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Reduction in faecal excretion of *Salmonella typhimurium* strain F98 in chickens vaccinated with live and killed *S. typhimurium* organisms

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SUMMARY

Chickens given orally at 4 days of age a smooth spectinomycin resistant mutant (Spc^r) of *Salmonella typhimurium* strain F98 excreted the organism in their faeces for approximately 4 weeks. Following oral administration of a nalidixic acid resistant (Nal^r) mutant of the same strain 4 weeks later when the chickens had virtually cleared themselves of the first infection, these chickens excreted far fewer salmonella organisms and for a shorter time than did a previously uninfected control group of chickens which were infected at the same time with the Nal^r mutant. Chickens inoculated intramuscularly at 4 days developed a similar immunity to challenge and also excreted the immunizing strain in their faeces. In contrast intramuscular inoculation or incorporation into the food of formalin-killed *S. typhimurium* organisms had little lasting effect on the faecal excretion of the challenge strain. Two attenuated mutants of strain F98 Nal^r were produced: one was a rough strain produced by lytic bacteriophage and the other was an *aro A* auxotrophic mutant which had been cured of the 85 kilobase-pair virulence-associated plasmid. These mutants were avirulent for chickens, mice, calves and man and when ingested by human volunteers did not persist in the faeces. When inoculated intramuscularly into chickens they produced an early reduction in faecal excretion of the challenge strain (Spc^r) which was not maintained. Oral administration of both strains produced reductions in faecal excretion of the challenge strain. This was much more noticeable with the rough strain which was itself excreted for a much longer period than the parent strain.

INTRODUCTION

The major sources of food-poisoning salmonella serotypes for poultry are thought to be infected feed and the poultry themselves [1]. Birds within a flock may become infected by the ingestion of faeces or contaminated litter or water containing salmonella organisms from other birds. Infection may also occur early

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in the chick's life by vertical transmission either from an infected ovary or oviduct or during passage of the egg through the cloaca [1]. It follows that increasing the immunity of chickens to oral infection with salmonella should reduce both horizontal and vertical transmission.

There is little evidence that vaccination of poultry can produce significant reductions in faecal excretion of salmonella organisms following challenge. In contrast, a considerable literature exists to show that following infection with a serotype that characteristically produces systemic disease, or with a vaccine strain derived from that serotype, a variety of animal species develop a strong immunity against reinfection. Live, attenuated or cell-subunit vaccines have been shown to be effective against *S. typhi* in man (2), *S. gallinarum* in chickens [3], *S. dublin* in cattle and *S. cholerae-suis* in pigs [4] and *S. typhimurium* and *S. enteritidis* in laboratory mice [5, 6].

In poultry the salmonella serotypes which cause food-poisoning in man are generally confined to the alimentary tract and rarely produce systemic disease unless very young chicks are infected. The evidence that vaccines can substantially reduce faecal shedding is not convincing. As with systemic disease [3], little effect was obtained by using killed bacteria [7, 8] although a reduction in faecal shedding of a number of serotypes was apparently produced by incorporating sonicated, lyophilized preparations from the homologous strains in the food [9]. Better protection appears to arise from immunizing with a live strain of the homologous serotype. Thus *galE* and streptomycin-requiring mutants of *S. typhimurium* can produce small reductions in faecal shedding of *S. typhimurium* under experimental conditions [10–12]. By contrast the live *S. dublin* 51 vaccine used in chickens protected against systemic multiplication of *S. typhimurium* but had no effect on faecal excretion [13, 14]. Unfortunately the *galE* vaccine of *S. typhi* retains some virulence for man [15] which must raise questions about the degree of attenuation of other *galE* vaccines.

