IMMUNOLOGICAL RESPONSE TO VACCINATION

Enhancement of the specific mucosal IgA response in vivo by interleukin-5 expressed by an attenuated strain of Salmonella serotype Dublin

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It has been shown that cytokines have potential as therapeutic adjuvants in vaccination. Interleukin-5 (IL-5) is a cytokine that regulates antibody production, in particular enhancing IgA production by activated mucosal B cells. This study examined the expression of a cloned cytokine gene encoding murine IL-5 (mIL-5) by an attenuated aroA strain (SL5631) of Salmonella serotype Dublin. The resulting strain, SL5631(pTRXFUS-mIL-5), expressed mIL-5 as a fusion with thioredoxin as demonstrated by immunological and biological assays. When strain SL5631(pTRXFUS-mIL-5) was used as a live vaccine in BALB/c mice, it colonised and multiplied at higher levels in spleens and livers than the strain carrying the empty plasmid. A reduction in invasiveness of SL5631(pTRXFUS-mIL-5) was observed in in-vitro invasion assays. Enhanced IgA response against Salmonella LPS in mucosal secretions and enhanced IgA and IgG responses were detected by ELISA and ELISPOT methods in sera of mice immunised with the strain expressing mIL-5. Results with IL-5-deficient mice showed that the enhanced IgA response was due to Salmonella-expressed mIL-5 rather than endogenous mIL-5.

Introduction

Attenuated strains of Salmonella have been shown to have potential as live vaccines against various foreign (i.e., non-Salmonella) pathogens [1]. Studies in animal models have demonstrated that immunisation with these heterologous vaccines is an effective and safe way to induce both serum and mucosal antibodies against Salmonella and the foreign antigen [2]. One important group of non-reverting Salmonella vaccine strains comprises the mutants dependent on aromatic compounds (aro-`) [3]. The aror` mutants are affected in the common pathway of aromatic biosynthesis, resulting in strains dependent on aromatic compounds that are available in limited amounts in host tissues. These mutants multiply for only a few generations in host tissues, but do persist in the spleen and liver for several weeks, inducing strong humoral and cellular immune responses [4].

Cytokines have been used in vaccination as immunological adjuvants, enhancing the protection induced by killed or recombinant vaccines. Salmonella auxotrophic mutants have proved to be effective live delivery vehicles for these therapeutic proteins. Interleukin (IL)-1β [5], IL-4 [6] and IL-6 [7] have been expressed in Salmonella and their effect on the immune response examined in a murine model system. Cytokines fall into two categories: those secreted by Th1 cells - interferon (IFN)-γ, IL-2 - and those secreted by Th2 cells - IL-4, IL-5, IL-6, IL-9, IL-10 [reviewed in 8]. Th1 cells are involved in providing help for macrophage activation [9] and delayed type hypersensitivity responses [10], whereas Th2 cells play a greater role in providing help for antibody responses [11].

When immunising against a particular pathogen, it is important to induce the appropriate type of immune response. Often, mimicking the natural response to a pathogen is not adequate when developing a vaccine. Many cytokines have been found to enhance the magnitude of the immune response against a given pathogen. In-vitro studies with purified IL-5 and in-vivo studies with recombinant IL-5 have revealed that this particular cytokine functions to enhance secretion of LPS-induced IgA, not promoting a switch to IgA [12] but acting as a secretory (s)IgA+B cell terminal differentiation factor [13, 14]. Promotion of IgA
production is thought to be regulated by IL-5 alone [13] or synergically with thymocyte growth factor (TGF)-β [15], IL-6 [16] and IL-4 [17]. Bertolini et al. [18] have reported that IL-5 can stimulate the secretion of IgM and IgG by staphylococcal protein A-activated B cells, suggesting that IL-5 can enhance the secretion of immunoglobulin classes other than IgA.

In the present study, the effects of IL-5 expressed by a live attenuated araA strain of Salmonella serotype Dublin (SL5631) were investigated in a murine model.

Materials and methods

Bacterial strains, plasmids and media

The bacterial strains used in this study are shown in Table 1. Salmonella strains were grown at 37°C in Luria-Bertani (LB) medium (Unipath) supplemented with appropriate antibiotics.

The coding region of murine IL-5 (mIL-5) was isolated from pEDFM-16 which carries nucleotides 28–627 of the mIL-5 cDNA [19] cloned into the HindIII site of pGEM-1 with HindIII linkers. pEDFM-16 was kindly provided by H. D. Campbell.

