Expression of disulphide-bridge-dependent conformational epitopes and immunogenicity of the carboxy-terminal 19 kDa domain of *Plasmodium yoelii* merozoite surface protein-1 in live attenuated *Salmonella* vaccine strains

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The 19 kDa carboxy-terminal domain of *Plasmodium yoelii* merozoite surface protein-1 (MSP1₁₉) was expressed in *Salmonella* vaccine strains as a carboxy-terminal fusion to fragment C of tetanus toxin (TetC). This study demonstrates that antibodies that recognize disulphide-dependent conformational epitopes in native MSP1 react with the TetC–MSP1₁₉ fusion protein expressed in *Salmonella*. The proper folding of MSP1₁₉ polypeptide is dependent on both the *Salmonella* host strain and the protein to which the MSP1₁₉ polypeptide is fused. Serum from mice immunized with *Salmonella typhimurium* C5aroD expressing TetC–MSP1₁₉ recognized native MSP1 as shown by immunofluorescence with *P. yoelii*-infected erythrocytes. Antibody levels to MSP1₁₉ were highest in out-bred mice immunized with *S. typhimurium* C5aroD carrying pTECH2–MSP1₁₉ and antibody was mostly directed against reduction-sensitive conformational epitopes. However, antibody levels were lower than in BALB/c mice immunized with a glutathione *S*-transferase (GST)–MSP1₁₉ fusion protein in Freund's adjuvant, and which were protected against *P. yoelii* challenge infection. In challenge experiments with *P. yoelii* the *Salmonella*-immunized mice were not protected, probably reflecting the magnitude of the antibody response. The results of this study have important implications in the design of live multivalent bacterial vaccines against eukaryotic pathogens.

Keywords: *Salmonella*, malaria, MSP1₁₉, vaccine, conformational epitopes

INTRODUCTION

The development of multivalent vaccines for human and veterinary use, based on the expression of heterologous antigens in live attenuated *Salmonella*, has received considerable attention in the last decade (Chatfield *et al.*, 1995; Hormaeche & Khan, 1996; Hormaeche *et al.*, 1995). Attenuated bacteria carrying precise deletions in their genome can be delivered to the immune system to

establish a self-limiting infection. During that time they can evoke humoral, secretory and cell-mediated immune responses to their own antigens as well as to recombinant heterologous antigens.

Malaria remains the most prevalent and devastating parasitic disease worldwide. Vaccination against the disease is an approach that will complement other strategies for prevention and control. Previous studies using *Salmonella* as the vaccine vector for malaria parasite antigens have focused primarily on the pre-erythrocytic stage (Aggarwal *et al.*, 1990; Flynn *et al.*, 1990; Gonzalez *et al.*, 1994). However, vaccines against the asexual blood stage that gives rise to the clinical symptoms of the disease will probably have the greatest

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Abbreviations: EGF, epidermal growth factor; GST, glutathione *S*-transferase; HRP, horseradish peroxidase; MSP1₁₉, *P. yoelii* merozoite surface protein-1 19 kDa carboxy-terminal domain.

impact on morbidity and mortality in malaria-endemic areas. Surface proteins of merozoites, the form of the parasite that invades red blood cells, are important vaccine candidates. Merozoite surface protein-1 (MSP1), a protein found in all *Plasmodium* species, has been extensively studied as a target of protective immunity. It has been used to vaccinate in animal models against parasite challenge, including that of *Plasmodium falciparum*, the major human malaria (Etlinger *et al.*, 1991; Siddiqui *et al.*, 1987).

The 19 kDa carboxy-terminal domain of MSP1 (MSP1₁₉) is of particular interest as a vaccine candidate. Studies with the human malaria *P. falciparum* *in vitro* (Blackman *et al.*, 1990; Cooper *et al.*, 1992; Pirson & Perkins, 1985) and the rodent malaria *Plasmodium yoelii* *in vivo* (Majarian *et al.*, 1984; Spencer Valero *et al.*, 1998) have shown that monoclonal antibodies to reduction-sensitive epitopes in this domain are effective at inhibiting invasion of erythrocytes. The cysteine-rich sequence of MSP1₁₉ contains at least five highly conserved disulphide bonds which generate a folded structure composed of two epidermal growth factor (EGF)-like modules (Blackman *et al.*, 1991). MSP1₁₉ has been expressed in *Escherichia coli* in the correctly folded state as judged by antibody binding (Burghaus & Holder, 1994; Chappel & Holder, 1993). Mice immunized with the *E. coli*-derived recombinant *P. yoelii* protein were protected against challenge infection (Daly & Long, 1993; Ling *et al.*, 1994), but the protection was abolished if the protein structure was destroyed by reduction and alkylation prior to use for immunization. This demonstrated that a recombinant MSP1₁₉ suitable for vaccine trials has to be correctly folded (Ling *et al.*, 1994). More recently, it has also been shown that both EGF-like motifs present in MSP1₁₉ are required for the induction of protective immunity (Ling *et al.*, 1995). Protection is predominantly mediated by antibodies (Daly & Long, 1995).

