Abstract
Objective of this study: to determine the ability of Salmonella enterica serovar Typhimurium STM1 as a delivery vehicle for the HIV p24 gene and HIV env gene. The STM1 delivery HIV-p24 vaccination was carried out in the form of a recombinant or a DNA vaccine whereas only a DNA vaccine was used for HIV env. Naked DNA vaccination was also tested and immune responses were evaluated following immunisation in mouse model. Results: vaccination cellular immune responses induced by recombinant p24 STM1 (STM1/pHly-p24) were greater than those elicited by the p24 DNA vaccine in STM1 (STM1/VR-p24), (but statistically not significance) than those induced by oral vaccination. However, IgA responses induced by oral vaccination with either a recombinant or DNA vaccine of p24 in STM1 are higher than those induced by IP vaccination. Conclusions: This result confirms other studies that Salmonella was able to deliver HIV antigens to the immune system and induced specific immune responses to the HIV antigen.

Keywords: Salmonella, Recombinant, DNA vaccine, HIV

Introduction
Mucosal sites are major portals of entry and primary sites of replication for HIV after sexual or oral exposure. The observation that CD8+ T cells that recognise HIV are found among peripheral blood and cervical lymphocytes in sex workers who are resistant to HIV infection, despite frequent vaginal exposures to HIV, suggest that protective CD8+ T cells against HIV may be induced at systemic and mucosal sites following natural HIV exposure (Vecino et al., 2002). The greatest impediment to the development of a HIV-1 vaccine that induces mucosal immune responses has been the poor immunogenicity of immunogens administered in this compartment (Fouts et al., 2003). Several strategies have been assessed to overcome this problem, including mucosal delivery vehicles (Shata et al., 2001, Vecino et al., 2002) and immunological adjuvant,(Biragyn et al., 2002).

Since HIV is transmitted either sexually or parentally, the desired vaccine should elicit protective responses against viral challenge by either route. The major goal of HIV vaccine development is constructing an immunogen that address and stimulate both the systemic and the mucosal immune systems. The ability of attenuated bacterial vaccine strains to transfer vaccines to host cells provides additional support for development of live oral bacterial vaccine vectors for delivery of HIV-1 vaccines (Bumann et al., 2000, Shata et al., 2001, Vecino et al., 2002). Studies showed that naked HIV-1 DNA injected intramuscularly did not in-
duce a measurable mucosal immune response (Shata et al., 2001) whereas, the Salmonella vector for delivery an HIV DNA vaccine is capable of inducing both mucosal and systemic HIV specific CD8+ T cells (Fouts et al., 2003).

The HIV-1 genome consists of the gag, pol and env genes, common with all members of the retrovirus family, as well as six accessory genes tat, vpr, vpu, nef, ref, and vif (Figure 1) (Nasioulas et al., 1999).

![Figure 1. Expression of the HIV-1 genome. At top, a schematic view of the linear proviral genome, with coding sequences of the HIV genes depicted as open rectangles. Potential splice donor sites are designated as D1-4 and potential splice acceptor sites as A1-7. The three major classes of viral RNA and the viral proteins that they encode are shown below. The Gag, Pol, and Env genes are expressed as precursor polyproteins, which are then cleaved to yield mature viral proteins. Abbreviations: LTR, long terminal repeat; RRE, Rev-Responsive Element; MA, matrix; CA, capsid; NC, nucleocapsid; Pro, protease; RT. From (www.hivinsite.com)](image)

Most HIV-1 gene products can be potentially used for vaccine target antigens. The viral envelope is particularly important as it is the only target antigen used for the generation of neutralising antibodies (Fouts et al., 2002, Biragyn et al., 2002, Muthumani et al., 2003). Antibodies, especially those directed against conformational epitopes of the CD4 ligand of gp120 or transmembrane protein gp41, can neutralise a wider range of HIV type 1 (HIV-1) isolates (reviewed in reference (Dimmock, 1993). However, these antibodies are rarely, if ever, induced by vaccination. Cytotoxic T lymphocytes (CTLs) are thought to be another important component of the antiviral immune response. Indeed, the capacity of HIV-specific CTLs to efficiently limit viral replication is suggested by a large decrease in HIV load following the initial appearance of CTLs during primary infection (reviewed in (Safrit and Koup, 1995) and by the temporal association between high CTL activity and stable viral load or CD4+ cell counts during asymptomatic stages (Harrer et al., 1996). Furthermore, HIV-exposed but seronegative individuals, as well as uninfected children born to HIV-1-infected mothers, have exhibited anti-HIV CD8+ CTL reactivity as a unique sign of virus exposure (Rowland-Jones et al., 1995). Thus, it is generally accepted that vaccination must induce CTLs as well as neutralizing antibodies, so that infected cells can be killed before they produce any virus. It also has been demonstrated that CTL numbers decline in association with progression of AIDS (Klein et al., 1995). Taken together, this evidence suggests that it is important for a potential HIV vaccine to induce a long lasting immune response and protect against HIV.