The vector pKK233.2 [20] carries the trc promoter and the lacZ ribosome binding site followed by an ATG initiation codon that is contained within a unique NcoI site. Digestion with NcoI exposes the start codon for direct inframe ligation of foreign genes. The mIL-5 coding region was inserted into this vector as described in the text.

The thioredoxin expression vector pTRXFUS [21] is based on pUC18 and contains a ColE1 origin of replication, a β-lactamase gene as a selectable marker and the bacteriophage λ P1 promoter located upstream of the Escherichia coli trxA gene. Expression studies with this plasmid were performed in E. coli strain GT724. This strain contains the bacteriophage λ cI repressor gene stably integrated into the chromosomesal ampC locus [21]. The cI gene is under the transcriptional control of the trp promoter, integrated upstream of cl in ampC. The mIL-5 coding region was inserted into this vector as described in the text.

DNA techniques

Small preparations of plasmid DNA were performed by the alkaline lysis method of Birnboim and Doly [24]. Ligations were performed as described by Maniatis et al. [25]. Electroporation procedures were performed as described by Dower et al. [26].

Expression of fusion protein

Strain GI724, carrying the thioredoxin fusion expression plasmid, was grown at 30°C in RM medium (M9 medium supplemented with glucose, casamino acids and ampicillin 100 mg/L) until the absorbance at 595 nm was 0.5. To drive expression of thioredoxin fusion, pTRXFUS uses the P1 promoter from bacteriophage λ. The bacteriophage λ cI repressor binds to the operator region in front of the P1 promoter and controls the level of transcription from this promoter. In strain GT724, the cI repressor gene is under the control of the trp promoter. Fusion protein expression in this strain is induced by the addition of tryptophan 100 mg/L to the medium, thereby preventing cI repressor synthesis and allowing transcription from the P1 promoter.

Expression of pKK233.2 recombinants in E. coli JM109 (Table 1) was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for various periods at 37°C.

Immunoblotting

Whole cell lysates were tested for their reactivity with antibodies by immunoblotting after SDS-PAGE. Bacteria (2 × 10^8 cfu) were boiled in sample buffer and the whole-cell lysates were separated on a polyacrylamide 10% w/v gel. For immunoblotting, the gel containing separated proteins was transferred electrophoretically to a nitrocellulose membrane [27]. The primary antibody used to probe Western blots was a mouse monoclonal anti-IL-5 antibody, TRFK-5 [28].

Table 1. Plasmids and strains used in experiments

<table>
<thead>
<tr>
<th>Plasmids and strains</th>
<th>Characteristics</th>
<th>Source [reference]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pEDFM-16</td>
<td>pGEM1/mIL-5, bp 28–627</td>
<td>[19]</td>
</tr>
<tr>
<td>pKK233.2</td>
<td>Pc, Amp⁹, PBR322 ori</td>
<td>[20]</td>
</tr>
<tr>
<td>pTRX</td>
<td>Pc, Amp⁹, ColE1 ori, Thioredoxin ORF</td>
<td>[21]</td>
</tr>
<tr>
<td>pTRXFUS</td>
<td>Pc, Amp⁹, ColE1 ori, Thioredoxin ORF, EK cleavage site</td>
<td>[21]</td>
</tr>
<tr>
<td>pKK233.2-mIL-5</td>
<td>pTRXFUS/SmaI-NcoI linkers/mIL-5 bp 28–627</td>
<td>This study</td>
</tr>
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<td>pTRXFUS-mIL-5</td>
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</tr>
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<td>[22]</td>
</tr>
<tr>
<td>JM109</td>
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<td></td>
</tr>
<tr>
<td>GI724</td>
<td>F, λ², lacP⁹, lacP18, ampC/VpadC</td>
<td>[21]</td>
</tr>
<tr>
<td>Salmonella</td>
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</tr>
<tr>
<td>SL5631</td>
<td>serotype Dublin (aroA148)</td>
<td>B. Stocker</td>
</tr>
<tr>
<td>LBS010</td>
<td>serotype Typhimurium (r 'm')</td>
<td>[23]</td>
</tr>
</tbody>
</table>
Immune complexes were detected with HRP-labelled anti-mouse antibodies for the ECL Western blotting detection system (Amersham Corporation).

