Protection against experimental salmonellosis in mice and sheep by immunisation with aromatic-dependent *Salmonella typhimurium*

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Summary. Mice immunised by the oral or intraperitoneal route with a live aromatic-dependent strain of *Salmonella typhimurium* exhibited significantly less protection against oral challenge with 50 LD₅₀ of an ovine isolate of *S. typhimurium* (12313) than when a bovine isolate with the same O antigens and phage-type as strain 12313 was used as the challenge organism. When challenged with 10 LD₅₀, however, protection against both strains was significantly better than that obtained when mice were vaccinated with killed vaccines (heat-killed, acetone-killed or irradiated) even when the antigenic mass of the killed vaccine was increased by up to 500-fold in an attempt to compensate for the expected limited multiplication of the mutant organism. Sheep immunised with the live mutant strain by either the intramuscular or oral route were protected against oral challenge with the virulent ovine isolate of *S. typhimurium*; unimmunised sheep died of acute enteritis within 7 days, although there was no evidence of systemic invasion by the challenge organism. After challenge, immunised animals ate more food than the unimmunised controls and suffered only transient, mild diarrhoea. Serum antibody titres against O and H antigens measured by direct or antiglobulin tests were significantly higher in sheep immunised by the intramuscular route than in those immunised orally. Sheep in both immunised groups developed skin swellings within 30 min after intradermal inoculation with purified homologous lipopolysaccharide indicating development of immediate-type hypersensitivity, but only those immunised by the intramuscular route showed significant indurated skin swellings characteristic of delayed-type hypersensitivity 48 and 72 h post-inoculation. Thus it appears that protection against salmonellosis in sheep immunised by the intramuscular or the oral route may involve different mechanisms.

Introduction

Salmonellosis of food producing animals is a serious public health hazard. It is also an economically important disease of sheep, causing infections and deaths in intensive husbandry conditions, which can result in huge economic losses under stressful conditions such as those encountered during the shipping of animals. Among the various serotypes of *Salmonella* isolated from cases of clinical salmonellosis in sheep in Australia, *S. typhimurium* usually predominates (Murray, 1984).

Numerous attempts have been made to protect mice and ruminant animals against salmonellosis with killed, subunit or live vaccines. Whereas the

use of non-living vaccines has been reported by some investigators to confer protection against experimental salmonellosis (Eisenstein and Angerman, 1978; Kuusi *et al.*, 1979, 1981; Svenson *et al.*, 1979; Tato *et al.*, 1979; Angerman and Eisenstein, 1980; Aitken *et al.*, 1982; Desiderio and Campbell, 1985), others found that live vaccines were superior to killed vaccines in protecting mice and calves against experimental challenge infections with virulent salmonellae (Mitsuhashi *et al.*, 1958; Ushiba *et al.*, 1959; Smith, 1965; Collins *et al.*, 1966; Germanier, 1972; Ivanoff *et al.*, 1980; Robertsson *et al.*, 1983; Smith *et al.*, 1984). A *galE* mutant characterised by a block in the enzyme uridine diphosphate-4-epimerase has been used in immunisation trials in ruminants. Calves immunised with this mutant were protected against

experimental salmonellosis (Wray *et al.*, 1977), although the protection reported in sheep was not as good (Jelinek *et al.*, 1982). Although reversion of the *galE* mutant to wild-type *in vivo* has not been reported (Germanier and Furer, 1971), the frequency of secondary mutations *in vitro* is significantly higher than that reported for the aromatic-dependent salmonella mutant (Hoiseth and Stocker, 1981). One such strain, SL1479, was derived from a virulent bovine isolate of *S. typhimurium* and constructed to have a complete, non-reverting defect in the common aromatic biosynthetic pathway, caused by a transposon-generated deletion or deletion-inversion mutation of gene *aroA* (Hoiseth and Stocker, 1981). Such mutant strains were shown to be avirulent and induced a highly effective immunity in mice (Hoiseth and Stocker, 1981) and calves (Robertsson *et al.*, 1983; Smith *et al.*, 1984).

The aims of the present investigation were, first, to compare the immunising efficacy of the live salmonella mutant SL1479 in mice against oral challenge with a virulent bovine or ovine isolate of the same phage type, and, second, to compare the immunising efficiency of live and killed vaccines prepared from strain SL1479 against challenge by the oral rather than the intraperitoneal route which has been used in most previous investigations. Although SL1479 has been reported to protect calves against challenge by the oral (Robertsson *et al.*, 1983) or intramuscular routes (Smith *et al.*, 1984), a comparison of the efficacy of these routes of immunisation cannot be made because of differences in the number of vaccine doses, presence of adjuvant, and the regimes of immunisation used. The third aim of this investigation, was to compare the clinical and immunological responses, and protection against experimental salmonellosis in sheep immunised by either the intramuscular or oral routes employing comparable regimes of immunisation.

