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Development and Evaluation of an Experimental Vaccination Program Using a Live Avirulent *Salmonella typhimurium* Strain To Protect Immunized Chickens against Challenge with Homologous and Heterologous *Salmonella* Serotypes

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A stable live avirulent, genetically modified Δ*cya* Δ*crp* *Salmonella typhimurium* vaccine strain, χ3985, was used in several vaccination strategies to evaluate its use in the control of *Salmonella* infection in chickens. Oral vaccination of chickens at 1 and at 14 days of age with 10^8 CFU of χ3985 protected against invasion of spleen, ovary, and bursa of Fabricius and colonization of the ileum and cecum in chickens challenged with 10^6 CFU of virulent homologous *Salmonella* strains from group B. Chickens challenged with heterologous *Salmonella* strains from groups C, D, and E were protected against visceral invasion of spleen and ovary, while invasion of the bursa of Fabricius and colonization of ileum and cecum was reduced in vaccinated chickens. Oral vaccination at 2 and at 4 weeks of age induced an excellent protection against challenge with virulent group B *Salmonella* serotypes and very good protection against challenge with group D or E *Salmonella* serotypes, while protection against challenge with group C *Salmonella* serotypes was marginal but significant. Vaccination at 2 and at 4 weeks of age also protected vaccinated chickens against challenge with 10^8 CFU of highly invasive *S. typhimurium* or *S. enteritidis* strains. The protection of chickens vaccinated with χ3985 against challenge with homologous and heterologous *Salmonella* serotypes is outstanding, and the complete protection against ovarian invasion in chickens challenged with 10^8 CFU of highly invasive *S. typhimurium* or *S. enteritidis* strains suggests that vaccination of chickens with χ3985 can complement the present hygiene- and sanitation-based *Salmonella* control measures. This paper reports a breakthrough in the use of live avirulent vaccine to control *Salmonella* carriers in chickens.

Food-borne enteric human pathogens continue to plague society on a worldwide scale and appear to be increasing despite research and management efforts to remedy the problem. In the United States, 80 to 90% of human *Salmonella* infections result from *Salmonella* transmission by persistent infection of farm animals and subsequent contamination of meat, eggs, and dairy products. In 1989, *Salmonella typhimurium*, *S. enteritidis*, *S. heidelberg*, *S. hadar*, and *S. agona* accounted for 57.9% of *Salmonella* serotypes isolated from human infections and accounted for 46.5% of isolations obtained from poultry (7). *S. enteritidis* isolates in the United States belong primarily to phage types PT8 and PT13A, with 48% of poultry and 64% of animal *Salmonella* isolates in the United States being PT8 (23).

The major obstacle to *Salmonella* control in the poultry industry is the ubiquitous presence of salmonellae. Once salmonellae get onto a farm, they spread rapidly because infected chickens and rodents serve as carriers. *Salmonella* carriers constantly shed salmonellae and contaminate the feeding and watering systems and the poultry farm environment. The present methods of controlling food poisoning-related salmonellae on the farm are inadequate or too expensive to enforce. The use of antibiotics has been reduced because of complications resulting from the development of antibiotic-resistant *Salmonella* strains (2, 18, 30), experimental implication of some antibiotics in enhancing *Salmonella* excretion (5), and the risk of feeding consumers with poultry products containing antibiotic residues. Inoculation of nonpathogenic gut flora from adult chickens into day-old chicks, a phenomenon known as competitive exclusion, has been shown to reduce colonization of young chicks by pathogenic organisms (27). Treatment of finished poultry products with irradiation is an emerging approach but is an expensive means of product sterilization.

The emergence of *S. enteritidis* infection of humans as a result of egg contamination by salmonellae led to the establishment in 1990 of a task force in the United States for *S. enteritidis* control in the poultry industry (35). Poultry feed is a major source of *Salmonella* infections for chickens, as a result of the use of *Salmonella*-contaminated raw materials from rendering plants. In the United States, the Food and Drug Administration Center for Veterinary Medicine has now focused on using microbiological and chemical standards instead of the old organoleptic criteria utilized in the inspection of rendering plants (24). The ultimate aim is to obtain *Salmonella*-free feed. The attainment of low levels of salmonellae in feed and strict adherence to sanitary regulation and biosecurity on farms will definitely reduce the load of salmonellae on the farm. These efforts will not lead to *Salmonella*-free chickens because the prolific nature of salmonellae in infected chickens will obliterate all control efforts if the chickens are unable to prevent *Salmonella* proliferation.

Therefore, there is a need for the induction of an inherent protective mechanism within chickens at the production level that will ensure a low level or elimination of *Salmonella* contamination. We believe that vaccination should be a major component of a *Salmonella* control program. Vaccination was used to control *S. gallinarum* (29), while *S. pullorum* was eliminated

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TABLE 1. Bacterial strains used

Strain	Genotype or phenotype	Reference or source
S. typhimurium		
χ3985	(Δ*crp-11* Δ[*zhc-1431*::Tn*10*] Δ*cya-12*Δ[*zid-62*::Tn*10*] FA^r Tc^r Ap^s Mal^-	10
F98	Wild-type group B (1, 4, 12)	5
2921-1	Wild-type group B (1, 4, 12)	19
S. agona NR1	Wild-type group B (1, 4, 12)	M. Rosenfeld, Borstel Institute
S. bredeney NR8	Wild-type group B (1, 4, 12, 27)	M. Rosenfeld, Borstel Institute
S. heidelberg NR99	Wild-type group B (1, 4, [5], 12)	M. Rosenfeld, Borstel Institute
S. infantis NR29	Wild-type group C_1 (6, 7)	M. Rosenfeld, Borstel Institute
S. montevideo NR35	Wild-type group C_1 (6, 7)	M. Rosenfeld, Borstel Institute
S. hadar NR14	Wild-type group C_2 (6, 8)	M. Rosenfeld, Borstel Institute
S. albany NR2	Wild-type group C_3 (8, 20)	M. Rosenfeld, Borstel Institute
S. enteritidis		
4973	Wild-type group D (1, 9, 12) PT13A	19
Y-8P2	Wild-type group D (1, 9, 12) PT8	28, 33
27A	Wild-type group D (1, 9, 12) PT8	28
B6996	Wild-type group D (1, 9, 12) PT13A	Charles Benson, University of Pennsylvania
S. panama NR38	Wild-type group D (1, 9, 12)	M. Rosenfeld, Borstel Institute
S. anatum NR3	Wild-type group E_1 (3, 10)	M. Rosenfeld, Borstel Institute

