Epidemiol. Infect. (1990), **104**. 427–441
Printed in Great Britain

Further studies on the inhibition of colonization of the chicken alimentary tract with *Salmonella typhimurium* by pre-colonization with an avirulent mutant

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(*Accepted 3 January 1990*)

SUMMARY

Oral administration to newly hatched chickens or to chicks up to 5 days of age with an avirulent, rough, spectinomycin-resistant mutant of *Salmonella typhimurium* strain F98 inhibited the colonization of a nalidixic acid-resistant mutant of the same strain administered by the same route 1 day later. The second strain passed rapidly through the alimentary tract and persisted in the caeca of only a few chickens. Resistance to colonization did not develop until 24 h after inoculation of the first strain but was still evident if the second strain was inoculated up to 7 days later. Resistance occurred in 5 different breeds of chicken and in chickens reared on 5 different diets. Protection was evident against a very high challenge dose and could be produced by the introduction of small numbers of the first strain. Pre-colonization of chicks with the first strain of F98 reduced faecal excretion of the second strain over many weeks, whether chickens were challenged directly or by contact with other infected chickens. The rough strain F98 produced protection against only a few *S. typhimurium* strains and not against other serotypes. However, strains of *S. infantis* and *S. heidelberg*, chosen because they colonized the chicken alimentary tract better than did F98, produced inhibition of a wider range of serotypes.

INTRODUCTION

Newly hatched chickens are highly susceptible to infection with salmonella organisms. Chickens infected at this time with salmonella serotypes characteristic of human food-poisoning excrete organisms in the faeces in greater numbers and for longer than do chickens infected when they are older [1–3]. This increase in resistance is explained by the gradual acquisition by the chickens of the micro-organisms that constitute its normal intestinal microflora [4–7]. The full complement is not reached until a few weeks after hatching, after which the chicken is as fully resistant as it will become without immunization.

Attempts to increase the resistance of the newly hatched chick to a level observed in the older chicken have centred on the administration of cultures or

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suspensions of caecal contents or faeces obtained from healthy adult chickens. Very soon after treatment with such material the chicks acquire the same degree of resistance to colonization by salmonella shown by the adult bird (for review see Mead and Impey [8]). To avoid the possibility of inadvertent introduction of avian pathogens with such material, mixtures of pure cultures of the appropriate micro-organisms can be used [9].

Searches for individual strains of protective bacteria have been largely unsuccessful. There have been reports of protection produced against salmonella by single strains of clostridium [10] or *Streptococcus faecalis* [11] although the latter study could not be reproduced by others [12, 13]. Barrow and Tucker [14] looked for an organism which possessed the colonization characteristics but not the virulence attributes of salmonella. They could not find an individual protective strain from a collection of over 600 strains of *Escherichia coli* or from 109 faecal and environmental samples, although three strains of *E. coli* administered simultaneously produced partial protection.

Barrow, Tucker and Simpson [15] found that intestinal colonization by *S. typhimurium* F98 could be virtually prevented by pre-colonization with a mutant of F98 or by other salmonella strains. They found that this inhibition was not the result of immunity or bacteriophage activity and that a homologous inhibition occurred between strains of other genera. Since there are now ways of introducing irreversible mutations into salmonella which reduce their virulence considerably [16–18] it seemed worthwhile exploring further the feasibility of using this genus-specific inhibition of colonization to protect chickens against salmonella infection. Immunity to salmonellosis can be increased by vaccination [19] but full immunity takes time to develop. The use of a single organism to protect chicks not only in the first few days of life, by colonization inhibition, but later, by immunization, would be an advantage. This study investigates the kinetics of the inhibition of colonization during the first weeks of life of the chicken.

MATERIALS AND METHODS

Chickens

Four different strains of chickens from salmonella-free flocks were used. These comprised strains of the Light Sussex, Rhode Island Red, White Leghorn and Brown Leghorn breeds. A commercial broiler hybrid was also used. Rearing conditions and diet have been described elsewhere [20]. The diet was based on wheat, maize and fish meal except for one experiment where four commercial diets were also used.

Bacterial strains

In most inhibition experiments, mutants of *Salmonella typhimurium* strain F98 (phage type 14) were used [15]. The inhibitory strain was a rough, spectinomycin-resistant (Spc^r) mutant designated F98 Spc^r ϕ^r which was avirulent for chickens. The rough mutant of F98 was prepared by selecting for a bacteriophage resistant mutant using a phage isolated from sewage as described by Barrow, Simpson and Lovell [3]. This was made Spc^r, while the fully virulent strain was made resistant to nalidixic acid (Nal^r), by the method of Smith and Tucker [2].

