***Chlamydia trachomatis* major outer membrane protein epitopes expressed as fusions with LamB in an attenuated *aroA* strain of *Salmonella typhimurium*; their application as potential immunogens**

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(Received 5 November 1990; revised 19 February 1991; accepted 2 April 1991)

The major outer-membrane protein (MOMP) of *Chlamydia trachomatis* is the focus of attention for chlamydial vaccine design, particularly those serovar- and subspecies-specific epitopes which provoke neutralizing immune responses. Selected surface-exposed B-cell epitopes of MOMP, incorporating B-subspecies specificities, were expressed as fusions with LamB, an inducible outer-membrane transport protein of *Escherichia coli*. These recombinant chlamydial–LamB proteins were correctly transported to the outer membrane of both *E. coli* and an *aroA* mutant of *Salmonella typhimurium*. The immunogenicity of the constructs was investigated in a mouse model of chlamydial salpingitis. After oral immunization, recombinant *S. typhimurium* were recovered from the livers of mice for up to two weeks, and a serum IgG response was induced both to the *Salmonella* and to the inserted chlamydial epitopes. By contrast, intravenous inoculation was ineffective. Although these LamB fusions proved only weakly immunogenic, this approach should be useful for investigating the ability of attenuated *S. typhimurium* vaccines incorporating chlamydial epitopes to stimulate protective mucosal immunity in the mouse model of chlamydial salpingitis.

Introduction

Chlamydia trachomatis serovars A, B and C are the infectious agents of trachoma, a major cause of preventable blindness world-wide. These three serovars belong either to the B complex (serovar B), or to the C complex (serovars A and C), according to which subspecies epitopes they express. Trachoma vaccines which use killed whole chlamydiae afford only short term, serovar-specific immunity associated with local tear antibody, and in some cases, when subjects subsequently become infected with heterologous serovars, increase disease severity (reviewed by Schachter & Dawson, 1978). A subunit vaccine which incorporated protective epitopes towards all three trachoma serovars but excluded antigens which induce damaging hypersensitivity responses (Morrison *et al.*, 1989) might be more successful.

Abbreviations: EB, elementary body; mAb, monoclonal antibody; MOMP, major outer membrane protein; PCR, polymerase chain reaction; RB, reticulate body; TBS, Tris-buffered saline; TTBS, Tween-20-Tris-buffered saline; VS, variable segment.

The nucleotide sequence data reported in this paper have been submitted to the EMBL, GenBank and DDBJ Nucleotide Sequence Databases and assigned the accession number M33536.

Ideally, such a vaccine should generate neutralizing mucosal antibody to the surface of the infectious EB, as well as systemic immunity, in order to prevent initial eye infection before the organisms become sequestered within host cells.

The most promising subunit vaccine candidate is the major outer membrane protein (MOMP), a porin which maintains structural rigidity of the outer envelope, regulates nutrient influx and which controls early intracellular differentiation of the chlamydiae (Bavoil *et al.*, 1984; Hatch *et al.*, 1984). Immunodominant serovar- and subspecies-specific epitopes on MOMP are surface-exposed, and antibodies to them are neutralizing *in vivo* (Zhang *et al.*, 1987). By contrast, most species- and genus-specific epitopes on MOMP are inaccessible to antibody (Batteiger *et al.*, 1986; Kuo & Chi, 1987; Zhang *et al.*, 1987; Conlan *et al.*, 1989) and thus non-neutralizing, although a surface-exposed-species-reactive MOMP epitope has been described (Collett *et al.*, 1989). Two B-complex-specific, surface-exposed linear epitopes (B₁ and B₂) have been identified within variable segment 4 (VS4) of serovar B MOMP (Conlan *et al.*, 1989). By contrast, a C-complex-specific, surface-exposed epitope is located within VS1 of serovar A

MOMP (Hayes *et al.*, 1990). Rabbit antisera to synthetic peptide analogues of epitopes B₁, B₂ and a modified analogue of the C-complex specific epitope react with the surfaces of live chlamydiae of all three trachoma-causing serovars, as demonstrated by immunogold transmission electron microscopy (Conlan *et al.*, 1989 and unpublished results). Thus, a subunit vaccine incorporating these epitopes might protect against trachoma infection, especially if such a vaccine was engineered to elicit local, as well as systemic immunity. The necessary antigenic persistence for stimulating mucosal immunity might be achieved using a suitable live, attenuated vaccine organism.

