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Effect of Vaccination of Hens with an Avirulent Strain of *Salmonella typhimurium* on Immunity of Progeny Challenged with Wild-Type *Salmonella* Strains

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The avirulent *Salmonella typhimurium* χ3985 was used to vaccinate white leghorn chickens at 16 and 18 weeks of age, and the effect of maternal antibody on *Salmonella* colonization of progeny of vaccinated hens was assessed with *S. typhimurium* F98 or χ3985. Progeny of hens that had been vaccinated at 1 and 3 or 2 and 4 weeks of age with χ3985 were used to determine the effect of maternal immunity on vaccine efficacy. Vaccination of hens induced long-lasting *Salmonella*-specific antibodies which were transferred into eggs and were detected as immunoglobulin G (IgG) in the egg yolk. Maternal antibody was detected in the progeny of vaccinated birds as IgG and IgA in serum and intestinal fluid, respectively. The titer of maternally transmitted IgG or IgA was highest in the first week of life of the progeny and declined with age. Maternal antibodies prevented colonization of the chicks by *S. typhimurium* χ3985 and reduced colonization by *S. typhimurium* F98. Overall, chicks from vaccinated hens had significantly higher antibody responses than did the progeny of nonvaccinated hens after oral infection with *Salmonella* strains. Maternal antibody reduced the efficacy of vaccination of progeny with χ3985 at 1 and 3 weeks of age. But vaccination at 2 and 4 weeks of age induced excellent protection against challenge with *S. typhimurium* F98 or *S. enteritidis* 27A PT 8 in birds from vaccinated hens and in specific-pathogen-free chickens. Vaccination of chickens at 2 and 4 weeks of age has been shown to protect the birds against challenge with homologous and heterologous *Salmonella* serotypes. A combination of vaccination of adult animals and use of the progeny of vaccinated birds will enhance effective control of *Salmonella* infections in the poultry industry. This will complement the present control of *Salmonella*-associated food poisoning caused by *Salmonella enteritidis* in eggs because the avirulent *S. typhimurium* vaccine strain χ3985 induced excellent protection against *S. enteritidis* in chickens.

The public health importance of *Salmonella* infection in poultry indicates a need for control of *Salmonella* infection of chickens on the farm. The use of vaccination to control *Salmonella* infection in chickens is inevitable. Live vaccines produce better protection than do killed vaccines (1, 2, 8, 19, 28, 31, 33, 40, 45), but killed vaccines appeal more to producers and regulators because they do not pose the possible public health risks that accompany the use of a live attenuated paratyphoid vaccine in the poultry industry. However, killed vaccines do not induce enough protection to eliminate the *Salmonella* carrier status in chickens (2). Live *Salmonella* vaccines replicate, colonize, and invade intestinal and visceral organs of inoculated chickens, thereby leading to the induction of strong immunity in the vaccinated chickens (1, 4–6, 22, 25, 26, 28, 39, 42, 45). In *Salmonella*-contaminated farms, proliferation of live wild-type *Salmonella* strains may result in fecal excretion of *Salmonella* cells by infected chickens. Fecal excretion of *Salmonella* cells may persist during the growth of chickens on the farm, with possible contamination of finished poultry products by *Salmonella* strains (22, 29, 44).

Advances in molecular genetics have led to the production of microbial pathogens with known genetic deletions that render virulent bacteria avirulent (21, 36). One such mutant is the Δ*cya* Δ*crp* *Salmonella typhimurium* vaccine strain χ3985 (11). We have shown that χ3985 is avirulent, stable, and immunogenic (10, 11, 25, 30); that it does not enhance the development of *Salmonella* carrier status in chickens (26); and that it effectively protects vaccinated chickens against challenge with homologous and heterologous virulent *Salmonella* serotypes (27).

Although vaccination of chickens with χ3985 protected against wild-type *Salmonella* challenge, chicks are at risk in the hatchery and during the first week of growth on the farm when raised in *Salmonella*-contaminated environments. It is therefore important to develop a vaccination program that will enhance the immunity of chicks early in life and to evaluate such a program for its effectiveness in reducing *Salmonella* infections of chicks during the hatching and brooding stages of production.

Passive immunity is engendered in chicks by the transfer of predominantly immunoglobulin G (IgG) antibody to the eggs (7, 13, 31, 32, 38). Maternal antibody is sequestered by the developing oocyte (14) and transported from the egg yolk across the yolk sac membrane into the embryonic circulation (7, 33). The protective effect of maternally derived IgG against Newcastle disease virus in the small intestine of birds is titer dependent and greatest during the first week of viral infection (40). The permeability of the chick intestine and transduction of circulating maternal IgG from serum into the intestine are greatest in the first week of life (12, 40).

Maternal antibody to avian leukosis virus may interfere with active immunization against avian leukosis virus (34, 43), but it can also delay infection and reduce viremia, virus shedding, and tumors in chickens (14, 15, 16, 20, 46). The influence of maternal antibody on *Salmonella* colonization, invasion, and fecal shedding in chickens obtained from eggs laid by vaccinated chickens has not been determined. This paper describes the effect of vaccination of hens on the colonization and inva-

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sion of virulent and avirulent *S. typhimurium* in the progeny of vaccinated hens (PVH), as well as the influence of maternal antibody on vaccine efficacy in the PVH injected with *S. typhimurium* χ3985 and challenged with *S. typhimurium* F98 or *Salmonella enteritidis* 27A PT8.

MATERIALS AND METHODS

Chickens. White leghorn hens and roosters were purchased from a specific-pathogen-free (SPF) stock supplied by Specific Pathogen-Free Avian Services (SPAFAS, Roanoke, Ill.). Following vaccination, the birds were bred in our animal facility. Fertile eggs were collected from the vaccinated hens and together with fertile SPF eggs (controls) from SPAFAS were incubated and hatched in Humidaire incubator-hatchers in our facility. All chickens were used as unsexed white leghorns.