Materials and methods

Experimental animals

Specific pathogen-free, Balb/c female mice (18–22g) aged c. 8 weeks and Merino wether sheep, aged 9–12 months were obtained from the CSIRO McMaster Farm, Badgery's Creek, NSW. Depending on the immunisation schedule, groups of 20–30 mice were used and were boxed in groups not exceeding 15 mice per cage post-vaccination and 10 mice per cage post-challenge. Mice were vaccinated at c. 8 weeks of age and mice aged c. 9 weeks were used for determination of the 50% lethal dose (LD50).

For experimental challenge infections, sheep were isolated and housed in separate pens with raised floors at the University of Sydney, Camden, NSW.

Bacterial strains and vaccine preparation

The auxotrophic mutant of *S. typhimurium* (strain SL1479; O antigens 1, 4, 12; phage-type 193) used as the vaccine was derived from *S. typhimurium* UCD 108–11, originally isolated from a calf that died of enteritis (Smith *et al.*, 1984). The *S. typhimurium* strains used for experimental challenge were a bovine isolate 7328 (O antigens 4, 5, 12; phage-type 9) and an ovine isolate, 12313 (O antigens 4, 5, 12; phage-type 9). These two strains were obtained from the Salmonella Reference Centre, Institute of Medical and Veterinary Science, Adelaide, South Australia, through the courtesy of Mr C. Murray.

The live vaccine was prepared by growing strain SL1479 in Brain Heart Infusion Broth (Oxoid) in shaking flasks at 37°C for 18 h. After centrifugation at 12350 g for 30 min the pellet was washed three times in phosphate-buffered saline (PBS) before final resuspension in PBS. Organisms in the vaccine were counted by the agar spread plate count technique. The killed vaccines were prepared by heating the washed suspensions of the live mutant SL1479 (10^{10} organism/ml) at 100°C for 1 h, or irradiation with 10^6 rads, or acetone treatment (Landy, 1953); these are referred to as heat-killed, irradiated and acetone-killed cells or vaccines respectively. Fresh vaccine preparations were used for every experiment and appropriately diluted before use.

Determination of the LD50 in mice

Mice were infected by gavage with varying numbers of the bovine (7328) or ovine (12313) isolates of virulent *S. typhimurium* and mortalities recorded for 15 days post-infection. The LD50 doses ($\pm$ SE) calculated according to Litchfield and Wilcoxon (1949) were $(7.2 \pm 2.2) \times 10^4$ and $(5.0 \pm 2.2) \times 10^5$ for the bovine and ovine isolates respectively.

Extraction of lipopolysaccharide (LPS)

The hot phenol-water extraction procedure (Westphal and Jann, 1965) was used for extracting LPS from the formalin-killed *Salmonella* mutant SL1479. Highly-purified LPS was obtained by preparative ultracentrifugation as described by Lindberg and Holme (1972).

Experimental reproduction of clinical salmonellosis in sheep

Sixteen Merino wethers were infected orally with varying numbers of the ovine isolate of *S. typhimurium* (12313) enclosed in gelatin capsules (10^7 organisms were given to two sheep, 10^8 organisms to three sheep, 10^9 organisms to three sheep, 10^{10} organisms to three sheep, 10^{11} organisms to three sheep, 10^{12} organisms to two sheep). Gelatin capsules were used for enclosing the

challenge organisms to avoid complications due to closure of the oesophageal groove (Prichard and Hennessy, 1981). These animals were monitored for a rise in body temperature, development of diarrhoea and faecal excretion of the challenge organism. Sheep that died during the course of infection were necropsied and the surviving animals were killed 15 days post-challenge. Various tissues including liver, spleen, ileal and caecal lymph nodes, bile and blood were plated directly on Xylose Lysine Deoxycholate (XLD) agar (Oxoid) and also cultured on cysteine-mannitol-selenite enrichment media.

Experimental design of immunisation and challenge studies

Mice. Groups of mice (20–30 per group) were immunised with either the live or killed mutant strain, SL1479, by the intraperitoneal (ip) or oral route and challenged 3 or 4 weeks later by oral administration of 50 LD₅₀ or 10 LD₅₀ of the bovine (7328) or ovine isolate (12313) of *S. typhimurium*. Unvaccinated mice (20–24 per group) were included in all challenge experiments as controls. Any mice in which there was any doubt about the precision of the vaccine or challenge delivery were culled from the experiment.

Sheep. Three groups each of five Merino wethers were used. Sheep in group I were immunised with the live *S. typhimurium* mutant SL1479, by the intramuscular (im) route, and those in group II by oral administration of the live-vaccine organisms in gelatin capsules. Animals in group III served as unimmunised controls. Sheep in groups I and II received three vaccinations at weekly intervals (10^8 , 10^{10} and 10^{10} organisms respectively). Twenty-one days later all animals were challenged with 1×10^9 of the ovine isolate of *S. typhimurium* (12313) administered in gelatin capsules. An additional group of 4 sheep was immunised with the heat-killed mutant strain, SL1479, by the im route using the immunisation regimen identical to that for sheep in group I. Sheep in this group were used only for comparative assessment of immunological parameters including agglutination titres and skin reactions.

