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Protection of chickens against experimental fowl typhoid using a *nuoG* mutant of *Salmonella* serotype Gallinarum

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A nuoG *mutation in NADH dehydrogenase I was introduced into a virulent strain of* Salmonella *serotype Gallinarum, the causative agent of fowl typhoid, using gene replacement with a* nuoG *open reading frame inactivated by insertion of DNA encoding a kanamycin resistance determinant. The* S. *Gallinarum* nuoG *mutant, named SG9NGK, was highly attenuated in chickens. SG9NGK colonized the caeca of chickens less efficiently than the* S. *Gallinarum parental strain, was less invasive and showed no evidence of multiplication in the liver or spleen. Using a single oral immunization with live bacteria SG9NGK reduced mortality in 2-week-old chickens following challenge with virulent* S. *Gallinarum from 75% to less than 8%.* © *1998 Elsevier Science Ltd. All rights reserved*

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Salmonella serotype Gallinarum is a cause of fowl typhoid in chickens, a systemic disease primarily involving the reticuloendothelial system[1]. The course of infection is either acute or chronic. Traditionally, control of *S.* Gallinarum and the biotype *S.* Pullorum, in flocks has involved elimination of carriers identified by serological detection, followed by improvements in hygiene and management to prevent re-infection. However, in countries where the ambient temperature necessitates the use of open-sided housing, the possibilities for control are relatively limited. Thus, although *Salmonella* serotype Gallinarum has been largely eradicated from some countries[2], it is still of considerable, and in some cases increasing, economic importance in many areas, including large parts of Latin America, the Middle East, the Pacific rim, Africa, and some parts of Southern Europe[3–5]. It is likely, therefore, that an effective live vaccine would be of considerable practical value.

A considerable amount of work has already been carried out into the feasibility of controlling fowl typhoid by vaccination. Early work on the development of vaccines against *S.* Gallinarum showed killed vaccines were of little value[6,7]. The live *S.* Gallinarum 9R vaccine was found to produce strong protection against experimental oral infection with a virulent *S.* Gallinarum strain[7], but it possessed residual virulence in some breeds of chicken[8–10]. In addition, the attenuating lesion of the *S.* Gallinarum 9R vaccine has not been identified. A strain of *S.* Gallinarum cured of the virulence-associated plasmid showed some attenuation, but was less protective than the 9R strain[11]. The development of live *Salmonella* vaccines for use in humans and other animals has resulted in the identification of a number of attenuating mutations[12–17], and some of these have been evaluated in *S.* Gallinarum. An *aroA* mutant of *S.* Gallinarum was shown to be attenuated for two-week-old chickens inoculated intramuscularly or orally. Although no immunity developed following oral administration of the vaccine, some protection followed intra-muscular inoculation, but this was less than with the 9R strain [2].

Previous studies with the 9R vaccine strain indicated that this vaccine could induce some protection also against experimental *S.* Enteritidis infection in laying hens[18,19]. Given the extent of *S.* Enteritidis infection in the poultry industry world-wide, there could be considerable advantage in using a vaccine strain which was effective against two serotypes of major importance.

During investigations in our laboratory into aspects of intestinal colonization in chickens, a Tn*phoA* mutant

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of the *S.* Typhimurium strain F98 was obtained which showed reduced virulence in one-day-old chickens compared with the wild-type (Turner *et al.*, in preparation). The Tn*phoA* insertion site was found to be located within *nuoG*, a gene in the *nuo* locus encoding NADH dehydrogenase I[20,21]. It was considered worthwhile to assess the virulence of a *nuoG* mutant of *S.* Gallinarum as well as the protective properties against fowl typhoid. This paper describes the construction of a defined non-polar *nuoG* mutant of *S.* Gallinarum which combines the desired phenotype of attenuation and immunogenicity, and reports on the potential of the strain as a live vaccine against fowl typhoid.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture media