Most work has been carried out with *S. typhimurium*, one of the more invasive serotypes for chickens [16, 17], strains of which, however vary considerably in their invasiveness [18]. Recent evidence suggests that more invasive salmonella strains are shed in the faeces for a shorter time than are less invasive strains, possibly as a result of the greater immunological stimulation arising from systemic dissemination [19]. As a practical corollary to this, chickens which have eliminated infection with an invasive strain of *S. typhimurium* should be relatively resistant to reinfection with the homologous strain. This paper presents the results of experiments which test this hypothesis using a strain of *S. typhimurium* F98 which is highly invasive for chickens [18, 19] and whose faecal excretion patterns have been well characterized [16, 19–23]. Results describing the protective effects and virulence of two attenuated mutants derived from strain F98 are also presented.

MATERIALS AND METHODS

Bacterial strains

The strain used throughout this study was *S. typhimurium* F98 which had been maintained at this Institute in the lyophilized state for a number of years. Broth cultures were made in 10 ml nutrient broth (Oxoid CM67) incubated for 24 h at

37 °C in a shaking water bath (100 strokes per min.). Such cultures contained between 8×10^8 and 2×10^9 c.f.u. per ml.

To kill bacteria, 0·2 ml 40% formaldehyde solution was added to a 10 ml broth culture or to 10 ml of a thick suspension in nutrient broth of bacteria obtained by harvesting the surface of 50 nutrient agar plates. Both suspensions were left at ambient temperature for 8 h and then at 4 °C overnight. The broth culture was centrifuged at 1500 g for 30 min and the pellet resuspended in fresh nutrient broth. A 0·1 ml aliquot was withdrawn and cultured in nutrient broth to check for sterility. The thick suspension also tested for sterility was air dried at 37 °C and mixed with a small amount of food with a pestle and mortar before being added to larger quantities of feed.

Production of bacterial mutants

Mutants resistant to nalidixic acid and spectinomycin. These were produced as described previously [16]. These authors showed that nalidixic acid resistant (Nal^r) mutants of *S. typhimurium* strains including F98 were as virulent for chickens as were the antibiotic sensitive parent strains. In preliminary experiments (unpublished) we have also determined that the spectinomycin resistant (Spc^r) mutant of strain F98 is also as virulent as the strain from which it was derived.

Production of a mutant cured of its virulence-associated plasmid and containing an aro A *deletion mutation.* The virulence-associated plasmid of strain F98 Nal^r was 'tagged' with transposon Tn *3* and cured by incubation at high temperature as described previously [19]. The *aro A* mutation was introduced into this strain by transduction with the high transducing frequency bacteriophage P22 HT *int* from *S. typhimurium* LT2 *aro A 554*:: Tn *10* kindly provided by Dr G. Dougan of Wellcome Biotechnology, Beckenham, Kent. The method was basically that described by Davis and colleagues [24]. In brief, the P22 phage was added to a broth culture of the LT2 strain at a multiplicity of infection (moi) of 0·05 which was reincubated for 24 h at 37 °C in an orbital shaking incubator. Bacteria were removed by centrifugation at 1500 g for 30 min and the supernatant filtered through a 0·45 μm pore size membrane filter (Millipore). After counting the phage preparation thus obtained it was added at a moi of 0·8 to a broth culture of the plasmid-cured mutant of F98 Nal^r which had been concentrated tenfold by centrifugation. After incubation with shaking at 37 °C for 30 min, the mixture was diluted 1 in 100 in phosphate buffered saline (PBS) and plated on Tryptose agar (Difco) containing ethyleneglycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetracetic acid (EGTA), 10 mM and tetracycline, 5 μg/ml. Colonies thus obtained were purified and checked for resistance to nalidixic acid. Their requirement for aromatic amino acids and *p*-amino benzoic acid (PABA) was tested by plating on minimial medium [25] containing glucose, 0·5%; PABA, 100 μg/ml and 2,3-dihydroxybenzoic acid, 100 μg/ml which either contained or did not contain tryptophan, phenylalanine and tyrosine each at 100 μg/ml. A deletion mutation was then produced at the point of insertion of the Tn *10* by positive selection for tetracycline sensitivity as described by Bochner and colleagues [26]. The mutant produced by this method was designed F98 *aro A*.

