Human immunodeficiency virus type 1 subtype C Gag virus-like particle boost substantially improves the immune response to a subtype C gag DNA vaccine in mice

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Human immunodeficiency virus type 1 (HIV-1) subtype C is the predominant HIV in southern Africa, and is the target of a number of recent vaccine candidates. It has been proposed that a heterologous prime/boost vaccination strategy may result in stronger, broader and more prolonged immune responses. Since HIV-1 Gag Pr55 polyprotein can assemble into virus-like particles (VLPs) which have been shown to induce a strong cellular immune response in animals, we showed that a typical southern African subtype C Pr55 protein expressed in insect cells via recombinant baculovirus could form VLPs. We then used the baculovirus-produced VLPs as a boost to a subtype C HIV-1 gag DNA prime vaccination in mice. This study shows that a low dose of HIV-1 subtype C Gag VLPs can significantly boost the immune response to a single subtype C gag DNA inoculation in mice. These results suggest a possible vaccination regimen for humans.

Human immunodeficiency virus type 1 (HIV-1) is probably the gravest infectious disease problem in sub-Saharan Africa, with over 29 million people currently infected (UNAIDS, 2002). Prophylactic vaccines that induce an effective, broad and long-lasting immune response may be the only way to prevent the spread of HIV-1; however, the presence of a number of subtypes potentially complicates the design of vaccines (McMichael et al., 2002). Subtype C virus infections predominate in southern Africa, and also represent the majority of HIV infections worldwide (UNAIDS, 2002): it is therefore a problem that vaccine development for this subtype has lagged behind work on subtype B and even subtype A (Mwau & McMichael, 2003).

For some years there has been great interest in using HIV-1 Gag as a candidate prophylactic vaccine. The HIV-1 Gag polyprotein (Pr55\textsuperscript{gag}) is the major structural protein which forms a submembrane shell in immature virus particles (Freed, 1998). Although in natural infections it associates with the env-encoded transmembrane glycoprotein (gp160) and viral RNA to mature into infectious virions, Pr55\textsuperscript{gag} expressed in isolation in a number of recombinant systems may also associate into non-infectious virus-like particles (VLPs) which are morphologically similar to immature HIV virions (Gheysen et al., 1989; Jowett et al., 1992; Mergener et al., 1992; Nermut et al., 1994, 2003; Overton et al., 1989; Royer et al., 1991, 1992; Shioda & Shibuta, 1990; Vernon et al., 1991; Wills & Craven, 1991).

Gag is relatively well conserved across diverse HIV-1 subtypes (van Baalen et al., 1996; Gaschen et al., 2002), and a potent cellular response is elicited to this protein in humans following infection with HIV-1 (Novitsky et al., 2001; Nabel, 2002). Robinson et al. (1999) have shown that a gag–pol DNA prime immunization followed by a recombinant fowlpox virus protein booster immunization afforded protection against a simian immunodeficiency virus (SIV)
challenge in rhesus macaques. Moreover, it appeared that this immunity was cell-mediated. Barouch et al. (2000) showed that rhesus macaques immunized with an HIV-1 gag DNA vaccine and boosted with IL-2/IG protein (a fusion protein having IL-2 functional activity) augmented the DNA vaccine-elicited cell-mediated immune response significantly.

Our group has shown in previous studies that an HIV-1 subtype C DNA gag vaccine induces a significant CTL response in mice (van Harmelen et al., 2003). In this study, we have investigated first whether the Pr55<sup>ag</sup> polyprotein of a typical southern African HIV-1 subtype C would form VLPs when expressed via recombinant baculovirus, and second, whether immunization with these Gag VLPs enhances the cellular immune response to our gag subtype C DNA vaccine candidate in mice.

