CONSTRUCTION AND IMMUNOGENICITY OF SALMONELLA VACCINE VECTOR EXPRESSING HIV-1 ANTIGEN AND MCP3

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This study aims to determine the efficacy of Salmonella enterica serovar Typhimurium STM-1 bearing MCP-3 gene 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 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 significant) 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. In addition, the numbers of cells secreting IL4 are reduced after oral vaccination with STM1/VR-p24/MCP3. However, for the HIV p24 antigen, STM1/MCP3 preferentially induces IFNγ-secreting splenocytes. 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 and for the HIV p24 antigen, STM1/MCP3 induces secretion of IFNγ.

Keywords: Salmonella, recombinant, DNA vaccine, MCP3, HIV

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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 recognize 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 [1]. The greatest impediment to the development of a HIV type 1 vaccine that induces mucosal immune responses has been the poor immunogenicity of immunogens administered in this compartment [2]. Several strategies have been assessed to overcome this problem, including mucosal delivery vehicles [1, 3] and immunological adjuvant [4].

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 [1, 5]. Studies showed that naked HIV-1 DNA injected intramuscularly did not induce a measurable mucosal immune response [5] whereas, the Salmonella vector for delivery of an HIV DNA vaccine is capable of inducing both mucosal and systemic HIV specific CD8+ T cells [2].

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 [6, 7]. 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 neutralizing antibodies [4]. Antibodies, especially those directed against conformational epitopes of the CD4 ligand of gp120 or transmembrane protein gp41, can neutralize a wider range of HIV-1 isolates (reviewed in reference [8]). 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 [9]) and by the temporal association between high CTL activity and stable viral load or CD4+ cell counts during asymptomatic stages [10]. 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 [11]. 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 [12]. 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 its epitopes are conserved among different HIV-1 clades [13]. This data suggests that HIV-1 gag is a promising target for an HIV-1 vaccine. The HIV-1 gag protein is localized 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 utilizing Salmonella delivery DNA vaccine.

Our previous study showed that the immunogenicity of the Salmonella, STM1 vaccine could be enhanced by endogenous expression of a murine chemokine, MCP3 [14]. Expression of the chemokine by STM1 increased uptake by cultured dendritic cells, and increased humoral and T-cell responses to bacterial antigens after intra-peritoneal, but more particularly, oral immunization. These results point to a generic method of increasing the immunogenicity of bacterial vaccines delivered by the oral route [14]. 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 only a DNA vaccine was used for HIV env. Naked DNA vaccination was also tested and immune responses were evaluated following immunization 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 was kindly provided by Dr. Johnson Mak, The MacFarlane Institute for Medical Research and Public Health Limited (Melbourne, Australia). Bacteria were grown at 37 °C in Luria-Bertani broth. For plasmid-bearing strains 100 µg/ml ampicillin and/or 50 mg/ml of kanamycin was added. The bacterial strains and plasmids used are listed in Table I.
Creation of plasmids

The HIV p24 sequence was amplified from plasmid pDRNL XMSXNB using the primers (5’ATAAGAATGCGGCGC
ATGCCTATAGTGCAGAAC
CTC3’) and (5’CGGGATCC

T TACAAAACTCTTGCTTTATGGC 3’). The PCR product was digested with NotI and BamH1 (sites underlined) and inserted into VR1012 digested with the same enzymes, creating VR-p24. Expression of P24 occurs in mammalian cells and the protein is directed to the cytoplasmic compartment.

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’CCTTAATTAA

CCCTATAGTGCAGAACCTCCAGGG 3’) and (5’GGTTAATTAA

AAAACTCTTGCTTTATGGC 3’).
TAT GGC 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. Bacteria were transformed with the above plasmids and also pCDNA3.1-Env (pCDNA.3.1.HindIII-EcoRV) and the empty vector (pCDNA3.1).

Testing and comparing the immunogenicity of STM1 carrying HIVp24 DNA with and without MCP3

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 µl of 1 mg/ml DNA was injected into each quadriceps muscle (total dose of 100 µg). For oral vaccination, a dose of 10^9 CFU of either STM1 or STM1 carrying DNA vaccine was prepared. Intraperitoneal vaccination with STM1 was administered at a dose of 10^9 CFU of either STM1 or STM1 carrying DNA vaccine. ELISA and ELISPOTS were performed as previously described in chapter 3.

Results

Sequence analysis

The expression plasmid clone bearing the correct insert is indicated in Figure 1. A successful expression plasmid clone was also obtained from a recombi-
nant 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 MCP3 and HIVp24 gene – Cellular immune response

Cellular responses to a passenger antigen were examined in vivo. The antigen was the HIVp24 protein, either delivered as a DNA vaccine or recombinant DNA in STM1. The effect of co-delivery MCP3 with recombinant p24 in STM1 in the increase of immune responses against p24 protein was also evaluated.

