Susceptibility of calves to challenge with *Salmonella typhimurium* 4/74 and derivatives harbouring mutations in *htrA* or *purE*

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***Salmonella typhimurium* 4/74 is highly virulent for cattle after oral challenge, causing severe diarrhoea, which is sometimes associated with systemic spread of the micro-organism. Although susceptible to oral challenge, groups of cattle were found to be relatively resistant to subcutaneous challenge with this strain. The virulence of *S. typhimurium* 4/74 harbouring mutations in *htrA* and *purE* was also assessed in cattle. Although *S. typhimurium* 4/74 *htrA* and *purE* are attenuated following oral challenge in mice, cattle were highly susceptible to oral challenge with these mutants. As with the parent *S. typhimurium* 4/74 strain, cattle exhibited greater susceptibility to oral compared to subcutaneous challenge with *S. typhimurium* *htrA* and *purE* mutants. Following subcutaneous challenge with sublethal levels of *S. typhimurium* 4/74, calves produced significant levels of antibodies to *S. typhimurium* soluble extract. No correlation was detected between interferon gamma levels in sera and susceptibility to infection by any route. The concentrations of the acute-phase-associated protein haptoglobin were increased in the sera of five of six cattle inoculated subcutaneously, although increases in concentration were smaller in cattle inoculated orally.**

Keywords: *Salmonella*, cattle, *htrA*, purine

INTRODUCTION

Salmonella spp. are responsible for a variety of enteric infections in humans and domestic animals. Disease signs may vary from mild diarrhoea to systemic disease. In humans, *Salmonella enterica* serovar Typhi (*Salmonella typhi*) is the cause of the systemic disease typhoid, whereas other *Salmonella* serovars, such as *S. enterica* serovar Typhimurium (*Salmonella typhimurium*), *S. enterica* serovar Dublin (*Salmonella dublin*) and *S. enterica* serovar Enteritidis (*Salmonella enteritidis*) cause mainly gastroenteritis. *S. typhi* is not pathogenic in mice but many *S. typhimurium* isolates can cause systemic infections, thus providing a model of human typhoid (Hormaeche *et al.*, 1995). In cattle, as in humans, *S. typhimurium* usually causes diarrhoea and

most infecting bacteria remain associated with tissues of the digestive tract (Jones *et al.*, 1988; Segall & Lindberg, 1993). The host and bacterial factors that determine whether diarrhoea will develop following challenge are poorly understood.

The murine model of salmonellosis has been exploited to identify genes required for survival and replication in this host (Chiang *et al.*, 1999). *Salmonella* strains harbouring such mutations are attenuated and therefore are potential live vaccine candidates against salmonellosis in other species (Mastroeni *et al.*, 1999). Thus, the murine salmonellosis model has been used to identify candidate mutations to generate attenuated *S. typhi* for live typhoid vaccine development. Mutations exploited in this way include those associated with aromatic compound dependence (*aro*) (Hosieth & Stocker, 1981; Tacket *et al.*, 2000), purine dependence (*pur*) (Levine *et al.*, 1987; O'Callaghan *et al.*, 1988; Everest *et al.*, 1997) and stress resistance (*htrA*) (Chatfield *et al.*,

Abbreviations: STSE, *Salmonella typhimurium* soluble extract; PBS-T, phosphate buffered saline/Tween 20; IFN γ , interferon gamma

1992; Strahan *et al.*, 1992; Tacket *et al.*, 2000). However, not all mutations that attenuate *S. typhimurium* in the murine model are fully attenuating for *S. typhi* in man. For example, although *galE* mutants of *S. typhimurium* are attenuated in mice, *S. typhi galE* strains can cause typhoid-like disease in humans (Hone *et al.*, 1988).