**Solubilisation of insoluble aggregates**

Insoluble aggregates were solubilised by the method of Van Kimmenade et al. [29]. The buffers used in the solubilisation of IL-5 were as follows: buffer A – 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA and 0.1 mM PMSF; buffer B – 5 M guanidine-HCl, 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA, 2 mM reduced glutathione and 0.2 mM oxidised glutathione; buffer C – 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA, 2 mM reduced glutathione and 0.2 mM oxidised glutathione. Strains carrying mIL-5 expression vectors were grown in LB broth with ampicillin 100 mg/L for 16 h at 37°C, diluted 1 in 50 and grown for a further 2 h under the same conditions. IPTG (0.5 mM) was then added to cultures which were then left for a further 3 h at 37°C with shaking. Cells were pelleted, resuspended in 2 ml of buffer A and sonicated for 1 min. Lysates were centrifuged for 15 min at 15 000 g and 4°C. The 1-g pellets were resuspended in 7 ml of buffer C and incubated at room temperature for 2 h. The lysate was centrifuged and the supernate was dialysed against phosphate-buffered saline (PBS) (three changes) for 16 h. Solubilised protein was concentrated with an Amicon-10 microconcentrator in a GSA rotor at 2000 g.

**IL-5 assay**

IL-5 was assayed with I3H cells (M. C. Fung, C. Loh and I. G. Young, unpublished) that express the mIL-5 receptor α subunit and are responsive to mIL-5 because of the presence of the mIL-5 receptor β subunit. This cell line was derived from FDC-P1 cells [30] by electroporation with the IL-5 receptor α subunit cDNA subcloned into the expression vector pcDNA1/Neo (Invitrogen). I3H cells were maintained in factor-free medium (RPMI 1640 with fetal bovine serum, FBS, medium with recombinant mIL-5 0.1 % and NaCl 585 mg/L) and adjusted to a concentration of monothioglycerol 6.2 μl/L; sodium pyruvate 110 mg/L; and washing the contents with 200 μl of PBS. The suspension was then tested for mIL-5 activity in the assay below.

**Immunisation protocol**

Female BALB/c, C57BL/6J (+/+) and C57BL/6J IL-5-deficient mice (–/–) [31] were obtained from John Curtin School of Medical Research, Australian National University, Canberra. BALB/c mice were obtained from the Animal Resources Centre, Western Australia, and IL-5-deficient (–/–) mice were obtained from the Animal Resources Centre, Western Australia. Mice were anaesthetised lightly before immunising with a 25-μl dose of live bacteria intranasally.

Blood was taken from the retro-orbital plexus before oral immunisation and then at weekly intervals. Rectal and vaginal samples were taken with absorbent wicks from killed mice that had been immunised orally. Wicks were then saturated with 50 μl of NaHCO3 5% w/v 5 min before oral administration of 50 μl of live bacteria by pipette. Mice were anaesthetised lightly before immunising with a 25-μl dose of live bacteria intranasally.

**Immunoassays**

ASC numbers in lung lymphoid cells isolated from mice that had been immunised nasally were determined using an enzyme-linked immunosorbent assay system.
with an ELISPOT assay [32] in 96-well nitrocellulose
U-bottom plates coated with Salmonella serotype
Enteritidis LPS (Sigma) 20 mg/L and incubated for
16 h at 4°C. Plates were washed with PBS and blocked
with PBS containing skimmed milk powder (Diploma)
5% w/v for 1 h. Lymphoid cells were collected and
purified as described by Ramsay et al. [33]. They were
then serially diluted in medium (100 µl of RPMI 1640
with PBS 10% v/v), added to plates and then left at
37°C in air with CO2 5% for 4 h. Plates were then
washed with PBS plus Tween-20 0.1% v/v to remove
cells. Goat anti-mouse IgA HRP conjugate (Silenus)
diluted 1 in 2000 in PBS with skimmed milk powder
1% w/v was added, 100 µl/well, and left for 16 h at
4°C. Plates were then rinsed in PBS with Tween-20
0.1% v/v and the colour was developed with 4-chloro-
1-naphthol as substrate.

Reactivity of immune sera and secretions with
Salmonella serotype Enteritidis LPS was examined by
standard ELISA, coating plates with salmonella LPS
5 mg/L and with reagents as for ELISPOT. Colour
development was in 100 µl of citrate-phosphate buffer
(0.1 M citric acid, 0.2 M Na2HPO4), H2O2 0.0008% v/v
and o-phenylene diamine dihydrochloride (OPD)
400 mg/L. The reaction was stopped by the addition
of 50 µl of H2SO4 25% v/v to each well. Plates were
read at 490 nm with an automated plate reader (Perkin
Elmer). Controls were performed with sera and
secretions collected from mice before immunisation.

