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Invasiveness and Persistence of *Salmonella enteritidis*, *Salmonella typhimurium*, and a Genetically Defined *S. enteritidis aroA* Strain in Young Chickens

GERARD L. COOPER,[1*] LINDSAY M. VENABLES,[1] MARTIN J. WOODWARD,[1] AND CARLOS E. HORMAECHE[2]

Bacteriology Department, Central Veterinary Laboratory, Addlestone, Surrey KT15 3NB,[1] and Division of Microbiology and Parasitology, Department of Pathology, University of Cambridge, Cambridge CB2 1QP,[2] England

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Newly hatched chicks were dosed orally with a *Salmonella typhimurium* wild-type strain, an *S. enteritidis* wild-type strain, and a genetically defined *S. enteritidis aroA* vaccine candidate, strain CVL30. The *S. typhimurium* strain, 2391 Nal^r, was virulent in newly hatched chicks and caused deaths in 7 of 20 chicks after an oral dose of 10^5 organisms. The *S. enteritidis* wild-type strain, LA5, caused death in 1 of 25 chicks and gross pathology including pericarditis and perihepatitis in 6 of the 24 survivors after an oral dose of 10^9 organisms. *S. enteritidis aroA* CVL30, attenuated by ca. 6.5 $\log_{10}$ in BALB/c mice, was nonvirulent when administered orally to chicks and did not cause morbidity. When newly hatched chicks were dosed, the pattern of invasion and colonization of the reticuloendothelial system by strain CVL30 was similar to that of its parent strain, LA5, irrespective of the dose. Oral inoculation of newly hatched chicks with <10 organisms of *S. enteritidis* LA5 or CVL30 was followed by multiplication in the cecal contents. Within 3 days of hatching, the pH of the cecal contents was reduced from ca. 7 to 5. Samples of gut contents were inoculated in vitro. The *S. enteritidis* strains multiplied in samples taken from the ileum and duodenum irrespective of age but multiplied in the cecal samples from newly hatched chicks only. Invasion from the gut by *S. enteritidis* LA5 and CVL30 was both age and dose dependent.

Salmonella enteritidis infection in humans remains a public health problem in many developed countries. It can be transmitted vertically in chickens (32), which contributes to its dissemination in poultry reared intensively.

Early exposure to invasive serotypes such as *Salmonella typhimurium* or *S. enteritidis* is associated with the subsequent development of clinical salmonellosis in young chickens (33). In particular, *S. enteritidis* phage type 4 is associated in Britain with pericarditis in broilers and broiler breeders infected naturally (21, 26) and in chickens infected experimentally (7). Infection with *S. enteritidis* phage type 34 caused mortality of up to 20% in some young broiler flocks in South Africa during 1993 for the first time (17). Chicks may be exposed to *S. enteritidis* in the hatchery, but this serotype has also been isolated in poultry houses up to 11 months after depopulation (15). This could be a source of infection for new stock. Mice in poultry houses may also be a significant reservoir of *S. enteritidis* (19).

Poultry products are a major source of *Salmonella* food-poisoning serotypes for humans. Efficacious vaccines may limit *Salmonella* infections in flocks, and live *S. enteritidis* vaccines might be an effective method to control this serotype in chickens should they reduce vertical transmission in breeding stock and colonization of the gut in young birds. Live *Salmonella* vaccine candidates may be generated by the rational mutation of a selected key gene or genes (4). Mutation of the *aroA* gene, a key gene in the chorismic acid pathway of bacteria (20), results in a severe metabolic deficiency affecting seven biochemical pathways (27). Vaccination of newly hatched chicks with *S. enteritidis aroA* imprecise-excision mutants conferred sufficient protection at the gut level to reduce invasion of the tissues and intestinal shedding after an oral challenge (7). Colonization of the spleens, livers, ceca, and ovaries in laying hens at 23 weeks old was also reduced (8).

The invasiveness and persistence of *Salmonella* vaccine candidates are a function of the genotype of the parent strain, the phenotypic effect of the attenuating mutation(s), and the genetic background of the host. In addition, chicks are essentially gnotobiotic when hatched (28) and have gastrointestinal tracts filled with meconium. These factors make chicks particularly susceptible to colonization of the gastrointestinal tract by salmonellae and other microflora. Likewise, they may also affect any attempt to control colonization by live oral vaccines.

For purposes of quality control, fully defined genetic deletions are preferable in live vaccines (16). We have prepared a genetically defined *S. enteritidis aroA* mutant, strain CVL30, which can be differentiated from the wild type by PCR and other tests. The aims of this study were to investigate the invasion and persistence characteristics of strain CVL30 compared with those of its parent strain and a strain of *S. typhimurium* after oral dosing. We have also investigated the effects of chick age on the in vivo capacity of these strains to invade from the gut and their survival and multiplication in vitro in samples of gut contents taken from chicks up to 14 days old.

MATERIALS AND METHODS

Bacteria. *S. enteritidis* phage type 4 LA5 was isolated post-mortem from a 4-day-old broiler chick in the United Kingdom. *S. typhimurium* 2391 was a chicken isolate obtained from the culture collection at the Central Veterinary Laboratory, Addlestone, United Kingdom. A nalidixic acid-resistant variant was isolated on a Luria-Bertani agar gradient plate con-

* Corresponding author. Mailing address: Bacteriology Department, Central Veterinary Laboratory, Addlestone, Surrey KT15 3NB, England. Phone: 44 932 357365. Fax: 44 932 347046.

taining up to 200 μg of nalidixic acid per ml. The preparation of *S. enteritidis* CVL30 from the parent strain LA5 will be described fully elsewhere. Briefly, a cosmid library of *S. enteritidis* LA5 was constructed, and a region of ca. 2 kb containing the *aroA* gene and flanking regions was identified. Approximately 400 bases of the *aroA* gene were excised, religated, and cloned in the suicide vector pGP704 (25), provided by G. Dougan (Imperial College, London, England). This was introduced into strain LA5 by electroporation, and following homogenotisation, a confirmed *aroA* strain was designated CVL30. Strain CVL30 has been administered to approximately 500 chickens and cultured postmortem. Strain CVL30 has been found to be stable.