by identification and removal of seropositive flocks. No effective vaccine is available for the control of *Salmonella* infection associated with human food poisoning in chickens. Killed *Salmonella* vaccines have not produced convincing levels of protection against wild-type *Salmonella* challenge (3, 11, 12, 34). Parenteral vaccination of chickens with outer membrane proteins of *S. enteritidis* was better than vaccination with killed bacteria in preventing colonization of immunized chickens by wild-type *S. enteritidis* after challenge (25). Oral vaccination with live homologous salmonellae has been shown to induce protection against visceral invasion by challenge strains, with reduction in the colonization of the gastrointestinal tract (3, 20, 26, 32). Intramuscular vaccination failed to protect vaccinated chickens against intestinal colonization (3).

Oral infection of 1-day-old chickens with *S. typhimurium* wild-type strain χ3761 induced lymphocyte depletion and immunosuppression, which facilitated establishment of *Salmonella* carrier status in infected chickens (15). This negative attribute of *S. typhimurium* χ3761 was overcome by the deletion of the *cya* and *crp* genes from χ3761 (15). The derived Δ*cya* Δ*crp* *S. typhimurium* strain, χ3985, is avirulent and immunogenic (10, 14). Immunization of chickens with χ3985 induced an effective protection against challenge with highly virulent *S. typhimurium* χ3761 (10, 14, 17). Prevention of *Salmonella* colonization in chickens is important for effective control of salmonellae in the poultry industry. It is therefore important to develop vaccination strategies that would enhance the ability of live avirulent *S. typhimurium* vaccine strains to protect effectively against invasive infection and colonization by homologous and heterologous *Salmonella* serotypes. This report describes the evaluation of the Δ*cya* Δ*crp* *S. typhimurium* strain χ3985 in various vaccination regimens in protecting chickens against challenge with virulent *Salmonella* strains from serotype groups B, C, D, and E that frequently colonize poultry and are implicated in food poisoning in humans.

MATERIALS AND METHODS

Chickens. Fertile eggs from specific-pathogen-free chickens, obtained from Specific Pathogen Free Avian Services (Roanoke, Ill.), were incubated and hatched in a Humidaire incubator hatcher in our facility. All chickens used were unsexed White Leghorns. Groups of experimental chickens were housed in separate isolators to prevent cross-contamination.

Bacterial strains. The *Salmonella* strains used are listed in Table 1. The Δ*cya* Δ*crp* *S. typhimurium* vaccine strain χ3985 is a derivative of a highly virulent *S. typhimurium* χ3761, with a 50% oral lethal dose of 3×10^3 CFU for 1-day-old chicks. *S. typhimurium* χ3985 is avirulent, with a 50% oral lethal dose of $>4 \times 10^9$ CFU for 1-day-old chicks (10). Representatives of *Salmonella* strains from group B, C, D, and E that are implicated in *Salmonella* infection of poultry, including highly invasive *S. enteritidis* strains that were isolated from farms implicated in human outbreaks of *Salmonella* food poisoning, were used as challenge strains. All *Salmonella* strains were maintained as frozen cultures suspended in 1% Bacto Peptone (Difco Laboratories, Detroit, Mich.) containing 5% glycerol and fast frozen in dry ice-ethanol for storage in duplicate at −70°C.

Growth of salmonellae for vaccination. *Salmonella* strains used for chicken inoculation were grown overnight as static cultures at 37°C in Luria broth (22). These cultures were diluted 1:50 into prewarmed Luria broth and grown with aeration at 37°C for approximately 4 h to an A_{600} of about 0.8 to 1.0. The cells were centrifuged at 8,000 × *g* for 10 min at 4°C and then suspended in buffered saline with gelatin (BSG) (8) to yield the required density. Serial dilutions of the suspended *Salmonella* strains were plated on Penassay agar (antibiotic medium 2; Difco) for titer determination, and χ3985 was plated on MacConkey agar (Difco) supplemented with 1% maltose to verify the Cya^- Crp^- phenotype.

Oral vaccination procedure and protection assessment. Vaccinated chickens were housed in Horsfall isolators equipped with HEPA filters and thermostatically regulated. Noninfected chickens used as controls were housed in standard growing cages until they were transferred into a Horsfall isolator before challenge with salmonellae. Chickens were deprived of food and water for 4 h before oral vaccination with χ3985. Chickens were vaccinated with 100 μl of 10^9 CFU of *S. typhimurium* χ3985 cells per ml suspended in BSG and delivered directly into the crop with a 1-ml syringe with 3-in.-long 18-gauge animal feeding biomedical needles (Popper and Sons, Inc., New York, N.Y.). Food and water were returned 30 min after vaccination. Food was not withdrawn before challenge with specified doses of various *Salmonella* serotypes because time of *Salmonella* infection on the farm cannot be predetermined. The degree of protection induced by oral vaccination was assessed by comparing the ability of the challenge *Salmonella* strain to colonize the gastrointestinal tract and invade visceral organs of challenged vaccinated chickens with the ability of the same *Salmonella* strain to