In-vitro cell invasion assays

The ability of recombinant Salmonella to invade tissue
culture cells in vitro was determined as described
previously [34]. Briefly, monolayers of c. 1 × 10^5
Mardin Darby Canine Kidney (MDCK) cells were
incubated with 1 × 10⁷ cfu of SL5631(pTRX) or
SL5631(pTRXFUS-mIL-5) at 37°C, in air with CO2
5% for 2 h. The monolayer was then washed twice with
PBS, covered with RPMI containing PBS 10% v/v and
gentamicin 100 mg/L and incubated at 37°C, in air
with CO2 5% for a further 2 h. The monolayer was
washed three times with PBS and lysed with 200 µl of
Triton X-100 1% v/v for 10 min. Samples were
collected in 800 µl of LB broth and plated on to LB
plates containing ampicillin 100 mg/L.

Results

Cloning of mIL-5 into prokaryotic expression
vectors and expression in Salmonella

Two prokaryotic plasmid vectors were employed for the
expression of mIL-5. mIL-5 cDNA was cloned initially
into plasmid pKK233.2. mIL-5 599-bp HindIII
fragment of pEDFM-16, carrying nucleotides 28–627 of
the mIL-5 cDNA [19], was excised and ligated into the
HindIII site of pKK233.2. The ligation product was
transformed into E. coli JM109. Clones carrying plasmid
were selected by growth on LB agar with ampicillin and
plasmids were screened for the presence of the mIL-5
insert by restriction endonuclease digestion. One clone
was identified and named pKK233.2-mIL-5. This clone
was passed through an intermediate restriction-minus
Salmonella serotype Typhimurium strain (LB50 10)
before being transformed into Salmonella serotype
Dublin strain SL5631. To induce maximum expression
of mIL-5 by strain JM109, the strain was grown at 37°C
for 16 h before addition of 0.5 mM IPTG and incubated
for a further 4 h. SDS-PAGE analysis of the strains under
reducing conditions revealed expression of a protein of
c. 13 kDa in the cell lysates of strain JM109(pKK233.2-
mIL-5), but no visible protein in the lysates of non-

![Fig. 1. SDS-PAGE 14% gel of whole-cell lysates from recombinant Salmonella and E. coli strains. (a) Lane 1, mol. wt
marker; 2, JM109(pKK233.2); 3, JM109(pKK233.2-mIL-5); 4, SL5631(pKK233.2); 5, SL5631(pKK233.2-mIL-5). (b) Lane 1, mol. wt
marker; 2, GI724(pTRX); 3, GI724(pTRXFUS-mIL-5); 4, SL5631(pTRX); 5, SL5631(pTRXFUS-mIL-5).](image)
inducible strain SL5631(pKK233.2-mIL-5) (Fig. 1a). Both strains were then analysed for the presence of biologically active mIL-5 by a tritiated-thymidine incorporation assay with an mIL-5-dependent cell line (1H3). Functional mIL-5 could not be detected in either strain. E. coli JM109(pKK233.2-mIL-5), therefore, appeared to be expressing insoluble mIL-5 that was not biologically active. Cell lysates were then solubilised in vitro and assayed for mIL-5 function once again. At this stage, low but positive mIL-5 bioactivity was recorded in lysates from strain JM109(pKK233.2-mIL-5) (Fig. 2a).

As only low functional activity of mIL-5 could be detected in strain JM109(pKK233.2-mIL-5) after solubilisation and no activity was detected in strain SL5631(pKK233.2-mIL-5), the expression vector pTRXFUS, previously shown to express soluble mIL-5 as a fusion product with E. coli thioredoxin [21], was used. To clone mIL-5 into this vector, NcoI linkers (8-mers) were ligated into the SmaI site of pTRXFUS. The restriction endonuclease NcoI was used to cut the clone pEDFM-16 at positions 102 and 458, excising a 356-bp fragment that encodes mIL-5 without including the signal peptide and polyA tail. The NcoI mIL-5 fragment was then ligated into the prepared NcoI site located downstream of the thioredoxin gene of pTRXFUS. This ligation mix was transformed into E. coli strain G1724. Ampicillin-resistant transformants were selected and plasmid DNA was checked by restriction analysis. Expression of the fusion protein was analysed by SDS-PAGE. To optimise growth and conditions for the maximum yield of protein, the strain was grown at 30°C for 16 h and then induced by the addition of tryptophan 100 mg/L followed by incubation for a further 4 h. Whole-cell lysates were analysed by SDS-PAGE under reducing conditions. A very intense protein band of c. 24 kDa was visualised (Fig. 1b). This was the expected size of the fusion protein (thioredoxin has a mol.wt of 11 kDa and mIL-5 monomer a weight of 13 kDa). pTRXFUS-IL-5 was transformed into SL5631 and when expression of the fusion was analysed on SDS-PAGE, a fine protein band of the expected size could be seen. After probing the above gels with the monoclonal antibody TRFK-5 using a Western blot procedure, a single band of 24 kDa was observed in cell lysates of both recombinant strains (data not shown).