Mice. Groups of 10 female, innately susceptible BALB/c mice of 6 to 8 weeks of age were infected by the intraperitoneal route with decimal dilutions of strain CVL30 in phosphate-buffered saline (PBS), pH 7.2. Survival over a period of 28 days was recorded, and the 50% lethal dose (LD_{50}) was determined (30).

Chickens. Fertile eggs were obtained from a specific-pathogen-free White Leghorn flock. After hatching, freedom from salmonellae was assured by the culture of meconium and organs from culled chicks. Sera from culled chicks were assessed by an indirect enzyme-linked immunosorbent assay method using a Westphal lipopolysaccharide (LPS) extract of *S. enteritidis* containing the O factors 9 and 12, as described elsewhere (6). The chicks were weighed, housed on wood shavings, and fed salmonella-free rations and tap water ad libitum. Chicks were observed regularly and killed in extremis.

Infection. Bacteria were grown overnight, with shaking, in tryptone soya broth (CM129; Oxoid) at 37°C. Optical density was determined by spectrophotometer at A_{595}, and decimal dilutions were made in PBS (pH 7.2). The viable count was determined on blood (5%) agar (CM331; Oxoid). Chicks were dosed orally with 0.1 ml of a PBS-washed culture containing 10^0 to 10^9 CFU delivered into the crop by gavage tube.

Enumeration of salmonellae in organ homogenates. Animals were killed by cervical dislocation or pentobarbitone overdose. The viable count in organ homogenates was determined (24) on brilliant green agar (BGA) (CM329; Oxoid) with or without 50 μg of nalidixic acid (Sigma) per ml, and the plates were incubated at 37°C overnight. The limit of detection was 200 CFU. Samples of organ homogenates were enriched in Selenite-F broth (CM395/L121; Oxoid), incubated at 42°C, and then subcultured on BGA. Novobiocin (Sigma) at a concentration of 1 μg/ml was included in BGA plates used for cecal samples to suppress gut bacteria other than salmonellae. Data points (see Fig. 1 to 6) were expressed as geometric means of viable counts expressed in $\log_{10}$ units.

The identity of strain CVL30 was confirmed routinely by its inability to grow on minimal salts agar (M9; BBL) and the inability to produce hydrogen sulfide from the sodium thiosulfate in triple sugar iron agar slopes (CM277; Oxoid). The inability to produce hydrogen sulfide is characteristic of the nonleaky *aroA* mutation, vitamin K being required as a cofactor for enzymatic reduction.

Infection of meconium and gut contents. Meconium from newly hatched chicks and the contents of the duodena, ilea, and ceca from older chicks were removed postmortem. The samples were diluted 1:1 (vol/vol) with sterile normal saline, and 0.1 ml of inoculum containing ca. 10^3 CFU of *S. enteritidis* LA5 or CVL30 or *S. typhimurium* 2391 Nal^r was added to 0.5 ml of each sample. The samples were incubated at 37°C overnight and plated on BGA, and the viable count was determined.

RESULTS

LD_{50} of strain CVL30 in BALB/c mice. The LD_{50} of *S. enteritidis* CVL30 was ca. 7.8 $\log_{10}$ by the intraperitoneal route. The intraperitoneal LD_{50} of parent strain LA5 was established previously as <12 organisms (7).

Oral dosing with *S. typhimurium* 2391 Nal^r and *S. enteritidis* LA5 and CVL30. The mortality was higher and the time to death was shorter with the *S. typhimurium* strain than with the *S. enteritidis* parent strain. An oral dose of 10^3 CFU of *S. typhimurium* 2391 Nal^r killed 3 of 20 chicks by day 5, whereas a dose of 10^5 CFU killed 7 of 20 chicks by day 4. This strain was therefore more virulent in newly hatched chicks than *S. enteritidis* LA5, which by day 6 postinfection killed 1 of 25 chicks dosed with 10^9 CFU. *S. typhimurium* 2391 Nal^r continued to multiply in the reticuloendothelial system (RES) following invasion (Fig. 1), and the numbers of this strain recovered from livers and spleens were greater than those from chicks given similar doses of *S. enteritidis* LA5 (Fig. 2).

S. typhimurium 2391 Nal^r and *S. enteritidis* LA5 multiplied in the gut of newly hatched chicks after oral inoculation with low doses of ca. 10^3 or 10^5 CFU, and by day 2, similar numbers were recovered from the cecal contents, irrespective of whether the chicks were dosed with 10^3 or 10^5 organisms (Fig. 1 and 2). In addition, when inocula containing very low numbers (2 or 20 CFU) of *S. enteritidis* LA5 were given orally to newly hatched chicks, they also multiplied in the gut, and ca. $\log_{10}$ 5.5 CFU/g were recovered from the cecal contents at postmortem 24 h later. Oral inocula of *S. typhimurium* 2391 Nal^r similar to those of LA5 also multiplied in vivo, and ca. $\log_{10}$ 4.0 CFU/g were recovered from the cecal contents 24 h later (data not shown).