We have previously described a system using the plasmid pTECH2 for the expression of guest antigens in *Salmonella* as C-terminal fusions to tetanus toxin fragment C (TetC) under the control of the anaerobically inducible promoter *nirB* (Chabalgoity *et al.*, 1996; Khan *et al.*, 1994a, b). However, the expression of a recombinant eukaryotic protein, in which formation of disulphide bridges produces a properly folded antigenic and immunogenic molecule, has not been demonstrated previously in *Salmonella*.

In the present study we have used pTECH2 to express MSP1₁₉ from *P. yoelii*, in a panel of different, live, attenuated *Salmonella* vaccine strains. We have evaluated the antigenicity and the immunogenicity of this recombinant protein and describe the delivery of MSP1₁₉ to the mouse immune system by a live Aro *Salmonella* vaccine strain.

METHODS

Plasmids, oligonucleotides and bacterial strains. Plasmid pTECH2 has been described (Khan *et al.*, 1994a). The DNA

sequence of the MSP1₁₉ EGF-like modules, corresponding to amino acids 1649–1754 in the *P. yoelii* YM MSP1 sequence (Lewis, 1989), was amplified by PCR from the *E. coli* expression vector pGEX-3X containing the *P. yoelii* sequence (Ling *et al.*, 1994). Oligonucleotide primers containing sites for *Bam*HI and *Spe*I cohesive ends (forward primer, 5' CACGGATCCGTCGACATGGATGGTATGGATTTAT-TAGG 3'; reverse primer, 5' CACACTAGTCTCGAGGCT-GGAAGAACTACAGAATAC 3') were used to amplify the DNA, allowing directional cloning of the MSP1₁₉ EGF-like modules into pTECH2 that had been previously digested with *Bam*HI and *Spe*I.

The bacterial strains used were *E. coli* Topp 10 (Stratagene), *E. coli* TG2 (*recA*) (Sambrook *et al.*, 1989), *Salmonella typhimurium* SL5338 (*galE* r⁻ m⁺) (Brown *et al.*, 1987) and the *S. typhimurium* vaccine strains: SL3261(*aroA*) (Hoiseth & Stocker, 1981), BRD726 (*ΔhtrA*) (Chatfield *et al.*, 1992), C5aroD (Miller *et al.*, 1989) and C5046 (*htrA::TnpHoA*) (Johnson *et al.*, 1991) (referred to in this study as C5*htrA*). Bacteria were grown aerobically in Luria–Bertani (LB) broth supplemented with 50 µg ampicillin ml⁻¹ as required. *Salmonella* and *E. coli* Topp 10 cells harbouring pGEX-3X-MSP1₁₉ (Ling *et al.*, 1994) were treated with 1 mM IPTG to induce expression of glutathione S-transferase-MSP1₁₉ (GST-MSP1₁₉). Plasmid DNA prepared in *E. coli* TG2 was first modified by transformation into SL5338 and then electroporated into the different vaccine strains.

Preparation of proteins. Glutathione-agarose (Sigma) was used to purify GST-MSP1₁₉ and prepare recombinant MSP1₁₉ (rMSP1₁₉) as previously described, and referred to as GST-MSP1EGF and MSP1EGF, respectively (Ling *et al.*, 1994). When necessary, proteins were reduced or reduced and alkylated. Protein (75 µg ml⁻¹) was reduced with 50 mM dithiothreitol. Reduced protein was carboxymethylated by addition of 150 mM sodium iodoacetate, in a solution adjusted to pH 8.5 with NaOH and incubated for 1 h at 37 °C as described in the section on Western blotting assays.