The experiment was terminated 20 days after the challenge infection. Excretion of the challenge strain in faeces was monitored by enrichment culture in mannitol-selenite broth containing cysteine followed by plating out on XLD media. Each animal was sampled on days one to 8 and on days 11, 14 and 20 after challenge. Sheep were considered to be free of infection when four consecutive faecal cultures were negative. Blood cultures were also monitored from days 2–6 post-challenge. The surviving sheep were killed with a captive bolt and a necropsy performed; various organs including liver, spleen, ileal and caecal lymph nodes, bile, segments of duodenal, ileal and caecal wall, and the associated intestinal contents were cultured for the presence of the challenge strain by direct plating on the XLD medium and also following enrichment in mannitol-selenite broth containing cysteine.

Clinical parameters. Rectal temperatures, total white blood cell (WBC) count and the differential WBC counts were determined pre-challenge and on days 1–8, 11, 14 and 20 days post-challenge with virulent *S. typhimurium*. Animals were weighed before challenge and at weekly intervals post-challenge. Food intake was assessed every 24 h during the first 8 days and also on days 11, 14 and 20 days post-challenge. The extent of diarrhoea in challenged sheep in all groups was estimated using the arbitrary scoring system described below: 0 = normal hard pellets with distinct conformation; 1 = pellets soft but pellet conformation still distinct; 2 = pellets soft and pellet conformation changed so as to result in partial coalescing of multiple pellets; 3 = soft faecal mass without discrete pellets; 4 = faecal mass a viscous paste; 5 = thin faecal paste, significantly reduced solid matter; 6 = watery diarrhoea with mucus, occasional blood stains and shreds of mucous membranes.

Immunological parameters

Agglutination tests were performed in microtitration plates by a standard procedure (Campbell *et al.*, 1970). Antigen was prepared from heat-killed mutant organisms (SL1479, 100°C, 1 h) to determine anti-O group-antigen titres, and a formalinised (0.3% final concentration) suspension of the same organisms was used for determination of anti-H antigen titres (Campbell *et al.*, 1970). Anti-globulin anti-O and H titres were determined by a modification of the method of Gel and Coombs (1968) described by Husband and McDowell (1978). Immediate and delayed skin reactions were measured with a Vernier caliper before intradermal injection of 0.05 ml of purified LPS (100 µg) prepared from the strain, SL1479, and at 30 min, 24, 48 and 72 h post-inoculation. The thickness of the double skin-fold was assessed one week after the last immunising dose and was represented as the difference between the double skin-fold thickness at the specified times and that measured immediately before intradermal injection.

Statistical analyses

Student's test was used to determine the significance of differences between mean log₁₀ values for the agglutination titres. X²-square test was used to determine the significance of protection observed in the mouse immunisation and challenge experiments except in tables with zero entries where significance was assessed by Fisher's exact test.

Results

Mouse experiments

S. typhimurium is considered to be a non-host adapted serotype, and it was of interest to compare the protection afforded by *S. typhimurium* mutant SL1479 (derived from a bovine isolate) against challenge with wild-type bovine or ovine isolates of

S. typhimurium. Immunised mice exhibited significantly less protection against challenge with the ovine isolate (12313) than with the bovine isolate (7328) even though both challenge strains had similar O antigens ($-1, 4^+, 5^+, 12^+$) and were both of phage type 9. At a low challenge dose (10 LD₅₀), however, the protection obtained with the ovine isolate was significant by greater ($p < 0.05$) than with the killed vaccines (tables I and II). Regardless of the method of inactivation used, however, immunisation with killed *S. typhimurium* strain SL1479, given ip even when the dose was 500 times that of the live mutant, failed to protect mice from challenge (table II). It is also evident (table I) that immunisation with living organisms by either the oral or ip route gave rise to similar levels of protection.

Experimental reproduction of clinical salmonellosis in sheep

The first experiment was designed to determine an approximate 25% (LD₂₅) or 50% (LD₅₀) lethal dose of the ovine isolate of *S. typhimurium* (12313) for sheep and at the same time confirm the manifestations of clinical salmonellosis reported previously (Brown *et al.*, 1976). With the exception of sheep that received 10^7 virulent *S. typhimurium*, all remaining animals developed elevated temperatures (1° – 2.2°C) and severe diarrhoea by days 4–5 post-challenge (details not shown). The diarrhoea, which was often blood-stained and contained mucus shreds, persisted until death of the animals on day 6–8 post-challenge. Administration of 10^7 organisms caused no deaths, 10^8 organisms killed 1 of 3 sheep, 10^9 organisms killed 3 of 3 sheep, 10^{10} and