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In one experiment formalin-killed organisms of F98 Spc^r and an *aro A* mutant of F98 Nal^r were tested for inhibitory activity. Their production is described elsewhere [19]. Because the *aro A* strain was Nal^r the challenge strain in this case was a double mutant, F98 Nal^r Spc^r.

Other strains of *S. typhimurium* and other *Salmonella* serotypes used to challenge chickens are listed in Table 5.

All bacterial strains were maintained on Dorset egg slopes at 4 °C. Broth cultures were prepared as described elsewhere [19].

Experimental design

Several experiments were carried out to investigate the inhibition under different conditions. In most cases these comprised minor variations in a standard procedure [15]. The basic experiment involved the oral administration of 10^8 organisms in 0·1 ml of the inhibitory strain to be tested to groups of 10 chickens within 24 h of hatching. One day later the chickens were given orally 10^5 challenge organisms in 0·1 ml. The chickens were usually killed after a further 3 days and the number of viable organisms of the challenge strain per gram of the caecal contents were estimated. For all experiments appropriate control groups were included.

The plans of the different experiments carried out are shown in Table 1. The experiments are described in brief below.

In experiment 1a the viable counts of both the inhibitory strain (*S. typhimurium* F98 Spc^r ϕ^r) and the challenge strain (F98 Nal^r) were made for samples taken from the crop, jejunum and caecum during the 3 days post-challenge to investigate if possible the mechanism of inhibition. In experiment 1b the inhibitory strain was F98 Nal^r and the challenge strain was F98 Spc^r ϕ^r. The experiment was performed to ensure that inhibition of F98 Nal^r by F98 Spc^r ϕ^r was not simply a reflection of their respective mutations.

Experiments 2a and 2b were designed to test the effects of varying the breed of chicken or the type of feed.

Experiments 3a and 3b examined the inhibitory effects of the *aro A* mutant of F98 Nal^r described by Barrow, Hassan and Berchieri [19] and of incorporating formalin-killed organisms in the feed during the course of the experiment.

The effect of varying the time (age of chicken) of administration of either the inhibitory strain or the challenge strain were tested in experiments 4a and 4b.

Experiments 5a and 5b tested the effect of varying the dose level of either the inhibitory or challenge strains.

In experiment 6 the effects of using different challenge strains were examined. Nal^r mutants of 9 strains of *S. typhimurium* and 8 strains of 7 other serotypes were tested (see Table 6 for strains used).

Experiment 7 looked at the inhibitory effect of a strain which colonized the alimentary tract of the chicken much better than did *S. typhimurium* F98. *S. infantis* 1326/28 Spc^r was selected for this purpose [3]. The challenge strains (7 serotypes) are shown in Table 6.

Since F98 did not inhibit *S. enteritidis* well a Spc^r mutant of *S. enteritidis* was tried for its inhibitory effect against Nal^r mutants of four other *S. enteritidis* strains in experiment 8.

In experiments 9a and 9b the inhibitory effect of F98 Spc^r ϕ^r on the prolonged

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Table 1. *Experimental plan*

Experiment no.	Breed of chicken (and diet)	Primary (inhibitory) infection		Secondary (challenge) infection		Bacteriological analysis
		Strain	Dose	Strain	Dose	
1a	Light Sussex (Standard diet)	F98 Spc^r ϕ^r	10^8 c.f.u. in 0·1 ml orally at < 24 h of age	F98 Nal^r	10^5 c.f.u. in 0·1 ml orally 24 h after first strain	Viable counts of F98 Nal^r and F98 Spc^r ϕ^r in crop, jejunum and caecal contents at 0–72 h after challenge
1b	Light Sussex (Standard diet)	F98 Nal^r	10^8 c.f.u. in 0·1 ml orally at < 24 h of age	F98 Spc^r ϕ^r	10^5 c.f.u. in 0·1 ml orally 24 h after first strain	Viable counts of F98 Nal^r in caecal contents at 3 days after challenge
2a	Commercial broiler Rhode Island Red White Leghorn Brown Leghorn	F98 Spc^r ϕ^r	10^8 c.f.u. in 0·1 ml orally at < 24 h of age	F98 Nal^r	10^5 c.f.u. in 0·1 ml orally 24 h after first strain	Viable counts of F98 Nal^r in caecal contents at 3 days after challenge
2b	Light Sussex (4 different diets) (Standard diet)	F98 Spc^r ϕ^r	10^8 c.f.u. in 0·1 ml orally at < 24 h of age	F98 Nal^r	10^5 c.f.u. in 0·1 ml orally 24 h after first strain	Viable counts of F98 Nal^r in caecal contents at 3 days after challenge
3a	Light Sussex (Standard diet)	F98 Nal^r *aro A*	10^8 bacteria/g in feed < 24 h of age	F98 Nal^r Spc^r	10^5 c.f.u. in 0·1 ml orally 24 h after first strain	Viable count of challenge strain in caecal contents 3 days after challenge
3b	Light Sussex (Standard diet)	F98 Spc^r (formalin killed)	10^8 bacteria/g in feed < 24 h of age	F98 Nal^r Spc^r	10^5 c.f.u. in 0·1 ml orally 24 h after first strain	Viable count of F98 Nal^r in caecal contents 3 days after challenge