Western blotting assays. Expression of the recombinant proteins was assessed by Western blotting. Overnight cultures of the different *Salmonella* constructs were diluted 1:100 and grown to mid-exponential phase (OD₆₅₀ approx. 0.6), harvested by centrifugation, and resuspended in one-tenth of the original culture volume of PBS-1% (v/v) Triton X-100. Samples were sonicated on ice with a Soniprep 150, MSE sonicator (Sanyo) set at 10 µm amplitude for 20 s. Aliquots (50 µl) of the sonicated samples were then mixed with an equal volume of twice concentrated loading buffer in the presence of 50 mM dithiothreitol (reduced) (two aliquots) or absence of dithiothreitol (non-reduced) and boiled for 5 min. One of the aliquots containing reduced protein was then carboxymethylated by addition of 150 mM sodium iodoacetate and the solution adjusted to pH 8.5 with NaOH, and incubated for 1 h at 37 °C. At the end of this incubation period, the pH of the sample was readjusted back to pH 7 for electrophoresis. Aliquots of the non-reduced, reduced or reduced and alkylated samples (20 µl) were resolved by SDS-PAGE (12.5% acrylamide) according to the method of Laemmli (1970). Proteins were transferred to a nitrocellulose membrane (Towbin *et al.*, 1979) and probed with either polyclonal or monoclonal antibodies (mAbs) or immune mouse serum. The polyclonal probes were rabbit anti-TetC serum (Khan *et al.*, 1994a, b) and mouse anti-MSP1₁₉ serum (Ling *et al.*, 1995). Blots were subsequently probed with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse immunoglobulin, respectively (Dako), and developed with 4-chloro-1-naphthol (Sigma). The mAb probes were F5

(1.2 µg ml⁻¹) and B10 (3.9 µg ml⁻¹) which recognize conformational epitopes either within the first single EGF-like module (F5) or spanning the two EGF-like modules (B10) of *P. yoelii* MSP1₁₉ (Spencer Valero *et al.*, 1998). Blots were subsequently probed with anti-mouse immunoglobulin (Amersham) and developed by enhanced chemiluminescence (Amersham).

Animals and immunization protocols. Either inbred female BALB/c or outbred female NIH Swiss mice were used for all immunization studies. Mice were purchased from Harlan Olac (Blackthorn, Bicester, UK) and used when at least 8 weeks old. Groups of 8–22 mice were injected intravenously (i.v.) in a lateral tail vein with 1×10^6 c.f.u. per mouse of the different recombinant *Salmonella*, and boosted with the same dose of *Salmonella* 4 and 9 weeks later. Serum samples were obtained at weeks 3, 5, 7, 11, 13 and 15 following the initial injection, and sera were stored individually at -20°C . Viable counts in organ homogenates were measured for mice injected with *Salmonella* 7 and 15 d earlier as described (Khan *et al.*, 1994a, b). Groups of animals were challenged i.v. with 10^3 *P. yoelii* YM-parasitized erythrocytes 3 d after the final 15 week bleed. The groups were: non-immunized mice, mice immunized with *Salmonella* strain C5AroD alone, mice immunized with C5AroD(pTECH2) and mice immunized with C5AroD(pTECH2-MSP1₁₉). Parasitaemia was assessed daily on blood films stained with Giemsa's reagent. Alternatively, female BALB/c mice were immunized intraperitoneally with the GST-MSP1₁₉ fusion protein administered with Freund's adjuvant, and serum obtained, as previously described (Ling *et al.*, 1994).

Antibody responses. Antibody responses were assessed by ELISA or by indirect immunofluorescence. Antibody responses were measured by ELISA as previously described (Khan *et al.*, 1994a, b). Briefly, each well of a 96-well microtitre plate (Nunc, Gibco-BRL, Life Technologies) was coated overnight at 4°C with either 0.1 µg recombinant TetC (rTetC) (Boehringer Mannheim) or 0.1 µg recombinant MSP1₁₉ (rMSP1₁₉) diluted in 0.1 M carbonate buffer, pH 9.6. For the ELISA under reducing conditions, plates were coated with rMSP1₁₉ that had been reduced with 0.05 M dithiothreitol at 37°C for 1 h before dilution into 0.1 M carbonate buffer, pH 9.6, containing 0.05 M dithiothreitol; the PBS solutions contained 0.01 M dithiothreitol. Individual serum or pooled serum samples were added at dilutions between 1/100 and 1/1638400 in PBS-1% BSA, and incubated for 1 h at 37°C . Serial dilutions of positive control sera were included on all plates. Rabbit anti-mouse total IgG HRP conjugate (Dako) was added diluted 1/1000, and incubated for another hour at 37°C . A solution of o-phenylenediamine (OPD, Sigma), prepared according to the manufacturer's instructions, was added for 15 min at 37°C . Development of the colour reaction was stopped with 3 M H₂SO₄ and the absorbance was measured at 490 nm.

