Mice vaccinated with a non-virulent, aromatic-dependent mutant of *Salmonella choleraesuis* die from challenge with its virulent parent but survive challenge with *Salmonella typhimurium*

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Summary. BALB/c mice given a live vaccine of an *aroA* mutant of *Salmonella choleraesuis* by intraperitoneal (i.p.) injection were not protected against i.p. challenge with its virulent parental strain but were protected against i.p. challenge with either of two virulent strains of *Salmonella typhimurium* (O [1],4,[5],12). Vaccination with a live vaccine of *S. typhimurium aroA* protected against challenge with *S. typhimurium* but not with *S. choleraesuis*. Intraperitoneal administration of either *aroA* strain evoked high levels of serum antibody against the homologous lipopolysaccharide (LPS) as determined by an enzyme immunoassay. Sera from vaccinated mice also reacted with heterologous LPS but at dilutions at least seven-fold lower than homologous end-point titres. The vaccination schedule employed with either live-vaccine strain conferred an equal degree of resistance to challenge with *Listeria monocytogenes*. After mixed infection of mice with equal numbers of virulent *S. typhimurium* and *S. choleraesuis* by the i.p. route, the former was isolated in numbers at least 50 000 times greater than the latter from the liver and spleen between days 1 and 5. When mice were vaccinated i.p. with *S. choleraesuis aroA*, *L. monocytogenes* or *P. multocida* before mixed infection, neither serotype showed more than a slight predominance in the liver and spleen during the same period. Thus, in relative terms, vaccination with *S. choleraesuis aroA* or inoculation with unrelated bacteria suppressed the growth of virulent *S. typhimurium* in mice but allowed virulent *S. choleraesuis* to multiply. These results clearly show that *S. choleraesuis* 38₁ can multiply to kill immunised BALB/c mice.

Introduction

Salmonella choleraesuis is a highly virulent member of the genus *Salmonella* which is host-adapted for swine. It is highly invasive in man, but unlike other invasive salmonellae, such as the typhoid and paratyphoid organisms, it infects man without involving the intestines (Weissbluth *et al.*, 1981). Human infection with *S. choleraesuis* is characterised by septicaemia, multiple abscesses in internal organs and osteomyelitis, and is associated with a higher mortality rate than are other salmonella infections (MacCready *et al.*, 1957; Saphra and Winter, 1957; Weissbluth *et al.*, 1981). Except in Hong Kong (Huang and Lo, 1967) *S. choleraesuis* is

not a common cause of disease in man. The O 6,7 antigen of *S. choleraesuis* is known to confer less virulence for mice than the O 4,12 antigen of *S. typhimurium* (Valtonen *et al.*, 1975; Valtonen, 1977). This is, at least partly, a result of O 6,7 activating complement by the alternative pathway more rapidly than O 4,12 (Liang-Takasaki *et al.*, 1983), which promotes rapid phagocytosis (Liang-Takasaki *et al.*, 1982) and intracellular killing of *S. choleraesuis* and other bacteria that carry the O 6,7 antigen by peritoneal macrophages (Nnalue and Stocker, 1987b; Saxen *et al.*, 1987). These facts are inconsistent with the unusually high mortality rates associated with infections caused by this organism.

Protective response against infection with such salmonella serotypes as *S. typhimurium* and *S. enteritidis* is largely serogroup-specific (Ornellas *et al.*, 1970; Lyman *et al.*, 1979). However, *Salmonella* strains of different serotypes have common anti-

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genic determinants in their outer-membrane proteins (Kuusi *et al.*, 1979; Svenson *et al.*, 1979) and in the LPS core (McCabe, 1972; McCabe *et al.*, 1977) which also elicit a protective response. Cross protection between serogroups (Smith, 1965; Smith and Halls, 1966; Collins, 1968; Schlecht, 1981) is, therefore, frequently observed. Moreover salmonellae (Blanden *et al.*, 1966; Eisenstein *et al.*, 1984), like other facultatively intracellular parasites (Mackness, 1964; Zinkernagel, 1976), elicit a non-specific protective response against unrelated bacteria. However, the strongest protective effects are usually directed against serotypes of the same O-antigen composition as vaccine strains (Ornellas *et al.*, 1970; Lyman *et al.*, 1979). This should also be the case in natural infections.