As strains carrying pTRXFUS-mIL-5 were expressing mIL-5 as a fusion product with thioredoxin, it was possible that the product would not be functional. Cell lysates of these strains were tested for the presence of functional mIL-5 in the biological assay described above. The recombinant Salmonella strain SL5631 (pTRXFUS-mIL-5) showed mIL-5 bioactivity, but lower than that of the mIL-5 expressed by E. coli strain G1724(pTRXFUS-mIL-5) (Fig. 2b). The different levels of activity are probably due to constitutive expression of the recombinant plasmid in Salmonella compared to the induced expression in E. coli.

Colonisation of mIL-5 recombinant Salmonella in mice

SL5631(pTRXFUS-mIL-5) demonstrated mIL-5 activity and, therefore, was chosen to study the effects that mIL-5 expression has on the colonisation of Salmonella in mice. Although mouse-to-mouse variation was evident, a higher level of colonisation was detected in the spleens (Fig. 3a) and livers (Fig. 3b) of mice immunised orally with the recombinant Salmonella strain SL5631(pTRXFUS-mIL-5) than those immunised with the control strain SL5631(pTRX). Numbers

![Fig. 2. Proliferation of the mIL-5-dependent cell line, 1H3, in response to Salmonella or E. coli carrying (a) pKK233.2-mIL-5 or (b) pTRXFUS-mIL-5. Results are expressed as mean cpm of tritiated thymidine incorporation over a 44-h culture period; SEM were <5%. (a) -□-, JM109(pKK233.2-mIL-5); -○--, SL5631 (pKK233.2-mIL-5); -△--, JM109(pKK233.2); -□- , SL5631(pKK233.2). (b) -□-, G1724(pTRXFUS-mIL-5); -○--, SL5631(pTRXFUS-mIL-5); -○--, SL5631(pTRXFUS-mIL-5); -○-- , G1724(pTRX); -△--, SL5631(pTRX).](image-url)
of bacteria present in the livers and spleens of mice immunised with strain SL5631(pTRXFUS-mIL-5) reached a maximum number of 2000 on day 7 after immunisation compared to a maximum number of 250 bacteria per organ on day 3 after immunisation of control mice. Both liver and spleen were cleared of bacteria by day 42, with higher numbers of bacteria occurring in the liver than the spleen of mice over the 6-week period. On day 5, 20% of the total bacteria were ampicillin resistant and by day 14 only ampicillin-sensitive bacteria remained in the organs of mice immunised with either strain (data not shown). The effect mIL-5 expression has on Salmonella colonisation in mice (−/−), where similar results were obtained (data not shown).

Invasion of epithelial cells in vitro by Salmonella expressing mIL-5

It was of interest to determine the ability of bacteria expressing mIL-5 to invade epithelial cells. A monolayer of MDCK cells was incubated with strain SL5631(pTRX) or strain SL5631(pTRXFUS-mIL-5) and intracellular organisms were recovered after treatment with gentamicin to kill extracellular bacteria. Reduced numbers of viable bacteria were found inside cells infected with strain SL5631(pTRXFUS-mIL-5) than in cells infected with the control strain following incubation for 1 h and 2 h (Fig. 4).