Indirect immunofluorescence assay. Antibody determination by indirect immunofluorescence was carried out as previously described (Holder & Freeman, 1984). Mouse serum was added to acetone-fixed *P. yoelii* YM blood stages and bound antibody was detected with FITC-labelled anti-mouse IgG.

RESULTS AND DISCUSSION

Expression of TetC-MSP1₁₉ fusion protein in *Salmonella* vaccine strains

The recombinant plasmid pTECH2-MSP1₁₉ was used to transform several attenuated *S. typhimurium* vaccine

Fig. 1. Expression of the TetC-MSP1₁₉ fusion protein as determined by SDS-PAGE and Western blotting. Bacterial lysates were fractionated by 12.5% SDS-PAGE in the absence of reducing agent and blots were probed with (a) polyclonal mouse anti-recombinant MSP1₁₉ antiserum or (b) polyclonal rabbit anti-recombinant TetC antiserum. Lysates were from SL3261 cells (lane 1), SL3261 cells harbouring pGEX-3X-MSP1₁₉ (lane 2), SL3261 cells harbouring pTECH2 (lane 3); and strains harbouring pTECH2-MSP1₁₉ (lanes 4–7): SL3261 (lane 4), BRD726 (lane 5), C5AroD (lane 6), C5htrA (lane 7). The sizes of molecular mass markers are indicated.

strains: SL3261, BRD726, C5AroD and C5htrA, and the level of expression of the fusion protein was assessed by Western blotting using polyclonal mouse anti-GST-MSP1₁₉ serum (Fig. 1a) and polyclonal rabbit anti-TetC serum (Fig. 1b) as probes. Lysates from each bacterial strain transformed with pTECH2-MSP1₁₉ (lanes 4–7) contained a polypeptide of the approximate size expected for the TetC-MSP1₁₉ fusion (approx. 55–60 kDa). Expression of the fusion protein appears to be considerably less in SL3261 relative to the other three vaccine strains, as evidenced by a fainter 55–60 kDa band (lane 4 compared to lanes 5–7). The lower level of expression in SL3261 (lane 4) is accompanied by increased amounts of a diffuse, doublet band with a slightly faster mobility than the fusion product, relative to the other three strains. This may represent breakdown products derived from the fusion, or the expression of truncated products. In Fig. 1(a), lane 2, the ~40 kDa GST-MSP1₁₉ is detected in a lysate of bacteria transformed with pGEX-3X-MSP1₁₉, and in Fig. 1(b), lane 3, the ~50 kDa TetC is detected in a lysate of bacteria

Fig. 2. Effect of reduction on electrophoretic mobility and antibody recognition of recombinant *P. yoelii* MSP1₁₉. Samples (either 2 µg pure protein or 20 µl bacterial lysates) were untreated (NR, non-reduced), reduced with dithiothreitol (R, reduced) or reduced with dithiothreitol and carboxymethylated with iodoacetate (RA, reduced and alkylated), separated by 12.5% SDS-PAGE and analysed by Western blotting. Blots were probed with (a) mAb F5, which recognizes a conformational epitope within the first EGF-like module of *P. yoelii* MSP1₁₉, (b) mAb B10, which recognizes a conformational epitope spanning the two EGF-like modules of *P. yoelii* MSP1₁₉, or (c) polyclonal rabbit anti-TetC antiserum. The blots in panels (a) and (b) were developed by enhanced chemiluminescence and the blot in panel (c) was developed with 4-chloro-1-naphthol. The constructs are in C5aroD (i) or BRD726htrA (ii). Shown are rMSP₁₉ purified from *E. coli* (lane 1), and lysates from cells containing pTECH2 (lane 2), cells containing pGEX-3X-MSP1₁₉ (lane 3), or cells containing pTECH2-MSP1₁₉ (lane 4). The various recombinant proteins are indicated: ●, rMSP1₁₉; ◆, GST-MSP1₁₉; △, TetC-MSP1₁₉; □, TetC. The sizes of molecular mass markers are indicated by dashes between the panels, corresponding to 97, 66, 46 and 30 kDa from top to bottom, respectively.

transformed with pTECH2. Some other polypeptides were detected non-specifically in all lanes.

These results demonstrate that a TetC-MSP1₁₉ fusion protein can be expressed in *Salmonella*. Since protection with a recombinant *P. yoelii* MSP1₁₉ is dependent upon correct conformation and disulphide bridge formation (Ling *et al.*, 1994), we then assessed whether or not the recombinant MSP1₁₉ expressed in *Salmonella* was capable of forming these conformational epitopes, as judged by reactivity to specific monoclonal antibodies.