We have found that live-vaccine strains (*galE* or *aroA* mutants) of *S. choleraesuis* did not protect salmonella-susceptible inbred mice (BALB/c and C57BL/6) against challenge with virulent strains (Nnalue and Stocker, 1987a), and that non-virulent bacteria with O 6,7 LPS did not persist *in vivo* after intraperitoneal (i.p.) inoculation (Nnalue and Stocker, 1987b). These mouse strains are readily infected with salmonellae because they carry the susceptible allele, *Ity*^s, of a genetic locus that determines ability to control multiplication of salmonellae in the liver and spleen. In this study I investigate whether the observed lack of protection was due to complete or relative failure of the protective response or to the ability of *S. choleraesuis* to multiply in mice that have mounted a good immunological response.

Materials and methods

Media

The media used were Blood Agar Base (CM55), Nutrient Broth (CM67) (Oxoid) and MacConkey Agar and Base (Difco) supplemented with the desired carbohydrate (5 g/L).

Bacterial strains

Bacterial strains and their sources or relevant references are listed in table I. The wild type strain of *S. choleraesuis*, 38₁ (Griffith and Kramer, 1981) was obtained from Dr R. Griffith of Iowa State University, Ames, Iowa. A mouse- and calf-virulent strain of *S. typhimurium*, strain UCD108-11 (Smith *et al.*, 1984), was obtained from Dr B. Smith of The University of California at Davis. *S. typhimurium* M7471 is of the FIRN biotype and was isolated from a case of food poisoning in England in 1958 (Ornellas and Stocker, 1974). The O-antigenic composition of these bacteria is: strain 38₁ O 6,7; UCD108-11 O 1,4,12; and strain

M7471 O 4,5,12. The construction of non-virulent, aromatic-dependent, derivatives of these strains has been described previously (table I).

Mouse vaccination and challenge

Specific-pathogen-free BALB/c mice came from the Department of Radiology, Stanford University, Stanford, CA. Live-vaccine strains were grown overnight in nutrient broth and held for 24 h at 4°C while viable counts were determined by plating. Broth cultures were then diluted in saline and used for inoculation. The live-vaccination regimen consisted of two doses, (2–5) × 10⁶ cfu/injection, given i.p. in 0.1 ml of saline 3 weeks apart. Vaccinated mice and unvaccinated controls were challenged i.p. with virulent strains 6 or 7 weeks after the second dose and observed for 6 weeks after challenge. Dead animals were examined *post mortem* for gross liver pathology and for re-isolation of the challenge strain. The growth of bacteria in the livers and spleens of vaccinated mice after i.p. challenge with virulent strains was studied. The mice were given three doses of live vaccine at 2-week intervals and challenged i.p. with 2.5 × 10⁵ cfu of each virulent strain 12 days after the last vaccination. Three mice each were killed on days 1, 3 and 6 after challenge to determine the numbers of each strain in the liver and spleen. Their livers and spleens were homogenised with a Colworth stomacher (Seward) in 5 ml of saline, then diluted and plated on MacConkey agar. MacConkey base agar with arabinose 5 g/L was used for plating in mixed infections to differentiate colonies of *S. choleraesuis* (arabinose negative) from those of *S. typhimurium* (arabinose positive). *Listeria monocytogenes* and *Pasteurella multocida* do not grow on this medium which allowed isolation and counting of salmonellae alone from livers and spleens also containing these bacteria.