In addition to their ability to confer protection against virulent challenge, live attenuated *Salmonella* vaccines can be used as vectors to deliver antigens from other pathogens, thus bringing the ideal single dose multi-valent vaccine closer to reality (Levine & Dougan, 1998). Few studies have been conducted using *Salmonella* as a vector for heterologous antigen delivery in species other than the mouse. Studies in cattle have used *Salmonella* strains harbouring *aro* mutations (Villarreal-Ramos *et al.*, 1998). However, in cattle the use of recombinant *Salmonella aro* mutants as vectors for foreign antigen delivery has been less successful than would have been expected from the results obtained in mice (d'Olivera *et al.*, 1997; Gentshev *et al.*, 1998; Villarreal-Ramos *et al.*, 2000). If attenuated salmonellae are to fulfil their potential as live vaccines against salmonellosis in different animal species it is necessary to evaluate different candidate *Salmonella* vaccines in the target animal species. Further, it will be essential to explore different delivery regimes and understand the mechanisms of immunity induced by *Salmonella* vectors in different species. In this work we examined the ability of different mutations that are known to attenuate salmonellae in mice to attenuate salmonellae in cattle. We have also examined the influence of the route of administration on the virulence of *Salmonella* and the immune response induced.

METHODS

Bacteria. The *S. typhimurium* strain 4/74 used for oral and subcutaneous challenge has been described elsewhere (Jones *et al.*, 1991; Rankin & Taylor, 1966). The *purE* and *htrA* mutations were introduced into *S. typhimurium* 4/74 as described by O'Callaghan *et al.* (1988) and Chatfield *et al.* (1992), respectively.

Calves. Four to five week old Friesian bull calves were used throughout. Calves were reared conventionally from birth and tested for the presence of *Salmonella* as described below. Blood samples were collected by venepuncture from the jugular vein.

Preparation of inocula. For oral challenge, bacteria were grown overnight in Luria-Bertani broth at 37 °C. Appropriate dilutions were made to obtain the number of bacteria required as indicated in the text and in Table 1 for each experiment. The concentration of the inocula was confirmed by plating dilutions on brilliant green agar (Oxoid). Bacteria were resuspended in 20 ml PBS and mixed with an equal volume of antacid solution (5 % sodium bicarbonate, 5 % magnesium carbonate and 5 % magnesium trisilicate; all from BDH) and delivered per os with a syringe (Jones *et al.*, 1988). Calves were inoculated subcutaneously in the prescapular area with bacteria suspended in a final volume of 1.5 ml PBS, preceded by intravenous injection of 2 ml flunixin meglumine at 50 mg ml⁻¹ (Schering-Plough Animal Health) to reduce the risk of hypersensitivity reactions.

Clinical observation of calves. Rectal temperature, faecal consistency, food consumed and general demeanour were recorded. Diarrhoea was scored on a scale from 0 to 3 depending on faecal consistency, with a score of 0 indicating normal faeces and a score of 3 indicating watery scour. One unit was added for the presence of blood and two units for the presence of sloughed mucosa or membrane formation (Jones *et al.*, 1988). Calves that reached a diarrhoea score of 20, failed to eat for two consecutive days, showed a sudden drop in temperature of more than 2 °C or failed to stand up unaided were killed by intravenous injection of pentobarbitone (May and Baker).

Bacteriological analysis. The presence of *Salmonella* in faeces before challenge was determined by enrichment in selenite brilliant green broth (SBG) (Difco) and Rappaport medium [0.45 % tryptone (Difco), 124 mM NaCl (BDH), 10 mM KH₂PO₄ (BDH), 180 mM MgCl₂·6H₂O (BDH), 0.012 % malachite green (BDH)] (Rappaport *et al.*, 1956) and by direct inoculation onto brilliant green agar (Oxoid) for colony counts. The presence of *Salmonella* in faeces after challenge was determined daily both by enrichment and by direct plating of dilutions of faecal homogenates. Briefly, 1 g faeces was placed in 9 ml PBS, pH 7.5 and mixed to obtain a homogeneous suspension. Five drops of 20 µl each were plated on brilliant green agar plates. The results were expressed as geometric mean counts (g sample)⁻¹. Bacteraemia was monitored daily by enrichment of 1 ml blood in SBG and Rappaport medium and subsequent inoculation onto brilliant green agar. After killing the following samples were removed from calves: ileum wall, ileum contents, ileal lymph node (the distal comma shaped node at the end of the chain of jejuno-ileal lymph nodes), caecum wall, caecum contents, caecal lymph node, colon wall, colon contents, colonic lymph node, liver, hepatic lymph node, spleen, lung, bronchial lymph node, blood and bile. From each sample, 1 g was placed in 9 ml H₂O and homogenized, where necessary, using mortar, pestle and sand. Dilutions of the homogenates were plated on brilliant green agar plates as described above. The presence of *Salmonella* was also determined by enrichment of 1 ml homogenate in SBG and Rappaport medium and subsequent inoculation onto brilliant green agar. Using this technique, the limit of detection by direct plating of bacterial homogenates is 500 bacteria g⁻¹ (i.e. 1 bacterium in a 20 µl droplet = 50 bacteria ml⁻¹ from the original 10 ml homogenate). Therefore, if bacteria were not detected by direct plating but were detected by enrichment in broth, the counts were assumed to be less than 500 bacteria (g sample)⁻¹.