A mouse model for chlamydial infection is available (Tuffrey *et al.*, 1986). When progesterone-treated C3H mice are inoculated into the uterus or ovarian bursa with a human genital tract isolate of *C. trachomatis*, they develop salpingitis and infertility. Since successful antigenic priming at any mucosal site, for example the gut, should protect against challenge at any other mucosal site, this model is suitable for preliminary trials of trachoma vaccine candidates.

The LamB protein of *E. coli* was chosen as a suitable vehicle for expression of chlamydial epitopes in the outer membrane of a live bacterial vaccine. LamB is a 47 kDa outer membrane protein which, like MOMP, is a porin and which regulates maltose transport in *E. coli*. The *lamB* gene has been cloned, placed under *tac* promoter control (Bouges-Bocquet *et al.*, 1984) and engineered to create a unique *Bam*HI site allowing insertion of foreign epitopes at amino acid position 153 of the mature LamB protein (Boulain *et al.*, 1986). Epitopes inserted at this site are located on a surface-exposed loop (Boulain *et al.*, 1986; Charbit *et al.*, 1991). As a vaccine vector for the modified LamB protein, we chose attenuated, non-reverting *aroA* strains of *Salmonella*. These organisms colonize the mouse gut but do not multiply, protecting mice against subsequent infection with the virulent parent strains (Hoiseth & Stocker, 1981). *AroA* strains have been used to elicit immune responses in mice to a variety of foreign antigens expressed both cytoplasmically (Brown *et al.*, 1987; Poirier *et al.*, 1988; Clements *et al.*, 1986; Fairweather *et al.*, 1990), and at the cell surface (Charbit *et al.*, 1987; Maskell *et al.*, 1987; Wu *et al.*, 1989). This approach is potentially useful in humans since variously attenuated strains of *Salmonella typhi*, including *aroA*, are undergoing trials as live oral vaccines for use in humans (Tacket *et al.*, 1990).

In this study, oligonucleotides encoding two B-subspecies epitopes of *C. trachomatis* were synthesized and cloned into the *lamB* gene. Both epitopes were incorporated into recombinant LamB proteins expressed in *E. coli* and *S. typhimurium*, and were recognized in immunoblots by polyclonal antibodies raised against

synthetic peptide analogues. When administered orally to the strain of mouse used to study chlamydial salpingitis, serum IgG responses to *S. typhimurium* and *C. trachomatis* were induced but neither mucosal response nor serum IgA was detected.

Methods

Bacterial strains and vectors. *Escherichia coli* JM109 (Yanisch-Perron *et al.*, 1985) Epicurean competent cells were purchased from Northumbria Biologicals Limited. *Salmonella typhimurium* strains LB5010 (*galE*⁻, *r*⁻, *m*⁺; Bullas & Ryo, 1983) and SL3261 (*aroA*; Hoiseth & Stocker, 1981) and bacteriophage P22 (Schmieger, 1972) were kind gifts of Dr G. Dougan (Wellcome Research Laboratories, Beckenham, UK). The *lamB* vector, pAJC264 (Boulain *et al.*, 1986) was a kind gift of Dr M. Hofnung (Institut Pasteur, Paris, France).

Enzymes. Restriction endonucleases, T4 DNA ligase and calf intestinal phosphatase were obtained from Boehringer Mannheim. T4 polynucleotide kinase was obtained from Gibco-BRL. All enzymes were used as recommended by the manufacturer.

Oligonucleotides. Complementary oligonucleotide pairs encoding epitopes B₁ and B₂ were synthesized which utilized *E. coli* high expression codon preferences (Grosjean & Fiers, 1982). The oligonucleotide pairs contained *Bam*HI-compatible ends which recreated a *Bam*HI site only at the 5' end of the insert when cloned into *Bam*HI cleaved vector. Positioning of cloning sites on the oligonucleotides resulted in the addition of a proline to the N-terminus and an asparagine to the C-terminus of each epitope. Epitopes B₁, B₂ and the corresponding oligonucleotide sequences are shown in Fig. 1. Oligonucleotides were synthesized using β -cyanoethyl phosphoramidite chemistry on an automated DNA synthesizer (Applied Biosystems Inc., Foster City, CA).