Antibody production and titration

Humoral response against O 6,7 and O 1,4,12 LPS was compared between mice inoculated with each live-vaccine strain. The mice were inoculated i.p. with two doses of live vaccine (5 × 10⁶ cfu), 3 weeks apart, and with one dose (5 × 10⁷ cfu) of heat-killed (2 h at 65°C) bacteria of the same strain, 6 weeks after the second live dose. They were bled 1 week after the last inoculation. This protocol was designed to simulate conditions in challenge experiments except that killed bacteria were used for the last dose to minimise the presence of live organisms in sera. Anti-LPS antibody in sera was determined by enzyme immunoassay (EIA) with LPS from *S. choleraesuis* 38₁ and *S. typhimurium* UCD108-11 prepared by the method of Darveau and Hancock (1983). The wells of a flat-bottomed microtitration plate were coated overnight at room temperature (c. 20°C) with 100 µl of LPS solution, 10 µg/ml in 0.1 M sodium carbonate buffer pH 9.6 (Na₂CO₃ 1.59 g/L; NaHCO₃ 2.93 g/L). The coating solution was discarded and the plates were washed three times (1 min/wash) with washing buffer (NaCl 8.0 g/L, KH₂PO₄ 0.2 g/L, Na-

HPO₄.12H₂O 1.15 g/L, KCl 0.2 g/L, Tween 20 0.05%). Sera for assay were diluted in two-fold steps up to 1 in 10 240 in incubation buffer (phosphate-buffered saline, pH 7.4, containing Tween 20 0.05% and bovine serum albumin 1%) and added to wells in 100- μ L volumes. Undiluted serum pooled from four unvaccinated mice of the same batch was used as the control. The plate was incubated for 3 h at 37°C and washed as before. Antibody that bound to the LPS in the wells was detected with a commercial EIA kit (Allelix Inc, Toronto, Canada). The urease-labelled rabbit antibody to mouse immunoglobulin was diluted as recommended by the manufacturer and 100 μ L was added to each well. The plate was incubated for 1 h at 37°C and washed as before. Substrate solution (100 μ L) was added and the plate was incubated for 30 min at room temperature, then scanned with an EIA reader (Dynatech Laboratories). EIA titres represent the average of results from four mice and are expressed as the serum dilutions that gave an A₅₉₅ of at least 0.1 unit above that of undiluted normal mouse serum.

Statistical methods

The data presented in the tables and figures are expressed as geometric means. Standard deviations are also geometric. Fisher's exact test was used at the 95% level of confidence to analyse the statistical significance of protection data from vaccinated animals compared to unvaccinated controls. It was assumed that the vaccination regimen used would provide some degree of protection against challenge with virulent strains; therefore, only one-tailed statistical tests were done.

Results

Tests of the protective effects of vaccination with *aroA* mutants

The protective effects of vaccination with the

aromatic-dependent derivatives of *S. choleraesuis* and *S. typhimurium* against homologous and heterologous virulent strains were compared in BALB/c mice (table I). An equal number of unvaccinated control mice of the same age as the test group was used in each challenge experiment. All control mice died from challenge with the doses of virulent bacteria used in this study (data not shown). Two doses of live vaccine were used because it had been shown in a previous study that single doses of *aroA* mutants of *S. choleraesuis* given as live vaccine i.p. gave no protection (Nnalue and Stocker, 1987a). As shown by Lyman *et al.* (1979), a live-vaccine would be expected to provide better protection against challenge with a virulent strain of the same O-antigen specificity than against that of another specificity. With the *S. choleraesuis* 38₁ *aroA* live-vaccine (hereinafter designated *S. choleraesuis aro*) the vaccination regimen partially but significantly protected mice against i.p. challenge with 500 LD50 doses of its virulent parent, but did not protect against i.p. challenge with 5000 LD50 doses (table II). Two of six vaccinated mice died from i.p. challenge with 500 LD50 doses of *S. choleraesuis* 38₁ ($p=0.03$) whereas eight of 11 mice died from similar challenge with 5000 LD50 doses ($p=0.11$). These results are comparable with those obtained in a previous study with these strains (Nnalue and Stocker, 1986). While not protected against i.p. challenge with 5000 LD50 doses of virulent *S. choleraesuis*, the vaccinated mice were significantly protected against i.p. challenge with at least 5000 LD50 doses of either of the two virulent strains of *S. typhimurium*. None of 11 vaccinated mice died from i.p. challenge with 5000 LD50 doses of M7471