ELISA. ELISA to detect antibodies to LPS was carried out as described by Demarco de Hormaeche *et al.* (1988). Each well of Microtest III 96-well plates (Falcon) was coated with 2.5 µg smooth Westphal LPS from *S. typhimurium* (Sigma) in 10 mM glycine, 20 mM NaCl, 0.2 mM EDTA, 0.01 mM NaF and 24 mM sodium deoxycholate. After overnight incubation at 37 °C, the plates were incubated with blocking buffer, consisting of 3 % BSA Fraction V (Sigma) in PBS and 0.05 % Tween 20 (Sigma) (PBS-T). After 1 h at 37 °C, the plates were washed with PBS-T. Fifty microlitres of cattle serum diluted in 0.3 % BSA in PBS-T was added to individual wells in duplicate. To detect IgG1, IgG2 and IgM, sera were diluted 1:1000, 1:100 and 1:100, respectively. After incubation for 1 h at 37 °C the plates were washed and 50 µl B37 (monoclonal antibody to bovine IgG1), B192 (monoclonal antibody to IgG2) or B67 (monoclonal antibody to IgM) diluted in 0.3 % BSA in PBS-T was added to each well. Monoclonal antibodies were diluted 1:250, a previously determined optimum concentration. After 1 h incubation at 37 °C, plates were washed

Table 1. Outcome of inoculation of calves with *S. typhimurium* 4/74, *S. typhimurium* 4/74 *htrA* and *S. typhimurium* 4/74 *purE*

ND, Not done; NA, not applicable. + indicates that bacterial cultures were positive for salmonellae after enrichment in SBG or Rappaport medium; – indicates that bacterial cultures were negative for salmonellae after enrichment in SBG or Rappaport medium.

Calf	Strain	Route of inoculation*	Log ₁₀ inoculum	Outcome (day of killing)†	Highest temp. (°C) (d)	log ₁₀ max. no. <i>S. typhimurium</i> (g faeces) ⁻¹ (d)	<i>Salmonella</i> in blood (time)
1	Wild-type	o	9.17	K (8)	41.5 (1)	7.23 (3)	+
2	Wild-type	o	9.17	K (3)	41.2 (3)	7.74 (6)	+
3	Wild-type	sc	9.25	K (3)	40.7 (2)	6.69 (3)	+
4	Wild-type	sc	9.25	K (3)	41.2 (1)	8.41 (4)	+
5	Wild-type	sc	9.17	K (7)	41.4 (1)	8.27 (4)	+
6	Wild-type	sc	9.17	s	41.0 (1)	4.07 (11)	+
7	Wild-type	sc	9.17	s	41.1 (1)	5.39 (8)	+
8	Wild-type	sc	9.25	s	40.4 (4)	4.30 (16)	+
9	<i>htrA</i>	o	10.9	K (1)	NA	NA	NA
10	<i>htrA</i>	o	10.9	K (1)	NA	NA	NA
11	<i>htrA</i>	o	10.9	K (1)	NA	NA	NA
12	<i>htrA</i>	sc	10	s	40.6 (1)	+	+
13	<i>htrA</i>	sc	10	s	41 (1)	+	+
14	<i>htrA</i>	sc	10	s	41.1 (2)	–	+
15	<i>htrA</i>	sc	10	s	40.9 (2)	–	+
16	<i>purE</i>	o	10.6	K (5)	40.5 (3)	5.77 (4)	ND
17	<i>purE</i>	o	10.6	K (5)	40.65 (3)	5.55 (4)	ND
18	<i>purE</i>	o	10.6	K (6)	40.75 (3)	6.32	ND
19	<i>purE</i>	o	10.6	K (6)	40.45 (3)	7.11	ND
20	<i>purE</i>	sc	8	s	40.15 (2)	–	ND
21	<i>purE</i>	sc	8	s	40.35 (2)	–	ND

* o, oral; sc, subcutaneous.