HIV-1 gag is one of the most conserved proteins of HIV-1 and epitopes which are conserved among different HIV-1 clades (Durali et al., 1998, McAdam et al., 1998). This data suggests that HIV-1 Gag is a promising target for a HIV-1 vaccine. The HIV-1 gag protein is localised inside of the capsid of virus, hence it is possible to construct the
HIV-1 gag vaccine in order to elicit a cellular response. The induction of efficient CD8+ T lymphocyte-mediated cellular immune responses requires the endogenous synthesis of the target protein, which can be achieved by utilising Salmonella, STM1 delivery DNA vaccine (Bachtiar et al., 2003).

This work was performed in order to determine the efficacy of STM1 as a delivery vehicle for the HIV gag gene (in particular p24 gene) and HIV env gene. The STM1 delivery HIV-p24 vaccination was carried out in the form of a recombinant or a DNA vaccine whereas a DNA vaccine was used for HIV env. Naked DNA vaccination was also tested and immune responses were evaluated following immunisation in mouse model.

Materials and Methods

**Bacterial strains and plasmids**

Plasmid pMOhly1 was a generous gift from Prof. I. Genstchev (Biozentrum der Univ. Wurzburg, Germany) and pDRNL XMSXNB (Figure 2) was kindly provided by Dr. Johnson Mak, The MacFarlane Institute for Medical Research and Public Health Limited (Melbourne, Australia).

![Figure 2. Plasmid pDRNL XMSXNB, the HIV NL43 based sequence encoding the complete HIV-1 Gag sequence, used as the template for p24 PCR amplification](image)

Bacteria were grown at 37°C in Luria-Bertani broth. For plasmid-bearing strains 100 mg/ml ampicillin and/or 50 mg/ml of kanamycin was added. The bacterial strains and plasmids used are listed in Table 1.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Features, use</th>
<th>Source or Reference</th>
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<tbody>
<tr>
<td>pMOhly1</td>
<td>Amp', papular expression vector with haemagglutinin signal sequence. Kan', CMV promoter.</td>
<td>(Strong et al., 1999)</td>
</tr>
<tr>
<td>VR1012</td>
<td>Amp', NL43 based which has 1/3 of the full length HIV-1 genome, encoding the complete HIV-1 gag sequence. VR10/2 expressing HIV-p24 pMOhly1 expressing HIV-p24 Neo', Amp', CMV promoter pCDNA3.1-haemolysis HIV-Env</td>
<td>This study</td>
</tr>
<tr>
<td>pDRNL XMSXNB</td>
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<td>This study</td>
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<td>VR-p24</td>
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<td>pCDNA3.1</td>
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<td>HSV coding HIV-Env</td>
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**Creation of plasmids**

The HIV p24 sequence was amplified from plasmid pDRNL XMSXNB using the primers 5'ATAAGAACCCCCCATACCGCGGAACTC3' and 5'CGGGAATCCTTACACTTATGCGG 3'. The PCR product was digested with NotI and BamH1 (sites underlined) and inserted into VR1012 digested with the same enzymes, creating VR-p24 (Figure 3). Expression of P24 occurs in mammalian cells and protein are directed to the cytoplasmic compartment.

![Figure 3. Schematic representative of pVR-p24, a new clone encoding the HIV p24 gene, expressed from the CMV promoter](image)
The prokaryotic expression vector pMOhly1, encoding with a hemolysin secretor apparatus was used to clone HIV p24 gene allowing the extracellular secretion of the p24 protein. To do this, the p24 gene was also PCR amplified from plasmid pDRNL XMSXNB, using primers (5’CC TTAATTAACCCTATAGTGCAGAACCTCCAGGG3’) and (5’GGTTAAA TTAAAAAATCTTTGCTTATATGC 3’). The PCR products were digested with the PacI enzyme (sites underlined) and cloned into a pMOhly1 vector resulting in a pHly-P24 recombinant plasmid (Figure 4).

Bacteria were transformed with the above plasmids and also pCDNA3.1-Env (pCDNA.3.1.HindIII-EcoRV) and the empty vector (pCDNA3.1) (Figure 5 and Figure 6) were also transformed as previously described (Bachtiar et al., 2003).

Testing and comparing the immunogenicity of STM1 carrying HIVp24 DNA

This study was approved by the animal ethic committee of RMIT University, Australia. To evaluate the ability of STM1 to deliver HIV-p24 vaccines and induce immune responses against the antigen, groups of 5 female Balb/c mice (7 weeks old) were vaccinated three times over a 9-week period. For IM immunization, 50 ul of 1 mg/ml DNA was injected into each quadriceps muscle (total dose of 100ug). For oral vaccination, a dose of 10⁹ CFU of either STM1 or STM1 carrying DNA vaccine was prepared. Intraperitoneal vaccination with STM1 was administered at a dose of 10⁶ CFU of either STM1 or STM1 carrying DNA vaccine. ELISA and ELISOTS were performed as previously described in (Bachtiar et al., 2003).