A nalidixic acid resistant (Nal^r) derivative of *S.* Gallinarum strain 9 was used to construct the *nuoG* mutant, and as the challenge strain in protection studies. This strain is highly virulent for chickens[1,22,23]. *E. coli* MC1016 harbouring plasmid pSFDK1 containing a non-polar kanamycin cassette was obtained from Dr Sansonetti of the Institute Pasteur, France. *E. coli* K12 strains SY327λpir and SM10λpir[24] were used as the host for suicide vector pGP704-derivatives[24] and for conjugative transfer of the relevant construct to *S.* Gallinarum 9. *E. coli* strains were grown on Luria-Bertani (LB) agar or in LB broth, and *S.* Typhimurium and *S.* Gallinarum strains were grown on MacConkey agar (CM7; Oxoid). Unless otherwise indicated, broth cultures contained $1-3 \times 10^9$ c.f.u. ml^{-1}. Antibiotics were used at the following concentrations: ampicillin, 100 $\mu g\ ml^{-1}$; kanamycin, 50 $\mu g\ ml^{-1}$; spectinomycin, 30 $\mu g\ ml^{-1}$; nalidixic acid, 20 $\mu g\ ml^{-1}$ and novomycin, 1 $\mu g\ ml^{-1}$.

DNA manipulations and reagents

Plasmid DNA was prepared using the Wizard DNA miniprep kit (Promega, UK) in accordance with the manufacturer's instructions. Restriction endonucleases and DNA-modifying enzymes were obtained from Boehringer (Mannheim, Germany) and used according to the manufacturer's instructions. Polymerase chain reactions (PCR) were performed according to standard procedures[25].

Construction of a *nuoG* mutant of *S.* Gallinarum

The defined *nuoG* mutation was constructed by PCR amplifying a 1.259 kbp DNA fragment from the *nuoG* gene of *S.* Typhimurium F98[22,23]. The nucleotide sequence of the 5′ primer was 5′-GCGAATTCTCCG-GTAACCT, and 3′ primer was 5′-GCTCTA-GAAAGCTTGCCGCGGAAAGCAC. The amplified DNA fragment was digested with restriction endonucleases *Eco*RI and *Xba*I and ligated into pGP704 digested with the same enzymes. A 850 bp non-polar cassette containing a kanamycin resistance determinant, *aphA-3*, was amplified by PCR from plasmid pSFB1[26] using 5′ primer (5′TCCCCCGGGT-GACTAACTAGGAGG) and downstream 3′ primer (5′-TCCCCCGGGGTCATTATTCCCT). The PCR products were digested with *Sma*I, and cloned into the *Sma*I site of the pGP704-*nuoG* fragment derivative 720 bp from the start of the *nuoG* open reading frame. This plasmid was transferred to *S.* Gallinarum strain 9 by conjugation, and kanamycin resistant, ampicillin sensitive transcongjugants were selected. One such transconjugant, named SG9NGK, was shown by Southern hybridization[25] and PCR analysis to have incorporated the *nuoG* mutation. SG9NGK was used in all subsequent vaccination experiments described in this report.

Experimental animals

Three-week-old specific pathogen-free Light Sussex chickens bred at the Institute for Animal Health were used throughout. These are outbred, of moderate sensitivity to salmonellosis[27], and were housed and reared as described previously[28].

Determination of virulence of the SG9NGK

Groups of 24 chickens were housed separately and inoculated orally with 0.3 ml of a 24 h LB broth culture (ca. 6×10^8 c.f.u.). Chickens were observed for typical signs of fowl typhoid (anorexia, ruffled feathers, cyanosis and diarrhoea) for a 3-week period, and those severely affected were killed humanely. For the estimation of LD_{50} values, groups of five chickens were inoculated intramuscularly with decimal dilutions of broth cultures. Chickens were observed for 1 week and the LD_{50} values were calculated[29].

Estimation of intestinal invasiveness

To assess the intestinal invasiveness of SG9NGK and the wild-type *S.* Gallinarum strains, two groups of 12 chickens were each inoculated orally with 0.3 ml of broth culture, as above, of SG9NGK or wild type. At intervals, three chickens from each group were killed and viable counts of Salmonellae in the liver, spleen, and caeca determined[23].

Persistence in the reticuloendothelial system

Groups of 20 or 30 chickens were inoculated intravenously with 10^4 c.f.u. of the wild-type strain or 10^6 c.f.u. of SG9NGK. Immediately and at intervals thereafter, groups of three chickens were killed and the viable counts of bacteria in the blood, livers and spleens were determined[23].