Murine immune response to live, attenuated Salmonella expressing the mIL-5/thioredoxin fusion product

To compare the titre of mucosal IgA produced in BALB/c mice immunised orally with strain SL5631(pTRXFUS-mIL-5) to those immunised with strain SL5631(pTRX), rectal and vaginal swabs and lung and intestinal lavages were performed on days 7, 14, 21 and 42 after immunisation. These mucosal samples were analysed for IgA specific to salmonella LPS in a standard ELISA (Table 2). The IgA titre increased in intestinal and lung samples up to day 42 and in vaginal and rectal samples up to day 21 (day 42 was not done), with at least a two-fold increase in IgA observed from mice immunised with strain SL5631(pTRXFUS-mIL-5) compared to strain
Table 2. Anti-salmonella LPS-specific IgA antibody levels in secretions from mice immunised orally

<table>
<thead>
<tr>
<th>Immunising strain</th>
<th>Intestinal IgA</th>
<th>Vagal IgA</th>
<th>Rectal IgA</th>
<th>Lung IgA</th>
</tr>
</thead>
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<tr>
<td></td>
<td>ELISA titre on given day after immunisation*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SL5631(pTRX)</td>
<td>&lt; 20†</td>
<td>30 (20–40)</td>
<td>60 (40–80)</td>
<td>ND</td>
</tr>
<tr>
<td>SL5631(pTRXFUS-mIL-5)</td>
<td>93 (40–160)</td>
<td>133 (80–160)</td>
<td>533 (320–640)</td>
<td>ND</td>
</tr>
<tr>
<td>SL5631(pTRX)</td>
<td>&lt; 20†</td>
<td>30 (20–40)</td>
<td>60 (40–80)</td>
<td>ND</td>
</tr>
<tr>
<td>SL5631(pTRXFUS-mIL-5)</td>
<td>80</td>
<td>267 (160–320)</td>
<td>533 (320–640)</td>
<td>ND</td>
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<tr>
<td>SL5631(pTRX)</td>
<td>40‡</td>
<td>120 (80–160)</td>
<td>120 (80–160)</td>
<td>ND</td>
</tr>
<tr>
<td>SL5631(pTRXFUS-mIL-5)</td>
<td>67 (40–80)</td>
<td>320‡</td>
<td>533 (320–640)</td>
<td>ND</td>
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ND, titre not determined.
†Titres are represented as mean (range) dilutions of secretions from three mice at which the OD490 was > 0.25 above the pre-immune secretions.

Table 3. Anti-salmonella LPS IgA-specific ASC among lymphoid cell isolates from mice immunised intranasally

<table>
<thead>
<tr>
<th>Immunising strain</th>
<th>Number of IgA-specific ASC on given day after immunisation*</th>
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<tbody>
<tr>
<td></td>
<td>7</td>
</tr>
<tr>
<td>SL5631(pTRX)</td>
<td>10</td>
</tr>
<tr>
<td>SL5631(pTRXFUS-mIL-5)</td>
<td>55</td>
</tr>
</tbody>
</table>

*Mice were immunised intranasally with 1 × 10⁸ cfu of recombinant Salmonella. Their lungs were removed on the days indicated and lung cell isolates were used in ELISPOT assays. Data represent mean counts from duplicate wells. Assays were performed with cells pooled from three mice in each group; SEM were < 10% in each case.

Table 4. Anti-salmonella LPS-specific IgA titres in sera of mice immunised orally

<table>
<thead>
<tr>
<th>Immunising strain</th>
<th>ELISA titre on given day after immunisation*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>7</td>
</tr>
<tr>
<td>SL5631(pTRX)</td>
<td>72 (40–80)</td>
</tr>
<tr>
<td>SL5631(pTRXFUS-mIL-5)</td>
<td>360 (160–640)</td>
</tr>
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</table>

ND, titre not determined.
†Titres are represented as mean (range) dilutions of sera from three mice at which the OD₄₉₀ was > 0.1 above the pre-immune sera.
††Titres from sera of all mice were the same.

Table 5. Anti-salmonella LPS-specific IgG titres in sera of mice immunised orally

<table>
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<tr>
<th>Immunising strain</th>
<th>ELISA titre on given day after immunisation*</th>
</tr>
</thead>
<tbody>
<tr>
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<td>7</td>
</tr>
<tr>
<td>SL5631(pTRX)</td>
<td>112 (80–160)</td>
</tr>
<tr>
<td>SL5631(pTRXFUS-mIL-5)</td>
<td>480 (320–640)</td>
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</table>

ND, titre not determined.
†Titres are represented as mean (range) dilutions of sera from three mice at which the OD₄₉₀ was > 1.5 above the pre-immune sera.
††Titres from sera of all mice were the same.
Anti-salmonella LPS IgA responses in IL-5-deficient mice immunised with the Salmonella strain expressing mIL-5