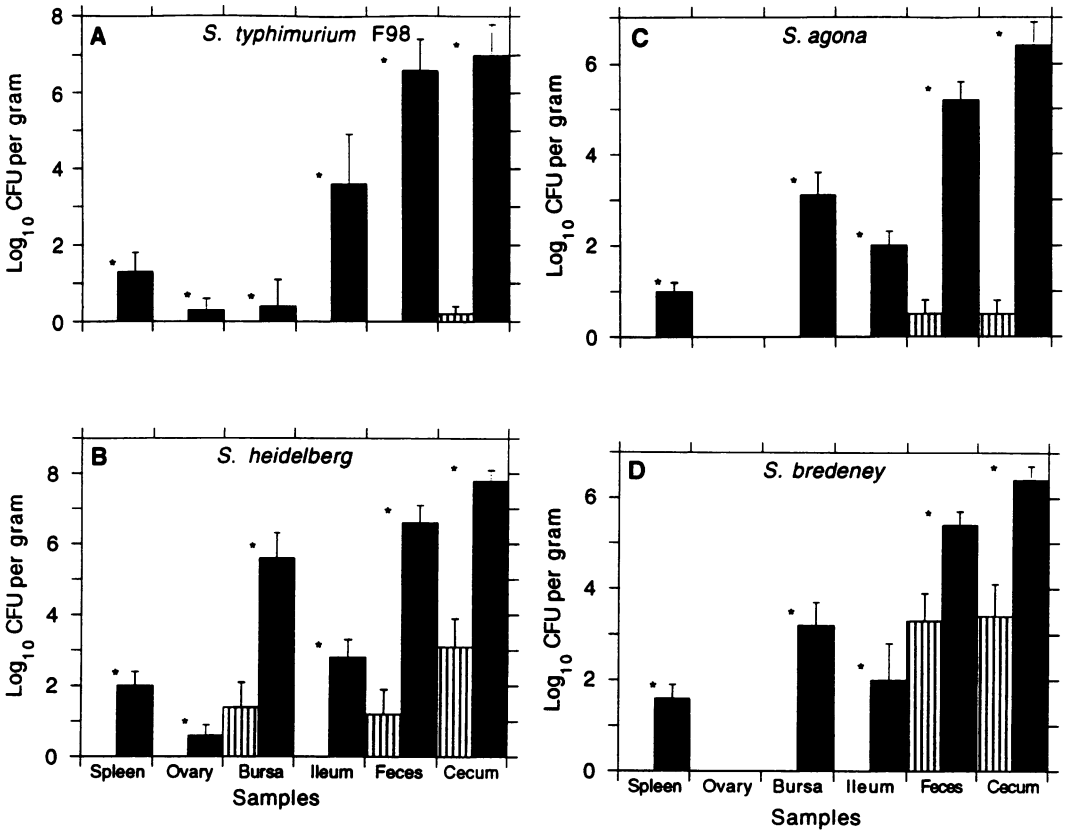

FIG. 1. Responses of chickens vaccinated with 10^8 CFU of Δ*cya* Δ*crp* *S. typhimurium* χ3985 at 1 and 14 days of age versus nonvaccinated chickens when challenged with 10^6 CFU of *S. typhimurium* F98 (A), *S. heidelberg* (B), *S. agona* (C), and *S. bredeney* (D) at 4 weeks of age. Samples were collected from individual chickens at 6 weeks of age, and values presented are means ± standard errors of the means of 10 samples. Each value represents $\log_{10}$ CFU of salmonellae isolated per gram of sample. *, significant difference at $P < 0.05$; ▥, vaccinated; ■, nonvaccinated.

colonize and invade nonvaccinated and challenged control chickens.

Vaccination experiments. (i) Oral vaccination at 1 day and 2 weeks of age. Two hundred 1-day-old chickens were vaccinated orally with 10^8 CFU of Δ*cya* Δ*crp* *S. typhimurium* χ3985 at 1 day of age and boosted with the same dose at 14 days of age. Twelve groups of vaccinated and twelve groups of nonvaccinated chickens (five chickens per group) were challenged orally at 4 weeks of age with 10^6 CFU of the following *Salmonella* serotypes: group B, *S. typhimurium* F98, *S. agona*, *S. heidelberg*, and *S. bredeney*; group C, *S. albany*, *S. hadar*, *S. infantis*, and *S. montevideo*; group D, *S. enteritidis* 4973 PT13A and *S. panama*; and group E, *S. anatum*. All chickens were necropsied at 6 weeks of age (i.e., 2 weeks after challenge with wild-type strains).

(ii) Oral vaccination at 2 and 4 weeks of age. Five groups of 10 specific-pathogen-free chickens were vaccinated at 2 weeks of age with 10^8 CFU of Δ*cya* Δ*crp* *S. typhimurium* χ3985 and boosted with the same dose at 4 weeks of age. When 6 weeks old, the vaccinated chickens and five groups of 10 nonvaccinated 6-week-old chickens were challenged with 10^6 CFU of *S. typhimurium* F98, *S. infantis*, *S. enteritidis* 27A PT8, *S. enteritidis* 4973 PT13A, or *S. anatum*. All the chickens were necropsied at 8 weeks of age.

Effect of vaccination on challenge with highly invasive *Salmonella* strains. One hundred 2-week-old chickens were vaccinated orally with 10^8 CFU of Δ*cya* Δ*crp* *S. typhimurium* χ3985 and boosted with the same dose when 4 weeks of age. The vaccinated chickens were divided into five groups of 10 chickens and challenged orally, along with five groups of 10 nonvaccinated chickens, at 6 weeks of age with 10^8 CFU of highly invasive *S. typhimurium* 2921-1 and *S. enteritidis* 4973 PT13A, B6996 PT13A, Y-8P2 PT8, and 27A PT8. We used 10^8 CFU of highly invasive *Salmonella* strains in order to test the limit of the protection induced by oral vaccination with χ3985 against visceral invasion by heterologous wild-type *Salmonella* strains. Five chickens from each group were necropsied at 4 days after challenge, and five were necropsied at 11 days after challenge. All vaccination and challenge experiments were repeated.

Sample collection and processing. Chickens were euthanized by CO_2 asphyxiation within 2 weeks after challenge with wild-type *Salmonella* strains. During necropsy, the chickens were laid on their backs with legs drawn away from the body. The skin was incised between the legs midway between the sternum and cloaca. The cut edge was forcibly reflected anteriorly to expose the ventral aspect of the body up to the neck region. The abdominal muscles and rib cage were cut from both sides anteriorly up to the clavicle, a bone shear was used to cut the clavicle, and the rib cage was reflected to one side to expose the organs in the abdominal and thoracic cavities. All samples were collected under strict aseptic conditions as described previously (15). All samples were collected into a sterile preweighed disposable polypropylene culture tubes with a snap cap and kept on ice after sample collection. The organs sampled from groups of vaccinated or nonvacci-