Folding and disulphide-bond-dependent conformation of MSP1₁₉ in *Salmonella* vaccine strains

Folding and formation of disulphide-bond-dependent epitopes in TetC-MSP1₁₉ was evaluated for all four *Salmonella* vaccine strains by SDS-PAGE and Western blotting. The Western blots of lysates from strains C5aroD and BRD726 are shown in Fig. 2; the other two strains gave identical profiles to that of C5aroD. Lysates were prepared from vaccine strains harbouring either

pTECH2, pGEX-3X-MSP1₁₉, or pTECH2-MSP1₁₉. The blots were probed with the mAbs F5 or B10 [panels (a) and (b) respectively], which recognize different conformational epitopes, and are capable of suppressing parasitaemia when used to passively immunize mice (Spencer Valero *et al.*, 1998). These mAbs recognize native MSP1 in the parasite, and properly folded recombinant MSP1₁₉ in which the appropriate conformational epitopes are formed, but not protein that is unfolded by reduction and alkylated to prevent refolding and reformation of conformational epitopes (Spencer Valero *et al.*, 1998). Alternatively, the blots were probed with polyclonal anti-TetC antibodies [panel (c)] that recognize non-reduced, reduced, and reduced and alkylated TetC containing polypeptides.

Both the mAbs [F5, panel (a) or B10, panel (b)] recognized a non-reduced recombinant MSP1₁₉ produced in *E. coli* (rMSP1₁₉) [lane 1, non-reduced (NR)]. The band representing a protein with the greatest mobility in lane 1 is the monomeric rMSP1₁₉, and the additional slower-migrating bands in this lane reflect the

ability of the protein to form intermolecular disulphide bonds in the non-reduced state. The reduced and alkylated rMSP1₁₉ (lane 1, RA) was not recognized by either mAb, as expected, since the protein had been unfolded and prevented from refolding so that the conformational epitopes were permanently destroyed. A similar result was obtained if the protein was not alkylated but kept in the continued presence of reducing agent during Western blotting (data not shown). The rMSP1₁₉ in the sample reduced prior to electrophoresis (lane 1, R) did react with both mAbs, presumably as a result of refolding after electrophoresis, perhaps as a result of oxidation during transfer from gel to nitrocellulose. The reduced rMSP1₁₉ had a greater mobility than the non-reduced protein, in close agreement with the results of others (Burghaus & Holder, 1994).

Both the mAbs tested [F5, panel (a) or B10, panel (b)] recognized the ~40 kDa, GST-MSP1₁₉ as indicated by a very heavy band in lanes containing non-reduced bacterial lysates from either *C5aroD* (i) or BRD726*htrA* (ii) harbouring the recombinant plasmid pGEX-3X-MSP1₁₉ (lane 3, NR). The additional band at ~80 kDa in these non-reduced samples may be a dimer resulting from intermolecular disulphide bond formation. The reduced protein had a lower mobility than the non-reduced protein (lane 3, R). This recognition by antibody and change in mobility are indicative of the production of a properly folded GST-MSP1₁₉ fusion protein in these two attenuated *Salmonella* strains, as has been previously reported for both the *P. yoelii* and the *P. falciparum* GST-MSP1₁₉ fusion proteins expressed in *E. coli* (Ling *et al.*, 1995; Burghaus & Holder, 1994).

Both the mAbs tested [F5, panel (a) or B10, panel (b)] recognized a ~55–60 kDa protein in lanes containing non-reduced bacterial lysates from *C5aroD* [(i), upper part of figure] harbouring the recombinant plasmid pTECH2-MSP1₁₉ (lane 4, NR), indicating the expression of a properly folded TetC-MSP1₁₉ fusion protein in this *Salmonella* strain. Reduction and alkylation completely abolished recognition of the protein (lane 4, RA), whilst the protein in the sample reduced prior to electrophoresis (lane 4, R) did react with both mAbs, presumably as a result of refolding after electrophoresis.

By contrast, in the other attenuated *Salmonella* strain BRD 726 [(ii), lower part of figure], mAb B10 [panel (b)] only marginally recognized the TetC-MSP1₁₉ fusion (lane 4, NR), suggesting that this fusion protein is not folded correctly in BRD726*htrA*.

