to yield the DREP plasmid. To ensure that neither of the modifications affected the function of the replicon, we inserted the HIVA antigen into both pBK-SFV-E-RZ and DREP for expression and immunogenicity studies. In addition, since one of the goals of this study was to benchmark SFV replicon DNA vectors against conventional DNA vaccines, pTH.HIVA was also included in these studies. The elements encoded by the pTH.HIVA, pBK-SFV-E-HIVA-RZ and DREP.HIVA vectors are shown in Fig. 2(a).

The expression of HIVA from the three vectors was assessed by transiently transfecting BHK-21 cells using Lipofectamine Plus (Invitrogen) and analysing the cells at 16–24 h post-transfection. Indirect immunofluorescence was carried out by fixing the cells in methanol and staining using an anti-Pk-TAG mAb (Serotec), followed by an FITC-conjugated anti-mouse IgG (Sigma). The Pk-TAG epitope is present at the C terminus of the HIVA antigen as described previously (Hanke & McMichael, 2000). Flow cytometry was performed by fixing transfected cell populations with Cytofix/Cytoperme (Pharmingen) and staining for p24 Gag expression using the FITC-conjugated KC57 mAb (Coulter). The immunofluorescence experiment demonstrated that HIVA was well expressed by all three vectors and localized to the cytoplasm (Fig. 2b, top).
Flow cytometry analysis revealed that the mean fluorescence intensity (MFI) of HIVA staining was almost identical for pBK-SFV-E-HIVA-RZ and DREP.HIVA (MFI = 751 and MFI = 801, respectively), and was about twofold higher than in cells transfected with pTH.HIVA (MFI = 531) (Fig. 2b, bottom panel). This difference in MFI most likely resulted from the more homogeneous copy number of antigen-encoding mRNA transcripts per cell in replicon-transfected cells (as a result of replicon-driven RNA amplification) compared with cells transfected with conventional DNA, as previously reported (Karlsson & Liljestrom, 2004). Similar immunogenicity between the original pBK-SFV-E-HIVA-RZ vector and the new DREP. HIVA vector was confirmed by analysing interferon-γ (IFN-γ) enzyme-linked immunospot (ELISPOT) responses stimulated by the two vaccines in a series of mice experiments (not shown).

To determine whether DREP.HIVA exhibited enhanced immunogenicity over the conventional DNA vaccine expressing HIVA, we performed a set of experiments using DREP.HIVA and pTH.HIVA vaccine preparations generated in the laboratory using Qiagen endotoxin-free kits. These experiments showed that DREP.HIVA stimulated detectable antigen-specific IFN-γ ELISPOT responses in mice after a single immunization using 10 μg DNA, while pTH.HIVA did not stimulate detectable responses using the same immunization and assay conditions (not shown). Since the HIVA plasmid used in the human clinical trials, pThr.HIVA, is not identical to the pTH.HIVA plasmid used in these initial experiments, we next performed a set of immunogenicity experiments using clinical batches of DREP.HIVA and pThr.HIVA. The clinical vaccine batches were produced by Cobra Biomanufacturing and provided for the current study by the International AIDS Vaccine Initiative. The pThr.HIVA plasmid was propagated under antibiotic-free conditions (Cranenburgh et al., 2001; Williams et al., 1998), while pTH.HIVA encoded an antibiotic-resistance gene (Fig. 2a) and could therefore be generated by using standard selection procedures. The clinical batch of DREP.HIVA retained the kanamycin-resistance gene as shown in Fig. 2(a). The availability of GMP-produced DNA batches allowed a direct comparison of the immunogenicity of two highly quality-controlled vaccine preparations. Previous experiments have shown that 50 or 100 μg pThr.HIVA elicits detectable T-cell responses in mice (Hanke et al., 1998, 2003). We therefore performed a single i.m. immunization using only 10 μg DREP.HIVA or pThr.HIVA DNA to obtain a highly sensitive measure of T-cell responses. Briefly, spleens were collected 12 days after the immunization and splenocytes were adjusted to 2 × 10^5 cells per well and added to pre-coated (anti-IFN-γ; MabTech) and blocked ELISPOT plates (Millipore). Cells were stimulated with 2 μg concanavalin A ml⁻¹ (ConA; Sigma), with 2 μg HIVA-specific peptide (RGPGRAFVTI; Ana Spec Inc.) ml⁻¹ (Takahashi et al., 1988) or were left unstimulated (medium only). After 20 h incubation in a 5 % CO₂ incubator at 37 °C, a biotinylated anti-IFN-γ antibody (MabTech) was added and the plates were incubated for 1 h at room temperature. After washing and incubating with an avidin–peroxidase complex (ABC kit; Vector Laboratories) for another hour, the spots were developed with aminoethyl carbazole substrate and the enzymic reaction was stopped after 4 min by washing the plates in water. Spots were counted using an ELISPOT reader (Axioplan 2 Imaging; Zeiss) and expressed as spot-forming cells (SFC) per 10^6 cells. All mice, eight immunized with DREP.HIVA and 10 immunized with pThr.HIVA, showed ConA responses well above 500 SFC per 10^6 cells confirming the viability of the splenocyte cultures (not shown). Using a criterion in the IFN-γ ELISPOT assay whereby 50 SFC per 10^6 cells was considered a positive response, this experiment showed HIVA-specific responses in six out of eight mice immunized with DREP.HIVA, while no responders were identified amongst the pThr.HIVA-immunized mice (Fig. 3). The group of naïve mice included in the experiment were negative for the HIVA peptide-specific stimulation (not shown). These data confirmed the enhanced immunogenicity of replicon DNA vaccines over conventional plasmid DNA previously observed for laboratory-grade plasmids.

The mechanisms underlying the enhanced immunopotency of replicon DNA vectors observed in mice remain to be defined. Replicon DNA vectors provide high antigen levels per cell; however, only about twofold more HIVA antigen was produced by DREP.HIVA compared with pTH.HIVA as shown in Fig. 2(b). In addition, replicon DNA plasmids are larger than conventional DNA vaccines (DREP.HIVA is 12 808 bp, pTH.HIVA is 5135 bp) and the transfection efficiencies of replicon DNA plasmids in vivo is therefore expected to be lower than those of smaller plasmids. Therefore, differences in total antigen levels expressed from replicon DNA and conventional DNA probably does not explain why replicon DNA vectors are more immunogenic.
Instead, it is likely that replicon-driven RNA replication inside the target cell contributes to the enhanced immunogenicity through the generation of double-stranded RNA intermediates. For example, RNA replication inside the cytoplasm of transfected cells may activate the protein kinase R and RNase L pathways, as shown for replicon DNA systems based on Sindbis virus (Leitner et al., 2003). Furthermore, replication-induced cell death may play an important role. Cellular components released from dying cells, such as heat-shock proteins, uric acid or cellular RNA, could alert the immune system and enhance the priming of antigen-specific T-cell responses (Kariko et al., 2004; SenGupta et al., 2004; Shi et al., 2003). In support of this, it was recently shown that co-delivery of an anti-apoptotic gene reduced the ability of a Sindbis-based replicon DNA vaccine to induce protective immunity (Leitner et al., 2004) and a cytopathic pestivirus-based replicon RNA vaccine induced cross-priming more efficiently than a non-cytopathic pestivirus replicon (Racanelli et al., 2004). Thus, there are several potential mechanisms for the increased immunogenicity observed for replicon DNA vaccines and these may be either dependent or independent of vector-induced cell death.

The results presented here support the evaluation of DREP.HIVA in non-human primate studies and in phase I clinical trials and should stimulate further investigations into the immunogenic mechanisms of replicon vaccine systems.

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