† K, killed; s, survived.

with PBS-T and 50 µl goat anti-mouse horseradish peroxidase conjugate (Dako) diluted 1:1000 in 0.3% BSA in PBS-T was added to each well according to the manufacturer's instructions. After 1 h at 37 °C, 100 µl 3,3',5,5'-tetramethylbenzidine (ICN), prepared according to the manufacturer's instructions, was added to each well. After 10 min, 25 µl 4 M H₂SO₄ was added to each well and plates were read at 450 nm in a Titretrek Multiskan MCC/340 ELISA reader.

To detect antibodies to a soluble extract of *S. typhimurium* (STSE), 96-well plates were coated with 50 µl STSE, prepared as described previously (Villarreal *et al.*, 1992), diluted to 2 µg ml⁻¹ in 0.1 M carbonate buffer [0.1 M Na₂CO₃, 0.1 M NaHCO₃; both from BDH], pH 9.6. After overnight incubation at 4 °C, the plates were incubated for 1 h at 37 °C before adding 100 µl blocking buffer [2% casein (Hammarsten, BDH) in PBS, 0.05% Tween 20 (Sigma), pH 7.0] to each well. Thereafter the protocol followed was essentially the same as for the LPS ELISA, except that 0.2% casein in PBS-T was used as the diluent.

Measurement of interferon γ (IFN γ) and haptoglobin in sera of cattle inoculated with *S. typhimurium* 4/74. IFN γ in sera was measured by ELISA (CSL, Bovigamm) according to the manufacturer's instructions.

The concentration of haptoglobin in sera was assessed using a method based on the preservation of the haemoglobin

peroxidase activity of haptoglobin in mild acidic conditions (Makimura & Suzuki 1982). The method was modified for use on an automated biochemical analyser (MIRA, Roche Diagnostics) to reduce interference from serum albumin (Eckersall *et al.*, 1999). In this method, using the automated biochemical analyser, 2.5 µl serum was mixed with 200 µl of methaemoglobin solution (0.06 mg ml⁻¹ in 0.9% NaCl), prepared as described by Makimura & Suzuki (1982). After 50 s, 90 µl SB-7 chromogen solution (Tridelta Development) was added. After a further 25 s, 50 µl 0.12% H₂O₂ substrate (diluted in H₂O) was added and the increase in absorbance at 600 nm over the following 50 s was monitored. The assay was quantified by comparison with dilutions of a bovine serum sample with a known concentration of haptoglobin, originally being calibrated with purified bovine haptoglobin (Horadagoda *et al.*, 1994).

RESULTS

Susceptibility of cattle to oral or subcutaneous challenge with *S. typhimurium* 4/74

Two calves, 1 and 2, were challenged orally with 1.5×10^9 *S. typhimurium* 4/74 bacteria and, as expected, both calves developed fever and diarrhoea (Table 1). Bacteriological culture of faeces showed that *S. typhi-*

Table 2. Bacterial concentrations at post-mortem in tissues of calves inoculated with virulent *S. typhimurium* 4/74

— indicates that bacterial counts were negative both by plate dilution and by enrichment in SBG or Rappaport medium; + indicates that bacterial counts were negative by plate dilution but positive after enrichment in SBG or Rappaport medium. A blank indicates that the tissue was unavailable.

Route of inoculation ...		log ₁₀ bacterial counts (g sample) ⁻¹							
		Oral		Subcutaneous					
Tissue	Calf...	1	2	3	4	5	6	7	8
Ileum									
Wall		4.00	3.96	6.79	4.53	6.73	—	—	—
Contents		4.38	+	4.20	1.00	9.04	—	—	—
Lymph node*		3.60	4.20	6.95	3.73	5.26	—	—	—
Caecum									
Wall		3.60	+	3.71	4.75	8.26	—	+	—
Contents		5.08	+	3.32	1.78	5.36	—	+	—
Lymph node		3.78	4.48	5.95	4.20	6.82	—	—	—
Colon									
Wall		4.15	+	3.41	4.98	3.94	—	+	—
Contents		+	+	2.57	+	8.52	—	+	—
Lymph node		3.78	3.70	6.58	4.11	4.71	—	—	—
Liver		3.26	+	5.57	2.18	3.60	—	—	—
Hepatic lymph node		+	+	7.72	3.04	4.76	—	+	—
Spleen		+	+	7.77	2.00	3.75	—	—	—
Lung		3.66	+	7.77	2.81	3.48	—	+	—
Bronchial lymph node		+	+	6.64	2.69	4.00	—	—	—
Blood		+	+	6.00	—	4.84	—	—	—
Bile		—	—	1.30	1.00	3.48	—	—	—