DNA manipulations. Complementary oligonucleotide pairs were simultaneously phosphorylated with T4 polynucleotide kinase and hybridized together in 70 mM-Tris/HCl (pH 7.6), 10 mM-MgCl₂, 5 mM-DTT and 500 μ M-ATP overnight at 37 °C. Oligonucleotide hybrids were ligated with *Bam*HI-cut, dephosphorylated pAJC264 in 66 mM-Tris/HCl (pH 7.5), 5 mM-MgCl₂, 5 mM-DTT and 1 mM-ATP overnight at 4 °C. Epicurean JM109 was transformed with the recombinant plasmids according to the supplier's instructions. The constructs made are summarized in Table 1. Recombinant plasmids were prepared by alkaline lysis (Birnboim & Doly, 1979) and purified through NACS Prepac cartridges (Gibco-BRL). *S. typhimurium* LB5010, a galactose epimerase mutant, was transformed with recombinant plasmids as follows: *S. typhimurium* LB5010, grown in LB-broth to an OD₅₅₀ of 0.5, was pelleted, resuspended in 0.5 vols of 100 mM-MgCl₂, incubated on ice for 20 min, pelleted again, then resuspended in one-fiftieth of the original volume of 100 mM-CaCl₂. Competent cells (200 μ l) were added to 20 ng of plasmid and kept on ice for 20 min, at room temperature for 20 min, and on ice for 40 min. The cells were heat-shocked for 2 min at 42 °C, then incubated for one hour at 37 °C with 800 μ l LB-broth, and plated onto LB-agar containing 100 μ g ampicillin ml⁻¹. Overnight cultures (200 μ l samples) of recombinant *S. typhimurium* were incubated with dilutions of bacteriophage P22 and plated onto LB-agar containing ampicillin (100 μ g ml⁻¹). Recombinant bacteriophage P22 were harvested from plates of confluent plaques. *S. typhimurium* SL3261 (*aroA*) was transduced with recombinant ϕ P22 as follows: 200 μ l of *S. typhimurium* SL3261 grown overnight in LB-broth

supplemented with 1 mM-CaCl₂ was incubated with 1 µl of the recombinant phage stock for one hour at 37 °C. The mixture was spread onto LB-agar containing ampicillin (75 µg ml⁻¹) and 5 mM-EGTA. In each of the three bacterial hosts, ampicillin resistant colonies were screened by immunoblotting for expression of the required epitopes. Inserts were checked for correct frame and orientation by DNA sequencing.

Antisera. Rabbit polyclonal antisera were raised against synthetic peptide analogues of epitopes B₁ and B₂ as previously described (Conlan *et al.*, 1989).

Immunoblotting. *E. coli* strains (400 µg protein ml⁻¹) were disrupted by boiling for 10 min in 0.0625 M-Tris/HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol and 0.001% (w/v) bromophenol blue. Whole cell lysates were separated by SDS-PAGE on 12.5% (w/v) polyacrylamide using the Laemmli (1970) buffer system. Denatured proteins were electroblotted onto nitrocellulose (Schleicher & Schuell) at 100 mA for 90 min using a semi-dry blotter (Ancos, Denmark) (Khyse-Andersen, 1984). The nitrocellulose was then blocked overnight at 4 °C in Tris-buffered saline (TBS; 2.24 g Tris l⁻¹, 26.24 g NaCl l⁻¹, pH 7.5) containing 0.05% (v/v) Tween-20 (TTBS) and 5% (w/v) dried skimmed milk (Marvel). Blots were incubated for 3 h at room temperature with primary antibodies diluted 1:200 in TTBS containing 5% (w/v) Marvel, then washed 3 × 5 min in TTBS. The blots were incubated for 1 h at room temperature with goat anti-rabbit IgG-alkaline-phosphatase conjugate (Bio-Rad) diluted 1:3000 in TTBS containing 5% Marvel, followed by washing for 4 × 5 min in TTBS, then 3 × 5 min in TBS. Antigen-antibody complexes were visualized using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Sigma) and the reactions were stopped by washing the blots in distilled water.