To compare the invasiveness of the strains groups of 20 to 30 chicks were dosed orally with *S. typhimurium* 2391 Nal^r (Fig. 1), *S. enteritidis* parent strain LA5 (Fig. 2 and 3), or *S. enteritidis* CVL30 (Fig. 4 to 6) in doses ranging from 10^3 to 10^9 CFU. To compare the effect of age on invasiveness, chicks ranging from 1 to 6 days old were inoculated.

Strain CVL30 invaded from the gut and reached the spleen and liver 1 day after the dosing of newly hatched chicks with 10^3 to 10^9 CFU and achieved plateau values which were ca. 1.0 $\log_{10}$ less than those of the parent strain LA5 in livers and spleens at approximately day 7 postinoculation. The auxotrophic nature of the mutant strain CVL30 did not restrict its invasiveness, it did not appear to undergo sustained replication within the RES, and there was no gross pathology at postmortem. In contrast, there was gross pathology in 6 of 24 newly hatched chicks dosed with 10^9 CFU of *S. enteritidis* parent strain LA5, which caused pericarditis, perihepatitis, and peritonitis. Chicks dosed with 10^5 to 10^9 CFU of strain LA5 also failed to thrive because of morbidity due to diarrhea and inappetence.

Invasion from the gut by *S. enteritidis* LA5 and CVL30 was age dependent. The numbers of strain CVL30 organisms recovered from the spleen and liver were similar when chicks were dosed orally at 1 day old and at 3 days old (Fig. 4 and 5). However, when chicks were dosed at 5 or 6 days old with strain LA5 (Fig. 3) or CVL30 (Fig. 6), the invasion and colonization of livers and spleens were dose dependent, with an inoculum of at least 10^9 CFU being required to ensure invasion. The persistence and multiplication in the ceca of both *S. enteritidis* strains were also reduced at this age.

By 3 weeks after the infection of newly hatched chicks *S. enteritidis* parent strain LA5 was still present in livers and spleens (Fig. 2). Clearance appeared to have begun at this age only in the group given the lowest dose, 10^3 CFU. In contrast,

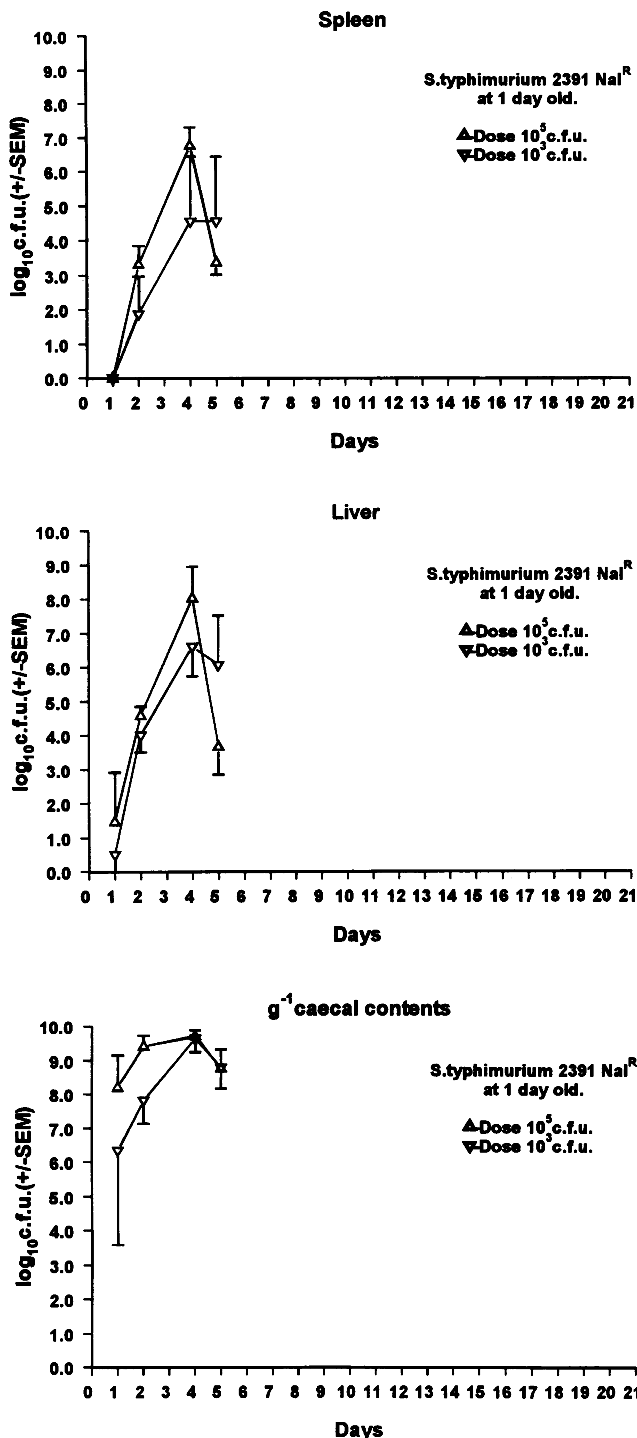

FIG. 1. Log$_{10}$ CFU of *S. typhimurium* 2391 Nal^r in the spleens, livers, and cecal contents of chicks dosed orally when newly hatched and then killed at intervals. Each point represents five chicks. SEM, standard error of the mean.