4a	Light Sussex (Standard diet)	F98 Spc^r ϕ^r	10^8 c.f.u. in 0·1 ml orally at < 24 h of age	F98 Nal^r Spc^r	10^5 c.f.u. in 0·1 ml at different times (0 h–7 d) after first strain	Viable count of F98 Nal^r in caecal contents 3 days after challenge
4b	Light Sussex (Standard diet)	F98 Spc^r ϕ^r	10^8 c.f.u. in 0·1 ml orally at < 24 h–8 d of age	F98 Nal^r Spc^r	10^5 c.f.u. in 0·1 ml orally 24 h after first strain	Viable count of F98 Nal^r in caecal contents 3 days after challenge
5a	Light Sussex (Standard diet)	F98 Spc^r ϕ^r	10^8 c.f.u. in 0·1 ml orally at < 24 h of age	F98 Nal^r Spc^r	10^8–10^2 c.f.u. in 0·1 ml orally 24 h after first strain	Viable count of F98 Nal^r in caecal contents 3 days after challenge
5b	Light Sussex (Standard diet)	F98 Spc^r ϕ^r	10^8–10^2 c.f.u. in 0·1 ml orally at < 24 h of age	F98 Nal^r Spc^r	10^5 c.f.u. in 0·1 ml orally 24 h after first strain	Viable count of F98 Nal^r in caecal contents 3 days after challenge
6	Light Sussex (Standard diet)	F98 Spc^r ϕ^r	10^8 c.f.u. in 0·1 ml orally at < 24 h of age	Different serotypes (see Table 6)	10^5 c.f.u. in 0·1 ml orally 24 h after first strain	Viable count of challenge strains in caecal contents 3 days after challenge
7	Light Sussex (Standard diet)	*S. infantis* 1326/28 Spc^r	10^8 c.f.u. in 0·1 ml orally at < 24 h of age	Different serotypes (see Table 6)	10^5 c.f.u. in 0·1 ml orally 24 h after first strain	Viable count of challenge strains in caecal contents 3 days after challenge
8	Light Sussex (Standard diet)	*S. enteritidis* Spc^r	10^8 c.f.u. in 0·1 ml orally at < 24 h of age	Nal^r mutants of 4 different *S. enteritidis* strains	10^5 c.f.u. in 0·1 ml orally 24 h after first strain	Viable count of challenge strains in caecal contents 3 days after challenge
9a	Light Sussex (Standard diet)	F98 Spc^r ϕ^r	10^8 c.f.u. in 0·1 ml orally at < 24 h of age	F98 Nal^r	10^5 c.f.u. in 0·1 ml orally 24 h after first strain	Cloacal swabs taken weekly from all chickens
9b	Light Sussex (Standard diet)	F98 Spc^r ϕ^r	10^8 c.f.u. in 0·1 ml orally at < 24 h of age	F98 Nal^r	Placed in contact with 5 chickens inoculated orally with 10^5 c.f.u. in 0·1 ml 24 h after first strain	Cloacal swabs taken from 'in-contact' chickens

faecal excretion of the challenge strain F98 Nal^r was examined. In experiment 9a groups of 50 chickens were given F98 Spc^r ϕ^r when less than 24 h old and challenged with F98 Nal^r 1 day later. In experiment 9b groups of 25 chickens were also given F98 Spc^r ϕ^r but were challenged by placing them in contact 1 day later with five chickens which had just been infected orally with 10^5 c.f.u. of F98 Nal^r. Chickens from experiments 9a and 9b were reared for 7 weeks. At weekly intervals cloacal swabs were taken from all the chickens in experiment 9a and from the 'in-contact' birds in 9b. These were treated as described below.