DNA sequencing. Cloning junctions and inserted DNA of recombinant plasmids were sequenced by the dideoxy chain termination method modified for double stranded DNA (Zhang *et al.*, 1988). Briefly, 3 µg of plasmid DNA was incubated for 5 min at room temperature in 0.2 M-NaOH, 0.2 mM-EDTA (total volume, 20 µl). The solution was neutralized by addition of 2 µl 2 M-NH₄-acetate (pH 4.6) and DNA was precipitated with 60 µl 100% ethanol. The DNA was pelleted, washed with 80% ethanol, repelleted, then sequenced using modified T7 polymerase (Sequenase; Cambridge Bioscience) and [α -³⁵S]dATP (410 Ci mmol⁻¹; 1.5 × 10¹³ Bq mmol⁻¹; Amersham). The 17mer oligonucleotides (5'AACTCTCTCTGGCAGCA and 5'TTCGCGGTTTCGTTGGT) used to prime sequences in both directions across the inserts were based on the published sequence of the LamB gene (Clement & Hofnung, 1981).

Separation of cytoplasmic and outer membranes of *E. coli* JM109 and *S. typhimurium* SL3261. Cytoplasmic and outer membranes of recombinant *E. coli* and *S. typhimurium* SL3261 expressing LamB containing B₁ + B₂ were separated by the method of Osborn & Munson (1974). Briefly, 250 ml cultures in LB broth, induced with 0.1 mM-IPTG were pelleted, resuspended in 15 ml 0.75 M-sucrose, 10 mM-Tris-acetate pH 7.5, 100 µg lysozyme ml⁻¹ and incubated on ice for 2 min. Cells were spheroplasted by addition of 30 ml ice-cold 1.5 mM-EDTA over 10 min, then sonicated to lyse the spheroplasts. After a clearing spin, membranes were washed twice by centrifuging through 0.25 M-sucrose, 3.3 mM-Tris/HCl and 1 mM-EDTA for 2 h at 100 000 g at 4 °C. Cytoplasmic and outer membrane fractions were separated by centrifugation at 130 000 g for 16 h at 4 °C through a 30–55% sucrose gradient. After centrifugation each gradient was collected in 30 fractions. Total protein content of each fraction was measured by modified Lowry assay (Ohnishi & Barr, 1978) and fractions containing protein peaks were subjected to PAGE followed by either silver

staining to detect LPS (Tsai & Frasch, 1982) or immunoblotting to detect recombinant LamB.

Immunization of mice with live *aroA* mutants of *S. typhimurium* expressing B₁ + B₂ recombinant LamB protein. Mice were immunized with a B₁ + B₂ recombinant which possessed smooth LPS. A stock of the immunizing strain was grown in LB broth containing ampicillin (125 µg ml⁻¹) and 0.1 mM-IPTG (16 h at 37 °C without shaking). This was harvested by centrifugation (600 g) and resuspended in PBS (Dulbecco A) containing 10% (v/v) glycerol as a cryopreservative to a total count of 2 × 10¹¹ ml⁻¹ (at 60% viability). Cells were stored frozen at -20 °C until required. A control culture of non-recombinant *S. typhimurium* was similarly prepared. Female C3H/He/Ola mice (Olac Ltd), 6–10 weeks old, were used in all experiments. This mouse strain was chosen because it is known to be susceptible to genital tract infections with human strains of *C. trachomatis* (Tuffrey *et al.*, 1986). On day one, 24 mice were immunized intravenously (i.v.) with 0.1 ml of the B₁ + B₂ LamB recombinant diluted in PBS to a total count of 2 × 10⁶ ml⁻¹. A further five mice received an equivalent dose of control *S. typhimurium aroA*. A second group of 24 mice were challenged *per os* (p.o.) with the recombinant (0.1 ml of culture at 2 × 10¹¹ ml⁻¹ total count) delivered using a gavage needle. A control group of five mice were similarly immunized with control *S. typhimurium aroA*. Six mice received no vaccine. All mice were housed in separate cages within the same room. At weekly intervals for six weeks, serum was obtained from mice (4 per group) which had received recombinant B₁ + B₂ (i.v. or p.o.) by tail-vein venepuncture whilst under metaflane anaesthesia. After blood samples had been taken the mice were killed by CO₂ anoxia. The liver and spleen were removed whole from each animal aseptically into 10 ml sterile distilled water. The small intestine between the caecum and stomach was removed intact and the contents collected by washing. Sera and clarified gut washes were stored at -80 °C. Livers and spleens were homogenized for 1 min in a Colworth stomacher and the resulting macerates plated on LB-agar containing ampicillin for recovery of organisms. On the sixth week following immunization, all control animals were processed as above.