Rough (phage resistant) mutant. Spontaneous rough mutants of strain F98 Nal^r

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were selected by their resistance to lytic bacteriophage obtained from sewage as described by Barrow and co-workers [19]. The strain thus produced was designated F98 Nal^r ϕ^r.

Chickens

Unsexed Light Sussex chickens obtained from a salmonella-free flock maintained at this Institute were used. Their rearing conditions and feed have been described previously [20]. Groups of experimental chickens were housed in separate rooms to prevent cross-contamination.

Virulence of mutants

The invasiveness for Vero cells of the parent strain F98 and the mutants F98 *aro A* and F98 Nal^r ϕ^r were tested as described previously [27].

Groups of 20 newly hatched chickens were given orally 0·1 ml of broth cultures of the parent strain or its mutants. Mortality was recorded over a period of 3 weeks.

Ten female Balb/c mice, approximately 6 weeks old, were given orally under anaesthesia 0·05 ml of a broth culture of the parent strain which had been centrifuged so that it contained approximately 10^{10} c.f.u./ml.

Two 3-day old male Friesian–Guernsey cross calves, which had received colostrum, were given orally 10 ml of a broth culture of the parent strain.

Faecal samples were obtained from four informed and consenting human volunteers for 3 days prior to ingestion of 10^8 organisms in 10 ml nutrient broth of F98 *aro A* or F98 Nal^r ϕ each mutant being taken by two of the volunteers. Following ingestion, bacteriological examination of the faeces was again carried out for several days. The general state of health and body temperature were also recorded.

Experimental design for vaccination experiments

In the first experiment, two groups of 31 and 29 chickens at 4 days of age were either given 10^8 organisms in 0·1 ml orally or 10^5 organisms in 0·1 ml by inoculation into the gastrocnemius muscle in each case with live cultures of *S. typhimurium* F98 Spc^r. Two similarly sized untreated control groups were also included in the experiment. These latter groups were housed separately and cloacal swabs were taken and cultured immediately prior to challenge to ensure that they were free of infection. When the rate of faecal excretion of F98 Spc^r in the infected chickens fell to low levels, all four groups of chickens were challenged orally with 10^8 organisms in 0·1 ml of F98 Nal^r. The size of the challenge dose was the same in all subsequent experiments.

In a second experiment two groups of 29 and 28 chickens also at 4 days of age were given either by mouth *via* the food or by the intramuscular route formalin-killed organisms of F98 Spc^r. Chickens given the organisms in the food received the equivalent of 10^8 salmonella organisms/g from 4 days of age for 5 days and 10^7/g for the remaining 33 days. Chickens were inoculated intramuscularly at 4 and 18 days of age with the 10^8 killed organisms in 0·1 ml. Two untreated groups were again included in the experiment. All four groups were challenged orally with F98 Nal^r 38 days after the initial immunization.

In a third experiment two groups of 29 chickens were inoculated at 4 days of age intramuscularly with 10^5 live F98 *aro A* or F98 Nal^r ϕ^r in 0·1 ml and reinoculated at 18 days of age with 10^8 organisms in 0·1 ml. These and an additional group of 28 uninoculated chickens were challenged orally 2 weeks later with F98 Spc^r.

In a final experiment two groups of 31 chickens at 4 days of age were given 10^8 organisms in 0·1 ml of F98 Nal^r ϕ or F98 *aro A* orally. When the rate of faecal excretion of the strain had fallen to low levels they and an untreated control group of 31 chickens, were challenged orally with F98 Spc^r.

Enumeration of bacteria in faeces

The faecal excretion of salmonella strains by chickens was assessed by the semi-quantitative method of Smith and Tucker [20]. This has been used on a number of occasions [16, 21, 23, 28] and found to give a consistent estimation of bacterial excretion. The brilliant green agar (Oxoid CM263) used contained either nalidixic acid, 20 μg/ml and novobiocin, 1 μg/ml or spectinomycin, 30 μg/ml. Statistical comparisons of rates of faecal excretion were carried out with a modified t test by Bayesian analysis [29].