Bacterial strains. The strains used were *S. typhimurium* F98 (3), with an oral 50% lethal dose (LD_{50}) of 5×10^5 CFU in 1-day-old chicks (unpublished data); *S. enteritidis* 27A PT8 (41); and *S. typhimurium* χ3985, an avirulent Δ*cya* Δ*crp* derivative of χ3761 with an LD_{50} of $>4 \times 10^9$ CFU for 1-day-old chicks (11). *S. typhimurium* χ4172 [*fli-8007*::Tn*10* (Fla$^-$ Mot$^-$ Tc^r) Δ(*galE-chl-uvrB*)*1005* (Bio$^-$ Gal$^-$ Chl^r UV^s)] (25) was used for extraction of *Salmonella* outer membrane proteins (OMPs). All bacterial 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 at −70°C.

Animal infectivity. Colonization, invasion, and humoral immune responses were assessed after oral inoculation of chickens with *Salmonella* strains. *Salmonella* strains for inoculation were grown overnight as static cultures at 37°C in Luria broth (L broth) (35). These cultures were diluted 1:50 into prewarmed L or brain heart infusion broth and grown with aeration at 37°C for approximately 4 h to an optical density at 600 nm 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 (9) to yield the required density. Serial dilutions were plated on Penassay agar for titer determination, and χ3985 was also plated on MacConkey agar (Difco) supplemented with 1% maltose to verify the Cya$^-$ Crp$^-$ phenotype. Chickens were vaccinated or challenged or both orally with 100 μl of *S. typhimurium* or *S. enteritidis* cells suspended in buffered saline with gelatin at the densities indicated in the different experiments (see below). The inocula were delivered directly into the crop with 1-ml syringes fitted with 18-gauge, 3-in. (ca. 8-cm) animal feeding biomedical needles (Popper and Sons, Inc., New York, N.Y.).

Experimental design. In experiment 1, 20 14-week-old white leghorn pullets and two mature roosters were obtained from SPAFAS. Fifteen of the pullets were vaccinated orally with 10^8 CFU of *S. typhimurium* vaccine strain χ3985 at 16 and 18 weeks of age, while eggs from the remaining five uninfected pullets were used as nonvaccinated controls. Eggs were collected from both groups of pullets between 22 and 52 weeks of age and analyzed for antibody content by enzyme-linked immunosorbent assay (ELISA). The vaccinated hens were introduced to the roosters on a continuous basis between 28 and 52 weeks of age. Fertile eggs were collected and along with equal numbers of SPF control eggs from SPAFAS were incubated and hatched in separate groups in our animal facility. The chicks obtained from these eggs were used in experiments 2 and 3. The five nonvaccinated layers that laid the eggs used as controls were killed at the end of experiment 1.

In experiment 2, 50 1-day-old chicks hatched from eggs laid by vaccinated hens (PVH) in experiment 1 and 50 1-day-old chicks hatched from eggs obtained from SPAFAS (SPF chickens) were divided into two groups of 25 each. One group of each was infected orally with 10^4 CFU of *S. typhimurium* F98, while the other group was infected orally with 10^8 CFU of *S. typhimurium* χ3985 (10^4 CFU of F98 and 10^8 CFU of χ3985 are both 1 log below the respective LD_{50} for orally infected, 1-day-old white leghorn chicks). A group of 25 PVH and 25 SPF chickens were used as uninfected controls. These doses were used to determine the effect of maternal antibody on colonization and invasion of *Salmonella* strains in orally infected 1-day-old chicks. From each of the groups of chicks infected with 10^4 CFU of F98 or 10^8 CFU of χ3985 and the controls, five birds were sampled at 3, 7, 14, and 21 days postinfection. Sera and intestinal fluids were collected and used for antibody detection by ELISA. The spleen, bursa of Fabricius, ileal content, ileal wall, and cecal content were taken for *Salmonella* quantification. Experiment 2 was repeated once.

In experiment 3, 50 PVH from experiment 1 and 50 SPF chickens were divided into groups of 25 each. One group of 25 PVH and one group of 25 SPF chickens were vaccinated orally at 1 and 3 weeks of age with 10^8 CFU of χ3985.

One chicken was removed from each group of 25 vaccinated chickens, and the rest of each group was divided into two groups of 12 chickens which were challenged with 10^6 CFU of *S. typhimurium* F98 or *S. enteritidis* 27A PT8 at 5 weeks of age. The remaining two groups of 25 PVH and 25 SPF chickens were vaccinated orally with 10^8 CFU of χ3985 at 2 and 4 weeks of age; later one chicken was removed from each group, and the rest of each group was divided into two groups of 12 each and challenged at 6 weeks of age with 10^6 CFU of *S. typhimurium* F98 or *S. enteritidis* 27A PT8. Two additional groups of 50 1-day-old PVH and SPF chickens were divided into two groups of 25 each. One chicken was removed from each group, and the rest of each group was divided into two groups of 12 chickens at 5 weeks of age and challenged with 10^6 CFU of *S. typhimurium* F98 or *S. enteritidis* 27A PT8. At 6 weeks of age, one chicken was removed from the remaining 25 chickens in each group, and the rest of each group was divided into two groups of 12 and challenged with 10^6 CFU of either F98 or 27A PT8. All birds were sampled at 2 weeks postchallenge. Samples were collected from the spleen, ovary, bursa of Fabricius, ileum, and cecum for *Salmonella* quantification. The level of detectable *Salmonella* cells was used to determine the degree of protection induced by vaccination, by comparing vaccinated and challenged groups with nonvaccinated and challenged control groups.