Polyclonal anti-TetC serum [panel (c)] recognized the ~50 kDa TetC in lanes containing bacterial lysate from either *C5aroD* (i) or BRD726*htrA* (ii) transformed with pTECH 2 (lane 2), and TetC-MSP1₁₉ (~55–70 kDa) in lanes containing bacterial lysate from either strain transformed with pTECH2-MSP1₁₉. The mobility of TetC does not change after reduction whilst the mobility of the fusion protein is dependent on its reduction state [panel (c), lane 4, NR, R, RA]: non-reduced > reduced > reduced and alkylated. Different mobilities of the

reduced and non-reduced forms of the recombinant MSP1₁₉ indicate that structural changes such as the cleavage of disulphide bonds have occurred in the protein as a result of reduction.

Further evidence for the disulphide-bonded conformation of MSP1₁₉ in *Salmonella* and production of a properly folded protein that appears antigenically similar to the natural antigen was obtained by probing blots similar to those in Fig. 2 with hyperimmune serum generated by infection of mice with *P. yoelii* parasites; this antibody is therefore generated against the natural antigen (Ling *et al.*, 1995). The recognition pattern of this serum was identical to the mAb recognition patterns with both *C5aroD* and BRD726*htrA* lysates (data not shown), suggesting that the hyperimmune serum only reacted with disulphide-dependent conformational epitopes in the purified rMSP1₁₉ and the different *Salmonella* constructs.

These results together clearly demonstrate that MSP1₁₉ can be expressed in *C5aroD* as a fusion with either GST or TetC, and contains epitopes that are reduction sensitive. We have shown that conformational epitopes within either the first EGF-like module or spanning the two EGF-like modules of MSP1₁₉ are formed in *Salmonella*.

It is particularly noteworthy that the proper folding of MSP1₁₉ appears dependent upon both the *Salmonella* strain (parental strain and attenuating mutation) and the protein to which the MSP1₁₉ is fused. It is not clear why BRD726 (SL1344 $\Delta htrA$), with an attenuating mutation within a heat-shock protein gene (Chatfield *et al.*, 1992; Johnson *et al.*, 1991), is only able to allow the production of a properly folded MSP1₁₉ when it is fused to GST whereas *C5aroD*, *C5htrA* and SL3261 (SL1344 *aroA*) all allow proper folding of the protein fused to either GST or TetC. Since BRD726 has the same parental background as SL3261 and a mutation in the same gene as *C5htrA*, it is possible that the combination of parental background and attenuating mutation in BRD726 is responsible. The results here demonstrate the importance of evaluating the antigenicity of vaccine strain panels when expressing different guest antigens in *Salmonella*.

To investigate the immunogenicity of MSP1₁₉ delivered by immunization with recombinant *Salmonella* we next determined the antibody response induced to the protein.

The influence of mouse strain and *Salmonella* vaccine strain on antibody responses

Groups of eight BALB/c or NIH Swiss mice were immunized with different recombinant *Salmonella* vaccine strains containing either pTECH2 or pTECH2-MSP1₁₉. Bacteria were recovered from mouse liver and spleen 7 or 15 d after i.v. immunization and grown on media with and without ampicillin to determine how many had retained the plasmid. More than 95% of

Table 1. Comparison of antibody responses against rMSP1₁₉ and rTetC as detected by ELISA analysis of sera from NIH Swiss and BALB/c mice immunized i.v. with various *S. typhimurium* strains

Mice were immunized with 1×10^6 c.f.u. per mouse of the recombinant *Salmonella* and boosted by repeat immunization with the same dose on weeks 4 and 9. Results are expressed as the geometric mean (GM) of A_{490} values for week 13 sera, diluted 1/100, from groups of four or five mice. Standard deviations (SD) are given. Antibody responses were measured against rMSP1₁₉ (a) or rTetC (b). Pooled sera from five non-immunized mice were included in each experiment; they had A_{490} values of <0.06 . ELISA measurements were made in duplicate.

	<i>Salmonella</i> strain and plasmid construct				
	SL 3261 pTECH2	SL 3261 pTECH2- MSP1 ₁₉	BRD726 pTECH2- MSP1 ₁₉	C5aroD pTECH2- MSP1 ₁₉	C5htrA pTECH2- MSP1 ₁₉
Swiss mice					
(a) MSP1 ₁₉					
GM	0.07	0.49	0.08	0.89	0.59
SD	0.04	1.45	0.20	1.34	1.41
(b) Tet C					
GM	2.43	2.32	1.68	2.52	2.34
SD	0.30	0.19	0.54	0.23	0.45
BALB/c mice					
(a) MSP1 ₁₉					
GM	0.09	0.08	0.16	0.31	0.43
SD	0.02	0.02	1.31	1.42	1.56
(b) Tet C					
GM	2.30	1.86	0.20	2.30	2.34
SD	0.29	0.56	1.34	0.10	0.22