C57BL/6J (+/+) and C57BL/6J IL-5-deficient mice (−/−) were immunised orally with $1 \times 10^{10}$ cfu of strain SL5631(pTRX) or strain SL5631(pTRXFUS-mIL-5). At days 7, 14 and 35, mucosal samples were collected and analysed for salmonella LPS-specific IgA by ELISA (Table 6). By day 35 after immunisation, IgA titres against LPS in intestinal secretions from mice immunised with strain SL5631(pTRX) were at least two-fold higher in normal (+/+) mice than in IL-5 deficient (−/−) mice. IgA titres against LPS, from both intestinal and lung secretions, peaked at least four-fold higher in mice immunised with the strain expressing mIL-5 than with the control. However, there was no significant difference between the titres from normal (+/+) mice and IL-5-deficient mice (−/−) immunised with the strain expressing mIL-5.

**Discussion**

The data presented here demonstrate that the attenuated *aroA* strain of *Salmonella* serotype Dublin, SL5631, could be used as a vehicle to deliver mIL-5 to the host immune system. Initially, the expression and functional activity of mIL-5 were compared with two different plasmid vectors, one carrying the gene for mIL-5 alone, and the other carrying the mIL-5 gene as a fusion with the *E. coli* thioredoxin gene. The data indicate that expression of mIL-5 alone, by either *E. coli* or *Salmonella*, produced insoluble, biologically inactive protein. This protein could be solubilised *in vitro*, but still gave very low activity. Apparently, expression systems that produce insoluble or aggregated proteins, such as pKK233.2, yield a small amount in a soluble, active form [35, 20]. Therefore, where only low doses of cytokine are needed, insolubility may not be a significant problem.

pTRXFUS-mIL-5 appears to be a more valuable system in several respects. The expression plasmid was found to express the fusion product constitutively in *Salmonella*. Also, the mIL-5 fusion protein was produced in a biologically active form. This is consistent with the report that the thioredoxin moiety appears to confer solubility to heterologous proteins [21].

The effects of mIL-5 expression on bacterial colonisation and persistence *in vivo* were examined. Bacterial counts were assessed after oral immunisation. It was of interest to find that four-fold greater numbers of bacteria were obtained from the livers and spleens of mice immunised with the strain expressing mIL-5 than from the organs of those immunised with the control strain. Denich et al. [6] reported a similar finding when mice were immunised with a *Salmonella* strain expressing IL-4. Several in-vitro experiments lead to the conclusion that IL-4 reduces the ability of macrophages to kill *Salmonella* [6]. It would appear that expression of mIL-5 also confers some advantage to the bacteria with respect to multiplication or survival in mice, whether by changes to the bacteria or modification of the host’s immune responses.

A Th1 response is usually stimulated during salmonella infection. This includes activation of macrophages by IFN-γ and TNF, resulting in an increase in the ability of macrophages to kill the intracellular bacteria. Prolonged colonisation by the recombinant *Salmonella* strain, SL5631(pTRXFUS-mIL-5), could be due to mIL-5 expression resulting in inhibition of the protective Th1 response and establishment of a non-protective Th2 population. It has been demonstrated that other Th2 cytokines, such as IL-4 and IL-10, inhibit several Th1 effector functions. For example, IL-4 and IL-10 act synergically with TGF-β to inhibit macrophage cytotoxicity [36]. In conflict with this hypothesis, evidence suggests that IL-5 is not

**Table 6. Anti-salmonella LPS-specific IgA titres in secretions of C57 (+/+) mice or C57 IL-5-deficient (−/−) mice immunised orally**

<table>
<thead>
<tr>
<th>Immunising strain</th>
<th>ELISA titre on days after primary immunisation*</th>
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<tr>
<td></td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>SL5631(pTRX)</td>
<td>17 (10-20)</td>
</tr>
<tr>
<td>SL5631(pTRXFUS-mIL-5)</td>
<td>22 (5-40)</td>
</tr>
<tr>
<td>SL5631(pTRX)</td>
<td>&lt; 5†</td>
</tr>
<tr>
<td>SL5631(pTRXFUS-mIL-5)</td>
<td>15 (5-40)</td>
</tr>
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<td></td>
<td>I, intestinal secretions; L, lung secretions.</td>
</tr>
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<td></td>
<td>Mice were immunised with $1 \times 10^{10}$ cfu on day 0 and day 21.</td>
</tr>
<tr>
<td>*Titres are represented as mean (range) dilutions of secretions from three mice at which the OD_{490} was &gt; 0.4 above the OD of the pre-immune samples.</td>
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<tr>
<td>†Titres from secretions of all mice were the same.</td>
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10-fold higher IgG titres than those infected with the control.