Table I. Bacterial strains and their i.p. LD50 in BALB/c mice

Strain	Description	LD50 (cfu, i.p.)	Source or reference
381	<i>S. choleraesuis</i> wild type	100	Nnalue and Stocker, 1986
UCD108-11	<i>S. typhimurium</i> wild type	5	Smith <i>et al.</i> , 1984; Nnalue and Stocker, 1987a
M7471	<i>S. typhimurium</i> FIRN wild type	c. 20	Ornellas and Stocker, 1974
SL2822	38 ¹ CRR426[<i>aroA</i> 544::Tn10 (Tcs, non-rev)] <i>zbfj</i> -903::Tn10.	$> 5 \times 10^6$	Nnalue and Stocker, 1987a
SL2888	UCD108-11 <i>hisC</i> 527 CRR426[<i>aroA</i> 544 ::Tn10 (Tcs, non-rev)] <i>zbfj</i> -903::Tn10.	$> 5 \times 10^6$	Nnalue and Stocker, 1987a
SL3237	M7471 <i>rpsL</i> 120 CRR401 [<i>aroA</i> 544::Tn10(Tcs, non-rev)]	$> 5 \times 10^6$	Hoiseith, S. K. Ph.D Thesis Stanford University, 1983
EGD	<i>L. monocytogenes</i> (virulent strain)	c. 2×10^3	Collins, 1979
	<i>P. multocida</i> (avirulent strain)	$> 10^7$	Wong and Kucera, 1982

CRR, complex genetic rearrangement mutation; non-rev, non-reverting; *zbfj*, silent locus at minute 20 of the salmonella chromosome.
* The LD50 of *L. monocytogenes* EGD for BALB/c mice was not determined. It was assumed to be c. 2×10^3 cfu because this dose killed 24 of 40 mice.

Table II. Tests of the protective effects of vaccination* with aromatic-dependent *Salmonella* strains

Challenge strain	Challenge dose, cfu (LD50)	Deaths/total†
Mice vaccinated with <i>S. choleraesuis aro</i>		
<i>S. choleraesuis</i>	5×10^4 (500)	[2/6]
	5×10^5 (5000)	8/11
<i>S. typhimurium</i> M7471	5×10^4 (5000)	0/11
<i>S. typhimurium</i> UCD108-11	5×10^4 (10 000)	3/11
Mice vaccinated with <i>S. typhimurium</i> M7471 <i>aro</i>		
<i>S. choleraesuis</i>	5×10^4 (500)	11/11
<i>S. typhimurium</i> M7471	5×10^4 (5000)	0/11
Mice vaccinated with <i>S. typhimurium</i> UCD108-11 <i>aro</i>		
<i>S. choleraesuis</i>	5×10^4 (500)	11/11
<i>S. typhimurium</i> UCD108-11	5×10^4 (10 000)	0/11

* Mice were vaccinated i.p. on days 0 and 21 with 5×10^6 cfu of the appropriate live-vaccine strain and challenged i.p. on day 70.

† Mortality data, except where enclosed in brackets, were pooled from two separate experiments with five and six mice/group.

and only three mice died in another group of 11 challenged with 10 000 LD50 doses of UCD108-11 ($p=0.0005$). Thus *S. choleraesuis aro*, used as a live vaccine in BALB/c mice, did not protect against challenge with its parental strain but protected against challenge with *S. typhimurium*.

In contrast, vaccination of mice with the *aroA* derivative of either strain of *S. typhimurium* protected against challenge with its virulent parent but not against challenge with *S. choleraesuis* (table II). Thus, all vaccinated mice survived i.p. challenge with 5000 or 10 000 LD50 doses of the virulent *S. typhimurium* parental strain (table II), but died from challenge with as low as 500 LD50 doses of *S. choleraesuis*. Thus, the cross-protective response between these strains, *S. choleraesuis* versus *S. typhimurium*, seems to be unidirectional. Because UCD108-11 is the more virulent of the two *S. typhimurium* strains and had previously been compared with *S. choleraesuis* 38₁ (Nnalue and Stocker, 1987a) it was chosen for subsequent comparisons in this study. Its *aroA* derivative is designated *S. typhimurium aro*.