Bacterial enumeration

Viable counts of bacteria in intestinal contents were carried out by the method of Miles, Misra and Irwin [21] using, as a solid medium, Brilliant Green agar (Oxoid CM263) containing either spectinomycin (30 μg/ml) or nalidixic acid (20 μg/ml) and novobiocin (1 μg/ml) or a combination of spectinomycin and nalixidic acid.

The faecal excretion of F98 Nal^r was assessed as described in the accompanying paper [19].

RESULTS

The effect on intestinal colonization by S. typhimurium *F98 Nal^r of pre-colonization with F98 Spc^r ϕ^r (Experiment 1)*

The viable counts of *S. typhimurium* F98 Spc^r ϕ^r and F98 Nal^r in the alimentary tract of chickens which had been infected orally with F98 Spc^r ϕ^r followed 24 h later by challenge with F98 Nal^r (Experiment 1a) are shown in Table 2. At the time of challenge F98 Spc^r ϕ^r was present in very high numbers in caecal contents and this persisted during the course of the experiment. Smaller numbers also persisted in the crop but the numbers in the jejunum gradually disappeared so that 48 h after hatching none was detectable. Immediately after challenge F98 Nal^r was isolated from the crop only. Within a few hours it was also isolated in small numbers from the caeca. However, this strain did not multiply once it had reached this organ, but was present in relatively low numbers in a small number of the birds during the remainder of the experiment. The occasional isolations made from the crop were possibly the result of environmental contamination. Subsequent short-term experiments used sampling at only 72 h after challenge.

To ensure that inhibition of F98 Nal^r by F98 Spc^r ϕ^r was not a reflection of their respective marker mutations, an experiment was carried out in which chickens were first given F98 Nal^r and then challenged with F98 Spc^r ϕ^r (Experiment 1b). Viable counts of the challenge organism were made only at 3 days post-challenge. Full inhibition of F98 Spc^r ϕ^r occurred.

The effect on inhibition of S. typhimurium *F98 Spc^r ϕ^r of varying the breed of chicken and type of feed (Experiment 2)*

Complete inhibition of F98 Nal^r by F98 Spc^r ϕ^r occurred when either Light Sussex, Rhode Island Red, White Leghorn, Brown Leghorn, or a commercial broiler hybrid were used with the standard diet. A similar degree of inhibition also occurred with Light Sussex chickens reared on four different commercial diets in addition to the standard diet based on fish meal, wheat and maize meal.

Table 2. *The number of organisms of* S. typhimurium *F98 in the alimentary tract of chickens given the Nal^r strain one day after the oral administration of the F98 Spc^r ϕ^r strain*

Strain	Organ sampled	Log_{10} viable number of F98 organisms per g of intestinal contents at the following times (h) after challenge with F98 Nal^r						
		0	3	6	12	24	48	72
F98 Spc^r ϕ^r	Crop	5·5* (5·1–6·4)	6·4 (5·4–6·3)	5·5 (4·5–6·3)	5·5 (5·0–6·4)	2·3 (N–3·5)	2·3 (N–3·5)	4·6 (3·5–5·3)
	Jejunum	3·5 (3·4–4·4)	4·1 (3·3–5·2)	3·0 (N–4·7)	N† (N–3·8)	N (N–2·8)	N (N)	N (N)
	Caecum	8·3 (7·6–9·0)	8·9 (7·6–9·2)	8·5 (6·3–9·0)	8·6 (7·5–8·9)	8·6 (8·1–8·6)	8·2 (7·4–8·6)	8·6 (6·8–8·7)
F98 Nal^r	Crop	4·3 (3·5–4·5)	N (N)	N (N–3·4)	N (N)	N (N)	N (N–2·7)	N (N)
	Jejunum	N (N)	N (N)	N (N)	N (N)	N (N)	N (N)	N (N)
	Caecum	N (N)	2·7 (N–3·3)	N (N–3·3)	N (N–2·3)	N (N–2·7)	N (N–3·6)	N (N–5·6)

* The median count per g of contents for five chickens is given with the range in parentheses.
† N, < 2·0.
Chickens were given 10^8 organisms of F98 Spc^r ϕ^r in 0·1 ml orally within 24 h of hatching followed 24 h later by 10^5 organisms of F98 Nal^r in 0·1 ml.