bacteria in all cases had retained the plasmid. Table 1 shows the geometric means and standard deviations of the antibody responses against rMSP1₁₉ (a) and rTetC (b) in groups of BALB/c or NIH Swiss mice. There was considerable variation in response against rMSP1₁₉ within the individual groups of both inbred and outbred mice, but the reason for this is unclear. The Swiss mice produced an overall greater response against rMSP1₁₉ than the BALB/c mice but the difference was not significant. Swiss mice immunized with C5aroD carrying pTECH2-MSP1₁₉ elicited the greatest response, achieving high antibody responses in 4/5 mice. These data indicate that attenuated recombinant *Salmonella* induce antibodies against rMSP1₁₉.

Both strains of mice developed high anti-TetC responses when immunized with recombinant *Salmonella* strains carrying either pTECH2 alone (column 2) or carrying pTECH2-MSP1₁₉ (columns 3–6), with slightly higher responses observed overall with the Swiss mice. The level of antibody response to TetC was apparently higher and less variable than that to MSP1₁₉, suggesting that the TetC part of the molecule was immuno-dominant.

It was important next to establish that the antibodies against recombinant MSP1₁₉ react with the native

protein in the parasite and recognize conformational epitopes.

Antibody responses induced by C5aroD(pTECH2-MSP1₁₉) compared to those induced by purified GST-MSP1₁₉

Antiserum from Swiss mice immunized with C5aroD (pTECH2-MSP1₁₉) was evaluated for its ability to recognize MSP1 expressed by *P. yoelii* parasites. Fig. 3 shows the reactivity of the antiTetC-MSP1₁₉ serum with acetone-fixed *P. yoelii*-infected erythrocytes as determined by immunofluorescence. Negative control serum from non-immunized mice or mice immunized with a *Salmonella* vaccine strain containing pTECH2 did not exhibit any specific reactivity (data not shown), while the positive serum gave a 'bunch of grapes like' fluorescence pattern corresponding to detection of MSP1 on the surface of budding merozoites in mature schizonts.

We then sought to establish the proportion of anti-MSP1₁₉ antibody from mice immunized with C5aroD (pTECH2-MSP1₁₉) that is directed against conformational epitopes, using an ELISA. Pooled serum from four mice immunized with C5aroD(pTECH2-MSP1₁₉) contained a very high level of antibody reacting with non-

Fig. 3. Immunofluorescence staining of *P. yoelii* parasites by immune antiserum against recombinant *Salmonella*. Acetone-fixed thin blood smears prepared from *P. yoelii*-infected erythrocytes were examined by indirect immunofluorescence assay. Shown is a typical fluorescence pattern observed for serum (diluted 1:100) from NIH Swiss mice immunized with *S. typhimurium* C5aroD harbouring the recombinant plasmid pTECH2-MSP1₁₉. The reaction of the antibodies with MSP1 on the surface of three schizont-stage parasites within erythrocytes is shown (bunch of grapes pattern). No specific reaction was observed with serum from non-immunized mice or those immunized with a vaccine strain containing the pTECH2 plasmid. Magnification $\times 800$.

reduced rMSP1₁₉ (A_{490} reading off the scale at 1/100 serum dilution) but a much lower level of antibody against the reduced, unfolded protein (A_{490} 0.53), representing less than 20% of the total MSP1-specific antibody. Serum from mice immunized with GST-MSP1₁₉ in the presence of Freund's adjuvant, and known to be protected against challenge infection with *P. yoelii* (Ling *et al.*, 1994), had a very high level of antibody reacting with non-reduced rMSP1₁₉ when measured at a 1/5000 dilution. These antibodies did not react with reduced MSP1₁₉, demonstrating that this method of immunization induces antibodies directed against conformational epitopes within the protein which is similar to the situation for protective antibodies following immunization with a recombinant GST-MSP1₁₉ in a strong adjuvant.