Immune response in vaccinated mice

Immune responses were tested in mice vaccinated in almost the same way as those challenged with virulent salmonellae. The average enzyme immunoassay (EIA) titre of antibody against O 6,7 LPS present in the sera of four mice vaccinated with *S. choleraesuis aro* was 2800 compared to 400 against O 1,4,12 LPS. The four mice vaccinated with *S. typhimurium aro* responded with an average

anti-O 1,4,12 LPS titre of $>10\ 240$ and an average anti-O 6,7 titre of 800. The level of non-specific resistance at the time of challenge was compared between mice vaccinated with *S. choleraesuis aro* or *S. typhimurium aro*. Mice vaccinated with either live-vaccine strain were found equally resistant to challenge with *L. monocytogenes* strain EDG (table III) and thus appeared to have developed similar levels of non-specific resistance. All of eight mice vaccinated with *S. choleraesuis aro* and seven of eight vaccinated with *S. typhimurium aro* survived challenge with 30 LD50 doses of listeria whereas none of the mice similarly vaccinated with either *aro* strain survived challenge with 3000 LD50 doses of listeria.

Survival and multiplication of virulent bacteria in vaccinated and unvaccinated mice

S. typhimurium was isolated in numbers at least 50 000 times greater than was *S. choleraesuis* from the livers and spleens of unvaccinated mice on days 1, 4 and 5 after i.p. administration of 6.5×10^3 cfu of each organism as a mixed infection (table IV). A similar comparison was done in mice vaccinated with *S. choleraesuis aro* to investigate further why such mice were better protected against challenge with *S. typhimurium* than with *S. choleraesuis*. In the first of two experiments, the mice had more *S. typhimurium* than *S. choleraesuis* in their livers and spleens on both of days 1 and 3 after challenge—36 times more on day 1 and 10 times more on day 3 (fig. 1). On day 6, however, there was 10 times more *S. choleraesuis* than *S. typhimurium* in these organs.

Table III. Tests of non-specific protective effects against listeria in mice vaccinated* with aromatic-dependent *Salmonella* strains

Vaccine strain	Challenge dose, cfu (LD50)	Deaths/total
<i>S. choleraesuis aro</i>	6×10^4 (30)	0/8
	6×10^6 (3000)	8/8
<i>S. typhimurium aro</i>	6×10^4 (30)	1/8
	6×10^6 (3000)	8/8
Control	6×10^4 (30)	8/8
	6×10^6 (3000)	8/8

* Mice were vaccinated i.p. on days 0 and 21 with 2×10^6 cfu and 5×10^6 cfu respectively of the appropriate live-vaccine strain and challenged i.p. with *L. monocytogenes* on day 65.

In the second experiment, more *S. choleraesuis* than *S. typhimurium* was recovered from the liver and spleen on similar days; four times more on day 1 after challenge, 10 times more on day 3 and about 400 times more on day 6 (fig. 2). It is not clear why the proportions of *S. choleraesuis* and *S. typhimurium* recovered from mice differed between the two experiments nor why the second group of mice appear to have responded better to vaccination and consequently suppressed the growth of challenge bacteria to a greater degree (by at least a factor of 10 on the respective days). Both groups of mice were purchased as a single batch from the same supplier but those used in the second experiment were 12 weeks old at the start of immunisation

Table IV. Growth of *S. choleraesuis* 38₁ and *S. typhimurium* UCD108-11 in unvaccinated mice after mixed-infection

Day	Geometric mean cfu in liver and spleen		Ratio (SL1260:SL2824)
	SL2824	SL1260	
1	< 30	1.3×10^4	> 80 000
4	< 10^4	3.6×10^8 SD 1.5	> 300 000
5	< 3×10^5	2×10^8 SD 7.2	> 50 000

Mice were inoculated ip with a mixed inoculum of 6.5×10^3 cfu of each strain. Figures represent results from two or three mice.