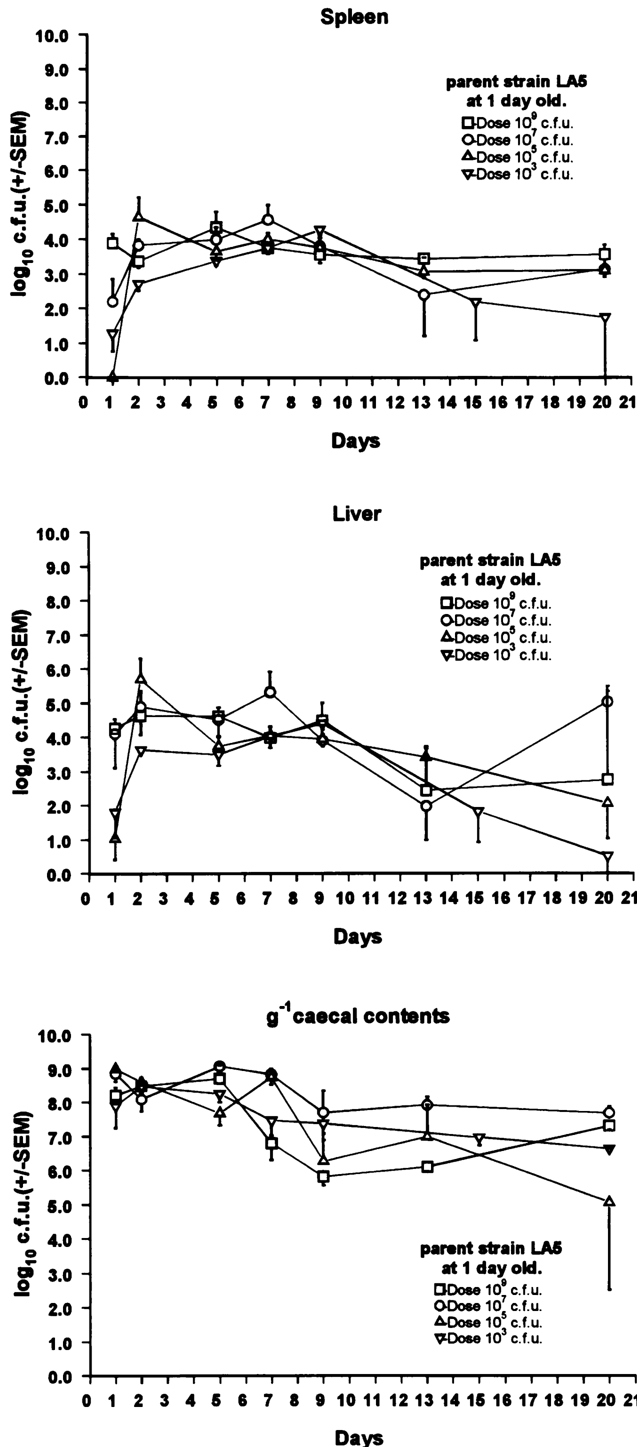

FIG. 2. Log$_{10}$ CFU of *S. enteritidis* parent strain LA5 in the spleens, livers, and cecal contents of chicks dosed orally when newly hatched and then killed at intervals. Each point represents five chicks. SEM, standard error of the mean.

the clearance of strain CVL30 from the livers and spleens of newly hatched chicks (Fig. 4) appeared to have begun by days 9 to 13 in the group dosed with 10^3 CFU. It was cleared from livers and spleens by ca. 3 weeks postvaccination, irrespective of the inoculum. Strain CVL30 was isolated in cloacal swabs from 3 of 30 chickens 4 weeks after they had been dosed as newly hatched chicks with ca. 10^9 CFU, and at 6 weeks, it was isolated postmortem from the ceca in 1 of 6 chickens, but only after enrichment in Selenite broth. Strain CVL30 was not recovered postmortem from three chickens at 9 weeks old.

