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The immunization of mice and calves with gal E mutants of *Salmonella typhimurium*

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SUMMARY

A galactose epimeraseless (gal E) mutant of *Salmonella typhimurium* was investigated in mice and calves for its suitability as a live vaccine. In mice, a very highly significant difference in the mortality rates was observed when vaccinated and non-vaccinated animals were challenged with virulent strains of *S. typhimurium* and *S. dublin*.

In calves, doses of 10^6 and above of gal E mutant injected subcutaneously provided highly significant protection both in terms of mortality and prevalence of symptoms when calves were challenged orally with *S. typhimurium*. However, there appeared to be a relation between the vaccine and the presence of renal lesions and before gal E mutants can be recommended, further work is necessary to determine the pathogenesis of these lesions.

INTRODUCTION

Salmonella typhimurium is the second most common serotype isolated from cattle and during the last 3 years has accounted for approximately 30 % of the incidents diagnosed (Sojka, Wray, Hudson & Benson, 1975). This ubiquitous serotype is very common in other species of animals and is also the most important cause of human salmonellosis (McCoy, 1976). Anderson (1968) suggested that an effective *S. typhimurium* vaccine for livestock might lead to a reduction in the incidence of disease caused by this organism.

Smith (1965) developed a live vaccine against *S. dublin* infection in cattle which Rankin, Newman and Taylor (1966) also found gave protection against *S. typhimurium* infection although it did not prevent scouring. Germanier (1970, 1972) and Germanier & Fürer (1971) described the use of galactose epimeraseless mutants of *S. typhimurium* as vaccines in mice.

These mutants are characterized by a block in the enzyme uridine diphosphate (UDP)-galactose-4 epimerase. Without an external supply of galactose, these mutants cannot synthesize UDP-galactose and because galactose is incorporated in the lipopolysaccharide (LPS) via UDP-galactose, only incomplete cell wall is formed, lacking the O-specific oligosaccharide repeat units. In other words, gal E mutants form LPS of the rough type. However, when galactose is supplied exogenously as it occurs *in vivo* UDP-galactose is synthesized by an alternative route

via galactose-1-phosphate and again smooth LPS can be synthesized. However, prolonged contact with galactose brings about lysis of the cells. Thus the properties of the gal E mutants *in vivo* are dependent upon two mechanisms acting in opposite directions; a virulence and immunogenicity increasing biosynthesis of cell wall lipopolysaccharide and a virulence lowering galactose induced bacteriolysis.

Because of the importance of *S. typhimurium* infection in calves the use of a Gal E mutant as a vaccine was investigated.

MATERIALS AND METHODS

Experimental animals

Mice. A total of 252 young CBA mice of both sexes and weighing approximately 25 g were used. The mice were kept in single groups of 10 in polythene cages. A standard diet of PRD* pellets was given.

Calves. In Expts 1–4, thirty-five 1–2 weeks old Jersey calves, 25–30 kg in weight and fed four pints of milk by bucket twice a day, were used (see Table 2). For Expt 5, twenty-five Friesian and Hereford × Friesian calves were purchased in a market. Their weights ranged from 42 to 64 kg and they were fed *ad lib.* by nipples attached to buckets. Five additional calves were used in toxicity experiments. All the calves were housed in groups of up to six calves in loose boxes and fed hay and water *ad lib.*

The calves were examined clinically and the body temperature recorded before vaccination. Freedom from salmonella infection was confirmed by bacteriological examination of faeces on arrival at the laboratory and before vaccination.

Bacterial strains

Salmonella typhimurium G30D (gal E mutant) and S2337 were kindly supplied by Professor R. Germanier, Swiss Serum and Vaccine Institute, Berne, and Mr R. J. Taylor, ARC, Compton, respectively. The former strain was used in mice and calves as a potential vaccine and the latter as the virulent challenge strain. *S. dublin* 188, kindly supplied by Dr H. Williams Smith, Houghton Poultry Research Station, was used as a challenge strain in mice.

The bacteriological examination of specimens for salmonellas

Faeces and contents from the gastro-intestinal tract were cultured on Shigella-salmonella (SS) agar and SS agar + 1 % galactose before and after enrichment in selenite broth for 24 h at 37 °C. The plates were incubated for 24 h at 37 °C and then examined for the presence of salmonellas.

Gal E mutants produce colourless colonies on the SS agar + galactose and this characteristic was used in differentiation of vaccine (G30D) from the challenge strain.

One- to two-gram portions of the organs were macerated in nutrient broth with a Colworth Stomacher and 1 ml samples treated as above.

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Inoculation procedures

Vaccination

Mice. Each mouse received 0·2 ml intraperitoneally (i.p.) of an 18 h nutrient broth culture of G30D, each dose contained approximately 10^6 organisms.

Calves. Each calf received 5 ml subcutaneously (s.c.) of an 18 h nutrient broth culture of G30D, which had been diluted so that the number of organisms in a given dose ranged from $10^{5·5}$ to 10^9.