Sample collection. Blood was collected by cardiac puncture, and chickens were euthanized by CO_2 asphyxiation. Necropsy and sample collection were performed as previously published (27). One milliliter of intestinal fluid was aspirated from the small intestine into a 3-ml syringe containing aprotinin and equipped with a 16-gauge needle. All samples were collected into sterile preweighed disposable polypropylene culture tubes with snap caps and kept on ice after sample collection.

Eggs were collected from the egg tray, cleaned, and kept at 4°C until processed for maternal antibody detection. The eggs were processed by wiping the tips with ethanol, allowing them to dry, and surgically removing the shell at the apex of each egg. The egg white was carefully aspirated under aseptic conditions into a sterilized tube before the egg yolk was removed aseptically into another sterilized tube. Thus, each egg was separated into egg white and egg yolk, five of each separated egg part were pooled separately and mixed, and 2 ml of each pool was collected as a sample for antibody titer detection in an ELISA.

Enumeration of viable *S. typhimurium* CFU. Collected samples from spleen, bursa of Fabricius, ileal and cecal contents, and ileal wall were weighed, and nine times their weight of buffered saline with gelatin was added. Samples were homogenized in a tissue homogenizer (Brinkman Instruments, Westbury, N.Y.) and analyzed by decimal dilutions of the sample in buffered saline with gelatin followed by plating of diluted samples on *Salmonella-Shigella* agar and incubation at 37°C for 24 h (permitting detection of 10^2 CFU/g). The presence or absence of *Salmonella* cells in the samples was confirmed by adding an equal volume of 2×-concentrated selenite-cysteine broth to the sample and incubating the mixture for 48 h at 37°C. Selenite-cysteine broth-enriched samples were subcultured on *Salmonella-Shigella* agar and incubated at 37°C for 48 h.

Preparation of OMPs. OMPs were prepared from *S. typhimurium* χ4172 [*fli-8007*::Tn*10* (Fla$^-$ Mot$^-$ Tc^r) Δ(*galE-chl-uvrB*)*1005* (Bio$^-$ Gal$^-$ Chl^r UV^s)] (25). OMPs were prepared and characterized by the modified method of Newell et al. (37). *S. typhimurium* χ4172 grown in L broth at 37°C was harvested and sonicated as previously described (24). Crude membranes were recovered from the sonicated cell supernatant (15,000 × *g*, 30 min). The membrane pellet was suspended in 2% (wt/vol) *N*-laurylsarcosine sodium salt (Sarkosyl; Sigma; 4.5 ml of suspended pellet plus 5 ml of 20% [wt/vol] Sarkosyl) in phosphate-buffered saline (PBS) at room temperature and centrifuged at 15,000 × *g* for 30 min at 4°C. The purified OMP pellet was suspended in PBS and stored at −20°C.

Antibody detection by ELISA. The methods for the indirect ELISA used to detect serum antibodies have been described elsewhere (24). OMPs, immunogenic surface antigens of *Salmonella* strains (23), were used as the test antigen in the ELISA. Serum and intestinal fluid samples were diluted in PBS–0.5% Tween 20 at 1:100 and in 0.5 N NaCl–1% bovine serum albumin (BSA)–0.1% Tween 20 at 1:10, respectively, and tested in duplicate. All incubation steps were at 37°C for 1 h. Goat anti-chicken IgM Fc, IgA Fc, or IgG Fc polyclonal antibodies (ImmunoVision Inc., Springdale, Ark.) at a dilution of 1:1,200 were used to detect chicken IgM, IgA, or IgG, respectively, in sera or intestinal fluids (from infected and noninfected chickens) bound to *S. typhimurium* OMPs coating ELISA plates (Dynatech Laboratories Inc., Alexandria, Va.). A rabbit anti-goat IgG–alkaline phosphatase conjugate (Sigma, St. Louis, Mo.) was used at a dilution of 1:1,000 with *p*-nitrophenyl phosphate (Sigma) (1 mg/ml) in diethanolamine buffer, pH 9.8, as the substrate. The reaction was stopped with 50 μl of 3 N NaOH. A_{405} values were read with an automated microplate reader (Bio-tek Instrument Inc., Burlington, Vt.). The above-described method was also used for detecting *Salmonella*-specific immunoglobulin isotypes in egg white or egg yolk at dilutions of 1:100 and 1:25, respectively.

Statistics. Statistical significance was calculated at the 0.05 level of probability by two-way analysis of variance (17).

RESULTS

Antibody transfer to eggs. Significant *Salmonella*-specific IgG was detectable in the egg yolk in high titers up to 30 weeks postvaccination, when the experiment was terminated (Fig. 1A). Low levels of IgM were also detectable at 6 weeks postvaccination in the egg yolk, and IgA was not detected at a significant level (Fig. 1A). Only IgG was detectable in egg white, but the titer was low (Fig. 1B).