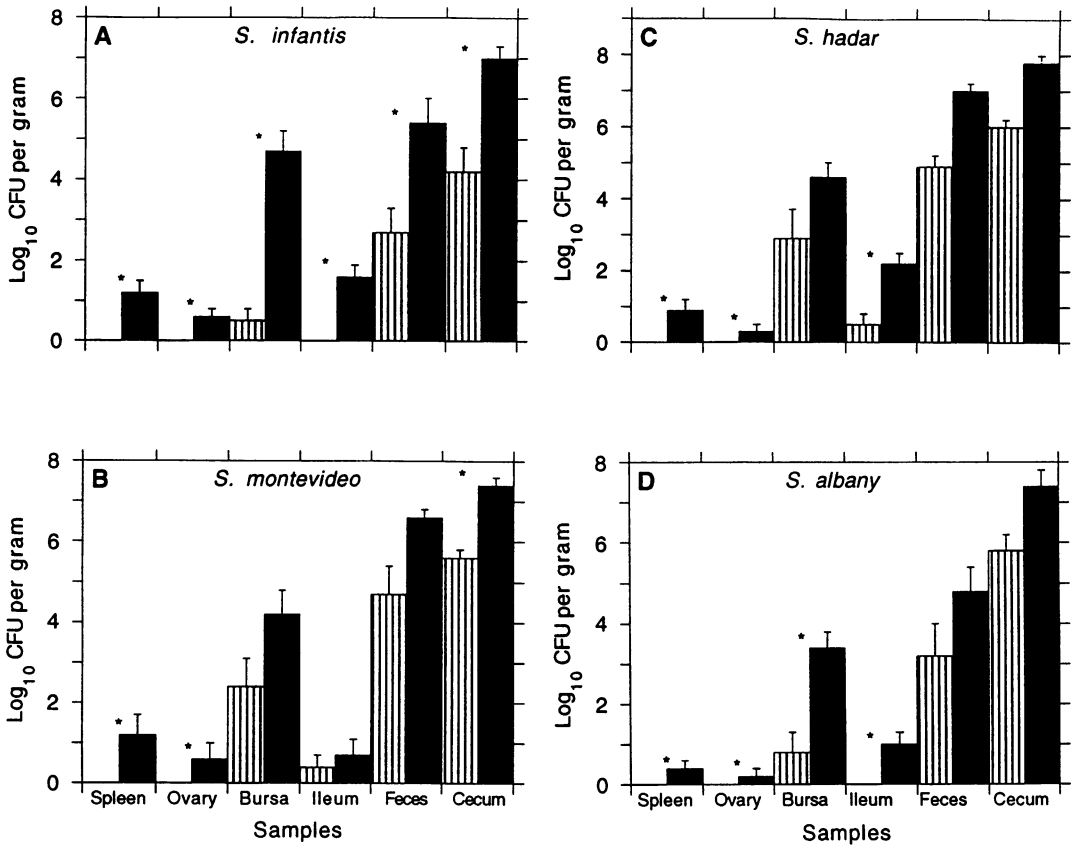

FIG. 2. Responses of chickens vaccinated with 10^8 CFU of Δ*cya* Δ*crp S. typhimurium* χ3985 at 1 and 14 days of age versus nonvaccinated chickens when challenged with 10^6 CFU of *S. infantis* (A), *S. montevideo* (B), *S. hadar* (C), and *S. albany* (D) at 4 weeks of age. Samples were collected from individual chickens at 6 weeks of age, and values presented are means ± standard errors of the means of 10 samples. Each value represents the $\log_{10}$ CFU of salmonellae isolated per gram of sample. *, significant difference at $P < 0.05$; ▥, vaccinated; ■, nonvaccinated.

nated chickens were spleen, ovary, and bursa of Fabricius. Contents from the ileum and cecum as well as feces were also collected. All samples were homogenized as 1:10 dilutions in BSG. The samples were analyzed by decimal dilutions in BSG. The diluted samples were plated in drops on *Salmonella-Shigella* agar and incubated at 37°C for 24 h (permitting detection of 10^2 CFU of salmonellae per g of sample). The presence or absence of salmonellae at concentrations below 10^2 CFU/g in the sample was tested by ability or inability to detect salmonellae in samples incubated in selenite cysteine broth at 37°C for 48 h, subcultured on *Salmonella-Shigella* agar, and incubated for 24 h at 37°C. Samples positive by selective enrichment in selenite cysteine broth were recorded as 10 CFU, and negative samples were recorded as 0 CFU.

Statistics. Statistical significance was calculated at the 0.05 level of probability, using two-way analysis of variance.

RESULTS

Protection induced by oral vaccination at 1 and 14 days of age. Colonization or invasion by the challenge *Salmonella* strain was determined by quantifying the amount of salmonellae in the gastrointestinal tract or visceral organs of vaccinated and nonvaccinated chickens after challenge with wild-type strains. The degree of protection was determined by comparing data obtained from vaccinated chickens with those obtained from nonvaccinated chickens. Oral vaccination of chickens at 1 and 14 days of age with Δ*cya* Δ*crp S. typhimurium* χ3985 induced significant protection against challenge with 10^6 CFU of wild-type homologous *Salmonella* strains from group B (Fig. 1). Solid protection was observed against visceral invasion by *S. typhimurium*, *S. agona*, and *S. bredeny*. Salmonellae were detected only by enrichment in the ceca of vaccinated chickens challenged with *S. agona* and *S. typhimurium* (<1 CFU/g). A low level of salmonellae was detected in the bursa of Fabricius of vaccinated chickens challenged with *S. heidelberg* (10 CFU/g) and in the feces and cecal contents of vaccinated chickens challenged with *S. heidelberg* and *S. bredeny* (1 to 10^3 CFU/g). This level is very low compared with *Salmonella* isolation from the visceral organs (10^1 to 10^5 CFU/g) or gastrointestinal tract (10^6 to 10^7 CFU/g) of nonvaccinated chickens challenged with the same *Salmonella* strains (Fig. 1). A lower level of protection was observed in vaccinated chickens challenged with *Salmonella* strains in group C compared with nonvaccinated but challenged chickens (Fig. 2). A significant level of protection was observed against challenge with *Salmonella* strains of group D (Fig. 3) and group E (Fig. 4) in vaccinated chickens compared with nonvaccinated chickens. No salmonellae were isolated from the spleen, ovary, and ileum of vaccinated chickens except for very low levels of *S. hadar* and *S. montevideo* in the ileum, but 10^1 to 10^5 CFU/g was detected in similar organs from nonvaccinated chickens. Higher levels of group C, D, or E *Salmonella* serotypes were detected from the bursa of Fabricius, cecal contents, and feces of vaccinated chickens compared with the isolation of group B *Salmonella* serotypes from vaccinated chickens. However,