Determination of antibody in sera and gut-washes of immunized mice by amplified ELISA. Antibodies to *S. typhimurium aroA* and *C. trachomatis* serovar B were detected by redox-amplified ELISA (Johannsson *et al.*, 1985). *S. typhimurium aroA* or *C. trachomatis* serovar B whole cells at a protein concentration of 10 µg ml⁻¹ were coupled to microtitre PVC microtitre trays (Flow laboratories) by overnight incubation in carbonate buffer at 18 °C (1.6 g l⁻¹ Na₂CO₃, 2.95 g l⁻¹ NaHCO₃, 0.2 g l⁻¹ NaN₃; pH 9.6). Excess antigen was removed by washing the trays three times with 50 mM-ethanolamine/100 mM-NaCl (pH adjusted to 10.5 with 100 mM-citric acid). The antigen-coated, washed trays were blocked for 1 h at 37 °C with PBS (Dulbecco A) containing 5% (v/v) normal goat serum (100 µl per well). The blocking solution was drained and test sera or gut washes added at 1:100 or 1:5 dilution respectively (50 µl per well). The diluent was blocking solution containing 0.05% (v/v) WI detergent (Sigma). The trays were sealed and incubated for 3 h at 37 °C, then washed four times. Goat anti-mouse alkaline phosphatase conjugates specific for IgA or IgG (Zymed laboratories) were added at 1:1000 dilution (50 µl per well) and incubation continued for 45 min. After a further six washes, NADP substrate (0.2 mM-NADP, 10 mM-MgCl₂, 1 mM-ZnCl₂, 150 mM-Na₂SO₄ in 50 mM diethanolamine buffer, pH 9.5) was added (50 µl per well). This solution was filtered through a 0.22 µm filter immediately before use. Incubation continued for 40 min at 18 °C. After this time 100 µl amplifier was added to each well. Amplifier solution consisted of 0.2 g l⁻¹ alcohol dehydrogenase, 0.15 g l⁻¹ diaphorase, 0.28 g l⁻¹ *p*-iodonitrotetrazolium violet (all reagents from Sigma) in 20 mM-sodium phosphate buffer (pH 7.2) containing 4% (v/v) ethanol. The reaction was stopped by addition of 3% (v/v) H₂SO₄ (25 µl per well) and the reaction product measured as A₄₉₅.

Fig. 1. (a) Sequence of serovar B VS4, showing position of epitopes B₁ and B₂. (b) Oligonucleotide pairs encoding B₁ and B₂. Brackets indicate non-native sequence added for cloning.

Results and Discussion

Expression of epitopes in *E. coli*

C. trachomatis epitopes B₁ and B₂ are shown in Fig. 1; they are identical to the sequences occurring in *C. trachomatis* serovar B/Jali-20/OT VS4 (Hayes *et al.*, 1990). Sequence B₁ contains the known B-complex-specific, surface-exposed epitope IFDV (Conlan *et al.*, 1988). Sequence B₂ contains a species-specific epitope, LNPTIA, which is not surface-exposed (Stephens *et al.*, 1988; Conlan *et al.*, 1988; Baehr *et al.*, 1988) as well as an additional surface exposed, B-complex specific region AGDV (Conlan *et al.*, 1989). Epitopes B₁ and B₂ were expressed as fusions with LamB both individually and in tandem, as shown in Table 1. An additional sequence from VS1 of serovar A, containing serovar A- and C-subspecies-specific epitopes (Hayes *et al.*, 1990) was expressed in *S. typhimurium aroA* but was not used for animal inoculations since it was poorly detected in Western blots and the recombinant salmonellae expressing this epitope consistently possessed rough LPS.