Antibody responses in PVH and SPF chickens. Intestinal *Salmonella*-specific maternal IgA was detected in noninfected PVH at significant levels at 3 and 7 days of age compared with

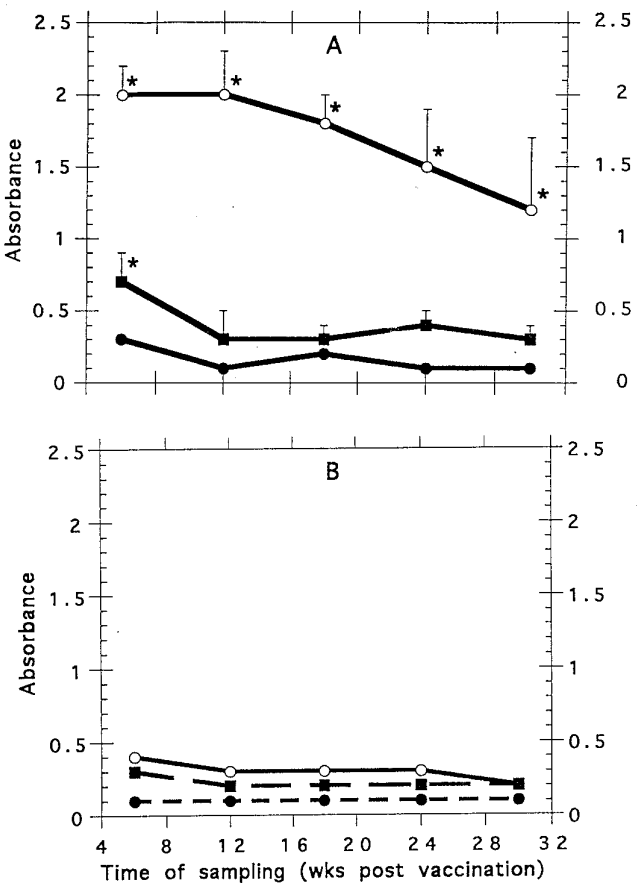

FIG. 1. Detection of *Salmonella*-specific IgM (■), IgG (○), and IgA (●) responses in the eggs of hens vaccinated at 16 and 18 weeks of age with 10^8 CFU of live avirulent *S. typhimurium* vaccine strain χ3985. Values are expressed as the A_{405} readings measured with 1:100 and 1:25 dilutions of egg yolk (A) and egg white (B), respectively. Two times the mean absorbance of samples from eggs laid by nonvaccinated hens serves as the baseline for the detection of *Salmonella*-specific chicken immunoglobulin isotypes by ELISA. Each datum point represents the mean for five samples ± the standard error of the mean (each sample is a pool of five egg parts). *, significant difference between results from eggs laid by vaccinated hens and results from eggs laid by nonvaccinated control hens ($P < 0.05$).

those in infected PVH and SPF chickens injected with F98 or χ3985 (Fig. 2A). By 2 and 3 weeks postinfection, *Salmonella*-specific IgA was detectable in infected PVH at a level significantly higher than that in infected SPF chickens. Chickens from the vaccinated groups infected with *S. typhimurium* F98 or χ3985 gave significantly higher titers of *Salmonella*-specific IgA at 3 weeks postinfection than did the nonvaccinated groups infected with *S. typhimurium* F98 or χ3985 (Fig. 2A).

Salmonella-specific maternal serum IgG was detectable in PVH at 3 and 7 days of age (Fig. 2B). This caused a significant antibody titer difference between infected PVH and infected SPF chickens at 3 and 7 days postinfection. PVH infected with *S. typhimurium* χ3985 produced a higher antibody titer at 2 and 3 weeks postinfection than did PVH infected with *S. typhimurium* F98. SPF chickens infected with χ3985 produced a higher *Salmonella*-specific serum IgG titer at 2 and 3 weeks postinfection than did SPF chickens infected with F98 (Fig. 2B). *Salmonella*-specific maternal antibody in the noninfected PVH decreased with time and was lowest at 3 weeks of age (Fig. 2A).

***Salmonella* colonization and invasion in PVH and SPF chickens.** PVH infected with *S. typhimurium* χ3985 were positive for *Salmonella* cells at 3 and 7 days postinfection, but no *Salmonella* cells were isolated from the spleen, bursa of Fabricius, ileal and cecal contents, and ileal wall at 2 and 3 weeks postinfection (Fig. 3). At 2 and 3 weeks postinfection, SPF chickens infected with *S. typhimurium* χ3985 and PVH infected with *S. typhimurium* F98 showed a significant decrease in the number of *Salmonella* cells isolated from their visceral organs and intestinal tract compared with that in SPF chickens infected with *S. typhimurium* F98 (Fig. 3). Significantly more *Salmonella* cells were isolated from the ileal wall (Fig. 3D) than from ileal contents (Fig. 3C) at 7 days postinfection in PVH infected with *S. typhimurium* χ3985 and at 2 weeks postinfection in all the other groups.

Effect of maternal antibody on vaccination efficacy. PVH vaccinated at 1 and 3 weeks of age were protected against invasion and colonization of the spleen, ovary, and bursa of Fabricius by *S. typhimurium* F98 challenge at 5 weeks of age compared with nonvaccinated PVH or SPF chickens chal-