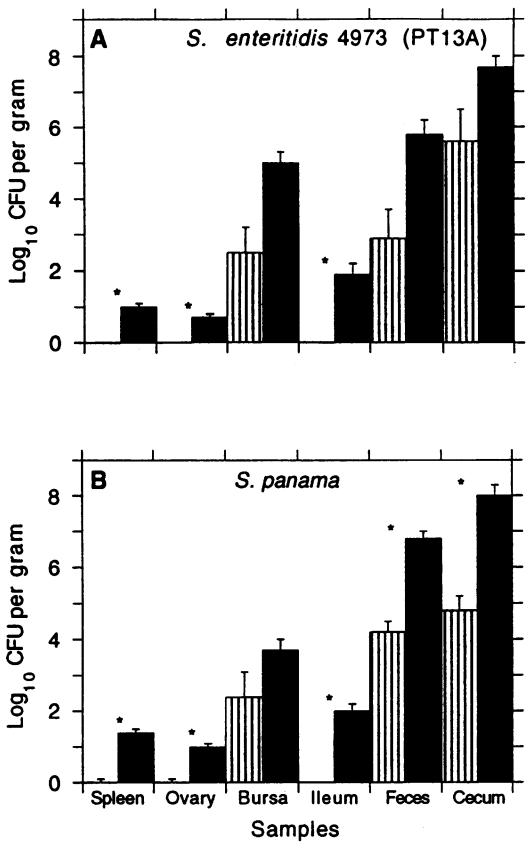

FIG. 3. Responses of chickens vaccinated with 10^8 CFU of Δ*cya* Δ*crp S. typhimurium* χ3985 at 1 and 14 days of age versus nonvaccinated chickens when challenged with 10^6 CFU of *S. enteritidis* 4973 PT13A (A) and *S. panama* (B) at 4 weeks of age. Samples were collected from individual chickens at 6 weeks of age, and values presented are the means ± standard errors of the means of 10 samples. Each value represents the $\log_{10}$ CFU of salmonellae isolated per gram of sample. *, significant difference at $P < 0.05$; ▥, vaccinated; ■, nonvaccinated.

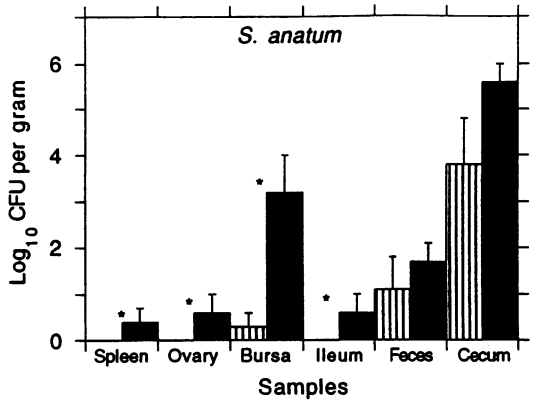

FIG. 4. Responses of chickens vaccinated with 10^8 CFU of Δ*cya* Δ*crp S. typhimurium* χ3985 at 1 and 14 days of age versus nonvaccinated chickens when challenged with 10^6 CFU of *S. anatum* at 4 weeks of age. Samples were collected from individual chickens at 6 weeks of age, and values presented are the means ± standard errors of the means of 10 samples. Each value represents the $\log_{10}$ CFU of salmonellae isolated per gram of sample. *, significant difference at $P < 0.05$; ▥, vaccinated; ■, nonvaccinated.

lower levels of group C, D, or E *Salmonella* serotypes were detected in the vaccinated chickens than in the nonvaccinated chickens, although only 8 of 21 comparisons yielded significantly lower titers.

Protection induced by oral vaccination at 2 and 4 weeks of age. Oral vaccination of chickens at 2 and 4 weeks of age with 10^8 CFU of Δ*cya* Δ*crp S. typhimurium* χ3985 precludes colonization of the ileum, cecum, bursa of Fabricius, spleen, and ovary by 10^6 CFU of wild-type *S. typhimurium* F98, *S. enteritidis* 27A PT8, and *S. anatum* (Fig. 5). Salmonellae were detected only in the ceca of vaccinated chickens challenged with *S. enteritidis* 4973 PT13A after selenite enrichment (Fig. 5). The amount of salmonellae isolated from the spleen, bursa of Fabricius, feces, and ceca of vaccinated chickens challenged with *S. infantis* was lower than the amount isolated from nonvaccinated chickens challenged with the same group C wild type (Fig. 5).

Protection against highly invasive *Salmonella* strains. Double oral vaccination of chickens when 2 and 4 weeks old prevented visceral invasion by 10^8 CFU of highly invasive *S. typhimurium* 2921-1 or *S. enteritidis* strains implicated in human *Salmonella* outbreaks caused by egg consumption. Challenge of vaccinated chickens with 10^8 CFU of *S. typhimurium* 2921-1 (Fig. 6) and *S. enteritidis* 4973 PT13A (Fig. 7), Y-8P2 PT8 (Fig. 8), 27A PT8 (Fig. 9), and B6996 PT13A (Fig. 10) showed that vaccination with *S. typhimurium* χ3985 prevented invasion and colonization of the spleen and ovary 4 days after challenge compared with invasion and colonization of nonvaccinated chickens challenged with the same *Salmonella* serotypes. By 11 days after challenge, salmonellae were detectable only by selenite enrichment from the bursa of Fabricius, ceca, and feces from all the vaccinated and challenged groups (0 to 10 CFU/g), compared with 5×10^4 CFU of salmonellae per g isolated from the nonvaccinated chickens challenged with similar *Salmonella* strains (Fig. 6 to 10).