In the human volunteer experiment faecal samples were processed within 2 h of collection. The numbers of coliforms and ingested salmonella mutants were counted using the method of Miles, Misra and Irwin [30], counting bacteria on brilliant green agar containing nalidixic acid and novobiocin and on MacConkey agar (Oxoid CM7).

Serum agglutination

In some experiments chickens were bled for the detection of serum agglutinins. They were bled from the wing vein immediately prior to challenge and after allowing the blood to clot the serum was removed and frozen at −20 °C until used. The bacterial suspension was obtained by resuspending the pellet from a centrifuged broth culture of F98 Nal^r in 0·5 ml PBS. One drop each of serum and bacterial suspension were mixed on a glass slide and the occurrence of agglutination within 1 min was recorded.

RESULTS

The effect of oral administration and intramuscular inoculation with live organisms on reinfection with the homologous strain

The results of examining the faeces of chickens which had been infected orally or intramuscularly with F98 Spc^r and reinfected orally with F98 Nal^r are shown in Table 1.

F98 Spc^r was excreted in the faeces of chickens infected by either route. The rate of excretion in chickens treated orally was initially high but decreased quickly so that at the time of challenge only two chickens were still excreting. Considerable faecal excretion was also observed after the first week in the chickens inoculated intramuscularly and at the time of challenge four chickens were still excreting F98 Spc^r. Following challenge the previously uninfected control chickens excreted F98 Nal^r for a period of 3–4 weeks but fewer chickens excreted a smaller number of salmonella organisms than occurred with F98 Spc^r at the beginning of the

Table 1. *The effect of oral administration or intramuscular inoculation of chickens with live* S. typhimurium *on the faecal excretion (percentage of chickens) of* salmonella *organisms following reinfection with the same strain*

Days of age		Oral immunization with: Nothing ⩾50	D	T†		Oral immunization with: Live F98 Spc^r ⩾50	D	T	P* value		After intramuscular immunization with: Nothing ⩾50	D	T		After intramuscular immunization with: Live F98 Spc^r ⩾50	D	T	P value
11		0	0	0	△	19	87	94			0	0	0	△	0	10	21	
18		0	0	0		6	52	55			0	0	0		17	48	59	
25		0	0	0		3	10	16			0	0	0		10	21	48	
32	▶	0	0	0	▶	0	0	6			0	0	0		3	7	21	
39		0	43	53		3	3	3	< 0·01	▶	0	0	0	▶	3	7	14	
46		0	35	48		0	0	0	< 0·01		0	0	20		0	0	0	0·023
53		0	8	15		0	0	0	~ 0·05		3	20	53		0	0	3	< 0·01
60		0	0	0		0	0	0			0	7	30		0	0	0	< 0·01
67			.....‡				.....				0	3	3		0	0	0	
74			.....				.....				0	0	0		0	0	0	

△ Immunizing infection. Chickens infected orally at 4 days of age with 10^8 organisms of *S. typhimurium* F98 Spc^r. Chickens infected intramuscularly into gastrocnemius muscle at 4 days of age with 10^5 organisms of F98 Spc^r. Bacterial enumeration made on brilliant green agar containing spectinomycin.

▶ Challenge infection. Chickens infected orally with 10^8 organisms of *S. typhimurium* F98 Nal^r at day 32. Bacterial enumeration made on brilliant green agar containing nalidixic acid.

Serum agglutinins present at 28 days in immunized chickens.

* P value of difference between T values of treated and control groups.

† ⩾50, ⩾50 colonies of *S. typhimurium* grew on the culture plate; D, *S. typhimurium* isolated by direct culture; T, *S. typhimurium* isolated by selenite enrichment or by direct culture.