Control mice and calves received 0·2 ml and 5 ml sterile nutrient broth respectively.

Challenge

All animals were challenged 3 weeks after vaccination.

Mice. Decimal dilutions of 18 h nutrient broth cultures of *S. typhimurium* S2337 and *S. dublin* 188 were injected i.p. in 0·2 ml doses, so that the number of organisms in each dose varied from 10^4–10^7 and 10^3–10^6 respectively.

For oral dosing, *S. typhimurium* S2337 was either administered for 4 days in the drinking water, in which the number of organisms was approximately 10^6 per ml or given by stomach tube in 0·2 ml doses of 10^8 organisms.

Calves. Each calf was dosed orally after overnight starvation with 50 ml of nutrient broth in which the number of organisms per dose varied from 10^7–10^9. Five calves were used in a toxicity experiment, all received 10^9 G30D s.c. behind the elbow and were examined daily for 10 days.

Examination of animals after challenge

Mice. The experiments were terminated 21 days after challenge. The liver, spleen and intestine of all the mice, including those that died, were examined bacteriologically for the presence of vaccine and challenge strains.

Calves. Clinical examination and faeces collection were carried out daily in each calf. All calves were examined *post mortem* for the presence of pathological lesions and cultures were made from the gastro-intestinal tract (abomasum, small and large intestine), from a representative number of mesenteric and hepatic lymph nodes as well as from liver, spleen, kidney, parotid, lungs and tonsil.

RESULTS

Mice

Table 1 summarizes the three experiments in which G30D was used as a vaccine in mice. When the mice were challenged i.p. with 10^6 organisms or less of S2337 all the vaccinated mice except one survived challenge whereas the majority of the non-vaccinated died. In the case of oral challenge, good protection was observed in the vaccinated group in contrast to the non-vaccinated group. When the mice were challenged with *S. dublin* in doses of 10^4 or less only three of the vaccinated died in contrast to all of the non-vaccinated (Table 1). The difference between the mortality rates of vaccinated and non-vaccinated in the three experiments was very highly significant.

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Table 1. *Experiments with mice*

(All mice vaccinated intraperitoneally (i.p.) with 10^6 G30D and challenged 3 weeks later.)

Expt.	Challenge organism, dose, route	Vaccinated		Non-vaccinated	
		Mortality	Carriage rate of survivors	Mortality	Carriage rate of survivors
1	*S. typhimurium* intraperitoneally				
	10^7	6/10	4/4	10/10	—
	10^6	1/10	8/9	10/10	—
	10^5	0/10	9/10	9/10	1/1
	10^4	0/10	7/10	5/10	4/5
2	*S. typhimurium* 10^8 orally	1/25	23/24	22/25	2/3
	S. typhimurium drinking water 10^6/ml	2/25	20/23	23/25	2/2
3	*S. dublin* i.p.				
	10^6	7/7	—	5/5	—
	10^5	10/10	—	10/10	—
	10^4	1/10	9/9	10/10	—
	10^3	2/10	8/8	10/10	—

Mortality, Expts 1, 2 and 3 ($P < 0{\cdot}001$).

In all three experiments the majority of survivors became carriers of the challenge strains and the vaccine strain was also isolated by enrichment from the spleen and liver of seven mice challenged orally.

In groups of mice killed at weekly intervals after vaccination, G30D was isolated until experiments were terminated at 60 days. The stability of G30D was tested both by passage in mice and by daily transfer on laboratory media, but galactose fermenting mutants were not detected.

Calves

Toxicity of G30D

Five calves which received 10^9 G30D s.c. behind the elbow developed swellings at the site of inoculation which persisted for 7–14 days. All the calves showed pyrexia, which reached a peak of 40·5° C, 2–3 days after the injection, and which persisted 4–5 days. No signs of ill-health were observed, the calves remaining lively and eating their food. The calves were killed at weekly intervals after vaccination but G30D was not isolated from any of them.

Vaccination with G30D

Table 2 summarizes the use of G30D as a vaccine. The smallest dose ($10^{5{\cdot}5}$) of vaccine (Expt 1) provided no protection in terms of mortality. In Expts 2–5, where larger doses of vaccine were used, significant protection was obtained. Similarly, the number of calves showing clinical signs in the non-vaccinated group was significantly higher than in the vaccinated; in terms of the presence of clinical signs on

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Table 2. *Gal E mutant* S. typhimurium *used as a vaccine in calves*

		Vaccinated calves				Unvaccinated calves		
				Number of days on which:			Number of days on which:	
Expt	*S. typhimurium* challenge dose	Dose vaccine	Mortality	Salmonella isolated after challenge	Calves showed clinical signs	Mortality	Salmonella isolated after challenge	Calves showed clinical signs
1	10^9	$10^{5\cdot5}$	4/5	36	17 (5)	1/2	23	11 (2)
2	10^9	10^6	0/2	13	—	2/2	12	12 (2)
3	10^7	10^9	0/7	43	8 (4)	0/5	32	8 (3)
4	10^9	10^9	0/6	24	2 (1)	3/6	53	41 (6)
5	10^9	10^9	0/13	38	15 (5)*	2/12	87	46 (8)
Totals			4/33	154	42 (15)	8/27	207	118 (21)

Results

Mortality: Expts 2–5 ($P < 0{\cdot}01$); Expts 1–5 (N.S.).
No. of calves which showed clinical signs: Expts 2–5 ($P < 0{\cdot}01$); Expts 1–5 ($P < 0{\cdot}05$).
No. of calves showing clinical symptoms on more than 2 days: Expts 1–5 ($P < 0{\cdot}001$).