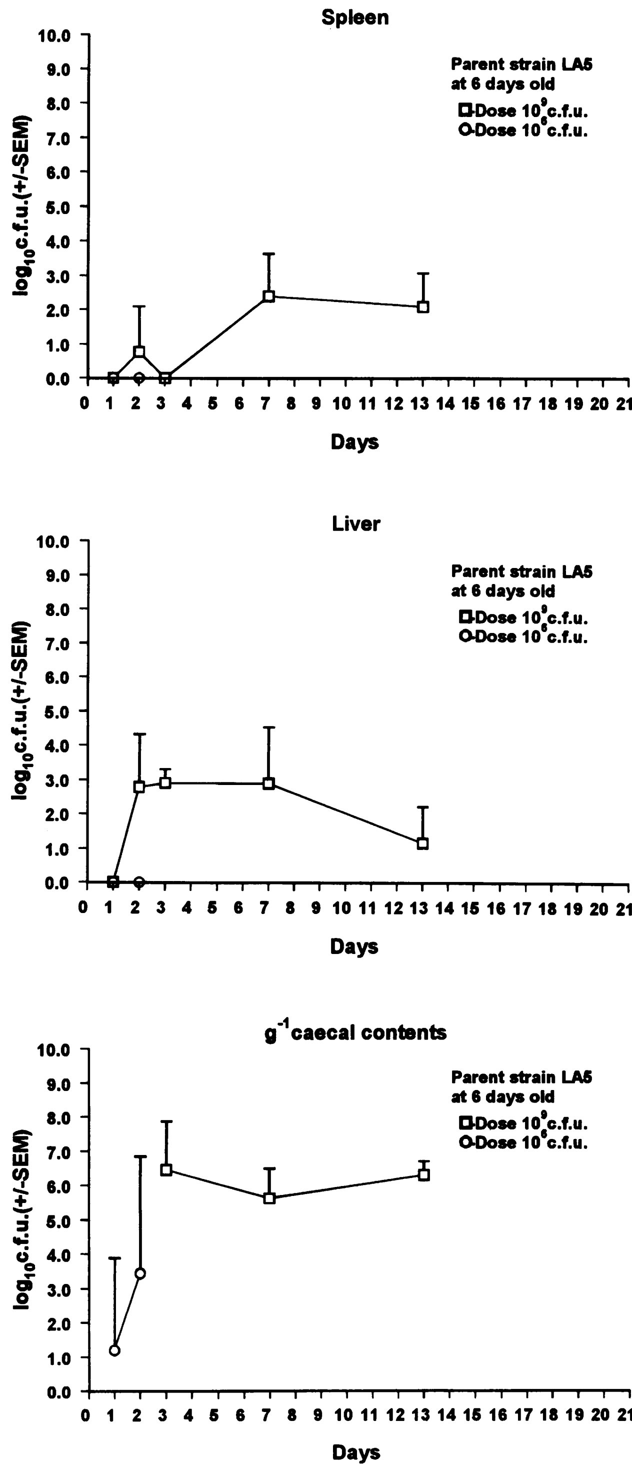

FIG. 3. Log_{10} CFU of *S. enteritidis* parent strain LA5 in the spleens, livers, and cecal contents of chicks dosed orally at 6 days old and then killed at intervals. Each point represents five chicks. SEM, standard error of the mean.

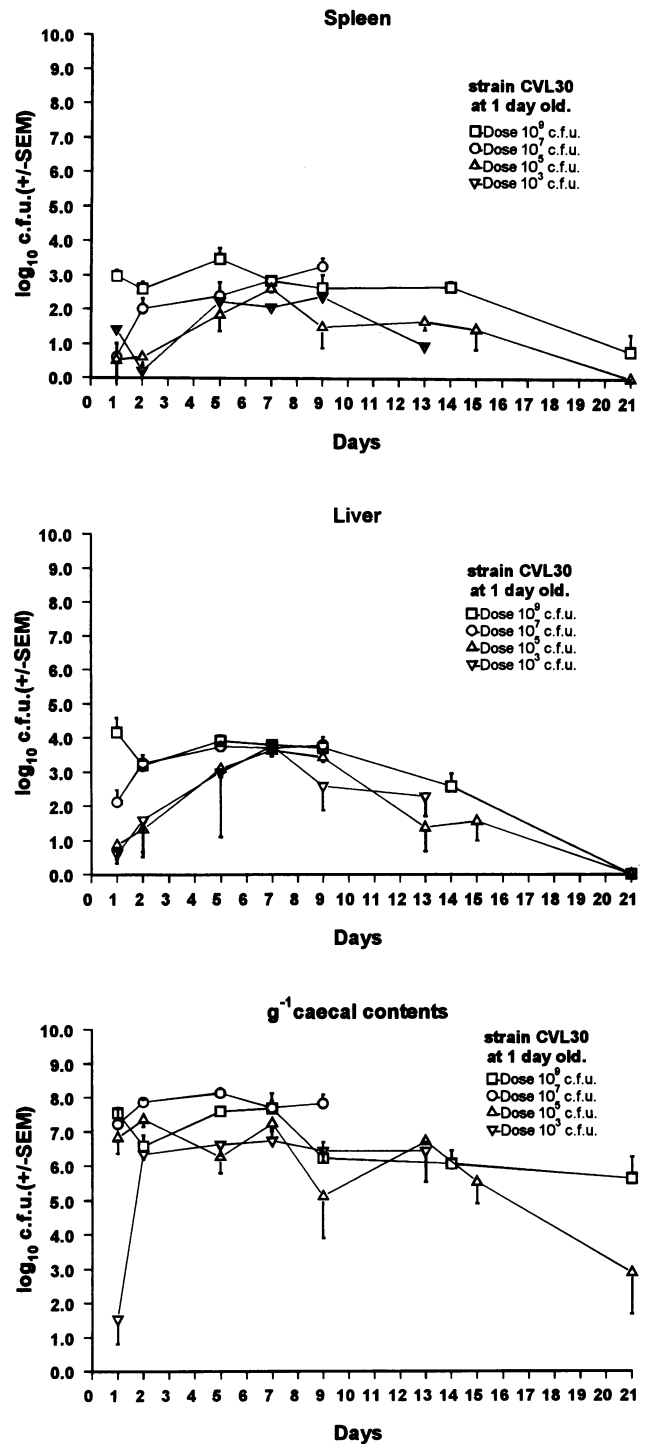

FIG. 4. Log_{10} CFU of *S. enteritidis aroA* CVL30 in the spleens, livers, and cecal contents of chicks dosed orally when newly hatched and then killed at intervals. Each point represents five chicks. SEM, standard error of the mean.

In vitro infection of meconium from newly hatched chicks. Meconium samples were taken postmortem from the duodena, ilea, and ceca of five chicks, and in each case, the pH was ca. 6.5. These samples were then spiked with salmonellae in vitro. After overnight incubation at 37°C, an inoculum of ca. 10^3 CFU of *S. enteritidis* LA5 or *S. typhimurium* 2391 Nal^r had multiplied to reach ca. 10^9 CFU in each sample. Strain CVL30 also multiplied in vitro in meconium from these sites but to levels ca. 0.5 to 1.0 $\log_{10}$ less than those of the wild-type strains.