whereas those for the first experiment were 8 weeks old. The difference in ages could, perhaps, have led to a difference in immunological maturity and consequently to better immune responses in the older mice, as evident from their greater ability to suppress the growth of virulent bacteria in the liver and spleen. Irrespective of the causes of these differences, both experiments show that after vaccination the growth of *S. typhimurium* was suppressed to a greater degree than that of *S. choleraesuis* with the result that the overwhelming growth advantage of *S. typhimurium* in non-vaccinated animals was decreased or even reversed. Although equal numbers of both organisms were used in the mixed-infection and mixed-challenge experiments, in fact each mouse was actually given 20 times more LD50 doses of *S. typhimurium* than of *S. choleraesuis*.

Figs. 1 and 2. Two experiments, comparing the growth of *S. choleraesuis* 38₁ (■—■) and *S. typhimurium* UCD108-11 (●—●) in the livers and spleens of mice vaccinated i.p. with *S. choleraesuis aro* and challenged i.p. with a mixture of 2.5×10^5 cfu of each virulent strain. Each point represents the geometric mean of determinations in three mice. Vertical bars indicate SD.

Table V. Effect of prior inoculation with *L. monocytogenes* on the growth of *S. choleraesuis* and *S. typhimurium* in the liver and spleen of mice

Day	Geometric mean cfu in liver and spleen		
	SL2824	SL1260	Ratio (SL1260:SL2824)
4	5×10^6 SD 1.92	9×10^7 SD 2.94	18
5	5×10^8 SD 1.90	3×10^9 SD 6.27	6

Mice were inoculated i.p. with *L. monocytogenes* as follows: 2×10^3 cfu on the first day, 2×10^5 cfu on day 7, and 2×10^6 cfu on day 14. More than half the mice died from the first dose but the survivors survived the subsequent doses. The mice were challenged on day 21 with a mixture of 5×10^5 cfu each of *S. choleraesuis* and *S. typhimurium* and 2×10^6 cfu of *L. monocytogenes*. Figures represent the geometric means of results from four mice.

Table VI. Effect of prior inoculation with *P. multocida* on the growth of *S. choleraesuis* and *S. typhimurium* in the liver and spleen of mice

Day	Geometric mean cfu in liver and spleen		
	SL2824	SL1260	Ratio (SL1260:SL2824)
5	3×10^5 SD 6.62	5×10^5 SD 8.11	1.7

Five mice vaccinated once i.p. with 10^7 cfu of *P. multocida* live vaccine strain were challenged i.p. 8 days later with 5×10^5 cfu each of *S. choleraesuis* and *S. typhimurium* and killed 5 days after challenge.

To identify more specifically the contribution of non-specific factors in the above results, I compared the growth of *S. choleraesuis* and *S. typhimurium* in mice pre-inoculated with micro-organisms that are antigenically unrelated to salmonellae. In one experiment, mice were given three i.p. injections of *L. monocytogenes*, 2×10^3 cfu on the first day, 2×10^5 cfu on the eighth day and 2×10^6 cfu on the fifteenth day. More than half of the mice died after the first injection but the survivors survived the subsequent inoculations. On day 21 they were challenged i.p. with a mixture of 5×10^5 cfu each of *S. typhimurium* and *S. choleraesuis* together with 2×10^6 cfu of listeria. When killed 4 or 5 days later, the mice had, on average, less than 20 times more *S. typhimurium* than *S. choleraesuis* on both days (table V). In another experiment, five mice were inoculated i.p. with 10^7 cfu of a *P. multocida* live-vaccine strain 8 days before these were given a mixed infection i.p. with *S. choleraesuis* and *S. typhimurium*. The mice were killed 5 days after challenge and found to have equal numbers of both organisms in their livers and spleens (table VI). Thus, as was observed in mice vaccinated with *S. choleraesuis aro*, the growth of *S. typhimurium* was suppressed more than that of *S. choleraesuis* in mice

pre-inoculated with other micro-organisms before mixed infection.