Table 3. *The number of organisms of* S. typhimurium *F98 Nal*^{r} *in the caecal contents of chickens given this strain at different times after the oral administration of F98 Spc*^{r} ϕ^r

Time after which Nal^r inoculated value	Log_{10} viable number of F98 Nal^r organisms per g of caecal contents in chickens which had been		Difference in Log_{10} median value
	Pre-treated with F98 Spc^r ϕ^r	Given F98 Nal^r only	
0 h	6·7 (5·2–7·5)*	8·6 (8·1–9·1)	1·9
1 h	7·0 (4·1–8·5)	8·8 (7·6–9·4)	1·8
3 h	7·3 (5·7–8·1)	8·8 (5·7–9·2)	1·5
6 h	5·4 (3·2–7·5)	8·8 (7·2–9·1)	3·4
9 h	5·0 (4·1–6·1)	8·7 (8·2–9·4)	3·7
12 h	5·8 (N–7·4)	8·1 (6·5–8·9)	2·3
24 h	2·0 (N–3·1)	5·9 (2·0–7·6)	3·9
2 d	N (N–3·6)	7·9 (N–8·8)	> 5·9
4 d	N (N–4·1)	4·9 (N–8·4)	> 2·9
7 d	N (N–3·9)	7·4 (4·4–8·5)	> 5·4

* See Table 2.
For other details of procedures on chickens see Table 1.

Table 4. *The number of organisms of* S. typhimurium *F98 Nal*^{r} *in the caecal contents of chickens given this strain 1 day after the oral administration of the Spc*^{r} ϕ^r *strain at different times after hatching*

F98 Spc^r ϕ^r given to chickens at following ages (d):	Log_{10} viable number of F98 Nal^r organisms per g of caecal contents in chickens which had been		Difference in Log_{10} median value
	Pre-treated with F98 Spc^r ϕ^r	Given F98 Nal^r only	
< 1	N (N–3·0)*	8·7 (7·9–9·0)	> 6·7
2	N (N–3·5)	6·0 (N–8·4)	> 4·0
3	N (N–2·6)	6·4 (N–8·4)	> 4·4
5	N (N–4·1)	3·0 (N–6·5)	> 1·0
8	N (N)	2·4 (N–2·9)	> 0·4

* See Table 2.
For other details of procedures on chickens see Table 1.

The effect on inhibition of caecal colonization by S. typhimurium *F98 Nal*^{r} *Spc*^{r} *of feeding an* aro A *mutant or formalin-killed organism of F98 (Experiment 3)*

The *aro A* plasmid-cured mutant was found to be fully protective against F98 Nal^r Spc^r but no inhibition of F98 Nal^r Spc^r was produced by incorporating formalin killed organisms of F98 Spc^r in the feed at a level of 10^8/g during the course of the experiment. The Log_{10} median counts of the challenge strain in the caecal contents of the 10 chickens (range in parentheses) in these two groups were 3·2(< 2·0–4·4) and 7·5(6·0–8·6) respectively compared with 7·0(< 2·0–7·7) in a group of chickens given F98 Nal^r Spc^r only.

https://doi.org/10.1017/S0950268800047440 Published online by Cambridge University Press

The effect on inhibition of caecal colonization of S. typhimurium *F98 Nal*^{r} *by F98 Spc*^{r} ϕ^r *of varying the interval between administration of the two strains (Experiment 4)*

The inhibitory effect of F98 Spc^r ϕ^r on F98 Nal^r when the latter strain was administered at different times after the first strain (Experiment 4a) is shown in Table 3. Very little inhibition of F98 Nal^r occurred when the two strains were given simultaneously or when the challenge strain was given 3 h later. A greater reduction occurred when the challenge strain was administered at least 6 h later. Inhibition also occurred when F98 Nal^r was given more than 24 h after the first strain. Challenge times of more than 7 days were not tested since a true immune response would begin to develop at this stage.

When the test was carried out using chicks at different ages (Experiment 4b) inhibition of F98 Nal^r also occurred (Table 3). As the chickens became older the viable count of F98 Nal^r in the control chickens also became reduced until there was little difference in the counts from the two groups when F98 Spc^r ϕ^r was administered at 8 days.

The effect on inhibition of caecal colonization of S. typhimurium *F98 Nal*^{r} *by F98 Spc*^{r} ϕ^r *of varying the challenge dose of F98 Nal*^{r} *(Experiment 5)*

The effect of varying the challenge dose of F98 Nal^r (Experiment 5a) is shown in Table 5. Good inhibition occurred when 10^4, 10^5, 10^6 or 10^8 organisms were given, although the median viable count per g of contents of F98 Nal^r in the pre-treated group given 10^8 organisms was (Log_{10}) 5·1. In the group given 10^2 organisms few salmonella bacteria were detected in either group.