DISCUSSION

The Δ*cya* Δ*crp S. typhimurium* vaccine strain is avirulent and immunogenic and induced protective immunity against *S. typhimurium* when used for oral vaccination in chickens (14) and mice (9, 36). The protection induced by χ3985 is dose dependent (17). In this study, oral vaccination of chickens at 1 and 14 days of age induced a strong protection against colonization and invasion of vaccinated chickens by homologous *Salmonella* strains with B-group O antigen. A lower level of protection was observed against cecal colonization of vaccinated chickens by heterologous *Salmonella* strains. The results obtained are better than those from earlier studies in which oral vaccination was used to prevent colonization of the ceca by homologous strains, using *S. typhimurium* F98 (3, 13, 16) or χ3985 (10, 14, 17) as the vaccine strain. Double oral vaccination of chickens with Δ*cya* Δ*crp S. typhimurium* χ3985 at 2 and 4 weeks of age induced a stronger protection against *Salmonella* serotypes from groups B, C, D, and E used in this study. Overall, protection against serotype B is excellent, protection against serotypes D and E is very good, and protection against group C strains is marginal. χ3985 also induced very good protection in vaccinated chickens against 10^8 CFU of highly invasive *S. typhimurium* and *S. enteritidis* strains. Most of the *S. enteritidis* strains used in this study are highly invasive and were implicated in food poisoning outbreaks in humans that were traced to eggs, with major economic loss and public health problems (19, 21, 31, 33). Most

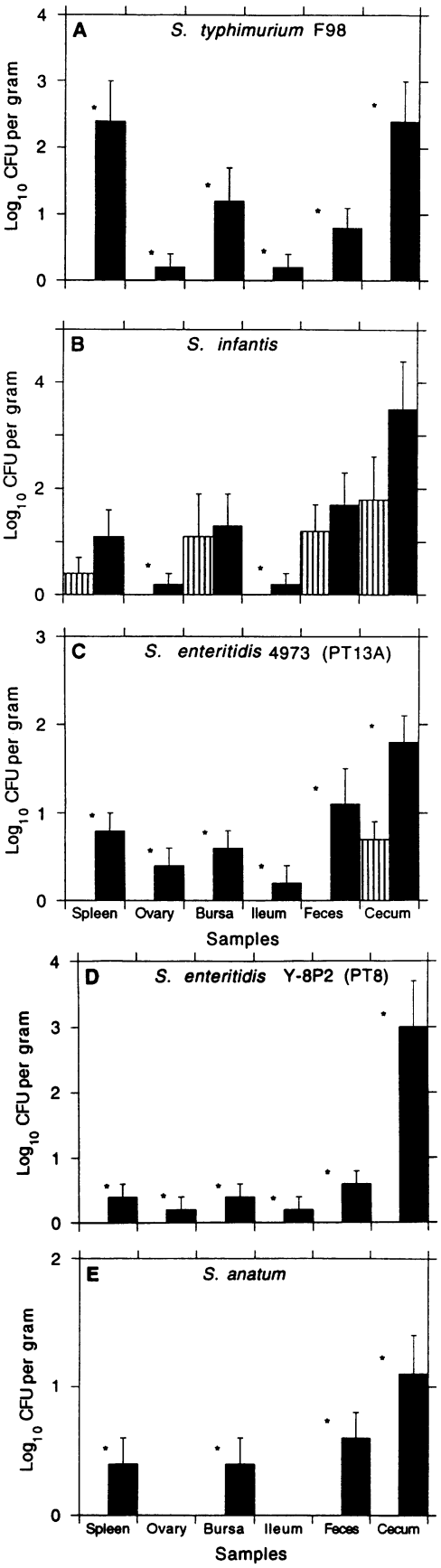

FIG. 5. Responses of chickens vaccinated with 10^8 CFU of Δ*cya* Δ*crp* *S. typhimurium* χ3985 at 2 and 4 weeks of age versus nonvaccinated chickens when challenged with 10^6 CFU of *S. typhimurium* F98 (A), *S. infantis* (B), *S. enteritidis* 4973 PT13A (C), *S. enteritidis* Y-8P2 PT8 (D), and *S. anatum* (E) at 6 weeks of age. Samples were collected from individual chickens at 8 weeks of age, and values presented are the means ± standard errors of the means of 10 samples. Each value represents the $\log_{10}$ CFU of salmonellae isolated per gram of sample. *, significant difference at $P < 0.05$; ▥, vaccinated; ■, nonvaccinated.

previous investigations involving the use of live oral *Salmonella* vaccine strains in chickens did not address cecal colonization or were not effective in preventing cecal colonization of vaccinated chickens by homologous *Salmonella* strains (1, 4, 6, 26).

The induction of excellent protection against homologous *Salmonella* serotypes and significant very strong protection against heterologous *Salmonella* serotypes induced by double oral vaccination of chickens with live avirulent χ3985 at 2 and 4 weeks of age showed that vaccination at 2 and 4 weeks of age is more effective than vaccination at 1 and 14 days of age. Comparison of results from a single vaccination at 3 days (10) and double vaccination with avirulent salmonellae at 1 and 14 days or 2 and 4 weeks shows that induction of protection

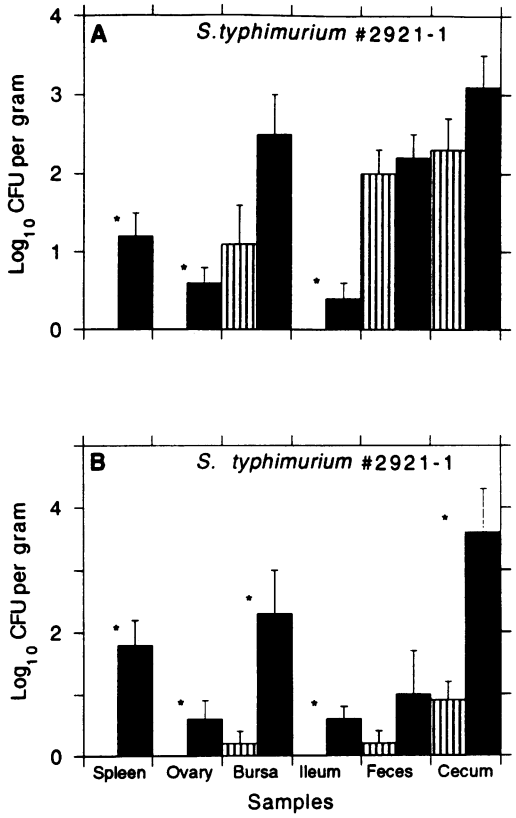

FIG. 6. Responses of chickens vaccinated with 10^8 CFU of Δ*cya* Δ*crp* *S. typhimurium* χ3985 at 2 and 4 weeks of age versus nonvaccinated chickens when challenged with 10^8 CFU of *S. typhimurium* 2921-1 at 6 weeks of age. Samples were collected from individual chickens at 4 days (A) and 11 days (B) after challenge, and values presented are the means ± standard errors of the means of 10 samples. Each value represents the $\log_{10}$ CFU of salmonellae isolated per gram of sample. *, significant difference at $P < 0.05$; ▥, vaccinated; ■, nonvaccinated.