‡ ... No samples taken.

experiment. When compared with the control groups the rate of excretion of F98 Nal^r in the chickens which had previously been infected with F98 Spc^r was very much lower, the differences being statistically highly significant. In the immunized groups small numbers of salmonella organisms were excreted by one bird only in each group and then on only one occasion.

No serum agglutinins were found in 10 chickens taken from each of the control groups at challenge when they were 32 days of age whereas all 10 of the chickens from both of the groups infected with F98 Spc^r contained agglutinins.

The effects of oral or intramuscular immunization with formalin-killed organisms on infection with the homologous strain

The results of examining the faeces of chickens which had either been given formalin-killed organisms of F98 Spc^r in their food or by intramuscular injection followed by oral challenge with live organisms of F98 Nal^r are shown in Table 2.

No isolations of F98 Spc^r were made during the period of immunization. Small reductions which were statistically significant on one occasion in the number of chickens excreting F98 Nal^r after in-feed immunization were observed at 14 days and 21 days after challenge. However, when compared with the reduction in excretion rate in the experiments with live organisms the differences were small. The patterns and rates of faecal excretion of F98 Nal^r in the chickens inoculated intramuscularly with F98 Spc^r were very similar to those in its control group.

The effect of intramuscular inoculation with attenuated strains of S. typhimurium *F98 Nal^r on infection with F98 Spc^r*

The effects of inoculating chickens intramuscularly with the attenuated strains of *S. typhimurium* F98 *aro A* or F98 Nal^r ϕ^r on the faecal excretion of F98 Spc^r given later orally are shown in Table 3.

Strain F98 *aro A* was detected in the faeces of a small number of the inoculated chickens and was still isolated from one at the time of challenge. Serum agglutinins were found in all 10 chickens examined at this time. Strain F98 Nal^r ϕ was not isolated from the faeces and no serum agglutinins were found in the 10 chickens examined.

When compared with the control group, both groups of pre-inoculated chickens showed a statistically significant reduction in faecal excretion of F98 Spc^r soon after challenge. However, in both cases the challenge strain persisted in the faeces of a small number of chickens beyond the point at which it had been eliminated by the control group.

The effect of oral administration of S. typhimurium *strains F98 Nal^r ϕ or F98* aro A *on infection with a Spc^r mutant of the homologous strain*

The results of examining the faeces of chickens inoculated orally with either *S. typhimurium* strain F98 Nal^r ϕ^r or F98 *aro A* followed later by challenge of these and of control groups of chickens with F98 Spc^r are shown in Table 4. Compared with the smooth strains examined, the rough mutant, F98 Nal^r ϕ^r, was excreted in the faeces for 6 weeks longer, a difference that has been observed previously [19]. Following challenge there was a great difference between the rates of excretion of F98 Spc^r in the control and immunized groups. The differences were

Table 2. *The effect of oral administration or intramuscular inoculation of chickens with formalin killed* S. typhimurium *organisms on faecal excretion (percentage of chickens) of* salmonella *organisms following challenge with the homologous strain*

Days of age	After 'in-feed' immunization with: Nothing ⩾ 50	Nothing D	Nothing T†	Killed F98 Spc^r ⩾ 50	Killed F98 Spc^r D	Killed F98 Spc^r T	*P** value	After intramuscular immunization with: Nothing ⩾ 50	Nothing D	Nothing T	Killed F98 Spc^r ⩾ 50	Killed F98 Spc^r D	Killed F98 Spc^r T
11	0	0	0	0	0	0		0	0	0	0	0	0
18	0	0	0	0	0	0		0	0	0	0	0	0
25	0	0	0	0	0	0		0	0	0	0	0	0
32	0	0	0	0	0	0		0	0	0	0	0	0
39	0	0	0	0	0	0		0	0	0	0	0	0
46	0	0	20	0	0	14		0	29	71	0	43	64
53	0	24	56	0	3	17	0·012	0	18	29	0	11	18
60	0	7	36	0	0	14		0	7	18	0	0	7
67	0	3	6	0	3	7			.....‡			.....	
74	0	0	0	0	0	7			.....			.....	
81	0	0	0	0	0	0			.....			.....	