Discussion

The finding that avirulent derivatives of *S. choleraesuis* were not effective as live vaccines in mice, unlike those of *S. typhimurium* (Nnalue and Stocker, 1987a), formed the basis of the present investigation. The results presented here show that *S. choleraesuis* can multiply to kill mice that have mounted a strong immune response to its antigens. Mice developed high levels of serum anti-O 6,7 LPS antibody after vaccination with *S. choleraesuis aro* but remained susceptible to infection with the virulent strain. The humoral response to LPS seems not to protect against infection by *S. choleraesuis*. This is not the case in infections caused by *S. typhimurium* or *S. enteritidis* in which adoptively transferred B cells (Hochadel and Keller, 1977), serum or monoclonal antibody (Colwell *et al.*, 1984; Carlin *et al.*, 1987; Saxen *et al.*, 1987) or killed or artificial vaccines (Herzberg *et al.*, 1972; Lindberg *et al.*, 1974; Ornellas *et al.*, 1974), which primarily elicit antibody, are protective. The O antigen is the main protective immunogen against infection with these serotypes and protection is O-antigen-specific

(Ornellas *et al.*, 1970; Lyman *et al.*, 1979; Carlin *et al.*, 1986). As would be expected from such specificity, vaccination with *S. typhimurium aro* protected against challenge with *S. typhimurium* and did not protect against challenge with *S. choleraesuis*.

The lack of protection against challenge with *S. choleraesuis* in mice vaccinated with *S. choleraesuis aro* is not due to poor elicitation of cellular immunity because such mice were well protected against challenge with *S. typhimurium* and expressed non-specific resistance against listeria to the same extent as mice vaccinated with *S. typhimurium aro*. The protective effects against *S. typhimurium* in such mice seems to be partly a result of immune response to shared antigens and partly from non-specific activation of immunological defences. Cross protection is often seen between *Salmonella* serotypes as well as between other enteric bacteria. It is believed to be due mainly to shared antigenic determinants in the LPS core (McCabe, 1972; McCabe *et al.*, 1977) and presumably also in the outer-membrane proteins. The effect of cross reactivity in this study is evident from the fact that the mice vaccinated with *S. choleraesuis aro* were not protected against 3000 LD₅₀ doses of listeria but were protected against 10 000 LD₅₀ doses of *S. typhimurium*. Non-specific protection can occur by two mechanisms: endotoxin (LPS)-induced resistance (Abernathy, 1957; Cluff, 1970; Morrison and Ulevitch, 1978; Vuopio-Varkila *et al.*, 1988) from generalised activation of cells of the reticulo-endothelial system; and cellular immunity mediated by activated macrophages which, though specifically elicited, is expressed non-specifically (Mackness, 1964; Levine and Hornick, 1981). Resistance induced by endotoxin is evident soon after inoculation with a gram-negative bacterium and is of short duration. It is probably the mechanism for the resistance to salmonella infection observed in mice vaccinated with *P. multocida*. Cellular immunity, on the other hand, takes at least 2 weeks to develop and should be largely responsible for resistance to salmonella in mice inoculated with listeria. That cross-reactivity and non-specific resistance, both relatively minor protective mechanisms, could protect mice vaccinated with *S. choleraesuis aro* against challenge with as many as 10 000 LD₅₀ doses of *S. typhimurium* is additional evidence that these mice have mounted a good immunological response to the antigens of *S. choleraesuis*. Apparently these protective mechanisms, as well as specific responses to the antigens of *S. choleraesuis*, could not prevent the multiplication of the virulent strain in vaccinated animals.

The deduction may be made that either a lesser degree of immunological activation is required to protect against *S. typhimurium* than against *S. choleraesuis* or that the latter organism is in some other way able to avoid an active specific immune response.

It is unlikely that the virulence of *S. choleraesuis* for mice vaccinated with its *aro* derivative is mainly due to immunosuppression, because neither *S. choleraesuis* nor *S. typhimurium* after mixed infection grew better in mice thus vaccinated than in those vaccinated with listeria. Moreover, the growth of *S. typhimurium* was suppressed to a greater extent than that of *S. choleraesuis* in mice vaccinated with *S. choleraesuis aro* and challenged simultaneously with both virulent strains. This shows that, even after challenge with virulent *S. choleraesuis*, the mice continued to manifest the augmented tissue bactericidal activity against intracellular bacteria expected after live-vaccine administration.