10^{11} organisms killed 2 out of 3 sheep, 10^{12} organisms killed 2 out of 2 sheep. Using probit analysis, the LD₅₀ and LD₂₅ were estimated to be 6.42×10^8 (95% confidence interval; 1.27×10^8 , 3.25×10^9) and 3.10×10^7 (95% CI; 2.90×10^6 , 3.31×10^8) respectively. In none of these animals was the virulent challenge strain of *S. typhimurium* cultivated from liver, spleen, bile or blood with or without enrichment cultures. In only 2 of 16 animals were the organisms recovered from the ileal and caecal lymph nodes. However, the organisms were always recovered from the gut contents, and the ileal and caecal walls by direct plating on XLD agar or after enrichment in cysteine-mannitol selenite broth. Therefore, it was apparent that salmonellosis, in this investigation, was characterised by acute enteritis without systemic involvement.

Immunisation experiments

In view of the failure of killed vaccines to confer protection in our mouse experiments and the poor performance of killed vaccines in calves in general, it was decided to evaluate the immunising efficacy of only the live mutant SL1479 in the sheep experiments in which im (group I) and oral (group II) immunisation was used.

Clinical responses. Immunisation of sheep with the live mutant by either route had no effect except for a temperature rise (0.4 – 0.8°C) in 3 of 10 immunised animals on days 2 and 3 after each immunisation. Sheep maintained their appetite and weight during the period of multiple immunisations. After challenge with the virulent organisms, there was an appreciable rise in rectal temperature (0.9 – 2.2°C) during the first 2–3 days in vaccinated and unvaccinated sheep. In immunised animals,

Table I. Protection of mice immunised with live aromatic-dependent *S. typhimurium* strain SL1479

Route of immunisation†	Time of challenge (weeks post-immunisation)	Number of mice surviving/total number challenged by the oral route with virulent <i>S. typhimurium</i> , phage type 9			
		Bovine isolate 7328		Ovine isolate 12313	
		3.6×10^6 cfu (50 LD ₅₀)	7.2×10^5 cfu (10 LD ₅₀)	2.5×10^7 cfu (50 LD ₅₀)	5×10^6 cfu (10 LD ₅₀)
Intraperitoneal	4	21/24**	24/24 ^b *	9/24 ^e	17/24 ^f *
Oral	3	20/26**	24/24 ^g *	10/24 ^g	18/24 ^h *
Unimmunised	4	0/24	0/24	0/20	0/20

† Mice immunised by the intraperitoneal route received 1×10^5 cfu whereas those immunised by the oral route received 1×10^5 cfu on day 0, 5×10^5 cfu on day 7 and 4×10^6 cfu on day 14.

* Significant protection afforded by vaccination: $p < 0.05$ (a vs. e; c vs. g; f and h vs. protection obtained with killed vaccines, see table II) and $p < 0.001$ (a, b, c, d, f and h vs. protection obtained with killed vaccines, see table II).

Table II. Protection of mice immunised intraperitoneally with killed aromatic-dependent *S. typhimurium* strain SL1479

Vaccine and dose	Time of challenge (weeks post-immunisation)	Number of mice surviving/total number challenged by the oral route with virulent <i>S. typhimurium</i> phage type 9			
		Bovine isolate 7328		Ovine isolate 12313	
		3.6 × 10 ⁶ cfu (50 LD50)	7.2 × 10 ⁵ cfu (10 LD50)	2.5 × 10 ⁷ cfu (50 LD50)	5 × 10 ⁶ cfu (10 LD50)
Heat-killed Cells	4				
(i) 1 × 10 ⁵ organisms		4/24	11/28	3/22	6/23
(ii) 1 × 10 ⁶ organisms		4/22	9/25	6/25	9/24
(iii) 1 × 10 ⁷ organisms		4/22	10/28	7/26	10/29
(iv) 5 × 10 ⁷ organisms		5/24	7/26	6/23	8/28
(v) Unimmunised		0/27	0/24	0/20	0/20
Irradiated Cells	4				
(i) 1 × 10 ⁵ organisms		6/24	10/25	4/26	9/26
(ii) 1 × 10 ⁶ organisms		4/25	9/25	5/27	10/25
(iii) 1 × 10 ⁷ organisms		3/21	9/30	2/22	4/23
(iv) 5 × 10 ⁷ organisms		7/20	9/20	4/20	6/22
(v) Unimmunised		0/20	0/20	0/20	0/20
Acetone-killed Cells	4				
(i) 1 × 10 ⁵ organisms		1/24	7/24	0/24	3/23
(ii) 1 × 10 ⁶ organisms		5/25	10/25	3/25	7/24
(iii) 5 × 10 ⁷ organisms		4/25	8/27	4/26	7/26
(iv) Unimmunised		0/20	0/20	0/23	0/23

rectal temperatures returned to normal by days 5–6 whereas, in the control group, temperatures continued to rise until shortly before death. There was a significant elevation in the total WBC count in all groups at 2–3 days post-challenge, followed by neutropenia at 5–7 days post-challenge and this was most severe in the control group. Appetite and body weight in the immunised groups did not change

appreciably during the 20-day period (data not shown) and only a transient mild-to-moderate diarrhoea (fig. 1) occurred. All animals (5 out of 5) in groups I and II survived challenge; they had relatively minor clinical signs. Although orally-immunised animals seemed to be slightly more affected on days 6, 7, 8 and 11 post-challenge than their im-immunised counterparts, there were no