Assessment of protection

Fifteen chickens were inoculated orally with 0.3 ml of an overnight culture of SG9NGK (10^9 c.f.u.), and another group of 12 birds were untreated. After three weeks three birds from the group inoculated with SG9NGK were killed to count the numbers of *Salmonella* in the tissues. All birds were then inoculated orally with 0.3 ml of a 24 h old culture of wild-type *S.* Gallinarum 9, and monitored for symptoms of disease, as above.

RESULTS

Comparison of virulence of SG9NGK and *S.* Gallinarum wild-type

S. Typhimurium strains harbouring *nuoG* mutations grow more poorly on acetate as the sole carbon

source[30], and a 24 h old LB broth culture will not suppress the growth of small numbers of wild-type cells inoculated into that culture[31]. SG9NGK was found to display the same phenotypes as the *S.* Typhimurium *nuoG* mutants (results not show). When 24 chickens were inoculated with wild-type *S.* Gallinarum, 18 died or were killed humanely within the following 3-week period as a result of fowl typhoid. In contrast, all 24 chickens in the group inoculated with SG9NGK remained healthy. At post-mortem examination, chickens inoculated with the wild-type *S.* Gallinarum strain showed grossly enlarged livers, and pure, heavy growth of the inoculated strain was obtained from this organ. By contrast, examination of the chickens inoculated with SG9NGK at the end of the experiment revealed healthy organs and no growth of the inoculated organisms from liver. By intra-muscular inoculation the $\log_{10}$ LD_{50} for the *S.* Gallinarum *nuoG* mutant was found to be > 7.08 compared to < 0.38 for the wild-type, indicating that SG9NGK was significantly attenuated by the *nuoG* mutation.

Comparison of invasiveness of SG9NGK and the wild-type *S.* Gallinarum

Wild-type *S.* Gallinarum was detected in the caeca from 6 h to 5 days after oral inoculation while SG9NGK was detected in the caeca up to 12 h post-inoculation, but not thereafter (*Table 1*). Wild-type *S.* Gallinarum were detectable in the livers 3 days after inoculation, but the numbers of SG9NGK were significantly less than the wild-type. Wild-type *S.* Gallinarum bacteria were detected in the spleens in significant numbers from three days post-inoculation, whereas SG9NGK was not detected at all in this organ (*Table 1*).

Persistence of SG9NGK and wild-type *S.* Gallinarum in the reticuloendothelial system

For this experiment chickens were inoculated with higher numbers of SG9NGK than wild-type *S.* Gallinarum to allow persistence over a longer period to be assessed. Immediately following inoculation, both strains were detected in the livers, spleens and blood of chickens (*Table 2*). The wild-type *S.* Gallinarum strain multiplied in the spleen and liver and eventually reappeared in the blood, producing a bacteraemia consistent with the later stages of disease. This was accompanied by the appearance of necrotic lesions in the liver and spleen. In contrast, SG9NGK gradually declined in numbers in the liver and spleen, and was not isolated from the blood after 2 days post-inoculation. Despite the gradual decline in numbers of the mutant in these organs, necrotic lesions developed within a few days of inoculation. SG9NGK persisted in the liver and spleen for 42 days after inoculation, when the experiment was terminated (*Table 2*).

Protection afforded by vaccination with SG9NGK

A group of chickens were immunized orally with SG9NGK and a similar control group were left unimmunized. Immediately prior to challenge, no SG9NGK bacteria were detected in the livers or spleens of three birds killed from the vaccinated group. By 3 weeks after challenge with wild-type *S.* Gallinarum, nine birds in the non-vaccinated group had died or been killed because of severe fowl typhoid. All nine birds, and two of the three survivors, had enlarged livers, and pure, heavy growth of wild-type *S.* Gallinarum was obtained from this organ. In the group vaccinated with SG9NGK, none of the birds showed any signs of disease during the course of the experi-

Table 1 Comparison of intestinal invasiveness of the *S.* Gallinarum *nuoG* mutant and wild type