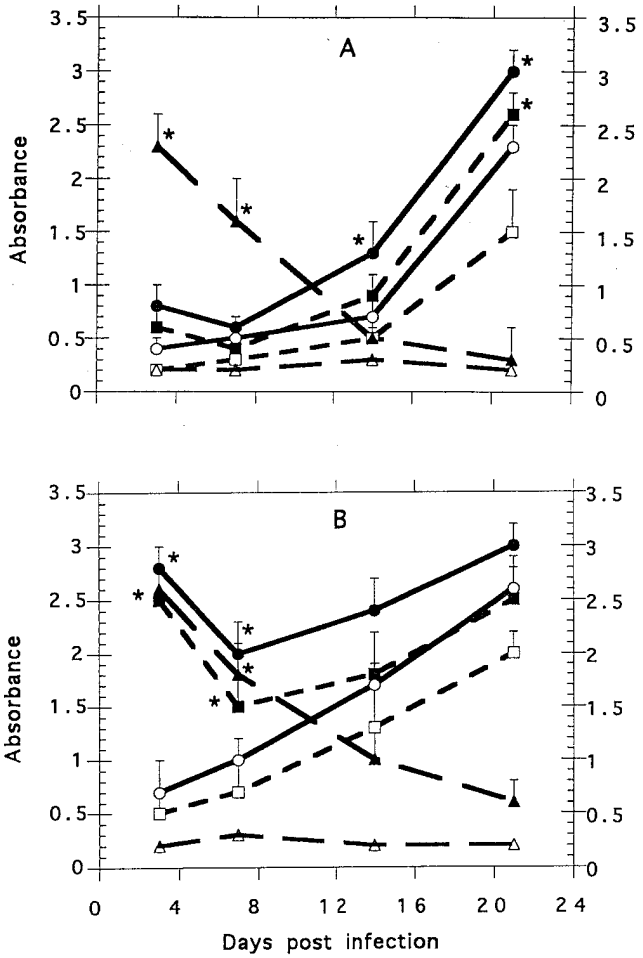

FIG. 2. Profile of *Salmonella*-specific IgA responses in the intestinal fluid (A) and IgG responses in serum (B) of PVH (■) and SPF chickens (□) orally infected at 1 day of age with 10^4 CFU of virulent *S. typhimurium* F98 or 1-day-old PVH (●) and SPF chickens (○) orally infected with 10^8 CFU of avirulent *S. typhimurium* vaccine strain χ3985. Samples from noninfected control PVH (▲) and SPF chickens (△) are also shown. Values are the means for 10 samples expressed as A_{405} values measured with 1:100-diluted sera and 1:10 intestinal fluid dilutions. Vertical bars indicate standard errors of means. *, significant difference between results from *Salmonella*-infected PVH and SPF chickens and results from non-*Salmonella*-infected PVH and SPF chickens, respectively ($P < 0.05$).

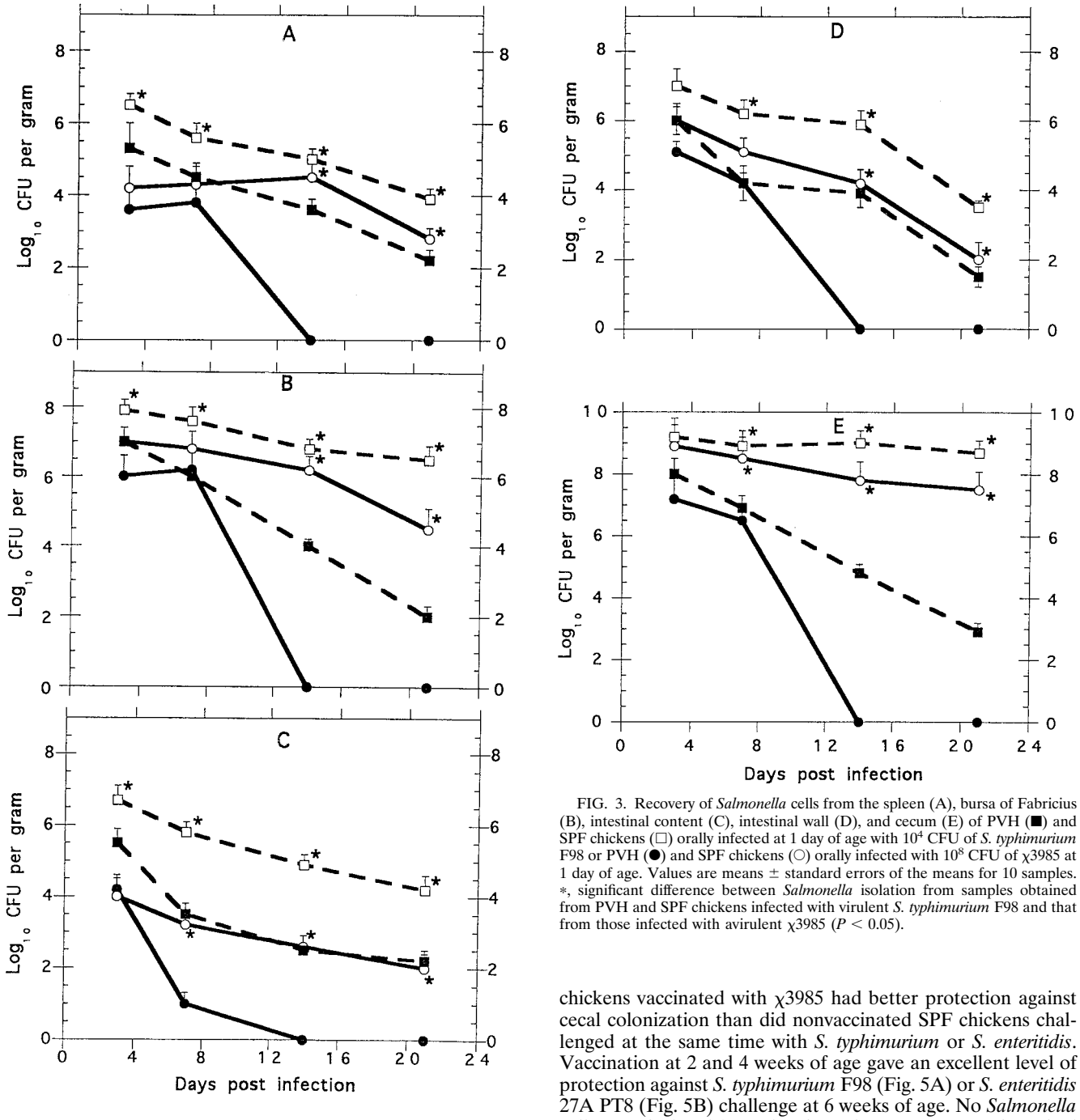

FIG. 3. Recovery of *Salmonella* cells from the spleen (A), bursa of Fabricius (B), intestinal content (C), intestinal wall (D), and cecum (E) of PVH (■) and SPF chickens (□) orally infected at 1 day of age with 10^4 CFU of *S. typhimurium* F98 or PVH (●) and SPF chickens (○) orally infected with 10^8 CFU of χ3985 at 1 day of age. Values are means ± standard errors of the means for 10 samples. *, significant difference between *Salmonella* isolation from samples obtained from PVH and SPF chickens infected with virulent *S. typhimurium* F98 and that from those infected with avirulent χ3985 ($P < 0.05$).