▷ Immunizing inoculation. Chickens immunized 'in-feed' by feeding formalin killed *S. typhimurium* F98 Spc^r in feed at 10^8/gm from 4 days of age for 5 days and at 10^7/g for remaining period up to 42 days. Chickens inoculated intramuscularly into gastrocnemius muscle with 10^8 formalin killed organisms of F98 Spc^r at 4 and 18 days of age. Bacterial enumeration made on brilliant green agar containing spectinomycin.

▶ Challenge infection 38 days after initial immunization. See Table 1.

* See Table 1.

† See Table 1.

‡ ... Samples not taken.

Table 3. *The effect of intramuscular inoculation with attenuated strains of* Salmonella typhimurium *on the faecal excretion (percentage of chickens) of* salmonella *organisms following challenge with the parent strain*

Days of age		After intramuscular immunization with: Nothing ≥ 50	Nothing D	Nothing T†		F98 *aro A* ≥ 50	F98 *aro A* D	F98 *aro A* T	*P** value		F98 Nal^r ϕ^r ≥ 50	F98 Nal^r ϕ^r D	F98 Nal^r ϕ^r T	*P** value
					▷					▷				
11		0	0	0		0	0	0			0	0	0	
18		0	0	0	▷	0	0	3		▷	0	0	0	
25		0	0	0		0	0	7			0	0	0	
32	▶	0	0	0	▶	0	0	3		▶	0	0	0	
39		0	26	69		0	7	17	< 0·001		3	7	14	< 0·001
46		0	18	32		6	6	6	0·041		0	0	0	0·002
53		0	3	15		3	3	7			3	3	10	
60		0	3	3		3	7	10			0	3	7	
67		0	0	0		3	3	3			3	3	7	
74		0	0	0		0	0	0			0	0	3	

▷ Immunizing infection. Chickens inoculated into the gastrocnemius muscle at 4 and 18 days of age with 10^5 and 10^8organisms respectively. Bacterial enumeration made on brilliant green agar containing nalidixic acid.

▶ Challenge infection. Chickens given orally 10^8 organisms of *S. typhimurium* F98 Spc^r. Bacterial enumeration made on brilliant green agar containing spectinomycin.

* See Table 1.

† See Table 1.

At 28 days serum agglutinins detected in chickens inoculated with F98 *aro A* but not in chickens inoculated with F98 Nal^r ϕ^r.

Table 4. *The effect of oral administration to chickens of the attenuated* Salmonella typhimurium *strain F98 Nal^r ϕ^r and F98* aro A *on the faecal excretion (percentage of chickens) of salmonella organisms following challenge with a Spc^r mutant of the parent strain*

Days of age	After oral immunization with: Nothing ⩾ 50	Nothing D	Nothing T		After oral immunization with: F 98 Nal^r ϕ^r ⩾ 50	F 98 Nal^r ϕ^r D	F 98 Nal^r ϕ^r T	*P* value	After oral immunization with: Nothing ⩾ D	Nothing D	Nothing T		After oral immunization with: F98 *aro A* ⩾ 50	F98 *aro A* D	F98 *aro A* T
				△								△			
11		.....			17	53	70			.....			17	60	97
18		.....			13	40	62			.....			20	33	63
25		.....			7	27	51			.....			0	7	10
32		.....			3	17	43			.....			0	7	17
39		.....			0	10	37		0	0	0		0	3	13
46		.....			0	7	30		▶ 0	0	0	▶	0	0	7
53		.....			0	13	26		27	47	77		0	10	47
60	0	0	0		0	7	17		3	7	33		0	0	10
67	▶ 0	0	0	▶	0	7	17		0	0	10		3	3	13
74	37	77	93		0	0	17	< 0·001	0	0	3		0	3	7
81	13	37	64		0	0	0	< 0·001	0	0	3		0	0	7
88	7	13	27		0	0	0	0·05		.....				.....	
95	0	0	7		0	0	0			.....				.....	
102	0	0	0		0	0	0			.....				.....	