As in the case of the in vitro experiment using ovalbumin encoded by a DNA vaccine, expression of the antigen can only occur after transfer of the plasmid to a eukaryotic cell. It is apparent that STM1 can deliver the HIV antigen-encoding plasmid to eukaryotic cells, and that an immune response is generated (Figure 2). Antigen-specific IFN\(_\gamma\) and IL4-secreting cells are enumerated. There is a significant increase in the IFN\(_\gamma\)-secreting cellular response to oral vaccination when STM1 secretes MCP3, however, the responses after IP vaccination

![Figure 2](image-url)

_Figure 2_. Results of the ELISPOT assay of the VR-p24 HIV experiment. Secretion of cytokines by splenocytes. Splenocytes were isolated from mice vaccinated with the various constructs shown, and the number of IFN\(_\gamma\)-secreting cells and IL4-secreting cells was measured after re-stimulation with p24 protein. Reactivity was evaluated against the control using the t-test. Oral vaccination with STM1/VR-p24/MCP3 significantly increased IFN\(_\gamma\) SFC (p < 0.0001) but decreased IL4 SFC (p < 0.05) compared to the vaccination without MCP3. Oral and IP vaccination with the DNA vaccine, VR-p24 in STM1 significantly increased IFN\(_\gamma\) and IL4 SFC over intramuscular vaccination (IM) with naked DNA vaccine (VR-p24)
are not significantly increased. The delivery of p24 by STM1 induced markedly increased immune responses compared to those induced by naked DNA vaccination. It is very interesting to note that the numbers of cells secreting IL4 are reduced after oral vaccination with STM1/VR-p24/MCP3. These results indicate that, at least for the HIV p24 antigen, STM1/MCP3 preferentially induces IFNγ-secreting splenocytes.

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 3 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 was shown in the number of IFNγ 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 Figures 4 and Figure 5.

![Figure 3. Comparison of cytokine secretion by the DNA vaccine and the recombinant p24 vaccine in STM1. Splenocytes were isolated from mice vaccinated with the various constructs shown, and the number of IFNγ-secreting cells and IL4-secreting cells was measured after re-stimulation with the p24 protein. Oral vaccination with recombinant STM1/pHly-p24 significantly increased IFNγ-SFC over STM1/VR-p24 (p < 0.005), whereas oral vaccination with STM1/pHly-p24 stimulated a significantly higher IL4 than those with IP vaccination (p < 0.005)]
Figure 4 shows the p24-specific humoral responses measured three weeks after the third vaccination. IgG responses induced by any intraperitoneal-STM1 vaccinated group are higher (not significantly) 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) that was given orally induced the highest IgA response amongst all other constructs when delivered with the same route of vaccination.

Figure 4. Comparison of antibody response by the DNA vaccine and recombinant p24 vaccine in STM1. There are no significant differences in the humoral IgG response to the p24 protein between the DNA vaccine and recombinant DNA from either oral or IP vaccination in STM1. A significant increase (p = 0.004) of serum IgG response was found from IP but not oral vaccination with STM1/VR-p24 over those vaccinated with STM1/Empty vector.

Figure 5. Serum IgA response to p24 protein. Sera were taken three weeks after the third vaccination and the reactivity was evaluated against the HIV-p24 protein. Oral vaccination with STM1/pHLy-p24 elicited higher IgA titre than those with STM1/VR-p24; p < 0.005. There was no significant difference between IgA levels of the two constructs when delivered via IP injections.

Figure 4 shows the p24-specific humoral responses measured three weeks after the third vaccination. IgG responses induced by any intraperitoneal-STM1 vaccinated group are higher (not significantly) 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) that was given orally induced the highest IgA response amongst all other constructs when delivered with the same route of vaccination.
Discussion

*Salmonella typhimurium* vectors have proven to be useful vaccine delivery systems [15]. These studies have led to increased efforts to develop *Salmonella* vectors for use in human vaccines. It has been long postulated that attenuated *Salmonella* could be used as vaccine vectors. The ability of *Salmonella* to elicit mucosal immune responses makes it a potentially useful vector for delivering HIV-1 antigens to the mucosal immune system. HIV-1 antigens delivered to the gut-associated lymphoid tissues (GALT) by *Salmonella* might stimulate protective immune responses against HIV-1. This study observed that when antigen was delivered to the gut mucosa as well as parenteral tissues of mice, using STM1 carrying plasmid DNA expressing HIV-envelope protein and p24, an immune response was induced. DNA immunization with HIV-envelope is targeting a humoral immune response, whereas immunization with DNA of p24 aimed at inducing a cellular immune response.