Fig. 1. Diarrhoeal scores of immunised and control sheep after challenge with virulent *S. typhimurium*. Histograms represent mean values + SEM (bars); ■ unimmunised; □ live, im; ▨ live oral.

statistically significant differences in any of the clinical parameters monitored. At termination of the experiment, the challenge strain of *S. typhimurium* was recovered from the faeces of 3 of 5 immunised sheep. In contrast, the unvaccinated control animals (Group III) quickly became seriously ill with profuse diarrhoea (fig. 1), dehydration and weight loss and all died within 7 days after challenge.

Antibody responses. The results for anti-O and anti-H agglutination titres, both direct and with antiglobulin are presented in figs. 2A-D. In animals immunised with the live mutant strain SL1479, there was a difference in the response between orally- and im-immunised animals; there were large increases in titres after im-immunisation and relatively smaller increases following oral immunisation. Substantial increases in the anti-O agglutination titres occurred in sheep immunised with the heat-killed vaccine by the im route. Direct and antiglobulin titres of both anti-O and anti-H antibodies increased significantly ($p < 0.001$) by day 14 (7 days after the second intramuscular injection of the live mutant).

Skin responses. Results of the skin-hypersensitivity tests are presented in table III. It can be seen that sheep immunised with the live mutant strain

Table III. Hypersensitivity reactions in immunised and unimmunised sheep after intradermal injection with purified homologous lipopolysaccharide

Immunisation group	Double skin-fold thickness (mm) at various times post-inoculation (mean value $\pm$ SEM) [†]			
	30 min	24 h	48 h	72 h
Live, oral	5.25* $\pm$ 1.09	3.05 $\pm$ 1.04	3.3 $\pm$ 0.65	1.5 $\pm$ 0.87
Live, im	6.63** $\pm$ 0.55	8.9*** $\pm$ 1.44	5.95* $\pm$ 0.92	4.43* $\pm$ 1.03
Heat-killed, im	3.5 $\pm$ 1.32	5.35 $\pm$ 2.23	2.65 $\pm$ 1.07	2.05 $\pm$ 1.22
Unimmunised	1.86 $\pm$ 0.42	1.68 $\pm$ 0.21	1.98 $\pm$ 0.58	0.62 $\pm$ 0.70

[†] Values differing significantly from those recorded in the unimmunised animals are indicated; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

SL1479 by the im route showed substantially greater swelling at all times after the injection of LPS than orally-immunised sheep. Significant immediate-type hypersensitivity reactions (30 min) were seen only in groups immunised with the live mutant; the

Fig. 2. Titres of antibody against O-(A, B) and H-(C, D) antigens in serum of immunised and control sheep at various times after immunisation. Histograms represent mean values $\pm$ SEM (bars); ■ unimmunised; □ live, im; ▨ live, oral; ▤ heat-killed, im.

im group showed the larger reactions. The swellings at this time were oedematous, hot, red and painful. The swellings reached a maximum in the group immunised with the live mutant by the im route at 24 h but a large, indurated swelling was still present at 48–72 h. This contrasted with the results in sheep immunised with the heat-killed vaccine by the intramuscular route, which showed distinct swelling at 24 h, but this was substantially reduced in size by 48–72 h.

Discussion

The observation of significant protection in mice immunised with the live *S. typhimurium* mutant SL1479 against challenge infection with a virulent bovine isolate (7328), but not an ovine isolate (12313), suggested that the challenge strains differed in their virulence. It was not clear whether the increased virulence of the ovine isolate was attributable to additional virulence characters not possessed by the bovine isolate, or was simply due to an amplified expression of the existing ones; the former explanation seems more likely because the LD50 of the ovine isolate was greater than that of the bovine isolate. The protection obtained in immunised mice when challenged with 10 LD50 was still significantly greater than that observed in mice immunised with three killed vaccines, and no significant increase in protection was achieved, even when the antigenic mass in the killed vaccines was increased. These results agree with some previous reports (Mitsushashi *et al.*, 1958; Ushiba *et al.*, 1959; Smith, 1965; Collins *et al.*, 1966) but contrast with those of others (Eisenstein and Angerman, 1978; Tato *et al.*, 1979; Angerman and Eisenstein, 1980; Desiderio and Campbell, 1985). We do not consider it valid to compare the results reported by the latter investigators with those presented here for two major reasons. First, the CD-1 strain of mouse was used in their investigations; it had intermediate-to-high resistance to salmonellosis (Hormaeche, 1979). Second, the challenge infection was administered by the ip rather than the oral route. We consider the latter route to represent the most natural route of infection in salmonellosis. Collins and Carter (1972) compared the immunogenicity of heat-killed and living *S. enteritidis* vaccines by the oral route in CD-1 mice and reported that whereas oral immunisation of mice with a heat-killed vaccine delayed the systemic signs of gastrically-introduced salmonella infection by 1–2 days, immunisation with a living attenuated vaccine resulted in immunity as judged by the rapid removal of organisms from the liver, spleen and