lenged at the same time with *S. typhimurium* F98 (Fig. 4A). A similar degree of protection was observed with the *S. enteritidis* 27A PT8 challenge, except that *Salmonella* cells were isolated from the spleen of PVH vaccinated with *S. typhimurium* χ3985 (Fig. 4B). The number of *Salmonella* CFU isolated from ileal and cecal contents was higher in PVH challenged with *S. typhimurium* than the number of *Salmonella* CFU isolated from the ileum and cecal contents of vaccinated SPF chickens challenged with *S. typhimurium*. PVH that were not vaccinated but were challenged at 5 weeks of age showed reduced *S. typhimurium* colonization in their cecal contents compared with *Salmonella* colonization of nonvaccinated SPF chickens challenged with the same *Salmonella* strain at the same time. SPF chickens vaccinated with χ3985 had better protection against cecal colonization than did nonvaccinated SPF chickens challenged at the same time with *S. typhimurium* or *S. enteritidis*. Vaccination at 2 and 4 weeks of age gave an excellent level of protection against *S. typhimurium* F98 (Fig. 5A) or *S. enteritidis* 27A PT8 (Fig. 5B) challenge at 6 weeks of age. No *Salmonella* cells were isolated from the visceral organs or gastrointestinal tract of either the vaccinated SPF chickens or vaccinated PVH after challenge with *S. typhimurium* or *S. enteritidis* (Fig. 5). Colonization of the visceral organs and intestinal tract by *Salmonella* cells was observed in all samples from nonvaccinated control groups challenged with *S. typhimurium* F98 (Fig. 5) or *S. enteritidis* 27A PT8 (Fig. 5) at 6 weeks of age.

DISCUSSION

Vaccination of hens with *S. typhimurium* χ3985 induced *Salmonella*-specific antibody responses which were detectable in eggs laid by the chickens. The predominant immunoglobulin

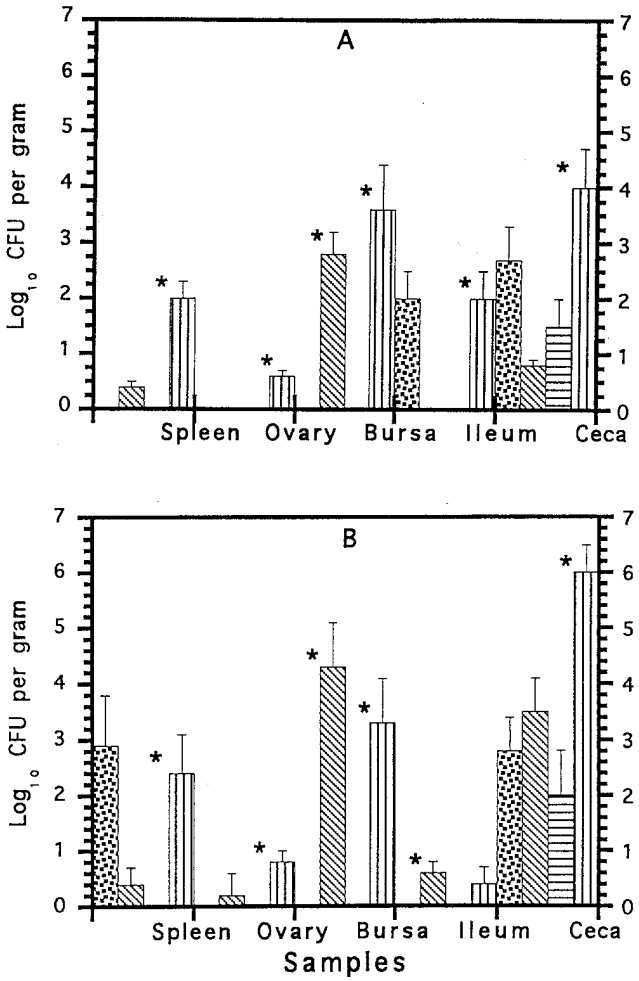

FIG. 4. *Salmonella* recovery at 5 weeks of age from the visceral and intestinal organs of PVH (▨) and SPF chickens (▤) vaccinated at 1 and 3 weeks of age with 10^8 CFU of live avirulent *S. typhimurium* χ3985 and orally challenged along with nonvaccinated PVH (▧) and nonvaccinated SPF chickens (▥) with 10^6 CFU of virulent *S. typhimurium* F98 (A) or *S. enteritidis* 27A (B) at 5 weeks of age. Values are expressed as means ± standard errors of means for 10 samples. *, significant difference between (i) *Salmonella* isolation from vaccinated PVH and results obtained from nonvaccinated PVH challenged with similar *Salmonella* serotypes or (ii) *Salmonella* isolation from vaccinated SPF chickens and results obtained from nonvaccinated SPF chickens challenged with similar *Salmonella* serotypes ($P < 0.05$).

isotype detected was IgG mainly from the egg yolk. Only antibody from egg was analyzed to avoid stressing the birds. It has been demonstrated that egg yolk antibody titers reflect serum antibody titers (6, 40) and that serum antibody levels and corresponding egg antibody levels are correlated, similar, and indistinguishable (35, 40). Therefore, we can presume that the level of IgG detected in eggs from the vaccinated hens is a reflection of the titer of *Salmonella*-specific IgG in the serum of the vaccinated birds. Injection of hens with 10^8 CFU of live avirulent *S. typhimurium* χ3985 induced a prolonged antibody response which was transferred to eggs laid by the vaccinated hens throughout the first 30 weeks of egg production.