* This is the distal, comma-shaped node at the end of the chain of jejuno-ileal lymph nodes.

murium was excreted from the day after inoculation until the day of death. Enrichment of bacteria from blood indicated that calf 2 developed bacteraemia on the third day after inoculation, while no bacteria were detected in the blood of calf 1. Calves 1 and 2 succumbed to infection on days 8 and 3 after inoculation, respectively, and were killed.

To assess the ability of *S. typhimurium* 4/74 to infect cattle via the parenteral route, six calves were inoculated subcutaneously with 1.5×10^9 (calves 5, 6 and 7) or 1.8×10^9 (calves 3, 4 and 8) *S. typhimurium* 4/74 (Table 1). Calves 3, 4 and 5 succumbed to infection while calves 6, 7 and 8 survived this high challenge dose. All the subcutaneously challenged cattle developed pyrexia and bacteraemia and secreted *S. typhimurium* in their faeces (Table 1). Calves 3, 4 and 5 succumbed to infection 3, 3 and 7 d after inoculation, respectively. Post-mortem bacteriological analysis of killed calves indicated that the salmonellae had spread systemically (Table 2). In calves 4 and 5, the number of *S. typhimurium* 4/74 cells associated with the digestive tract was greater than the number of salmonellae associated with systemic sites such as the spleen, lung and liver. In calf 3, the number

of *S. typhimurium* 4/74 cells isolated from the spleen, lungs and hepatic nodes was greater than the number of bacteria isolated from the digestive tract tissues. Four to five weeks after inoculation, the calves that survived (6, 7 and 8), were killed and a post-mortem bacteriological analysis performed as indicated in Methods (Table 2). *S. typhimurium* 4/74 was only detected in one animal after enrichment of organ homogenates.

Susceptibility of calves to challenge with *S. typhimurium* 4/74 derivatives harbouring mutations in *htrA* or *purE*

To assess the virulence of mutant *S. typhimurium* 4/74, calves were inoculated orally with 8×10^{10} and subcutaneously with 1×10^{10} *S. typhimurium* *htrA*. All three calves inoculated orally (9, 10 and 11), succumbed to salmonellosis the day after inoculation (Table 1). Post-mortem bacteriological analysis revealed that most of the *S. typhimurium* 4/74 *htrA* bacteria were associated with digestive tract tissues. After enrichment of organ homogenates, *S. typhimurium* 4/74 *htrA* was also found in the spleen of calf 10 and the lungs of calf

Table 3. Bacterial concentration at post-mortem in tissues of calves inoculated orally with *S. typhimurium* 4/74 mutants

+ indicates that bacterial counts were negative by plate dilution but positive after enrichment in SBG or Rappaport medium; — indicates that bacterial counts were negative both by plate dilution and by enrichment in SBG or Rappaport medium. A blank indicates that the tissue was unavailable.

Tissue	Inoculated with... Calf...	$\log_{10}$ bacterial counts (g sample) ⁻¹						
		<i>S. typhimurium htrA</i>			<i>S. typhimurium purE</i>			
		9	10	11	16	17	18	19
Ileum								
Wall		+	4.8	+	5.6	—	6.7	6.3
Contents		4.9	—	+	—	—	—	6.3
Lymph node*		3.9	4.2	3.9	3.9	3.0	3.3	5.0
Caecum								
Wall		3.8	3.6	—	—	1.8	3.0	5.0
Contents		2.6	—	—	2.3	—	6.5	6.9
Lymph node		3.8	4.3	3.7	4.0	—	2.5	5.0
Colon								
Wall		4.2	—	5.1	3.7	2.6	3.0	6.3
Contents		3.6	—	+	—	—	—	5.8
Lymph node		4.2	4.3	+		2.5	2.5	6.3
Liver		—	—	—	—	—	—	2.0
Hepatic lymph node		+	—	4.2	—	—	—	5.0
Spleen		—	+	—	+	—	—	+
Lung		—	—	+	—	—	—	—
Bronchial lymph node		—	—	—	—	—	—	—
Blood		—	—	—	—	—	—	—
Bile		—	—	—	—	—	—	—

* This is the distal, comma-shaped node at the end of the chain of jejuno-ileal lymph nodes.