When F98 Spc^r ϕ^r was given to chickens at different doses (Experiment 5b), namely 10^8, 10^6, 10^4, 10^2 and nothing (control group), the viable counts (median with range in parentheses) of F98 Nal^r in these groups after challenge were N(N–5·4), N(N–6·3), 2·7(N–4·4), 7·9(7·1–8·6) and 7·4(2·5–9·0) respectively. This indicated that 24 h was sufficient to allow relatively small numbers of F98 Spc^r ϕ^r to multiply in the alimentary tract to produce an inhibitory effect. The reason for the absence of inhibition following the administration of 10^2 organisms may have been that this number of organisms is less than an infective dose.

The effect on caecal colonization by other salmonella strains of pre-treatment with S. typhimurium *F98 Spc*^{r} ϕ^r *(Experiment 6)*

The inhibitory effect of F98 Spc^r ϕ^r on Nal^r mutants of a variety of strains of *S. typhimurium* (including F98) and on several other serotypes is shown in Table 5. Very little inhibition was observed except with five of the *S. typhimurium* strains.

The effect on caecal colonization by other salmonella strains of pre-treatment with S. infantis *1326/28 Spc*^{r} *(Experiment 7)*

Because the last result suggested that inhibition was greater between homologous than between heterologous serotypes, and that F98 was perhaps not the most inhibitory strain, additional experiments were carried out to find a more inhibitory salmonella strain. *S. infantis* strain 1326/28 was selected because it

Table 5. *The number of organisms of* S. typhimurium *F98 Nal^r in the caecal contents of chickens given different doses of this strain 1 day after the oral administration of F98 Spc^r ϕ^r*

Number of F98 Nal^r organisms in challenge dose	Log_{10} viable number of F98 Nal^r organisms per g of caecal contents in chickens which had been: Pre-treated with F98 Spc^r ϕ^r	Given F98 Nal^r only	Difference in Log_{10} median value
10^8	5·1 (3·4–7·3)*	8·2 (6·8–8·7)	3·1
10^6	3·0 (N–5·2)	7·8 (7·0–8·6)	4·8
10^5	2·7 (N–3·8)	8·2 (6·6–8·6)	5·5
10^4	2·0 (N–2·8)	4·9 (N–8·0)	2·9
10^2	N (N–2·3)	N (N–3·1)	$\simeq 0$

* See Table 2.
For other details of procedures on chickens see Table 1.

Table 6. *The number of* Salmonella *organisms in the caecal contents of chickens given Nal^r mutants of different strains one day after the oral administration of F98 Spc^r ϕ^r*

Challenge strain tested (Nal^r)	Log_{10} viable number of Nal^r strains per g of caecal contents in chickens which had been: Pre-treated with F98 Spc^r ϕ^r	Given challenge strain only	Log_{10} reduction
S. typhimurium			
Bangor	N (N–4·5)*	8·2 (7·4–8·4)	> 6·2
F98	N (N–3·7)	7·3 (7·0–8·8)	> 5·3
1116	3·6 (N–4·3)	8·7 (6·5–8·9)	5·1
Neale	4·2 (N–5·6)	8·8 (8·4–8·9)	4·6
575	5·1 (N–5·8)	9·1 (7·4–9·1)	4·0
Beauville	6·9 (6·5–7·8)	8·6 (7·4–9·1)	1·7
Swindon	6·3 (4·5–7·1)	7·8 (7·6–8·3)	1·5
Norwich	7·7 (7·3–8·7)	8·0 (8·1–9·0)	0·3
3866	6·9 (3·2–7·2)	7·1 (6·5–8·2)	0·2
S. infantis	6·3 (3·5–9·2)	9·0 (8·8–9·1)	2·7
S. montevideo	6·5 (3·5–8·8)	8·7 (8·1–8·9)	2·2
S. virchow	7·3 (6·3–8·8)	8·8 (8·2–9·1)	1·5
S. enteritidis strain 1	7·2 (4·1–8·6)	8·9 (8·8–8·9)	1·5
S. enteritidis strain 2	7·4 (5·4–7·7)	8·8 (8·2–9·1)	1·4
S. agona	7·5 (6·1–9·0)	8·9 (7·3–9·3)	1·4
S. hadar	8·3 (6·9–8·9)	9·1 (8·8–9·2)	0·8
S. heidelberg	8·5 (7·2–8·9)	9·1 (8·9–9·2)	0·6