Immunoblots of *E. coli* strains expressing epitopes B₁ and B₂ as recombinant LamB proteins are shown in Fig. 2 (a and b). In VS4 of serovar B MOMP, the sequence 288SAETIFDVTTLNPTIAGAGDV³⁰⁸ contains epitopes B₁ (residues 288–298) and B₂ (residues 298–308) which overlap each other by one amino acid. Interestingly, the double construct B₁ + B₂ in the reversed order, ie (P)LNPTIAGAGDV(DP)SAETIFDVTTL(D) ap-

Table 1. Summary of chlamydial epitopes fused with LamB
Brackets indicate non-natural sequences.

Epitopes	Amino acid sequence	No. of residues
B ₁	(P)SAETIFDVTTL(D)	13
B ₂	(P)LNPTIAGAGDV(D)	13
B ₁ + B ₂	(P)SAETIFDVTTL(DP)LNPTIAGAGDV(D)	26

peared to express both epitopes, since a non-truncated recombinant protein was obtained. However, only the proximal B₂ was detected by immunoblotting (data not shown). DNA sequencing confirmed the correct orientation of both epitopes in the double recombinant. Presumably the lack of reactivity of B₁ in this construct reflects stoichiometric constraints on the ordering of epitopes within the LamB vector.

Expression of epitopes in *S. typhimurium* SL3261

S. typhimurium SL3261 (*aroA*) was transduced with ϕ P22 carrying recombinant plasmids, since direct transformation of smooth *Salmonella* strains is very inefficient (MacLachlan & Sanderson, 1985) and selects for rough mutants (Brown *et al.*, 1987). The receptor for ϕ P22 is not present on rough salmonellae, ensuring that transductants are smooth. Only smooth salmonellae will

colonize mice. Fig. 2c shows immunoblots of *S. typhimurium* SL3261 expressing recombinant LamB fused with epitopes B₁ + B₂. These epitopes were

expressed in *S. typhimurium* SL3261 at equivalent levels to those observed in *E. coli* JM109.

Location of recombinant LamB proteins within the bacterial outer membrane

To verify the location of the expressed proteins, cytoplasmic and outer membrane fractions of recombinant *E. coli* and *S. typhimurium* were separated by sucrose density gradient centrifugation, then subjected to PAGE followed by silver staining (Fig. 3a and b). The outer membrane component LPS was located in fractions towards the bottom of the gradient. In identical gels immunoblotted and probed with anti-B₁ (Fig. 3c and d), the recombinant LamB protein was located exclusively in those fractions which contained LPS, indicating that it is present in the outer membrane in both *E. coli* and *S. typhimurium*. From the proposed model of LamB folding (Boulain *et al.*, 1986; Charbit *et al.*, 1991) it would be expected that the inserted *C. trachomatis* epitopes should reside within a surface-exposed loop. However, attempts to label these epitopes at the surfaces of recombinant *E. coli* and *S. typhimurium* with immunogold were unsuccessful. The long side chain of smooth LPS may prevent antibody binding to the surface-exposed LamB, blocking surface labelling of the recombinant bacteria with immunogold. However, Brown *et al.* (1987) demonstrated that expression at the surface of salmonellae is not necessary to provoke B- and T-cell responses to cloned proteins.

Fig. 2. (a, b) *E. coli* recombinants expressing LamB fusion proteins containing: lane 1, B₁; lane 2, B₂; lane 3, B₁ + B₂. (a) Probed with anti-B₁. (b) Probed with anti-B₂. (c) *S. typhimurium* aroA recombinant expressing LamB fusion protein containing B₁ + B₂, probed with anti-B₁. Approximate molecular masses are shown.