11 (Table 3). The four calves inoculated subcutaneously with *S. typhimurium htrA* (12, 13, 14 and 15) developed bacteraemia and pyrexia but survived (Table 1). *S. typhimurium* 4/74 *htrA* was detected in the blood of these calves between 1 h and 1 d after inoculation, and bacteria were also shed in the faeces of three of these calves. As salmonellae were only detected in faeces by broth enrichment, less than 500 bacteria (g faeces)⁻¹ were being secreted.

Groups of calves were inoculated orally (16, 17, 18 and 19) or subcutaneously (20 and 21) with 3.8×10^{10} or 1×10^8 *S. typhimurium* 4/74 *purE* respectively. Calves 16 and 17 succumbed to salmonellosis on the fifth day post-inoculation and calves 18 and 19 succumbed on the sixth day after inoculation (Table 1). These calves developed pyrexia and secreted salmonellae in their faeces. Post-mortem bacteriological analysis showed that *S. typhimurium* 4/74 *purE* was mainly associated with digestive tract tissues. In two calves, bacteria were isolated either by enrichment or by direct plating from the spleen or the liver. (Table 3). Calves 20 and 21, inoculated subcutaneously, developed pyrexia but did not secrete *S. typhimurium* 4/74 *purE* in their faeces (Table 1).

Measurement of IFN γ and antibodies to LPS in the sera of calves challenged with *S. typhimurium* 4/74

In a preliminary attempt to relate immunological parameters to susceptibility to infection, the concentrations of IFN γ were measured in the sera of infected calves (Table 4). There was no obvious correlation between the concentration of IFN γ , the route of inoculation or susceptibility to infection. Calves 1 and 2, inoculated orally, had the highest serum IFN γ concentrations on days 2 and 1 respectively. The IFN γ concentrations remained higher than they were before inoculation until both calves died. Calves 3, 4, 6 and 7, which were inoculated subcutaneously, had the highest serum IFN γ concentrations 1 d after inoculation, whereas calf 5 had detectable serum IFN γ on day one with the highest concentration on day two after inoculation. Calf 8 had detectable serum IFN γ on day one after infection with the highest concentration at 6–7 d after inoculation.

Antibodies to LPS and STSE in the sera of calves that survived subcutaneous inoculation of virulent *S. typhimurium* 4/74 were measured by ELISA. Calf 8 had detectable levels of anti-LPS antibodies prior to chal-

Table 4. IFN γ concentration (ng ml⁻¹) in serum of animals inoculated with *S. typhimurium* 4/74

ND, not done.

Route of inoculation...	Time	Calf...	Oral		Subcutaneous				
			1	2	3	4	5	6	8
–1 d			0.619	1.71	0	0.074	2.733	0.892	1.301
0			1.642	0.824	0	0	1.506	2.255	1.165
1 h			1.778	0.347	0	0	1.028	1.165	0.551
4 h			1.097	0.415	0	0	2.119	0.483	1.983
1 d			17.525	0.483	4.914	27.682	8.595	10.027	12.89
2 d			10.64	11.526	1.369	6.618	9.14	3.073	2.528
3 d			8.936	9.754	1.097	3.482	5.664	2.324	2.596
4 d				6.755		1.437	4.846	2.528	5.527
5 d				2.937			3.755	2.733	5.459
6 d				ND			2.187	2.664	6.073
7 d				2.528			4.641	2.664	7.436
15 d								1.301	0.892

Fig. 1. ELISA to detect antibodies to *S. typhimurium* 4/74 LPS. (a) IgG1 to LPS; (b) IgG1 to STSE; (c) IgG2 to STSE; (d) IgM to STSE. To detect IgG1, IgG2 and IgM sera were diluted 1:1000, 1:100 and 1:100, respectively. ♦, calf 6; ■, calf 7; ▲, calf 8.**Table 5.** Haptoglobin (mg ml⁻¹) in serum of cattle inoculated with *S. typhimurium* 4/74