**Table 6. Anti-salmonella LPS-specific IgA titres in secretions of C57 (+/+) mice or C57 IL-5-deficient (−/−) mice immunised orally**

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only involved in B-cell differentiation, but also in the generation of cytotoxic T lymphocytes (CTL) as a killer helper factor (KHF) [37, 38]. Therefore, it is uncertain what role the recombinant mIL-5 is playing in the prolonged colonisation of the liver and spleen by the bacteria. Cell lysis may play an important part in liberating the cellular contents [39] such that cell-free active mIL-5 could then interact with mIL-5 receptors on target cells. It is not known at this stage whether mIL-5 is secreted by the recombinant Salmonella in vivo.

To further understand the interaction of the mIL-5 expressing Salmonella strain with host cells, the invasiveness of the bacteria was investigated in vitro. From these studies, it appears that expression of mIL-5 affects the bacteria or the host cells in such a way that it slows bacterial invasion of the non-polarised epithelial cells in vitro. Therefore, it seems that the Salmonella strain expressing mIL-5 is slowed at the invasion stage of infection, but once inside the cells, persists longer than the control strain.

Expression of soluble, active mIL-5 by strain SL5631(pTRXFUS-mIL-5) in vivo not only appears to enhance mucosal and serum IgA, but serum IgG also. Levels of salmonella LPS-specific serum IgA and IgG from mice immunised with an mIL-5 expressing strain were examined. A significant increase in IgA and IgG titres was observed in mice immunised orally with the strain expressing mIL-5 compared to the control. Similar reports have described mIL-5 as an inducer of IgG, IgA and IgM secretion [18, 40]. A low increase in the number of IgA ASCs was detected on day 7 after infection which peaked by day 14. The low number of IgA ASCs observed on day 7 may suggest that other secretory B cells are produced in an early response to mIL-5. Janardhana et al. [40] reported that IL-5 stimulated the early appearance of IgM- and IgG-secreting B cells, with IgA-secreting B cells appearing in the lungs of mice 2 weeks after immunisation.

This study has important implications for the use of live vector vaccines expressing immunogenic proteins and for improved mucosal immunisation strategies. It has shown that the IgA response can be enhanced by expression of mIL-5 in Salmonella. This system will now be used to enhance the immunogenicity of Salmonella in vivo. Whether mIL-5 is secreted by the recombinant Salmonella in vivo.


31. Aumann E, Brosius J. "ATG vectors" for regulated high-level expression of soluble, active mIL-5 by strain SL5631(pTRXFUS-mIL-5) in vivo not only appears to enhance mucosal and serum IgA, but serum IgG also. Levels of salmonella LPS-specific serum IgA and IgG from mice immunised with an mIL-5 expressing strain were examined. A significant increase in IgA and IgG titres was observed in mice immunised orally with the strain expressing mIL-5 compared to the control. Similar reports have described mIL-5 as an inducer of IgG, IgA and IgM secretion [18, 40]. A low increase in the number of IgA ASCs was detected on day 7 after infection which peaked by day 14. The low number of IgA ASCs observed on day 7 may suggest that other secretory B cells are produced in an early response to mIL-5. Janardhana et al. [40] reported that IL-5 stimulated the early appearance of IgM- and IgG-secreting B cells, with IgA-secreting B cells appearing in the lungs of mice 2 weeks after immunisation.

This study has important implications for the use of live vector vaccines expressing immunogenic proteins and for improved mucosal immunisation strategies. It has shown that the IgA response can be enhanced by expression of mIL-5 in Salmonella. This system will now be used to enhance the immunogenicity of particular antigens by co-expressing them with mIL-5 and possibly other cloned cytokines. Therefore, this system will be particularly useful in immunisation against infectious agents where the mucosal immune response plays an important role in host defense. Further studies will be required to gain a clearer understanding of the effect mIL-5 expression has on Salmonella pathogenesis and host responses.

We thank B. Stocker for the Salmonella strains, H. Campbell for the pEDFM-16 plasmid, and Sally Ford for assistance with IL-5 biological assays. This work was supported by a grant from the National Health and Medical Research Council.

References