mesenteric lymph nodes, although the challenge organisms still persisted in the gut.

One possible reason for the superiority of the live vaccine could be due to the fact that the host is exposed to protective antigens for a longer period of time, thus allowing longer stimulation of the sites responsible for induction of immunity. We conducted an experiment (data not shown) in which mice were immunised with the heat-killed salmonella mutant, SL1479 (1×10^5 bacteria/mouse) every day for 5 days. Exposure for periods longer than 5 days resulted in the development of ruffled coats and loss of body weight, perhaps because of the effect of endotoxins. These mice were challenged with 50 LD50 and 10 LD50 doses of the bovine and ovine isolates of *S. typhimurium*, but no significant protection was observed.

Therefore, it is clear from the data presented in this investigation that killed *S. typhimurium* organisms are inferior to the live mutant SL1479 as vaccines for protection against experimental mouse typhoid.

The manifestation of clinical salmonellosis in sheep as enteritis in this investigation contrasts with the results reported previously for *S. typhimurium* (Brown *et al.*, 1976) in which the challenge organisms were recovered from practically all the internal organs, gut wall and contents, and various lymph nodes. A lack of systemic involvement was also reported by Hall *et al.* (1978) during their investigations on the pathogenesis of intra-ruminal infections of cows with *S. dublin*. The primary sites of bacterial penetration appeared to be the lower ileum and caecum. In these sites, the infection was considered to spread from the intestinal contents to the intestinal wall and to the associated lymph node. No spread to the liver and spleen occurred. In contrast, systemic involvement almost always occurs whenever the challenge dose of salmonellae is administered in such a way that the organisms come in contact with the oropharynx (De Jong and Ekdahl, 1965; McCaughey *et al.*, 1971; Brown *et al.*, 1976; Wray and Sojka, 1978; Smith *et al.*, 1979). Although no direct experimental evidence is available, it is tempting to speculate that the oropharyngeal region may be involved in the process of systemic invasion.

In this investigation we found that when sheep were immunised with the live *S. typhimurium* mutant, SL1479, by the oral route, the organisms could be recovered in faeces for only 4 days post-immunisation. Essentially similar results were reported previously for calves immunised orally with the same live mutant (Robertsson *et al.*, 1983). In contrast, the live mutant organism could not be

cultured from the liver and spleen. Similar results were obtained when sheep were immunised by the intramuscular route.

Although killed vaccines have been reported to protect calves to varying degrees against experimentally-induced salmonellosis (Cameron and Fuls, 1976; Bairey, 1978; Smith *et al.*, 1980; Aitken *et al.*, 1982), surviving animals, in general, had moderate to severe manifestations of clinical salmonellosis. Recently, Robertsson *et al.* (1983) reported that calves immunised with an aromatic-dependent *S. typhimurium* mutant, SL1479, by the oral route, were protected against experimentally-induced acute salmonellosis, whereas those immunised with an heat-inactivated vaccine succumbed to the challenge infection. In our investigation, sheep immunised with the live, aromatic-dependent *S. typhimurium* mutant SL1479, by the im or the oral route and challenged with the virulent ovine isolate of *S. typhimurium*, were protected against the enteric form of salmonellosis. This is significant because the cellular and humoral immune mechanisms against *Salmonella* infection would have to operate at the intestinal mucosa and in the gut lumen. The generation of these immune mechanisms by oral immunisation is understandable (McGhee *et al.*, 1984), but the mechanisms by which im immunisation provided the host with protective factors operative at the intestinal mucosal level are not known at present. It is clear, however, that there are marked differences in the antibody and skin reactions of sheep immunised by the two routes of immunisation.

Increases in the anti-O and anti-H agglutination

titres were much larger in animals immunised by the im route than those in orally immunised animals. Assuming that the anti-O agglutination test measured antibodies to the LPS structure of *Salmonella* species, the finding that these antibodies, in sheep immunised with the heat-killed vaccine, were significantly higher than in those immunised with the live mutant by oral route were in agreement with results reported previously (Lindberg and Robertsson, 1983). The anti-H titres rose substantially only in the group immunised intramuscularly with the live mutant. Another significant aspect in which the results obtained in this investigation differed from those reported earlier was that sheep immunised by the oral route developed no delayed-type hypersensitivity, suggesting that the protection observed, may have been mediated by localised protective factors and possibly cell-mediated immune mechanisms operative at the intestinal mucus surface and in the gut lumen. A comparative study of the mechanisms of protection stimulated by the intramuscular or oral routes of immunisation is currently being performed.