Route of inoculation...	Days	Calf...	Oral		Subcutaneous				
			1	2	3	4	5	6	8
0			0.03	0.02	0.07	0	0.02	0.02	0.87
1			0.04	0.03	0.02	0.56	0.28	0.32	0.56
2			0.03	0.12	0.24	0.55	0.29	0.13	0.84
3			0.02	0.11	0.01	0.1	0.23	0.21	0.5
4				0.03		0.08	0.08	0.09	0.36
5				0.04			0.04	0.04	0.28
6							0.03	0.02	0.57
7							0.02	0.02	0.85

lenge and the concentration declined after inoculation (Fig. 1a). Calf 7 did not produce detectable levels of anti-LPS throughout the experiment, while calf 6 had a slight increase in the relative concentration by weeks 3 and 4. IgG2 or IgM antibody to LPS was not detected in any of

the calves. We assayed for the presence of antibodies to STSE by ELISA (Fig. 1b–d) in the same sera. Although there were differences in the responses of individual animals, all calves produced detectable IgG1, IgG2 and IgM to STSE after challenge.

Measurement of haptoglobin in sera of inoculated calves

In five out of six cattle inoculated subcutaneously with *S. typhimurium* 4/74, an increase in the concentration of serum haptoglobin was apparent, reaching a peak of between 0.15 mg ml⁻¹ and 0.56 mg ml⁻¹ on days one and two after infection (Table 5). A relatively high concentration of serum haptoglobin was detected in calf 8 prior to inoculation. In this calf the concentration of haptoglobin in serum did not increase beyond that detected on day 0. In the calves inoculated orally (1 and 2) the haptoglobin response was low in one animal (< 0.12 mg ml⁻¹) and hardly detectable in the other (Table 5). Thus, there was a detectable difference between the route of inoculation and the acute phase reaction as identified by alterations in the concentration of serum haptoglobin.

DISCUSSION

We have compared the outcome of inoculation of calves using either the oral or the subcutaneous route of challenge with wild-type *S. typhimurium* 4/74 and *S. typhimurium* 4/74 *htrA* and *S. typhimurium* 4/74 *purE* mutants. Interestingly, in contrast to results obtained in mice, both the *S. typhimurium htrA* and *S. typhimurium* 4/74 *purE* derivatives were virulent for calves after oral challenge. Although calves inoculated subcutaneously with *S. typhimurium* 4/74 generally survived, those inoculated orally mostly succumbed to salmonellosis. The outcomes of the inoculation of calves with *S. typhimurium* 4/74 *purE* or *htrA* indicated a major difference in the behaviour of these *S. typhimurium* mutants in calves and mice in terms of the level of attenuation. Taking these observations together with the different influences of the route of challenge between mice and cattle, the data suggest a fundamental difference in the pathogenesis of salmonellosis between the two species. We confirmed previous observations (Jones *et al.*, 1991; Watson *et al.*, 1998) showing that calves of four weeks of age inoculated orally with 10⁸–10⁹ *S. typhimurium* 4/74 succumb to salmonellosis. In contrast, calves inoculated subcutaneously with around 10⁹ *S. typhimurium* 4/74 generally survived. Other workers have suggested that salmonellosis is more reliably induced by the subcutaneous route than by oral inoculation (Bailey, 1978). However, the dose they used (5 × 10⁹ *S. typhimurium*), although affecting the animals, failed to induce diarrhoea. We assessed the potential of parenteral use of attenuated *Salmonella* strains, as this would facilitate immunological evaluation of vaccine candidates and help in measuring immune responses in local draining lymph nodes. In our preliminary experiments, calves were inoculated subcutaneously with 1 × 10⁹ *S. typhimurium* 4/74, which induced clinical symptoms and diarrhoea, and in some cases death. Although *S. typhimurium* can spread systemically in cattle, systemic organs do not appear to be the primary site of bacterial growth. Thus, in cattle, the primary target organ may be the gut and associated lymphoid tissues, and this targeting may be partially

independent of the route through which *S. typhimurium* enters the host. These findings are consistent with *S. typhimurium* being mainly a systemic, rather than a gastroenteric, pathogen in mice, but mainly a gastroenteric pathogen in calves. In agreement with this hypothesis, Tsolis *et al.* (1999) have reported that virulence-associated factors linked with intestinal penetration, such as SPI-1, seem to be more important for pathogenicity in calves than genes required during the systemic phase of infection, such as SPI-2. These differences have also been supported by studies in cattle (Watson *et al.*, 1998) and rabbits (Everest *et al.*, 1999), both of which develop diarrhoea associated with *S. typhimurium* infections. Either the gut lumen or the gut-associated tissues may provide a suitable site for replication of salmonellae. In addition, the function of intestinal cells in cattle appears to be more susceptible to disruption compared to mice, leading to fluid loss and diarrhoea.