* Figures in parentheses indicate the number of calves affected.

more than 2 days there was a very highly significant difference between the two groups. In Expts 4 and 5, the difference between the two groups was highly significant in terms of the isolation of salmonella, but this was not observed in Expts 1–3. G30D was isolated from the faeces of 4 calves on one occasion only. In another calf, G30D was isolated for 8 days but this calf died of pneumonia 13 days after vaccination when G30D was recovered from liver, spleen and lungs at post-mortem.

When the calves were examined at autopsy, lesions of pyaemic nephritis were observed in 12 of 38 vaccinated calves but in only 2 of 27 non-vaccinated and the difference was significant. Salmonellas were not isolated from the kidneys. At post-mortem examination, the challenge strain was isolated by enrichment from lymph nodes of 4 vaccinated calves and 3 unvaccinated calves.

In Expt 5, the daily weight gain of the vaccinated did not differ significantly from the controls which survived challenge.

DISCUSSION

The experiments in mice showed that G30D produced good protection against challenge with a virulent strain of *S. typhimurium* but, in our experiments, both the vaccine and challenge strains persisted in the survivors. Germanier (1970), however, reported that the vaccine strain did not persist for more than 5 weeks and that the challenge strain was eliminated rapidly from vaccinated mice. He also considered that the time required for elimination may relate to the breed of mice because s.p.f. mice (F2 Charles River × BALB C) eliminated the vaccine strain more rapidly than ordinary JCR Swiss White Mice. Similarly, Robson & Vas (1972) and Plant & Glynn (1976) found that different breeds of mice differed in their susceptibility to salmonella infection. Thus the difference between the results of the present investigations and those of Germanier may relate to the breeds of mice used.

The role of cellular and humoral factors in the immunity produced by the vaccine has been studied in mice using immunosuppressive drugs (Morris, Wray & Sojka, 1976). These experiments demonstrated the importance of humoral factors in protecting mice against intra-peritoneal challenge with *Salmonella*. In calves vaccinated with G30D we were unable to demonstrate delayed hypersensitivity using lymphocyte migration tests (Morris, unpublished results) and these findings suggested that humoral factors are involved in the immunity of calves to salmonellosis.

Protection against *S. dublin* infection was observed when mice were vaccinated with G30D and Germanier (1972) observed protection against *S. enteritidis*. He suggested that this cross protection was based on a non-specific infection – immunity which resulted from the intracellular persistence of the vaccine. Smith & Halls (1966) found that the use of *S. dublin* vaccine in mice protected them against *S. typhimurium* infection. He suggested that the protection possessed some specificity because no protection was obtained when mice were challenged with *Erysipelothrix* and *Escherichia coli*. Although *S. dublin* and *S. typhimurium* share common antigens, Smith & Halls (1966) considered that these antigens were not involved in the immune process.

Smith (1965) also demonstrated that results obtained with mice are not necessarily capable of being extrapolated to another species. In our experiments doses of 10^6 of G30D produced protection in mice and calves. In calves, a highly significant difference in mortality was observed between the vaccinated and controls in Expts 2–5. Similarly in all the experiments a significant difference was observed in the number of calves in the 2 groups showing clinical signs. Adverse clinical reactions were not observed following vaccination but the incidence of pyaemic nephritis was higher than in the non-vaccinated. No salmonellas were isolated from the kidneys of any of these calves but it is possible that the lesions may have developed as a consequence of vaccination. *S. choleraesuis* infection in pigs has been shown to produce a Shwartzman reaction which is characterized by renal cortical necrosis (Lawson & Dow, 1964). However, glomerular thrombosis which is a feature of the Shwartzman reaction was not observed and the nephropathy had characteristics of a toxaemia and possibly hypersensitivity reaction (S. Terlecki, personal communication).

The experiments in calves showed that G30D produced significant protection and because the duration of clinical signs and excretion of salmonella may be reduced, its use would be likely to decrease the spread of salmonella. In Expt 5, calves purchased at a market were used and this experiment may be considered to provide some indication as to the vaccine's efficacy in the field. However, there appeared to be a relation between the vaccine and the presence of renal lesions and, before gal E mutants can be recommended, further work is necessary to determine the pathogenesis of these lesions.

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