We are very grateful to Mr J. Edwards, Ms. Suellen Tomkins and Mrs Helen Carpenter of McMaster Laboratory for their excellent technical assistance. We also acknowledge the competent assistance of Mr N. Catt and Mr. K. McKean during the sheep experiments. Finally, we are grateful to Mr C. Murray, Institute for Medical and Veterinary Science, for providing the clinical *Salmonella* isolates which were used as challenge strains in this investigation, and to Dr G. Eagleson for his advice on statistical analyses. Financial assistance for the work was provided from a CSIRO/University of Sydney Collaborative Research Fund.

REFERENCES

- Angerman C R, Eisenstein T K 1980 Correlation of the duration and magnitude of protection against *Salmonella* infection afforded by various vaccines with antibody titers. *Infection and Immunity* **27**: 435–443.
- Aitken M M, Jones P W, Brown G T H 1982 Protection of cattle against experimentally induced salmonellosis by intradermal injection of heat-killed *Salmonella dublin*. *Research in Veterinary Science* **32**: 368–373.
- Bairey M H 1978 Immunization of calves against salmonellosis. *Journal of the American Veterinary Medical Association* **173**: 610–613.
- Brown D D, Ross J G, Smith A F G 1976 Experimental infections of sheep with *Salmonella typhimurium*. *Research in Veterinary Science* **21**: 335–340.
- Campbell D H, Garvey J S, Cremer N E, Sussdorf D H 1970 *Methods in Immunology*, 2nd edn. Benjamin, New York pp 128–131.
- Cameron C M, Fuls W J P 1976 Immunization of mice and calves against *Salmonella dublin* with attenuated live and inactivated vaccines. *Onderstepoort Journal of Veterinary Research* **43**: 31–37.
- Collins F M, Carter P B 1972 Comparative immunogenicity of heat-killed and living oral *Salmonella* vaccines. *Infection and Immunity* **6**: 451–458.
- Collins F M, Mackaness G B, Blanden R V 1966 Infection-immunity in experimental salmonellosis. *Journal of Experimental Medicine* **124**: 601–619.
- Gell P G H, Coombs R R A 1968 *Clinical Aspects of Immunology*, 2nd edn. Blackwell, Oxford, p 19.
- De Jong H, Ekdahl M O 1965 Salmonellosis in calves—the effect of dose rate and other factors on transmission. *New Zealand Veterinary Journal* **13**: 59–64.
- Desiderio J V, Campbell S G 1985 Immunization against experimental murine salmonellosis with liposome-associated O-antigen. *Infection and Immunity* **48**: 658–663.
- Eisenstein T K, Angerman C R 1978 Immunity to experimental *Salmonella* infection: studies on the protective capacity and immunogenicity of lipopolysaccharide, acetone-killed cells and ribosome-rich extracts of *Salmonella typhimurium* in C3H/HeJ and CD-1 mice. *Journal of Immunology* **121**: 1010–1014.
- Germanier R 1972 Immunity in experimental salmonellosis III. Comparative immunization with viable and heat-inacti-