* See Table 2.
For other details of procedures on chickens see Table 1.

colonized the alimentary tract much better than did F98 [3]. A Spc^r mutant of this strain was tested for inhibitory activity in newly hatched chickens against Nal^r mutants of five *S. typhimurium* strains and the seven other serotypes (eight strains) from Table 6. The median number of challenge organisms of the 13 strains

Table 7. *The effect of oral administration of* S. typhimurium *F98 Spc*^{r} ϕ^r *on the faecal excretion by chickens of* S. typhimurium *F98 Nal*^{r}

	The percentage of chickens excreting F98 Nal^r after infection by												
	Direct oral administration*							Contact†					
	Chickens pre-treated with F98 Spc^r ϕ^r			Chickens given F98 Nal^r only			P§ value of difference	Chickens pre-treated with F98 Spc^r ϕ^r			Chickens given F98 Nal^r only			P value of difference
Time (weeks) after inoculation with F98 Nal^r	> 50	D	T‡	> 50	D	T		> 50	D	T	> 50	D	T	
1	6	8	12	34	60	98	< 0·001	0	0	8	20	28	56	0·001
2	4	6	8	14	44	78	< 0·001	0	0	4	0	24	48	0·001
3	2	4	10	18	44	70	< 0·001	0	0	0	4	8	12	
4	0	4	6	2	6	28	0·006	0	0	0	0	4	16	
5	2	2	6	4	12	18		0	0	0	0	4	4	
6	0	0	0	0	2	6		0	0	0	0	0	0	
7	0	0	0	0	2	4			..... ‖			.....		

* Groups of 50 chickens pre-treated orally with 10^8 organisms of F98 Spc^r ϕ^r in 0·1 ml within 24 h of hatching. Challenge by oral inoculation with 10^8 organisms of F98 Nal^r in 0·1 ml 24 h later.

† Group of 25 chickens pre-treated orally with 10^8 organisms of F98 Spc^r ϕ^r in 0·1 ml within 24 h of hatching. Challenge by placing them in contact with 5 chickens infected orally with 10^8 organisms of F98 Nal^r in 0·1 ml 24 h later.

‡ > 50, > 50 colonies isolated per plate; D, > 1 colony per plate; T, salmonella organisms isolated by direct or enrichment culture.

§ P value of difference in T values calculated by the method of Walters (1966).

‖ ...not done.

in the chickens pre-treated with *S. infantis* 1326/28 (with the range in parentheses) was Log_{10} $< 2 (< 2–2·3)$ whereas that in the unprotected control groups was 6·7(3·9–7·2). Inhibition of the homologous strain was greater than between heterologous strains.

The effect on caecal colonization by S. enteritidis *strains of pre-inoculation with* S. enteritidis *KMS Spc*^{r}

Since F98 did not inhibit two *S. enteritidis* strains (Table 6) a single *S. enteritidis* strain, KMS, was tested for protective capability against the four others. The median and range of the challenge strains in the pre-treated chickens were (Log_{10}) $< 2·0 (< 2·0–2·6)$ and in the control chickens were 8·0(4·3–8·0).

Inhibition of faecal excretion of S. typhimurium *F98 Nal*^{r} *by F98 Spc*^{r} ϕ^r *(Experiment 9)*

The inhibitory effect of pre-treatment of chickens with F98 Spc^r ϕ^r on the faecal excretion of F98 Nal^r when chickens were infected either individually or by contact with other infected chickens is shown in Table 7.

Chickens which were not pre-treated with F98 Spc^r ϕ^r and which were given individually broth cultures of F98 Nal^r (Experiment 9a) excreted large numbers of this organism for several weeks and small numbers of chickens were still excreting when the experiment was terminated at seven weeks. The number of pre-treated chickens excreting F98 Nal^r was much lower and they had ceased to excrete this organism by 6 weeks after challenge. The differences in the excretion rates calculated by Bayesian analysis were statistically significant from 1–4 weeks after challenge.

The excretion rate of F98 Nal^r in chickens infected by contact (Experiment 9b) and not pre-treated with F98 Spc^r ϕ^r was considerably lower and no chickens were excreting this organism when the experiment was terminated at 6 weeks. The pre-treated chickens ceased to excrete F98 Nal^r by 3 weeks after contact with the infected birds. The differences in excretion rates were statistically significant during the 2 weeks that the pre-treated chickens were excreting F98 Nal^r.