The HIV-1 subtype C gag gene from the South African HIV isolate DU422 (Williamson et al., 2003) was cloned into the multiple cloning site pFastBac1, and transposed into competent E. coli DH10Bac cells which were then screened for successful transposition into the baculovirus shuttle vector (bacmid). Gag VLPs were produced in Spodoptera frugiperda (SF21) cells via recombinant baculovirus expressing the full-length myristylated Pr55<sup>ag</sup> precursor protein, according to the manufacturer’s protocols (Gibco Life Sciences). The cells were incubated in TC100 medium (Gibco Life Sciences) supplemented with fetal calf serum at 28 °C for 84 h. Transfected SF21 cells were separated from VLPs which had budded into the culture medium by centrifugation at 3000 g. Putative Pr55<sup>ag</sup> VLPs were purified from the culture fluid on sucrose gradients as described by Nermut et al. (1994). Purified VLPs were dialysed for 16 h in 1× PBS at 4 °C, and Gag content and integrity were evaluated by Western blotting using antisera to HIV-1 p17 (ARP431, NIBSC) diluted 1 in 1000 in 1× PBS (pH 7.4) after SDS-PAGE on 10 % gels.

The process of VLP production by SF21 cells was visualized by transmission electron microscopy (TEM). Recombinant virus-infected cells were prepared for ultrathin sectioning by fixing cells sequentially in 2·5 % glutaraldehyde and 1 % osmium tetroxide in 1× PBS (pH 7·4). Fixed cells were washed in 1× PBS and water, and then dehydrated in graded ethanol solutions and 100 % acetone, after which they were embedded in Spurr’s resin and sectioned. Sections were stained with both 2 % uranyl acetate and Reynolds’ lead citrate and viewed using a Zeiss S1109 electron microscope at magnifications of 12000× to 100 000× using an accelerating voltage of 80 kV.

Gag VLPs harvested from the extracellular medium were prepared for TEM by adsorption onto carbon-coated copper grids and staining with 2 % uranyl acetate or 2 % methylamine tungstase.

The preparation of the HIV-1 subtype C gag DNA candidate vaccine pTHgagC has been reported previously (van Harmelen et al., 2003). Briefly, the gag gene from the subtype C isolate DU422 (European Collection of Cell Cultures provisional accession no. 01032114) was resynthesized for human codon usage, and inserted into the pTH DNA vaccine vector (T. Hanke, Oxford, UK) to result in the vaccine construct pTHgagC. The DNA vaccine was manufactured for animal immunizations by Aldevron (Fargo, USA) and resuspended at 1 mg ml<sup>−1</sup> in sterile PBS (Sigma).

To test the immune response in mice to the gag DNA prime/Gag VLP boost vaccination strategy, BALB/c mice (five per group) were inoculated and subsequently boosted 4 weeks later, as indicated in Table 1. DNA inoculations were intramuscular with 100 μg of plasmid, and VLP inoculations were intraperitoneal with 2 μg of protein. This strategy allowed the following prime/boost combinations: gag prime/empty vector boost, gag prime/gag boost, gag prime/VLP boost and vector prime/VLP boost. Ten days after the boost inoculation, mice were killed and splenocytes isolated from harvested spleens. Effector cells were generated by stimulating splenocytes in a bulk culture for 5 days with the Gag-specific MHC class I-restricted peptide AMQMLKDTI, followed by a restimulation with or without Gag peptide for 4 h. Functional cytotoxicity of these effector cells was then detected in a standard 4 h <sup>51</sup>Cr release assay using P815 cells as antigen presenting cells. In addition, Gag peptide-specific IFN-γ<sup>+</sup>/CD8<sup>+</sup> cells were detected by flow cytometry after the 4 h restimulation of these effector cells with or without the Gag peptide and P815 cells as antigen presenting cells at a 1 : 1 ratio in the presence of 2 μg Brefeldin A. A 4 h restimulation with an irrelevant peptide was included as an additional control. Cells were stained with anti-CD8 (PharMingen), and then permeabilized using the cytofix/cytoperm kit before staining with anti-IFN-γ according to the manufacturer’s instructions (PharMingen). Labelled cells were acquired on a FACSCalibur flow cytometer (500 000 gated events acquired per sample) and analysed using Cellquest software (Becton Dickinson).