- vated cells of *Salmonella typhimurium*. *Infection and Immunity* **5**:792-797.
- Germanier R, Fürer E 1971 Immunity in experimental salmonellosis II. Basis for the avirulence and protective capacity of *galE* mutants of *Salmonella typhimurium*. *Infection and Immunity* **4**:663-673.
- Hall G A, Jones P W, Aitken M M 1978 The pathogenesis of experimental intra-ruminal infections of cows with *Salmonella dublin*. *Journal of Comparative Pathology* **88**:409-417.
- Hoiseth S K, Stocker B A D 1981 Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature* **291**:238-239.
- Hormaeche C E 1979 Natural resistance to *Salmonella typhimurium* in different inbred mouse strains. *Immunology* **37**:311-318.
- Husband A J, McDowell G H 1978 Immunity to experimental enteritis in lambs vaccinated prenatally. *Research in Veterinary Science* **25**:343-349.
- Ivanoff B, André C, Robert D, André F, Fontanges R 1980 Protective immunity and the cell-mediated immune response in mice following oral administration of an avirulent strain of *Salmonella typhimurium*. *Annales de Microbiologie (Paris)* **131B**:153-161.
- Jelinek P D, Robertson G M, Millar C 1982 Vaccination of sheep with live *galE* mutant of *Salmonella typhimurium*. *Australian Veterinary Journal* **59**:31-32.
- Kuusi N, Nurminen M, Saxen H, Mäkelä P H 1981 Immunization with major outer membrane protein (porin) preparations in experimental murine salmonellosis: effect of lipopolysaccharide. *Infection and Immunity* **34**:328-332.
- Kuusi N, Nurminen M, Saxen H, Valtonen M, Mäkelä P H 1979. Immunization with major outer membrane proteins in experimental salmonellosis of mice. *Infection and Immunity* **25**:857-862.
- Landy M 1953 Enhancement of the immunogenicity of typhoid vaccine by retention of the Vi antigen. *American Journal of Hygiene* **58**:148-164.
- Lindberg A A, Holme T 1972 Evaluation of some extraction methods for the preparation of bacterial lipopolysaccharides for structural analysis. *Acta Pathologica et Microbiologica Scandinavica* Section B, **80**:751-759.
- Lindberg A A, Robertsson J A 1983 *Salmonella typhimurium* infection in calves: cell-mediated and humoral immune reactions before and after challenge with live virulent bacteria in calves given live or inactivated vaccines. *Infection and Immunity* **41**:751-757.
- Litchfield J T, Wilcoxon F 1949 A simplified method of evaluating dose-effect experiments. *Journal of Pharmacology and Experimental Therapeutics* **96**:99-113.
- McCaughy W J, Kavanagh P J, McClelland T G 1971 Experimental *Salmonella dublin* infection in sheep. *British Veterinary Journal* **127**:557-566.
- McGhee J R *et al.* 1984 Mucosal immunoregulation: environmental lipopolysaccharide and GALT T lymphocytes regulate the IgA response. *Microbiology and Immunology* **28**:261-280.
- Mitsuhashi S, Kawakami M, Yamaguchi Y, Nagai M 1958 Studies on the experimental typhoid 1. A comparative study of living and killed vaccines against the infection of mice with *S. enteritidis*. *Japanese Journal of Experimental Medicine* **28**:249-258.
- Murray C J 1984 Isolates of salmonella and *Escherichia coli* serotyped at the Salmonella Reference Laboratory in 1982 and 1983 from veterinary and human sources. *Australian Veterinary Journal* **61**:273-274.
- Prichard R K, Hennessy D R 1981 Effect of oesophageal groove closure on the pharmacokinetic behaviour and efficacy of oxfendazole in sheep. *Research in Veterinary Science* **30**:22-27.
- Robertsson J A, Lindberg A A, Hoiseth S, Stocker B A D 1983 *Salmonella typhimurium* infection in calves: protection and survival of virulent challenge bacteria after immunization with live or inactivated vaccines. *Infection and Immunity* **41**:742-750.
- Smith B P, Habasha F, Reina-Guerra M, Hardy A J 1979 Bovine salmonellosis: experimental production and characterization of the disease in calves, using oral challenge with *Salmonella typhimurium*. *American Journal of Veterinary Research* **40**:1510-1513.
- Smith B P, Habasha F G, Reina-Guerra M, Hardy A J 1980 Immunization of calves against salmonellosis. *American Journal of Veterinary Research* **41**:1947-1951.
- Smith B P, Reina-Guerra M, Hoiseth S K, Stocker B A D, Habasha F, Johnson E, Merritt F 1984 Aromatic-dependent *Salmonella typhimurium* as modified live vaccines for calves. *American Journal of Veterinary Research* **45**:59-66.
- Smith H W 1965 The immunization of mice, calves and pigs against *Salmonella dublin* and *Salmonella cholerae-suis* infections. *Journal of Hygiene* **63**:117-135.
- Svenson S B, Nurminen M, Lindberg A A 1979 Artificial *Salmonella* vaccines: O-antigenic oligosaccharide-protein conjugates induce protection against infection with *Salmonella typhimurium*. *Infection and Immunity* **25**:863-872.
- Tato P, Flisser A, Gavilanes M, Molinari J L 1979 Immunogenic complexes obtained from *Salmonella typhimurium* and *Salmonella typhi* Ty2 by the bacterial acetone powder method. *Annales de Microbiologie (Institut Pasteur)* **130A**:47-60.
- Ushiba D, Saito K, Akiyama T, Nakano M, Sugiyama T, Shirono S 1959 Studies on experimental typhoid: bacterial multiplication and host cell response after infection with *Salmonella enteritidis* in mice immunized with live and killed vaccines. *Japanese Journal of Microbiology* **3**:231-242.
- Westphal O, Jann K 1965 Bacterial lipopolysaccharides. Extraction with phenol-water and further applications of the procedure. In: Whistler R L *et al.* (eds) *Methods in Carbohydrate Chemistry* vol. 5. Academic Press, New York. pp 83-91.
- Wray C, Sojka W J 1978 Experimental *Salmonella typhimurium* infection in calves. *Research in Veterinary Science* **25**:139-143.
- Wray C, Sojka W J, Morris J A, Morgan W J B 1977 The immunization of mice and calves with *galE* mutants of *Salmonella typhimurium*. *Journal of Hygiene* **79**:17-24.