The mechanism by which *S. choleraesuis* multiplies to kill apparently immunised animals remains unknown. Though able to cause rapidly lethal septicaemia in man and animals, *S. choleraesuis* is readily phagocytosed and killed by mouse peritoneal macrophages *in vivo* and *in vitro*. The result is that <1% of *S. choleraesuis* or other O 6,7 bacteria injected i.p. are recovered from the liver and spleen after 24 h, compared to ≥30% of O 4,12 strains (Nnalue and Stocker, 1987b). The greater virulence of *S. typhimurium* (O 1,4,12) for mice after i.p. injection and its overwhelming predominance in mixed infections of mice is explained, at least in part, by the fact that it is not so readily phagocytosed and killed by peritoneal macrophages. It seems a reasonable deduction that in mice inoculated i.p. with moderate numbers of both organisms (e.g., 6.5×10^3 cfu of each) most of the *S. choleraesuis* would be rapidly internalised by phagocytic cells while much of the *S. typhimurium* inoculum would remain and multiply extracellularly. As bacteria generally multiply more rapidly extracellularly than intracellularly (Collins, 1979) the non-phagocytosed fraction of an i.p. inoculum of *S. typhimurium* must contribute greatly to the net increase in bacterial numbers *in vivo*. This implies that mechanisms, immunological or otherwise, that simply enhance phagocytosis, e.g., non-specific resistance, would greatly reduce its virulence. One piece of evidence for the importance of extracellular multiplication for the virulence of *S. typhimurium* UCD108-11 is the fact that replacement of its O 1,4,12 antigen with the O 6,7 antigen of *S. choleraesuis* 38₁ causes a 10 000-fold reduction in i.p. virulence (Nnalue

and Stocker, 1987a). The activation of macrophages as a consequence of vaccination, even with unrelated bacteria, should also produce a similar result by reducing or even eliminating the contribution of extracellular multiplication to virulence. Thus, in an immunised mouse most, if not all, of a small inoculum of *S. choleraesuis* or *S. typhimurium* should be quickly phagocytosed after i.p. inoculation. My data therefore agree with theoretical considerations and suggest that *S. choleraesuis* grew better than *S. typhimurium* when most of the bacteria were intracellular and, thus, that it is better adapted for life within mammalian phagocytes. Ability to survive and multiply within phagocytes would seem to be a requirement for an organism such as *S. choleraesuis* that is readily phagocytosed *in vivo*.

It is of interest that while >99% of an i.p. inoculum of *S. choleraesuis* is killed within 24 h, the organism has an i.p. LD₅₀ of 100 cfu in mice. It is very unlikely that a micro-organism could be so virulent by a route in which it is so efficiently destroyed unless the fraction that survived initial killing were sheltered from further bactericidal action and multiplied rapidly. This suggests that even one cfu of *S. choleraesuis* surviving the initial

killing in the peritoneal cavity could multiply to kill a mouse. Published work (Nnalue and Stocker, 1987b; Saxen *et al.*, 1987) suggests that anti-phagocytic properties play little or no role in the survival and multiplication of *S. choleraesuis in vivo*; thus, antimicrobial mechanisms that simply increase the rate of phagocytosis should have little effect on the outcome of infection. The perfused rat or mouse liver can trap bacteria extracellularly within sinusoids (Moon *et al.*, 1975) and both Kupfer and endothelial cells can trap salmonellae (Friedman and Moon, 1977). An interesting question is whether, despite its efficient killing by macrophages, *S. choleraesuis* remains virulent for vaccinated mice because it can invade cells that lack bactericidal properties or can multiply extracellularly at sites within the liver and spleen not accessible to cellular infiltration. This organism offers a unique experimental tool for investigation of the strategies of microbial persistence and growth in an immunised host as well as of host mediation systems against intracellular parasites.

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