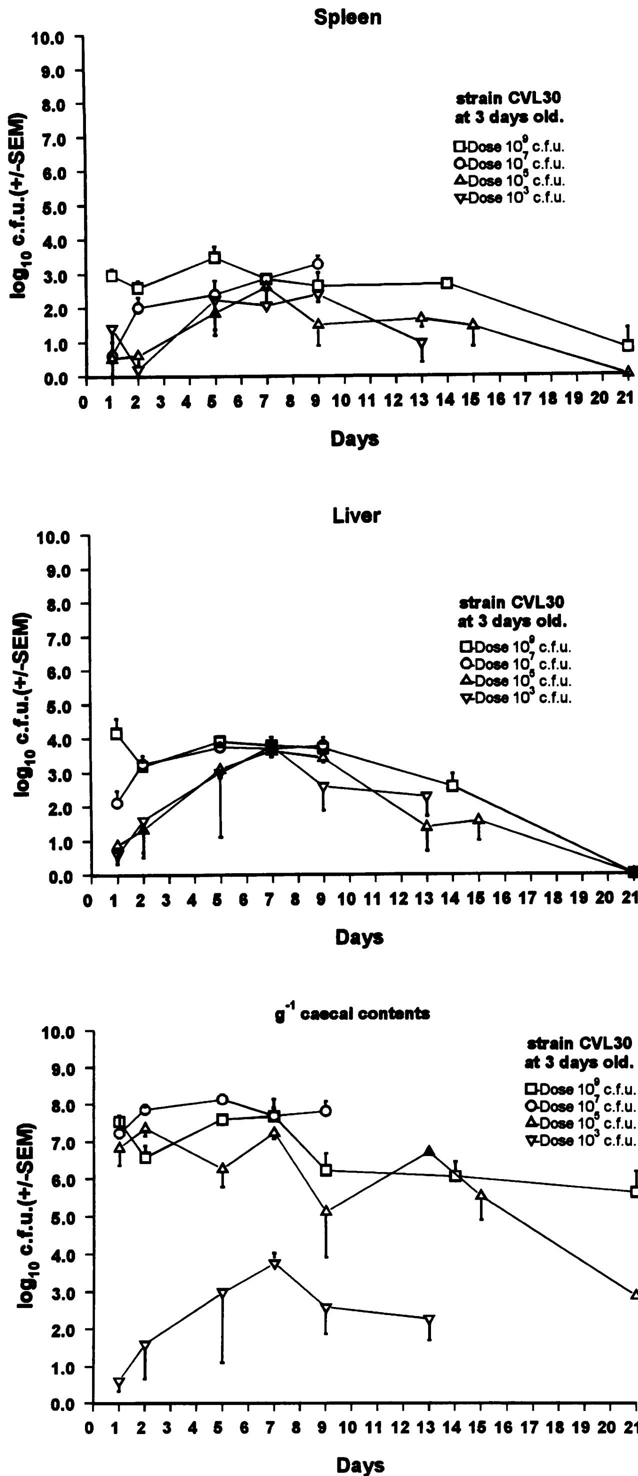

FIG. 5. Log_{10} CFU of *S. enteritidis aroA* CVL30 in the spleens, livers, and cecal contents of chicks dosed orally at 3 days old and then killed at intervals. Each point represents five chicks. SEM, standard error of the mean.

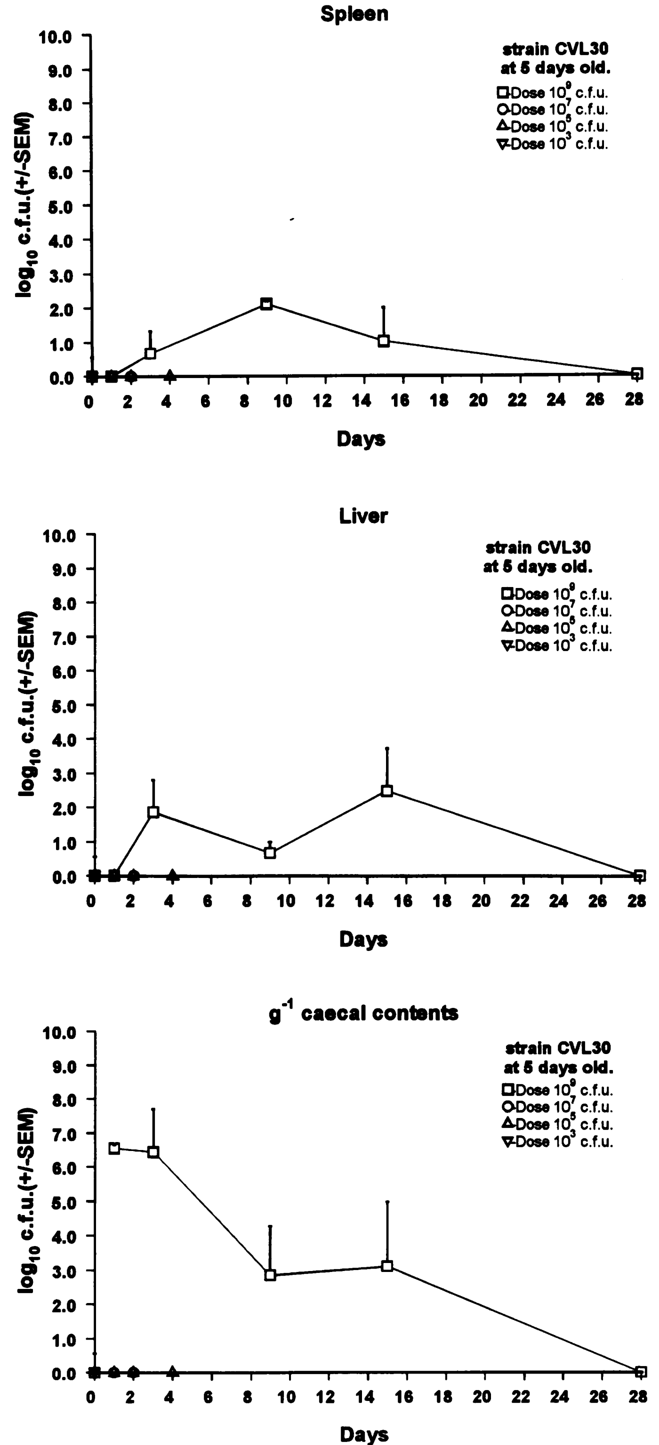

FIG. 6. Log_{10} CFU of *S. enteritidis aroA* CVL30 in the spleens, livers, and caecal contents of chicks dosed orally at 5 days old and then killed at intervals. Each point represents five chicks. SEM, standard error of the mean.