The magnitude of the antibody response against MSP1₁₉ is also a critical factor in determining the level of protection against challenge infection with *P. yoelii* parasites (Ling *et al.*, 1994). Therefore, the antibody titres of 12 of a group of 22 mice immunized with C5aroD(pTECH2-MSP1₁₉) were determined by ELISA against rMSP1₁₉ and rTetC. These 12 mice were a representative sample from the group. Fig. 4 shows the titration curves of the individual sera measured against rMSP1₁₉ (a) and rTetC (b). Each of the mice immunized with the C5aroD(pTECH2-MSP1₁₉) had a much lower level of antibody to rMSP1₁₉ than mice immunized with GST-MSP1₁₉ administered with Freund's adjuvant, which had a titre at least twofold greater (Fig. 4a, dotted line). By indirect immunofluorescence an even greater difference was observed between serum from mice immunized with the recombinant *Salmonella* (titres

Fig. 4. Comparison of the titres of specific antibodies in sera from individual NIH Swiss mice immunized i.v. with *S. typhimurium* C5aroD(pTECH2-MSP1₁₉) as detected by ELISA. Mice were immunized with 1×10^6 c.f.u. per mouse of the recombinant *Salmonella* and boosted by repeat immunization with the same dose on weeks 4 and 9. Results are expressed as A_{490} values of the twofold serial serum dilutions (1/800 to 1/1638400), and plotted on a semi-logarithmic scale for clarity. The serum samples were obtained on week 15. Antibody responses are measured against rMSP1₁₉ (a) and TetC (b). The dotted lines with filled circles indicate the titres of pooled serum from BALB/c mice immunized with rGST-MSP1₁₉ (a) or rTetC (b). ELISA measurements were recorded in duplicate and averaged.

1/25–1/800) and the serum from the mice immunized with GST-MSP1₁₉ (titre approximately 1/10000) and protected against challenge infection.

ELISA analysis of the IgG subclass distribution of the anti-MSP1₁₉ antibody response in mice immunized i.v. with C5aroD(pTECH2-MSP1₁₉) showed that the mice developed specific IgG1 and IgG2b together with IgG2a, a similar subclass distribution to that observed with antiserum from mice immunized with the GST-MSP1₁₉ (data not shown).

Despite the quantitative differences in the antibody levels in mice immunized by *Salmonella* infection and those immunized with purified recombinant protein administered with adjuvant, we determined whether or

not the response induced by *Salmonella* immunization was sufficient to protect against parasite challenge.

Parasite challenge

Groups of Swiss mice were challenged by inoculation of 10^3 *P. yoelii*-infected erythrocytes and monitored daily. Immunization with C5aroD(pTECH2-MSP1₁₉) slightly delayed (by 1 d) the onset of patent parasitaemia and prolonged the survival of 4/12 mice compared to control groups (from 10 to 11 d), but did not protect the mice (data not shown). There was no correlation between antibody titre and onset of parasitaemia or survival time.

Overall, these data suggest that the reason for the lack of protection observed in the challenge study is likely to be based on the magnitude of the antibody response. The amount of antigen delivered to the mouse immune system by infection with recombinant *Salmonella* is unknown but may be less than that delivered by immunization with purified recombinant GST-MSP1₁₉ and this may be responsible for the lower antibody response. The correctly folded TetC-MSP1₁₉ fusion protein expressed in C5aroD appears to induce antibody that is primarily directed against conformational epitopes within the recombinant protein, suggesting that the fine specificity of the immune response may not be responsible for the lack of protection we observed. However, it is possible that the humoral response is directed to irrelevant epitopes, which are conformational but are not important in protection. Previous workers have demonstrated that the route of inoculation, and the adjuvant employed, have a profound effect on the fine specificity of the antibodies elicited against *P. yoelii* (Daly & Long, 1996). Furthermore, others have shown that mAbs, specific for *P. falciparum* MSP1 and able to competitively inhibit the binding of each other have dramatically differing abilities to inhibit parasite growth *in vitro*. Some of these mAbs effectively inhibit invasion of red blood cells whilst others that can block this inhibition do not prevent invasion (Blackman *et al.*, 1994). It is possible that the carboxy-terminus of *P. yoelii* MSP1 also induces the production of antibodies that exhibit similar properties, although this has not been demonstrated.

In summary, the data show that antigenic MSP1₁₉ can be expressed in *Salmonella* in a form that reacts with protective mAbs. In addition, infection with an attenuated strain expressing TetC-MSP1₁₉ induces antibodies that recognize the native parasite protein and are directed against conformational epitopes shown to be important targets of a protective immune response. However, the magnitude of the antibody response was lower than that achieved by immunization with a recombinant protein and adjuvant. Therefore, the balance of evidence suggests that the magnitude of the antibody response is the most likely reason for the lack of protection against challenge infection (Daly & Long, 1995; Ling *et al.*, 1994). Further studies are now required

to improve the immunogenicity of this antigen in the attenuated bacterial delivery system.

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