DISCUSSION

Inhibition of colonization between similar micro-organisms has not only been demonstrated with salmonella and *Escherichia coli* in chickens [15, 22] but also with *E. coli* in gnotobiotic mice and new born infants [23, 24] and between enterotoxigenic *E. coli* in pigs [25]. This approach has also been used to reduce colonization by skin staphylococci [26–28], α-haemolytic streptococci [29] and *Clostridium difficile* [30].

Despite the fairly extensive use of this phenomenon, like conventional competitive exclusion [8], the mechanisms of inhibition are poorly understood. Inhibition between skin staphylococci is thought to be mediated by antibiotic-like substances [27] whereas that occurring between homologous *E. coli* and salmonella organisms is likely to involve the protective strain occupying the niche required by the challenge organism. This is known to be the mechanism for the inhibition of K88$^+$ enterotoxigenic *E. coli* by K88$^+$ non-enterotoxigenic *E. coli* where

https://doi.org/10.1017/S0950268800047440 Published online by Cambridge University Press

attachment site occupation is involved [25]. Whether such a physical niche is involved with salmonella (which do not attach to the caecal epithelium in large numbers) is unclear.

A greater understanding of the mechanism of inhibition should assist in elucidating the mechanisms whereby salmonella organisms colonize the alimentary tract of the chicken. Conventional competitive exclusion is thought to involve a bacteriostatic mechanism [31]. Since inhibition of colonization occurs between different mutants of the same salmonella strain there is no reason to believe that this type of inhibition is other than bacteriostatic.

In the present work inhibition occurred rapidly after challenge; the challenge strain passed quickly through the alimentary tract and did not establish itself, implying that the inhibitory principle was already present in the alimentary tract at the time of challenge. However, the full inhibitory effect did not develop for several hours after the introduction of the first strain. This was surprising since after oral administration of broth cultures the crop and caecal lumen are very rapidly colonized [3]. This perhaps indicates that reaction with the host is essential in some way or that time is required for complete functional establishment in the caeca.

Chickens could be protected even if the first strain was not given until several days after hatching. However, by this time even chickens which had not been pre-colonized had begun to acquire resistance, presumably as a result of gradual acquisition of a normal inhibitory microflora. Inhibition of colonization occurred even when the second strain was presented as an undiluted culture (10^8 organisms). Whether the reduction in the caecal count of the second strain following challenge with this high number of organisms would be translated into reduced faecal excretion is unclear and would require longer-term experiments. Under commercial conditions the number of salmonella organisms ingested by chickens must vary immensely and will occasionally include large doses by, for example, consumption of food contaminated with caecal contents.

Strain F98 is not an ideal protective strain for general use since it protected against only five of nine *S. typhimurium* strains and none of the other serotypes tested. This may be because of its relatively poor colonizing ability [3]. The other strain tested, *S. infantis* 1326/28 was far more inhibitory but even so the greatest inhibition occurred against the homologous strain. Future studies on this phenomenon should be carried out using a strain which colonizes the alimentary tract very well.

Pre-colonization with the first strain produced a good reduction in faecal excretion of the second strain whether the chickens were challenged directly or by contact with infected chickens. Pre-treated-chickens challenged by the latter method were cleared of the second strain 4 weeks before the control birds, suggesting that good protection also occurs against environmental challenge. Protection also occurred in a variety of breeds (including one broiler line) and in chickens reared on different feeds. There is therefore reason to believe that this form of inhibition could occur under field conditions. It would be useful to know whether inhibition also occurred in turkeys and ducks and in other domestic animals such as calves where salmonellosis is also a major problem.

The first strain protected against challenge for at least 7 days. After this time

true immunity should begin to develop in response to infection with the first strain [19]. It is possible therefore that by choosing a single strain which is both inhibitory and immunogenic, increased resistance to salmonella infection could be produced from 24 h after hatching and for many weeks thereafter, both mechanisms of inhibition being generated by the same strain.

The problem of acceptability to the public and the regulatory authorities must be overcome for this approach to be used commercially. F98 *aro A* and F98 Nal^r ϕ^r (similar to F98 Spc^r ϕ^r) were avirulent for chickens and man [19] but the parent strains, F98, was already of reduced virulence for mammals. It should soon be possible to allay completely fears over the use of salmonella derived strains by eliminating toxigenicity by transposon and deletion mutagenesis.

ACKNOWLEDGEMENTS

The authors wish to acknowledge Mr B. Wells and Miss V. Peters for assistance in various ways. A.B. wishes to thank CNPQ and FUNDUNESP-SP, Brazil for financial support.

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