Fig. 3. Cytoplasmic and outer membranes of recombinant (B₁ + B₂) *E. coli* (a, c) of *S. typhimurium* aroA (b, d) separated by sucrose gradient centrifugation and collected as fractions, which were then subjected to PAGE and either silver stained (a, b) or immunoblotted and probed with anti-B₁ (c, d). Fractions from the top of each gradient to the bottom were loaded from left to right on the gels. Arrows indicate LPS and recombinant LamB.

Recovery of B₁ + B₂ LamB recombinant S. typhimurium aroA mutants from immunized mice

These were the first experiments to use recombinant chlamydial antigens expressed in salmonellae to elicit immune responses in the mouse model of chlamydial infection. Ampicillin-resistant bacteria were recovered from the livers of mice immunized i.v. for up to two weeks post-challenge and from the spleens of these animals for up to four weeks (Table 2). Bacteria were also recovered from the livers of p.o. immunized mice for up to two weeks. However, no bacteria were isolated from the spleens of p.o. immunized animals after the first week. In all cases the numbers of bacteria recovered were low (10–30 organisms per organ) and they were confirmed to be carrying the recombinant *lamB* plasmid by PCR, using as primers the *lamB*-specific oligonucleotides described above (data not shown).

Detection of antibodies in immunized mice to S. typhimurium and C. trachomatis

A live attenuated *S. typhimurium aroA* mutant containing recombinant LamB protein incorporating *C. trachomatis* epitopes B₁ + B₂ was assessed for its ability to generate anti-chlamydial antibodies when inoculated into mice. Two routes of administration were tried; i.v. immunization with *S. typhimurium aroA* expressing LamB (B₁ + B₂), which produced circulating IgG antibody to *S. typhimurium* and to *C. trachomatis* in only 5/24 and 3/24 animals respectively (Fig. 4a), and oral immunization, which produced circulating IgG to *S. typhimurium* in 15/19 animals although only 5/19 had circulating IgG to *C. trachomatis* (Fig. 4b). Antibody titres to *C. trachomatis* were generally much lower than for *S. typhimurium*, as would be expected given the much lower mass of chlamydial epitopes. There was little correlation between the levels of antibody to *S. typhimurium* and *C. trachomatis* in the p.o. immunized animals. Neither IgA nor IgG antibodies to either *S. typhimurium* or *C. trachomatis* were detected in the gut washes, nor was IgA antibody to either antigen detected in the sera.

Brown *et al.* (1987) elicited high-titre antibodies to β -galactosidase expressed as cytoplasmic inclusions in *S. typhimurium aroA* when intravenously administered. However, this group used a different strain of mouse (BIOXA/J) to that used in the present study. Moreover, the β -galactosidase in their immunogen represented up to 2% of the total protein whereas the *C. trachomatis*-specific epitope present in our vaccine strain was much less than this as judged subjectively by the quantity of LamB present on SDS-PAGE profiles.

By contrast to i.v. immunization, p.o. administration of the recombinant LamB *aroA* strain resulted in

Fig. 4. Detection of IgG antibodies to *S. typhimurium* and *C. trachomatis* serovar B in mice. Control group 1, normal control sera; same mice in (a) and (b). Experimental groups; immunized i.v. (a) or p.o. (b) with recombinant *S. typhimurium aroA*, sampled 1–6 weeks after challenge as shown. Control group 2, inoculated i.v. (a) or p.o. (b) with non-recombinant *S. typhimurium aroA*, sampled 5 weeks post challenge. Each pair of columns represents a single mouse. Filled columns represent IgG to *S. typhimurium*; hatched columns represent IgG to *C. trachomatis*.

Table 2. Recovery of bacteria from livers and spleens of mice immunized with *S. typhimurium* LamB (B₁ + B₂)

Time (weeks)	No. of mice positive			
	i.v. immunized		p.o. immunized	
	Liver	Spleen	Liver	Spleen
1	2/4	2/4	2/4	1/4
2	1/4	1/4	1/4	0/4
3	0/4	2/4	0/4	0/4
4	0/4	1/4	0/4	0/4
5	0/4	0/4	0/3*	0/3*
6	0/4	0/4	NT*	NT*

* Five mice in the p.o. immunized group died during the experiment and were not available for testing.