Antibodies that are passively transferred from the breeder to the eggs are absorbed from the egg yolk across the yolk sac membrane into the embryonic circulation as maternal antibody (7, 13, 31, 32). *Salmonella*-specific IgG and IgA titers in the sera and intestinal content of PVH were significantly higher than the titers observed in SPF chickens. The maternal IgG transported to the PVH was derived from the egg yolk, since we detected significantly high titers of IgG in the egg yolk. *Salmonella*-specific IgA antibodies were detected in the intestines of the PVH, but we were unable to detect *Salmonella*-specific IgA in the eggs laid by vaccinated hens. Since the eggs laid by vaccinated birds are free of *Salmonella* cells (data not shown), we excluded the possibility of production of antibodies to *Salmonella* infection during embryonic development. The gradual decline of the *Salmonella*-specific IgA level in PVH within 2 weeks posthatching also suggests that the detectable IgA is acquired passively and not by active immunity. We are sure that the various immunoglobulin isotypes detected by our ELISA are authentic on the basis of preliminary standardization. The *Salmonella*-specific IgA titer in the intestinal fluid of noninfected PVH was significantly higher 1 week posthatching compared with the IgA titer in intestinal fluid of PVH infected with *Salmonella* strains. The difference in IgA titer may be due to increased intestinal motility or fluid production induced by *Salmonella* invasion of the intestine and/or mopping up of *Salmonella*-specific IgA by proliferating *Salmonella* cells in the gastrointestinal tract.

PVH showed reduced colonization of both avirulent *S. typhimurium* vaccine strain χ3985 and virulent strain F98 in the visceral organs and the intestine for up to 2 and 3 weeks postinfection. This protection can be attributed to the presence of maternal antibody. The precise protective mechanism of maternal antibodies is not clearly understood, but specific antibodies can inhibit, neutralize, and prevent colonization of pathogens. Maternal antibody is known to delay viral infection and reduce viremia and virus shedding in infected chickens (16, 20). The protective effect of maternally derived *Salmonella*-specific antibodies observed early in the PVH was reflected by the inability of *S. typhimurium* χ3985 to colonize PVH by 2 weeks postinfection. *Salmonella* isolation from PVH infected with *S. typhimurium* F98 was also reduced compared with *Salmonella* isolation from SPF chickens infected with F98. It can be deduced that the avirulent, live *S. typhimurium* vaccine strain χ3985 is more susceptible to the bactericidal action of maternal antibody than is virulent *S. typhimurium* F98, since χ3985 colonized and proliferated at a lower rate than F98. Although maternal antibody was detectable in the serum at 2 weeks postinfection, it may not be available in the intestinal lumen at a high titer because the ability of serum maternal antibody to transduce the intestinal epithelium after 1 week of age is reduced (40). The importance of maternal antibody in the intestine may therefore be limited to the early few weeks of life. Reduced *Salmonella* colonization of the intestinal lumen may be responsible for the isolation of more *Salmonella* cells from the ileal wall compared with isolation of *Salmonella* cells from the ileal content between 1 and 2 weeks postinfection. We have reported similar observations within a few days postinfection in SPF chickens infected with *Salmonella* strains, and the difference was less apparent by 1 week postinfection in our earlier report (23).

Susceptibility of chicks to *Salmonella* infection is age dependent: day-of-hatch chicks with an immature immune system are very susceptible to *Salmonella* infection (3, 18, 25, 42). We have previously demonstrated that early exposure of chicks to *Salmonella* strains causes transient lymphocyte depletion of lymphoid organs, which enhances the development of *Salmonella* carrier status in chickens (26). The presence of maternal antibody in PVH reduced *Salmonella* colonization. Vaccination of hens with the avirulent live *S. typhimurium* vaccine strain χ3985 will reduce *Salmonella* proliferation in PVH and may subsequently reduce lymphocyte depletion, which is asso-