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<thead>
<tr>
<th>Prime Inoculation</th>
<th>Boost Inoculation</th>
<th>IFN-γ&lt;sup&gt;+&lt;/sup&gt;/CD8&lt;sup&gt;+&lt;/sup&gt; (%) Cells + Peptide</th>
<th>IFN-γ&lt;sup&gt;+&lt;/sup&gt;/CD8&lt;sup&gt;+&lt;/sup&gt; (%) Cells - Peptide</th>
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<tr>
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<td>pTHgagC DNA</td>
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<td>1·8</td>
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<tr>
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<td>Gag VLPs</td>
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<td>0·8</td>
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<tr>
<td>pTH</td>
<td>Gag VLPs</td>
<td>9·4</td>
<td>1·1</td>
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Table 1. Flow cytometry results following the prime/boost schedule of immunogens in mice

Booster inoculations followed 4 weeks after priming inoculations. IFN-γ<sup>+</sup>/CD8<sup>+</sup> cells were detected by flow cytometry after the 4 h restimulation with or without the Gag peptide AMQMLKDTI and P815 cells as antigen-presenting cells at a 1 : 1 ratio in the presence of 2 μg Brefeldin A. Results shown are from one of three repeats with similar results (not shown).
Fig. 1(a) shows HIV-1 subtype C Gag VLPs produced in transfected Sf21 cells. These are budded into the extracellular medium, with envelopes of the budding VLPs being derived from Sf21 plasma membranes (Fig. 1b). The diameter of the VLPs ranged from 110 to 120 nm (Fig. 1a, b), which is consistent with Gag VLP sizes reported by other researchers (Nermut et al., 1994). Nearly all published work on Gag VLPs has been done on protein from subtype B, and there is very little published on any other subtype: a notable exception is the proof by Buonaguro et al. (2001) that subtype A Pr55gag efficiently forms VLPs. Our work extends the catalogue of virus isolates which can be used for this purpose to DU422, a typical southern African HIV-1 subtype C, elements of which are being incorporated into a number of subtype C vaccines (Williamson et al., 2003).

Gag peptide-specific functional cytotoxicity and IFN-γ+/CD8+ T cells arising in response to the various inoculation regimens are shown in Fig. 2 and Table 1, respectively. A single dose of baculovirus-produced Gag VLPs was found to be immunogenic, with both Gag peptide-specific CTL lysis (Fig. 2a) and IFN-γ-producing CD8+ T cells (Table 1) being generated. More importantly, the VLPs boosted the response to a single pTHgagC prime far more substantially than a boost with pTHgagC: a 4-fold increase in the number of Gag peptide-specific CD8+/IFN-γ+ memory cells was

Fig. 2. Functional cytotoxicity and IFN-γ+/CD8+ T cells of the following prime/boost regimens: (a) pTHgagC/pTH; (b) pTHgagC/pTHgagC; (c) pTHgagC/Gag VLPs; (d) pTH/Gag VLPs. + Peptide and − peptide values are represented by ◆ and □, respectively.
seen when a single inoculation of pTHgagC was boosted after 4 weeks with Gag VLPs, compared to a 1.5-fold increase when the pTHgagC prime was boosted with pTHgagC (Table 1). The magnitude of the response to the pTH prime/VLP boost was similar to the pTHgagC/pTHgagC combination (Table 1). The same trend was seen in the cytotoxicity assays, with lysis increasing markedly with a VLP boost of a pTHgagC prime (Fig. 2c), compared to two DNA immunizations (Fig. 2b) or a single VLP immunization (Fig. 2d). Mice inoculated with the empty vector pTH did not respond until they were boosted with Gag VLPs, and there was no response to the irrelevant peptide (results not shown). We did not determine here whether the breadth of the CTL response increased with Gag VLP boosting; however, we have seen in preliminary experiments where baboons immunized with the same gag-containing DNA vaccine were boosted with identical VLPs that a significantly wider range of peptides were recognized after VLP boost, compared to the response to DNA alone (G. Chege, E. Shephard, J. van Harmelen, C. Williamson, A.-L. Williamson, A. Jaffray & E. P. Rybicki, unpublished results).

The results shown here prove the concept that a primary gag DNA vaccine inoculation followed by a VLP protein boost regimen significantly enhances the cellular immune response to gag DNA in mice. The results are all the more significant when the size of the VLP boost is considered: only 2 ng of protein was used per dose, which was still sufficient as a single dose to elicit a similar magnitude of response to a gag vaccine prime/boost combination. The ability to significantly boost a response to a DNA vaccine with such a low dose of VLPs could have a major impact on the cost of vaccination regimens, as DNA vaccines are more costly to produce than subunit proteins. We are hoping to further reduce this cost by producing VLPs in plants (A. Jaffray & E. P. Rybicki, unpublished).

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