NT, not tested.

seroconversion of IgG antibody to *S. typhimurium* in the majority of mice. This may simply be a reflection of the larger dose of bacteria given p.o. compared to i.v.; however, it was not accompanied by increased colonization of the liver or spleen. Oral immunization produced an antibody response to *C. trachomatis* in significantly more animals than did i.v. immunization. However, the

total number of mice responding and the antibody titres remained low. Charbit *et al.* (1987) also found only low levels of antibody to a hepatitis B virus epitope inserted into the LamB protein, although this group used *E. coli* rather than *S. typhimurium* as the bacterial host. Leclerc *et al.* (1989) found the route of inoculation to be important; i.v. and i.p. administration of live recombinant *E. coli* being more effective than subcutaneous administration, however, mucosal immunity was not sought by those authors.

Surprisingly, no IgA antibody to *C. trachomatis* or to *S. typhimurium* was found in any of the test samples, including the sera. Maskell *et al.* (1987) using *S. typhimurium aroA* mutants expressing the B subunit of *E. coli* heat labile toxin, demonstrated a consistent IgA response in both the sera and gut washes of recipient Balb/C mice. In that study, the livers and spleens of orally-immunized mice were colonized by bacteria for up to 5 weeks, longer than in the present study, which might partly explain the differences in IgA production. Sigwart *et al.* (1989) also demonstrated IgA antibodies to *S. dublin aroA* in orally immunized Balb/C mice. In their study, colonization of the liver and spleen was similar to the results reported here, but *S. dublin* was recovered from the small intestine for up to 3 weeks following oral administration. In our study, the recombinant *S. typhimurium* was cleared rapidly (8 d) from the small intestine of C3H/He/Ola mice. Long-term colonization of the small intestine and particularly the gut-associated lymphoid tissue (GALT) should favour a local IgA response, thus the transient passage of *S. typhimurium* through the gut observed here is probably an insufficient stimulus to elicit secretory IgA. This may reflect the mouse strain we used; C3H/He/Ola mice are susceptible to chlamydial salpingitis (Tuffrey *et al.*, 1986) but are less susceptible to natural *S. typhimurium* infections than Balb/C mice (Hormaeche, 1979). Moreover, surface expression of the recombinant LamB protein might also alter the colonization properties of *S. typhimurium*. Thus the mouse and *Salmonella* strain, the carrier protein and the properties of the inserted epitopes are all likely to influence the immunogenicity of attenuated *Salmonella* constructs in mice.

At least one attenuated *Salmonella*, *S. typhi* Ty21a, has already proved acceptable for use in man. In some systems *Salmonella* vectors have achieved adequate mucosal immunity. Our results suggest this may not be true of all systems, indicating a need to modify our vaccination strategy if effective mucosal immunization is to be achieved. The choice of mouse strain is limited by murine genotypic factors affecting the establishment of colonization and chlamydial salpingitis. We believe that the strategy should be to identify a stable *Salmonella* host capable of colonizing the mouse intestine for 3–4 weeks

following oral administration. There is a considerable range of *S. typhimurium* and *S. enteritidis* strains to choose from (reviewed by Curtiss *et al.*, 1989). Secondly, the ability of the chlamydial-*Salmonella* recombinant to colonize the mouse gut should be determined prior to immunization and challenge studies. Finally, incorporation of T-cell epitopes of MOMP (Allen *et al.*, 1990; Holland *et al.*, 1990) as well as important neutralizing epitopes from other chlamydial surface antigens may enhance the immunogenicity of the constructs. Brown *et al.* (1987) have demonstrated that cloned antigens expressed in *aroA* mutants of *S. typhimurium* can induce cellular as well as humoral immune responses. Each model system of human infection may pose its own problems. We believe such studies to be of fundamental importance, given the present inability to control a large number of diseases affecting respiratory, mucosal and ocular surfaces by immunization.

We wish to thank Dr M. Hofnung for the gift of pAJC264 and Dr G. Dougan for the gifts of *Salmonella* strains and bacteriophage P22. This work was supported by a grant from the Edna McConnell Clark Foundation, New York.

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