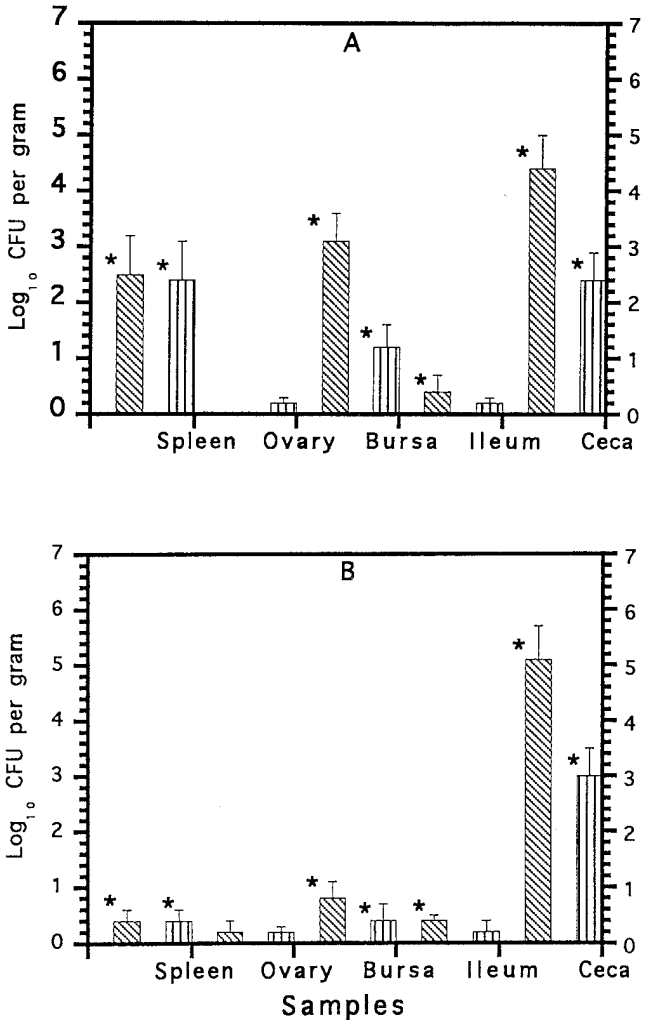

FIG. 5. *Salmonella* isolation at 6 weeks of age from the visceral and intestinal organs of PVH (▨) and SPF chickens (▤) vaccinated at 2 and 4 weeks of age with 10^8 CFU of live avirulent *S. typhimurium* vaccine strain χ3985 and orally challenged along with nonvaccinated PVH (▧) and nonvaccinated SPF chickens (▥) with 10^6 CFU of virulent *S. typhimurium* F98 (A) or *S. enteritidis* 27A (B) at 6 weeks of age. Values are expressed as the means ± standard errors of the means for 10 samples. *, significant difference between (i) *Salmonella* isolation from vaccinated PVH and results from nonvaccinated PVH challenged with a similar *Salmonella* serotype or (ii) *Salmonella* isolation from vaccinated SPF chickens and results from nonvaccinated SPF chickens challenged with the same *Salmonella* serotype ($P < 0.05$).

ciated with enhanced *Salmonella* proliferation. Reduced lymphocyte depletion will prevent the development of *Salmonella* carrier status by chickens raised in *Salmonella*-contaminated environments. A reduction in the number of *Salmonella* carriers in the poultry industry will reduce environmental and poultry product contamination by salmonellae.

Nonvaccinated PVH challenged with *Salmonella* strains at 5 weeks of age showed reduced *Salmonella* colonization compared with *Salmonella* isolation from SPF chicks challenged at the same time, but this protective effect was reversed at 6 weeks of age. It is difficult to interpret this observation without extensive experimental investigations which are beyond the scope of this paper. Our observation was duplicated in a repeat experiment. We are inclined to speculate that the presence of maternal antibody induced some nonspecific immune responses that were active up to 5 weeks of age in PVH. The presence of maternal antibody may destabilize the normal intestinal flora that is important in competitive exclusion. This may give the SPF chickens with stabilized normal intestinal flora an advantage over PVH, especially after maternal immunity wanes, as observed in this group of chickens infected with *Salmonella* strains at 6 weeks of age.

Protection was also enhanced in the ileal and cecal contents of vaccinated SPF chickens compared with that in PVH that were challenged 2 weeks postimmunization at 1 and 3 weeks of age. The major difference lies in the fact that the PVH have high titers of maternal antibody in their intestine during the first week posthatching. Maternal antibody may have reduced the efficacy of the first vaccination by preventing the proliferation of χ3985 in vaccinated PVH. The organism in the first vaccination of SPF chickens effectively proliferates and induces primary immunity in vaccinated SPF chickens. A second vaccination will induce a stronger anamnestic response in the vaccinated SPF chickens than in the vaccinated PVH and therefore will induce better protection against *Salmonella* infection. The difference in the affinity, intensity, and effectiveness of the induced immune responses in PVH and SPF chickens was reflected by the inability of vaccination of PVH at 1 and 3 weeks of age to prevent cecal *Salmonella* colonization. Both groups of vaccinated chickens were able to protect against visceral *Salmonella* colonization because a single vaccination is protective against visceral invasion by homologous *Salmonella* challenge but not against heterologous challenge (27). This may explain why *S. enteritidis* is isolated from the spleen of PVH vaccinated at 1 and 3 weeks of age and challenged with *S. enteritidis* at 5 weeks of age, since the first vaccination may have been rendered less effective by the presence of maternal antibody. The immune system of chickens improves with time posthatching. The introduction of virulent pathogens can retard successful development of the immune system. Vaccination at 1 and 14 days of age has been shown to be less effective than vaccination at 2 and 4 weeks of age (27). Vaccination at 1 and 3 weeks of age was less effective in chickens with maternal antibody compared with vaccination at 2 and 4 weeks of age. Vaccination at 2 and 4 weeks of age induced excellent protection against *S. typhimurium* F98 or *S. enteritidis* 27A PT8 in the presence or absence of maternal antibody. We therefore conclude that *Salmonella*-specific maternal antibody may reduce *Salmonella* vaccine efficacy if vaccination is carried out in the first week posthatching. The observed difference in the efficacy of vaccination at 1 and 3 weeks or 2 and 4 weeks of age is related to a higher maternal antibody titer at the first week of life and age-enhanced immunocompetence of 2-week-old chickens at the time of primary vaccination.

Double vaccination of hens at 16 and 18 weeks of age with live avirulent *S. typhimurium* vaccine strain χ3985 induced *Salmonella*-specific antibody which was passively transferred in eggs and was detected as *Salmonella*-specific maternal intestinal IgA and serum IgG in PVH. Maternal antibody reduced *Salmonella* colonization of chicks and did not affect the efficacy of vaccination in chickens vaccinated at 2 and 4 weeks of age. We have reported earlier that vaccination of SPF chickens at 2 and 4 weeks of age protected the vaccinated chickens from challenge with homologous and heterologous *Salmonella* serotypes (27). We therefore conclude that a combination of hen and PVH vaccination with avirulent live *S. typhimurium* vaccine strain χ3985 may protect the poultry industry from *Salmonella* infection of broilers, pullets, and breeders raised in *Salmonella*-contaminated farms. The duration of protection induced by vaccination with avirulent live *S. typhimurium* vaccine strain χ3985 is being investigated.

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