Strain CVL30 grew, apparently under stress, as pinpoint colonies after overnight incubation at 37°C on BGA. (BGA is a rich medium which normally allows strain CVL30 to grow as profusely as its parent strain, LA5.) However, after 2 days of incubation at 37°C, strain CVL30 appeared to recover and the growth was as profuse as that of the wild-type strains (data not shown).

Infection of gut samples from chicks 3 to 14 days old. Samples taken postmortem from the contents of the duodena,

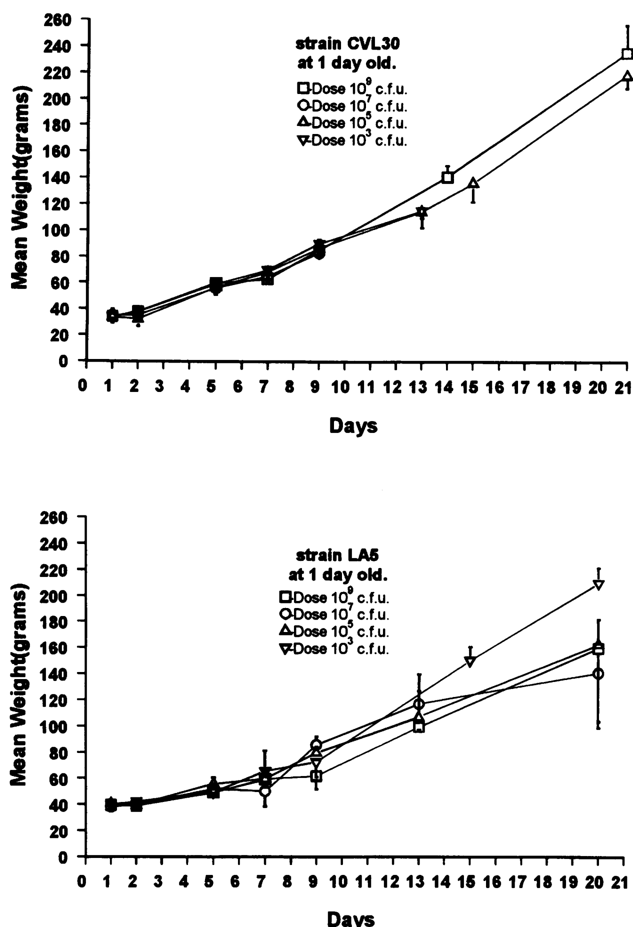

FIG. 7. Mean weights (grams) of chicks dosed when newly hatched with *S. enteritidis* parent strain LA5 or *S. enteritidis aroA* CVL30 and then killed at intervals.

ilea, and ceca of three chicks at 3, 7, 10, and 14 days old were pooled and diluted 1:1 (vol/vol) in normal saline. The pH was ca. 5.5 in the duodenum sample, ca. 6 to 6.5 in the ileum sample, and ca. 4.5 to 5 in the cecal sample, irrespective of age. The samples were spiked with ca. 10^3 CFU of strain LA5, 2391 Nal^r, or CVL30 and incubated at 37°C overnight. The wild-type strains LA5 and 2391 Nal^r multiplied to reach ca. 10^4 to 10^9 CFU in samples from the duodenum and ileum, but they were not recovered from the cecal samples. Strain CVL30 also multiplied in the duodenum and ileum samples but to levels ca. 0.5 to 1.0 $\log_{10}$ less than those of the wild-type strains. Once again, it grew slowly as tiny pinpoint colonies after overnight incubation on BGA at 37°C when cultured from the spiked duodenum and ileum samples, but it did not appear to grow in the cecal samples. Strain CVL30 grew slowly at first on BGA but recovered after 2 days at 37°C to give the normal colonial appearance on BGA when plated directly from broth culture (data not shown).

Weight gain. The lack of pathogenicity of strain CVL30 was suggested by the mean weights of vaccinated chicks (Fig. 7). At 3 weeks old, their mean weight of ca. 220 to 235 g, irrespective of vaccine dose, was similar to that of unvaccinated control chickens weighing ca. 220 g. In contrast, there was considerable morbidity in chicks dosed with *S. enteritidis* parent strain LA5, which caused a significant difference in weight between chicks dosed with 10^5 to 10^9 CFU and controls ($P < 0.01$). There was no significant difference in weight between those inoculated with the lowest dose 10^3 CFU of *S. enteritidis* LA5 and the controls at 3 weeks old.

DISCUSSION

The ideal *S. enteritidis* live oral vaccine for chickens should be derived from a highly virulent parent strain, invade from the gut, and persist for a sufficient length of time to stimulate maximum immunity but carry a mutation which will prevent its continued growth in vivo (3). A live *S. enteritidis* vaccine candidate should be completely avirulent in the host and other animals, highly immunogenic, and capable of colonizing the intestinal tract and the gut-associated lymphoid tissues (14). An efficacious live *Salmonella* vaccine should establish a limited infection in the host and mimic closely the early stages of natural infection (4). Strain CVL30 was well tolerated in chickens with no adverse effect on weight gain, mimicked closely the early stages of infection by its parent strain, LA5, and invaded rapidly from the gut to colonize the RES after the oral dosing of newly hatched chicks. Therefore, this strain fulfills some of the criteria required for a live *S. enteritidis* oral vaccine outlined above, and an article on the protection generated by strain CVL30 in chickens accompanies this article (9).