△ Immunizing infection. Chickens given orally at 4 days of age 10^8 organisms of *S. typhimurium* F98 Nal^r ϕ^r or F98 *aro A*. Bacterial enumeration made on brilliant green agar containing nalidixic acid.

▶ Challenge infection. See Table 3.

At challenge serum agglutinins detected in chickens given F98 *aro A* but not in chickens F98 Nal^r ϕ^r.

For meaning of other symbols, see Table 1.

Table 5. *The viable count of coliform and* salmonella *organisms in the faeces of four human volunteers*

Days after ingestion of attenuated salmonella	Log_{10} viable count per g following ingestion of F98 *aro A* by Volunteer 1 Mc*	BG†	Volunteer 2 Mc	BG	F98 Nal^r ϕ^r by Volunteer 3 MC	BG	Volunteer 4 MC	BG
−3	4·9	...	4·5	...	8·2	...	4·5	...
−2	6·1	...	7·3	...	8·2	...	6·8	...
−1	5·2	N	6·9	N	7·2	N	2·9	N
0	7·4	N	7·1	N	6·2	N	2·0	N
+1	5·6	2·0	8·6	3·8	6·5	3·1	3·0	N
+2	6·3	N	6·4	N	8·2	4·5	4·5	N
+3	6·2	N	8·3	N	6·7	2·3	5·0	N
+4	6·1	N	7·4	N	6·7	N	4·5	N
+5	...	...	...	...	7·5	N	4·8	N

* Viable count of lactose fermenters on MacConkey agar.

† Viable count of non-lactose fermenters on brilliant green agar containing nalidixic acid and novobiocin.

N. < 2·0.

All four volunteers remained completely healthy.

statistically highly significant. F98 Spc^r was isolated from a small number of immunized chickens for 1 week only. No serum agglutinins were found in the 10 chickens bled at the time of challenge. By contrast F98 *aro A* produced reductions in the incidence of faecal excretion soon after the challenge which were not statistically significant but the challenge strain persisted in the faeces of both immunized and control groups at similar frequencies. Serum agglutinins were present in the immunized chickens at challenge.

Virulence of attenuated strains of F98 Nal^r

The invasiveness of *S. typhimurium* strains was assessed by quantitating the recovery of bacteria from a Vero cell monolayer following lysis [27]. The log_{10} counts of F98 Nal^r, F98 *aro A*, F98 Nal^r ϕ^r and a non-invasive *Escherichia coli* K12 strain recovered were 5·4, 5·4, 5·0 and < 2·0 respectively. The two mutants were therefore as invasive as the parent strain.

When introduced orally into newly hatched chickens, F98 Nal^r produced 90% mortality after 3 weeks. By contrast both F98 *aro A* and F98 Nal^r ϕ^r produced no mortality or any signs of morbidity. Strain F98 Nal^r produced no deaths or morbidity when given to 10 Balb/C mice and two calves orally. The attenuated mutants were not tested in these animals.

The results of examining the faeces of human volunteers before and after ingestion of F98 *aro A* or F98 Nal^r ϕ^r are shown in Table 5. All four volunteers remained healthy with normal temperatures and faecal consistency. In all cases the coliform counts, identified as lactose fermenting colonies on MacConkey agar, were variable. Following ingestion, F98 *aro A* was isolated from the faeces in small numbers for one day only and strain F98 Nal^r ϕ^r from one volunteer only on three occasions.