	$\log_{10}$ viable count per gram of organs[a]					
	Liver		Spleen		Caeca	
Time after infection	*nuoG* mutant	Wild type	*nuoG* mutant	Wild type	*nuoG* mutant	Wild type
6 h	<2	<2	<2	<2	4.6	5.1
12 h	<2	<2	<2	<2	2.9	2.5
3 days	2.6	4.4	<2	4.5	<2	3.3
5 days	2	5.1	<2	4.7	<2	2.8

[a]Mean value from three birds

Table 2 Comparison of presence in the tissues of the *S.* Gallinarum *nuoG* mutant and wild-type following intravenous inoculation

	$\log_{10}$ viable count per gram of organs[a]					
	Liver		Spleen		Blood	
Days after infection	*nuoG* mutant	Wild type	*nuoG* mutant	Wild type	*nuoG* mutant	Wild type
0	4.5	2.5	6.3	4.3	3.6	0.7
2	5.1	4.6	5.7	5.0	0.8	<0.5
4	4.2[b]	4.9[b]	5.6	5.4[b]	<0.5	1.0
7	3.4[b]	5.5[b]	5.3[b]	5.0[b]	<0.5	1.7
10	2.7[b]	5.7[b]	4.6[b]	5.6[b]	<0.5	2.4
14[c]	3.3[b]		4.5[b]		<0.5	
21	3.3[b]		5.0[b]		<0.5	
35	2.7		4.5		<0.5	
42	1.7		2.6		<0.5	

[a]Mean value from three birds[b]Necrotic lesions visible[c]At 14 days birds infected with the wild-type were killed

ment. On completion of the experiment, the vaccinated birds were healthy and no bacterial growth was obtained from the livers.

DISCUSSION

We have introduced a mutation into the *nuoG* gene of *S.* Gallinarum which codes for a subunit of the NADH dehydrogenase I complex. The *nuoG* mutation attenuates this serotype such that inoculation with 10^7 viable organisms produced no outward effects. The mechanism for the attenuation is unclear. However, it is known that *nuoG* mutants grow very poorly on acetate as the sole carbon source[30]. Since the NADH dehydrogenase I complex is the first component of the electron transport chain, it is likely that *nuo* mutants are less efficient than the wild-type at extracting energy from non-fermentable carbon sources, and this may be the reason for the attenuation.

Three days after oral inoculation SG9NGK had not colonized the caeca in detectable numbers, unlike the wild-type *S.* Gallinarum. In addition, SG9NGK was less invasive, as indicated by reduced numbers of salmonellae detected in the liver and none detected in the spleen (Table. 1). Unpublished data using the mouse virulent *S.* Typhimurium C5 strain also indicates that the *nuoG* mutation may confer partially reduced invasiveness in tissue culture cells (Barber, unpublished results). Following inoculation of chickens intravenously, SG9NGK exhibited no evidence for multiplication in the liver or spleen, nevertheless, necrotic lesions were observed on these organs several days post-inoculation after the bacterial numbers had begun to decline.

SG9NGK has a number of potential advantages over existing live vaccine candidates[2,11]. The degree of attenuation was similar to that observed with an *S.* Gallinarum *aroA* mutant[2] (LD_{50} values in excess of 10^7 for both). However, the *S.* Gallinarum *aroA* strain did not induce a strong protective immunity, reducing mortality after challenge with the wild-type strain from 63% to 30%, and then only after intra-muscular administration. No protection was obtained by oral vaccination with the *S.* Gallinarum *aroA* strain. The decline in numbers of salmonellae in the liver and spleen was slower for SG9NGK than the *S.* Gallinarum *aroA* mutant[2], and this may be the reason for the stronger protection obtained with the former. The 9R *S.* Gallinarum vaccine strain reduced mortality to 11.5% by oral vaccination[2]. Although fewer birds were used in the present experiments none of them showed any signs of illness, and this represents a mortality of less than 8%. Also, here we used 3-week-old rather than 2-week-old birds, but previous experience indicates that this makes little difference to the virulence of this serotype[2,11] (Barrow, unpublished observations). Thus, SG9NGK conferred a degree of protection at least similar to, or possibly better than, the 9R vaccine strain. However, the attenuating lesion in the 9R strain has not been identified, and in this respect SG9NGK is probably more acceptable as a live vaccine.

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