S. enteritidis CVL30 and LA5 were of comparable invasiveness in newly hatched chicks, and this may be related to the lack of an established microflora in the chicks (23). When chicks were dosed at 5 or 6 days old, the invasiveness of both the wild-type parent strain LA5 and the attenuated *aroA* strain CVL30 was greatly affected. Colonization of the spleen and liver could be detected only when an inoculum of 10^9 CFU was given. We consider this to be dose related, because in each case sodium bicarbonate was administered orally to neutralize the low pH of gastric acid prior to oral inoculation.

The multiplication of 10^3 CFU of strain CVL30 in vivo was suppressed in the cecal contents of chicks dosed at 3 days old. This suppression became more apparent in 5-day-old chicks when strain CVL30 and strain LA5 did not multiply in the ceca. In contrast to strain CVL30, which did not cause morbidity in chicks dosed orally, the wild-type strain LA5 invaded rapidly in newly hatched chicks, causing limited mortality and gross pathology in some survivors. However, there was no mortality and no evidence of gross pathology postmortem in chicks dosed with the *S. enteritidis* wild-type strain LA5 at 6 days old. Because the cecal tonsils, located at the ileo cecal junction and acting as secondary lymphoid tissue, are not functional in the newly hatched chick (28), this may have contributed to the invasion by these *Salmonella* strains. Infection of newly hatched chicks with small numbers of the *S. enteritidis* wild-type strain was followed by rapid multiplication in the meconium-lined gut and invasion. Theoretically, this could progress to the colonization of internal organs, such as those of the immature reproductive tract, followed by a persistent infection and may be a factor in the relative success of this serotype in chickens due to its eventual vertical transmission via the egg.

S. typhimurium is more virulent for chickens than other food-poisoning serotypes (31), and *S. typhimurium* 2391 Nal^r was certainly more virulent than *S. enteritidis* LA5 in White Leghorn chicks. After invading, 2391 Nal^r multiplied in the livers and spleens more rapidly to reach greater numbers than any *S. enteritidis* poultry strain which we have investigated in chicks at this laboratory (unpublished observations). We reported (7, 8) that an oral challenge at 8 weeks old with an *S.*

enteritidis wild-type strain, 109 Nal^r, or *S. typhimurium* 2391 Nal^r was followed by invasion from the gut. Invasion was followed by rapid seroconversion to LPS in nonvaccinated control chickens within 7 to 10 days of challenge. However, at this age, there were no deaths and very little morbidity in controls after an oral dose, with antacid, of 10^9 CFU of *S. typhimurium* 2391 Nal^r. This was in marked contrast to the mortality in chicks following a dose of 10^3 CFU of the same strain reported here.

The reduction of pH in the cecal contents of chicks older than 1 day may have been due to the action of a developing gut microflora (1), anaerobes and lactobacilli in particular. A variable response to inoculation with an *S. typhimurium aroA* mutant in weanling BALB/c mice, which are innately susceptible to salmonella infection, has also been reported (5). Among 3-week-old mice of various sublines dosed orally with the *aroA* strain, there was mortality ranging from <5 to 22%, although mortality was absent from older mice. This variable susceptibility may be related to the development of a more complex gut microflora in the older mice.

In vitro, samples of the contents of the duodena and ilea but not the ceca from chicks 3 days old and more supported the multiplication of strain CVL30. However, when plated on BGA medium, this strain appeared to grow under stress as tiny pinpoint colonies, resuscitated only by further incubation at 37°C. A reduction of the pH in defined laboratory media has been shown to affect the growth of salmonellae, reducing the viable count of *S. typhimurium* (13, 18), and the suppression of growth described here may have been due to a more acidic pH in the ceca of the older chicks.

There was a delay after inoculation during which small numbers of LA5 and 2391 Nal^r organisms multiplied in the gut, and this was followed by invasion, colonization, and multiplication in the RES and, ultimately, deaths. Such a phenomenon has been reported for chicks (2). Therefore, it is difficult to see how an oral LD_{50} (29) or a 50% oral colonization dose (12) can be established in newly hatched chicks if small numbers of salmonellae are able to multiply rapidly in the gut irrespective of the loading dose. An oral LD_{50} established in newly hatched chicks may reflect the capacity of a strain to multiply in the conditions in the intestinal tract at the time of inoculation, e.g., its capacity to withstand acid pH or osmotic shock. Such an LD_{50} may not accurately reflect the strain's virulence, i.e., the capacity to evade destruction in the RES, quantifiable preferably by a parenteral route.

Chicks hatch with immature T lymphocytes which become fully responsive when the chicks are around 4 days old (22), and the deliberate impairment of B- or T-lymphocyte responses did not appear to influence resistance to colonization of the ceca by *S. typhimurium* (10). Therefore, it is likely that systemic cellular immunity and mucosal responses in the gut would develop significantly only after approximately 4 days of age, which would leave the chick relatively unprotected for the first few days of its life. From a practical point of view, when chicks hatch in commercial hatcheries, between days 18 and 20 of embryonation, they are often exposed to aerosols and infected dust bearing salmonellae. In a survey of commercial hatcheries in the United States, salmonellae were recovered from 80% of the posthatch egg fragments and 88% of swabs from conveyor belts (11). Significantly, several *Salmonella* serotypes were recovered from chicks before the ingestion of feed or the entry of salmonellae from any other source. Prompt vaccination, as soon as possible after hatching, may prevent colonization of the gut by small numbers of wild-type salmonellae, which, as described here, may multiply extensively in the gut, with the consequent threat to public health. To be fully effective, live *S. enteritidis aroA* vaccines should likely be administered soon after hatching or even in ovo.

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