Our previous study demonstrated that STM1 could potentially be used as a vehicle to deliver heterologous antigens when delivered orally or IP in mice [14]. In order to investigate the ability of STM1 to deliver medically important antigens, 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 (CMV) promoter. A recombinant vaccine of HIV-p24 in STM1 was 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 for immunogenicity in mice and the MCP3 carrying plasmid, pMOMCP-3, was used as a genetic adjuvant of the DNA vaccine of HIV when delivered by STM1.

This experiment demonstrates that all STM1 carrying DNA encoding HIV antigens elicited a specific immune response against their particular antigens. STM1 vaccine expressing the 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 that were vaccinated with a recombinant p24 in STM1 mounted a moderate IgG and IgA titre. This result was 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 immunized mice to elicit a humoral immune response [16]. There have been previous reports that naturally exposed but uninfected individuals have HIV-1 specific CTLs without detectable antibodies [11,17]. CTLs have not been assessed in this experiment, but the ability of
STM1 carrying the p24 gene of HIV-1 to induce 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.

This study observed that STM1 carrying a DNA vaccine expressing the p24 protein (under the control of a CMV promoter), stimulates specific immune responses and these responses were augmented by co-delivery with MCP3. A previous study reported that co-delivery of cytokine gene adjuvants of GM-CSF cDNA with DNA vaccine constructs increases antigen-specific antibody and T helper cell proliferation [18]. Another study demonstrated that the β-chemokines can both modulate the immune response induced by a HIV-1 DNA vaccine [19]. This result correlates to the study that DNA vaccines encoding HIV-1 glycoprotein 120 fused with MCP3 enhance systemic and mucosal immune responses [4].

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 IFNγ SFC (P < 0.005) secreting cells than those from p24 DNA vaccine immunized mice. Similar evidence also occurs in terms of the IgA response, as shown by the observation that the recombinant form of p24 in STM1 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. This demonstrates that antigen-specific immune responses can be modulated positively in mice through the use of the MCP3 gene adjuvant. 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.

The ideal vaccine would be one that can be administered orally and yet will induce broad immune responses. Attenuated bacterial strains such as *Salmonella*, *Shigella* and *Listeria* spp. have these properties. These attenuated strains have been used experimentally to express recombinant proteins, using prokaryotic expression vectors, such that the foreign protein is expressed within the bacterium after infection and is able to stimulate an immune response [20–22]. Attenuated bacteria can also deliver a DNA vaccine to the cell that they infect. One of the best studied is *Salmonella*. The antigen encoded by the DNA vaccines is expressed in
the mammalian cell and an immune response is induced [20, 23]. This strategy is able to overcome the limitation of relatively low levels of immune responses induced by DNA vaccination.

Recently, several studies have exploited the properties of DCs, as these cells have been thought to be the first antigen-presenting cells that can capture antigen in their immature stage, in the port of antigen entry such as mucosa and skin. Vaccine antigens are not presented directly to the immune system but must first be captured, processed, and bound to antigen presenting molecules, typically those of the class I or class II MHC [24]. Hence, an increase of antigen presentation of the encoded protein that has been delivered by DNA vaccine might be a good strategy to enhance immune response in DNA vaccination. There are some methods designed to increase the exposure of encoded antigen to DCs, including fusing the coding region of the antigen to chemotactic protein, MCP3 [4]. The overall aim of this thesis was to evaluate the ability of STM1 to deliver heterologous antigen particularly in the form of DNA vaccine. This study focused on three areas; 1) the use of *Salmonella*, STM1 for delivery of foreign DNA; 2) the central role of DCs in providing an immune response to STM1 and 3) attempts to improve the immunogenicity of this genetic vaccine delivered by STM1.

The humoral immune response analysis reveals that there was no significant difference in humoral IgG response to p24 protein between DNA vaccine and recombinant DNA from either oral or IP vaccination in STM1. Interestingly, oral vaccination with STM1/pHLy-p24 elicited a higher IgA titre than that with STM1/VR-p24; *p*<0.005. Furthermore, there was no significant difference between IgA levels of these two constructs when they were delivered intraperitoneally. It was expected that STM1/pHLy-p24, which was constructed to secrete p24 protein, and oral vaccination of this vaccine in STM1 enabled gut mucosa to the secretion of specific IgA to HIV-p24 protein. 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 [3]. This is the first report showing that MCP3 was able to act as a genetic adjuvant in delivery of an HIV antigen by *Salmonella*.

References

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