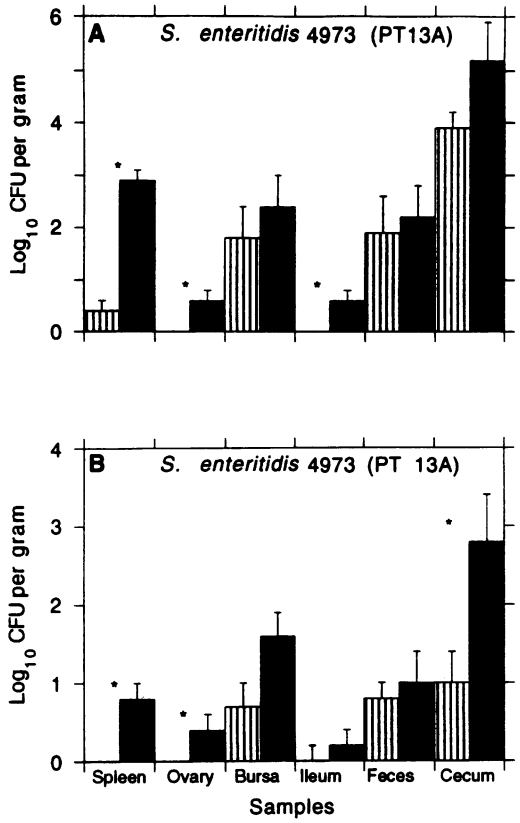

FIG. 7. Responses of chickens vaccinated with 10^8 CFU of Δ*cya* Δ*crp* *S. typhimurium* χ3985 at 2 and 4 weeks of age versus nonvaccinated chickens when challenged with 10^8 CFU of *S. enteritidis* 4973 PT13A at 6 weeks of age. Samples were collected from individual chickens at 4 days (A) and 11 days (B) after challenge, and values presented are the means ± standard errors of the means of 10 samples. Each value represents the $\log_{10}$ CFU of salmonellae isolated per gram of sample. *, significant difference at $P < 0.05$; ▥, vaccinated; ■, nonvaccinated.

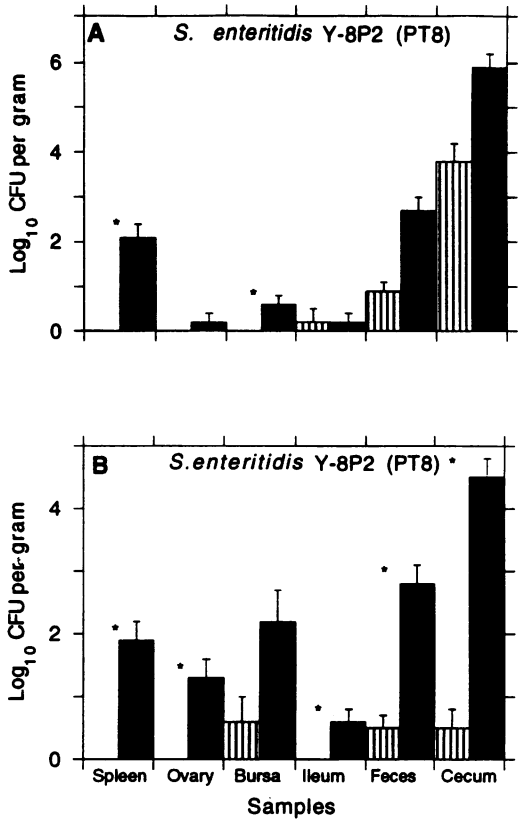

FIG. 8. Responses of chickens vaccinated with 10^8 CFU of Δ*cya* Δ*crp* *S. typhimurium* χ3985 at 2 and 4 weeks of age versus nonvaccinated chickens when challenged with 10^8 CFU of *S. enteritidis* Y-8P2 at 6 weeks of age. Samples were collected from individual chickens at 4 days (A) and 11 days (B) after challenge, and values presented are the means ± standard errors of the means of 10 samples. Each value represents the $\log_{10}$ CFU of salmonellae isolated per gram of sample. *, significant difference at $P < 0.05$; ▥, vaccinated; ■, nonvaccinated.

depends on age and vaccination schedule. Double vaccination at 1 and 14 days of age or at 2 and 4 weeks of age prevented visceral invasion of wild-type salmonellae in vaccinated chickens, but double vaccination at 1 and 14 days was less effective than vaccination at 2 and 4 weeks in preventing cecal colonization. This may be related to the age at vaccination such that at 1 day of age, when chicks are less immunocompetent, vaccination efficacy may be reduced. Interference in the efficacy of primary vaccination may also prevent development of adequate memory cells required for the induction of an anemnestic response to secondary vaccination. This may reduce the ability of vaccination at 1 and 14 days of age to induce cross-protection against intestinal colonization by heterologous *Salmonella* serotypes. The intestinal colonization observed in chickens vaccinated at 1 and 14 days of age and challenged with *S. enteritidis* PT13A (Fig. 3A) is probably due to the virulence and highly invasive nature of *S. enteritidis* PT13A.