Results and Discussion

Sequence analysis

The expression plasmid clone bearing the correct insert is indicated in Figure 7. A successful expression plasmid clone was also obtained from a recombinant construct, pHlyP24. Sequence analysis was performed and revealed an identity match of 100% for VRp24 and pHly-p24 with HIV-1 vector pNL4-3 from the database.
Immunogenicity testing of STM1 carrying HIVp24 gene.

Cellular immune response

*In vivo* experiment cellular responses to a passenger antigen, HIVp24 protein were examined. STM1 can deliver the HIV antigen-encoding plasmid to eukaryotic cells, and that an immune response is generated (Figure 8). Antigen-specific IFNα and IL4-secreting cells are enumerated by both oral and IP vaccination. The delivery of p24 by STM1 induced markedly increased immune responses compared to those induced by naked DNA vaccination.

The comparison of immune response elicited against p24 between DNA vaccine and a recombinant molecule expressed in STM1 was also evaluated. The result shown in Figure 9 indicates that following vaccination cellular immune responses induced by recombinant p24 STM1 (STM1/pHLy-p24) were greater than those elicited by the p24 DNA vaccine in STM1 (STM1/VR-p24). This as shown in the number of IFNα and IL4 secreting cells enumerated after either oral or IP vaccination.

Humoral responses

Humoral responses to p24 were assessed three weeks after the third vaccination (i.e. at week 9). These responses are depicted in Figure 9 and Figure 10. Figure 9 shows the p24-specific humoral responses measured three weeks after the third vaccination. IgG responses induced by any intraperitoneal-STM1 vaccinated group are higher (but statistically not significance) than those induced by oral vaccination. However, IgA responses induced by oral vaccination with either a recombinant or DNA vaccine of p24 in STM1 are higher than those induced by IP vaccination. Furthermore, a recombinant p24 in STM1...
(pHly-p24/STM1) which was given orally induced the highest IgA response amongst all other constructs when delivered with the same route of vaccination.

Our previous study showed that STM1 can potentially be used as a vehicle to deliver heterologous antigens when delivered orally or IP in mice (Bachtiar et al., 2003). In order to investigate the ability of STM1 to deliver medically important antigens, an envelope and p24 vaccines expressing proteins of HIV were tested. In the present study STM1 containing a DNA vaccine of HIV-envelope or HIV-p24 were employed under the control of a Cytomegalovirus promoter (CMV). A recombinant vaccine of HIV-p24 in STM1 also constructed in a prokaryotic expression vector (pMOHly1), that was constructed to be under the control of a hemolysin secretorial signal of E. coli. These constructs were tested to assess of their immunogenicity in mice.

This experiment demonstrates that all STM1 carrying DNA encoding HIV antigens elicited a specific immune response against their particular antigens. STM1 vaccine expressing p24 protein of HIV induced specific T cell memory as determined by analysis of the ELISPOT results. The humoral response was also evaluated, and sera from mice which were vaccinated with a recombinant p24 in STM1 mounted a moderate IgG and IgA titre. This result is expected as vaccination with p24 targets cellular immune response rather than an antibody response. Studies of Salmonella as a carrier in mice have found that the level of heterologous expression affects the ability of immunised mice to elicit a humoral immune response (Fairweather et al., 1990). There have been previous reports that naturally exposed but uninfected individuals have HIV-1 specific CTLs without detectable antibodies (Rowland-Jones et al., 1995, Rowland-Jones et al., 1999). CTLs have not been assessed in this experiment, but the ability of STM1 carrying the p24 gene of HIV-1 to induced a T-cell and an antibody response reveals that STM1 bearing a recombinant HIV antigen can provide a suitable starting point for vaccination against HIV.
Further analysis specifies that oral or IP vaccination with recombinant STM1 p24 mounted greater cellular responses than vaccination with the DNA vaccine in STM1. Analysis of ELISPOT results reveals that after re-stimulating with p24 protein, splenocytes from recombinant STM1 p24 orally vaccinated mice stimulated more IFNα SFC’s (P<0.005) secreting cells than those from p24 DNA vaccine immunised mice. Similar evidence also occurs in term of the IgA response, as shown by the observation that the recombinant form of p24 in STM 1 induced a higher immune response than those induced by vaccination with DNA vaccine of p24 in STM1. Conversely, there are comparable IgG responses following vaccination with the p24 DNA vaccine in STM1 and with recombinant p24 in STM1.

These results further support the utility of S. typhimurium as a delivery vehicle for foreign antigens and the use of the genetic adjuvant approach. However, additional studies are needed to further improve the potency and consistency of these approaches in primates; these results have important implications for the development of human vaccines.

References