DISCUSSION

These experiments indicate that experimental infection of 4-day-old chickens with the invasive *S. typhimurium* strain F98 produced an immunity to reinfection via the oral route which can be easily demonstrated once the chickens have eliminated the immunizing strain. It was unclear whether oral or intramuscular immunization conferred greater protection on the chickens since the immunizing organisms were excreted in the faeces of both groups of chickens. Strain F98 is highly invasive for chickens [18] and persists in the faeces for several weeks [16, 20, 21]. It is thus likely that both secretory and systemic immunity are induced following either route of introduction but the contribution of either to faecal clearance is unknown. The ability to produce such a profound reduction in faecal excretion of the challenge strain suggests that immunity may also be at least partially responsible for clearance of the first strain and thus by implication all naturally occurring salmonella infections.

It is clear that, as has previously been reported for fowl typhoid [3] and for disease-free intestinal carriage of salmonella [7, 8], killed bacteria do not evoke as strong a protective response as do live cells. Whether this is because relevant antigens are destroyed during preparation of the bacteria or because persistent presentation of the antigen on actively multiplying bacterial cells is essential for stimulation is unclear.

We were able to stimulate good responses with the parent strain and with one of the mutants (F98 Nal^r ϕ^r) attenuated in the laboratory. The mutant stimulated a good protective response following oral introduction but was less effective by the parenteral route. The protection it afforded was as good as that reported previously for a *gal E* mutant of *S. typhimurium* [10, 12]. It is unclear why F98 *aro A* produced a poorer immunity by the oral route since it was excreted in the faeces for several weeks and was invasive *in vitro*.

Strain F98 itself is not virulent for mice or calves by the oral route and the two attenuated mutants were not virulent for chicks or for human volunteers. Both mutants were still invasive for Vero cells. Later results to be published elsewhere indicate that the mutants did not persist in the tissues of infected chickens for longer than a few weeks. Nevertheless both strains were excreted for some time in the faeces and F98 Nal^r ϕ^r persisted for many weeks as reported previously [19]. This is unsatisfactory from a practical view point because salmonella organisms isolated from the faeces of poultry in the field must be reported under the Zoonoses Order [31]. Therefore should live attenuated vaccines be used to reduce faecal excretion in poultry in the future it is essential that some means are available to differentiate easily the vaccine and field strains. Rough strains are occasionally encountered in the field but the Nal^r marker should make differentiation relatively easy since resistance to this antibiotic is very rare amongst natural poultry isolates from the United Kingdom [32]. Other drug resistance markers could also be introduced into these strains to create double mutants. Since the rough strain did not stimulate the production of serum agglutinins this would allow vaccinated chickens to be differentiated from naturally infected birds. Whether or not such a strain would stimulate the production of antibodies distinguishable from those stimulated by a field strain when tested in a more sensitive system such as the ELISA remains to be demonstrated.

Because of the duration of faecal excretion of F98 Nal^r ϕ^r it might be more useful used for vaccinating breeder and laying chickens rather than broilers. However, considerably more work is required on the efficacy of the mutant before that stage is reached. Such experiments might include tests for the duration of immunity, cross-protection against other serotypes and effect on vertical transmission. Cross protection against other strains and serotypes could pose a problem since many strains of *S. typhimurium* are less invasive for chickens [18] and might not stimulate a strong secondary response and the *Salmonella* genus is antigenically heterogeneous. It is clear that progress on a comprehensive vaccine for salmonella for poultry will be slow without fundamental work on the basis of colonization and the antigenic determinants involved. Further tests on the stability of the attenuated strains will be required. This should pose no problems since the *aro A* mutation is a deletion mutation and although the nature of the rough mutation is unknown, other rough strains such as the *S. gallinarum* 9R vaccine [3] have been used for many years without reverting to virulence. In future it should be possible to attenuate salmonella strains safely by deleting the gene(s) for toxigenicity. It is unlikely that invasiveness would be one of the characteristics to be eliminated since for immunization by the oral route to be effective, invasion is probably necessary in order to stimulate systemic and secretory immune responses.

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