Vaccination at 2 weeks of age induced a better immune response than was induced by vaccination at 1 day of age, and this was augmented by the booster dose at 4 weeks of age. The booster vaccination possibly expanded the memory responses by increasing the level of immune response to *Salmonella* common immunogens, thereby leading to the induction of strong cross-protection observed against *Salmonella* serotypes from groups D and E. The lower level of protection against *Salmonella* serotypes from group C in chickens vaccinated at 2 and 4 weeks of age may be due to a unique antigen that is important in group C *Salmonella* pathogenesis that is lacking or not well expressed in *S. typhimurium*. Chickens vaccinated at 2 and 4 weeks of age and then challenged with highly invasive *Salmonella* strains were less protected against intestinal colonization by wild-type challenge at 4 days postchallenge compared with the observed protection 1 week later (Fig. 6 to 10). This may be due to time of sampling postchallenge. The reduction in protection is more prominent in chickens infected with *S. typhimurium* 2921-1 and *S. enteritidis* 4973 PT13A because they are highly invasive and more virulent than other challenge strains used. The increase in level of protection with time after vaccination (Fig. 6 to 10) shows that protection is due to specific immunity, since nonspecific immunity occurs earlier in an infection and declines with time after infection, whereas specific immune responses are induced some days after infection and improve with time after infection. The induction of effective cross-protection against other *Salmonella* serotypes implies that a strong level of immune response has been elicited against a common immunogenic *Salmonella* antigen(s). Outer membrane proteins, lipopolysaccharide core, and other common antigens of members of the family *Entero-*

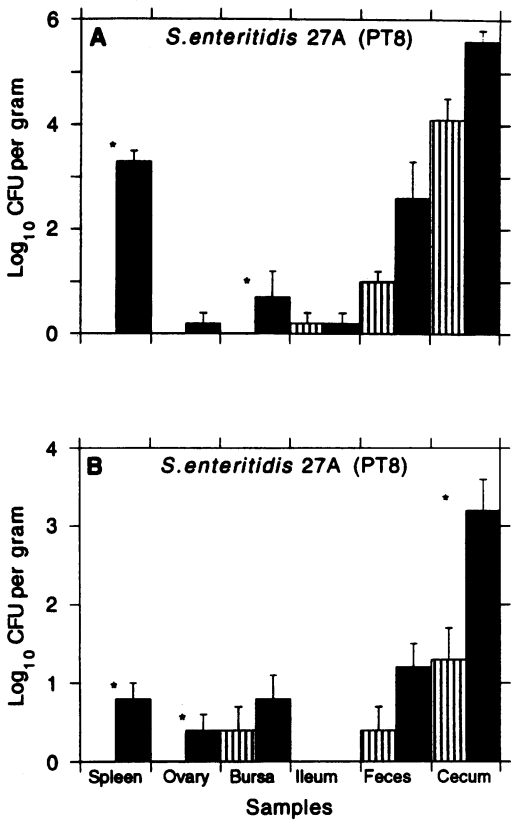

FIG. 9. Responses of chickens vaccinated with 10^8 CFU of Δ*cya* Δ*crp S. typhimurium* χ3985 at 2 and 4 weeks of age versus nonvaccinated chickens when challenged with 10^8 CFU of *S. enteritidis* 27A PT8 at 6 weeks of age. Samples were collected from individual chickens at 4 days (A) and 11 days (B) after challenge, and values presented are the means ± standard errors of the means of 10 samples. Each value represents the $\log_{10}$ CFU of salmonellae isolated per gram of sample. *, significant difference at $P < 0.05$; ▥, vaccinated; ■, nonvaccinated.

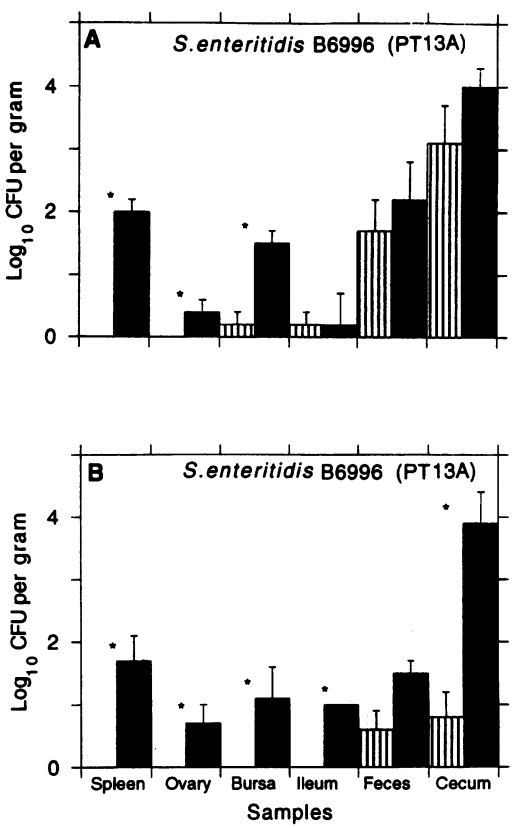

FIG. 10. Responses of chickens vaccinated with 10^8 CFU of Δ*cya* Δ*crp S. typhimurium* χ3985 at 2 and 4 days of age versus nonvaccinated chickens when challenged with 10^8 CFU of *S. enteritidis* B6996 PT13A at 6 weeks of age. Samples were collected from individual chickens at 4 days (A) and 11 days (B) after challenge, and values presented are the means ± standard errors of the means of 10 samples. Each value represents the $\log_{10}$ CFU of salmonellae isolated per gram of sample. *, significant difference at $P < 0.05$; ▥, vaccinated; ■, nonvaccinated.

bacteriaceae may play a role in the induction of cross-protection immunity.

The vaccination protocol evaluated focused on the production of broilers, which are processed as table birds at 6 weeks of age, pullets, which become layers that produce eggs for an average of 1 year after 22 weeks of age, and breeders, which produce fertile eggs. There is need to control *Salmonella* infection at all levels of production, but contamination of meat during processing and eggs before or after being laid are the major sources of poultry-associated *Salmonella* infection in humans. The exposure of chickens to salmonellae from the hatchery may prevent the use of vaccination at 2 and 4 weeks of age in broiler vaccination. There will therefore be need for the development of a comprehensive vaccination program that involves vaccination of breeders and their progeny. The vaccination schedule described in this report needs to be subjected to a field trial under normal production conditions to ascertain its practicality on the farm. This report shows that avirulent live *S. typhimurium* vaccine strain χ3985 can be useful in the control of *Salmonella* carriers in chickens. We are currently investigating the duration of immunity in layers and the effect of maternal antibody on colonization of a breeder's progeny by salmonellae and vaccination of a breeder's progeny.

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