The difference in pathogenesis of *S. typhimurium* in cattle and mice is of relevance in the design and development of safe and effective vaccines. This is particularly important as the mouse is now most frequently used as the animal of choice for identifying potentially attenuating gene mutations in *Salmonella* spp. Indeed, mutations in both the *aro* and *htrA* genes were originally identified as attenuating in mice and were subsequently used as components of rationally attenuated *S. typhi* vaccine candidates. Mutations in *aro* genes have been shown to significantly attenuate *Salmonella* strains in several animal species including cattle (Smith *et al.*, 1984; Jones *et al.*, 1991) and humans (Tacket *et al.*, 1992). Mutations in *htrA* were also used to prevent the detectable systemic spread of *S. typhi* in volunteers challenged with *S. typhi aro* vaccines (Tacket *et al.*, 2000). The *htrA* gene encodes a periplasmic protease which increases the resistance of salmonellae to peroxide and which appears to contribute to the ability of salmonellae to resist the bactericidal action of macrophages (Johnson *et al.*, 1991). The apparent virulence of *S. typhimurium* 4/74 *htrA* in calves after oral challenge may reflect differences in the ability of bovine macrophages to kill *S. typhimurium*. Alternatively, *S. typhimurium* may grow in different host sites in bovine tissues. However, at this time we cannot rule out the possibility that other *Salmonella* strains may be attenuated by *htrA* or *purE* mutations in cattle.

IFN γ can be produced by T cells and NK (natural killer) cells in response to exposure to bacterial antigens. In naïve animals IFN γ is produced predominantly by NK cells. In mice IFN γ can be detected *in vivo* in the sera of mice inoculated intravenously with salmonellae 3 d after inoculation (Mastroeni *et al.*, 1996; Ramarathnam *et al.*, 1991). Depletion of IFN γ during the early stages of salmonellosis in mice led to an increase in the rate of growth of virulent salmonellae (Muotiala & Makela, 1990). Equally, administration of exogenous IFN γ in the early stages of salmonellosis in mice leads to a decrease in the rate of growth of salmonellae (Muotiala & Makela, 1990). We looked for a relationship between

the route of infection and production of IFN γ in cattle inoculated with *S. typhimurium* 4/74, but found no correlation. Calves inoculated orally or subcutaneously that either survived or succumbed to the infection produced IFN γ . An acute phase reaction has been demonstrated in cattle infected with *S. typhimurium*. The production of haptoglobin is stimulated during the acute phase reaction by a combination of pro-inflammatory cytokines such as interleukin 1 (IL-1), IL-6 and TNF α being released from macrophages and other cells at the site of lesions or infection. Potentially, IFN γ can be involved in this pathway by activation of macrophages, but in our studies, with the difference between the subcutaneous and oral routes of inoculation it would appear that the IFN γ and haptoglobin responses were not correlated. The acute phase response detected in the subcutaneously inoculated cattle was moderate. Recent studies show that the acute phase response to conditions such as clinical mastitis, pulmonary thromboembolism and acute respiratory disease can lead to serum concentrations of 2–3 mg haptoglobin ml⁻¹ (Horadagoda *et al.*, 1999) and here we observed a rise to only about 25 % of these levels. The acute phase response is not likely to be a useful diagnostic tool in orally infected cases of salmonellosis (e.g. natural infection), but could be a useful tool in experimental infection, especially for monitoring the response to subcutaneous inoculation.

In conclusion, our studies, taken together with those of others, highlight significant differences in the pathogenesis of salmonellosis between mice and cattle. This conclusion is supported by data obtained using different routes of challenge and different mutations that influence the growth of salmonellae *in vivo*. Further studies using different *Salmonella* strains deleted in different genes should be of value in identifying the differences in basic pathogenic mechanisms and improve our understanding of host specificity in *Salmonella*, a poorly understood area at the present time.

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