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Virulent *Salmonella typhimurium*-Induced Lymphocyte Depletion and Immunosuppression in Chickens

JUBRIL OLU HASSAN AND ROY CURTISS III*

Department of Biology, Washington University, St. Louis, Missouri 63130

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The effects of experimental *Salmonella* infection on chicken lymphoid organs, immune responses, and fecal shedding of salmonellae were assessed following oral inoculation of 1-day-old chicks or intra-air-sac infection of 4-week-old chickens with virulent *S. typhimurium* wild-type χ3761 or avirulent *S. typhimurium* Δ*cya* Δ*crp* vaccine strain χ3985. Some 4-week-old chickens infected intra-air-sac with χ3761 or χ3985 were challenged with *Bordetella avium* to determine the effect of *Salmonella* infection on secondary infection by *B. avium*. *S. typhimurium* χ3761 caused lymphocyte depletion, atrophy of lymphoid organs, and immunosuppression 2 days after infection in 1-day-old chicks and 4-week-old chickens. The observed lymphocyte depletion or atrophy of lymphoid organs was transient and dose dependent. Lymphocyte depletion and immunosuppression were associated with prolonged fecal shedding of *S. typhimurium* χ3761. No lymphocyte depletion, immunosuppression, or prolonged *Salmonella* shedding was observed in groups of chickens infected orally or intra-air-sac with χ3985. Infection of chickens with salmonellae before challenge with *B. avium* did not suppress the specific antibody response to *B. avium*. However, *B. avium* isolation was higher in visceral organs of chickens infected with χ3761 and challenged with *B. avium* than in chickens infected with *B. avium* only. Infection of chickens with χ3985 reduced *B. avium* colonization. We report a new factor in *Salmonella* pathogenesis and reveal a phenomenon which may play a critical role in the development of *Salmonella* carrier status in chickens. We also showed that 10^8 CFU of χ3985, which is our established oral vaccination dose for chickens, did not cause immunosuppression or enhance the development of *Salmonella* carrier status in chickens.

Increased consumer interest in the microbiological safety of meat products necessitates the adoption of production practices that have a higher probability of yielding *Salmonella*-free products. The U.S. poultry industry processes approximately 7.5 billion hatching eggs through incubation facilities each year (4). Economically, *Salmonella* paratyphoid infections are among the bacterial diseases that are most important to the hatching industry, with an estimated annual loss of $77 million (31). The cost associated with human salmonellosis in the United States is estimated at $4 billion annually (45). Adverse publicity from *Salmonella* outbreaks due to contamination of eggs or poultry meat has generated severe economic stress on the poultry industry.

The virulence of various *Salmonella* isolates can be assessed by inoculating 1-day-old chicks. As the chicks mature, their resistance to *Salmonella* infection rapidly increases (43). Low levels of salmonellae colonize the intestinal tract of newly hatched chicks (41), but the minimum infective dose rises steadily thereafter (32). Adherence to the intestinal mucosa is the first step in the establishment of persistent *Salmonella* colonization of the gut (44). Invasion of the mucosa and dissemination to the visceral organs decrease as the age at exposure of the chick to salmonellae increases. High challenge doses of *S. typhimurium* in 1-day-old chicks lead to high mortality and postinoculation persistence of infection (16). Most infected adult birds excrete salmonellae for a variable period of time, but some become lifelong carriers. Salmonellae persist longer and in higher titers in the cecum and bursa of Fabricius of infected chickens (2, 3, 21, 22, 43). Although the colonization, invasion, and persistence of salmonellae in infected chickens have been well studied, the development of the carrier status is not well understood.

The days immediately following hatching may be critical for the development of resistance to *Salmonella* infection. Exposure of chickens to large doses of salmonellae can result in the establishment of intestinal colonization with the potential of excretion of salmonellae until market age. The probability of systemic infection resulting from mucosal invasion is directly related to the number of salmonellae that initially colonize the intestinal epithelium (40). High levels of *Salmonella* organisms can be isolated repeatedly from samples taken at broiler hatcheries. The conditions that exist during the incubation of hatching eggs favor the proliferation of microorganisms (9). Muira et al. (34) found that over 50% of chick fluff sampled in commercial hatcheries was contaminated with salmonellae and that hatcher fluff kept for 4 years at room temperature still had between 1 and 1,000,000 viable salmonellae per g of fluff. As few as two *Salmonella* cells led to colonization of day-of-hatch chicks via the cloaca (8). A chick that becomes colonized in the hatchery can spread salmonellae to other chicks in the hatchery and to flockmates during rearing. When such a flock reaches the processing plant, salmonellae can be released into the processing facility and contaminate the processing line and finished products.

In a recent study in our laboratory, we observed that *S. typhimurium* wild-type strain χ3761 colonized better than *S. typhimurium* χ3985, which is a derivative of χ3761 (11, 23). Also, χ3761 induced a lower level of humoral responses and did not reduce fecal excretion during experimental infection of chickens when compared with χ3985 (23). We suspected that highly virulent *S. typhimurium* may have an adverse effect on chicken immunocompetence and may encourage the development of a *Salmonella* carrier status.

This work sought to investigate the effect of experimental *Salmonella* infection of chickens on chicken lymphoid organs,

* Corresponding author. Mailing address: Department of Biology, Campus Box 1137, Washington University, St. Louis, MO 63130. Phone: (314) 935-6819. Fax: (314) 935-4432.

immune responses, and *Salmonella* excretion and to assess the effect on immune responses to secondary infection with *Bordetella avium*.

MATERIALS AND METHODS

Chickens. Fertile eggs from a specific-pathogen-free stock supplied by Specific Pathogen-Free Avian Services (Roanoke, Ill.) were incubated and hatched in Humidaire incubator-hatchers in our facility. All chickens were unsexed white leghorns.

Bacterial strains. The strains used were *S. typhimurium* χ3761, with an oral 50% lethal dose (LD_{50}) of 2×10^3 CFU in 1-day-old chicks, 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)] (21) was used for extraction of *Salmonella* outer membrane proteins (OMPs). *B. avium* 197 was provided by Y. M. Saif (Ohio Agricultural Research and Development Center, Ohio State University, Wooster). 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, organ weight, and humoral and cellular immune responses were assessed after oral or intra-air-sac inoculation of chickens with salmonellae. *Salmonella* strains for inoculation were grown overnight as static cultures at 37°C in Luria broth (30). *B. avium* was grown in brain heart infusion broth statically at 37°C. These cultures were diluted 1:50 into prewarmed Luria or brain heart infusion 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) (10) to yield the required density. Serial dilutions were plated on Penassay agar for titer determination, and χ3985 was plated on MacConkey agar (Difco) supplemented with 1% maltose to verify the Cya^- Crp^- phenotype. Chickens were immunized with 100 μl of *S. typhimurium* or *B. avium* cells suspended in BSG and delivered directly into the crop with 1-ml syringes fitted with 3-in. (1 in. = 2.54 cm) 18-gauge animal feeding biomedical needles (Popper and Sons, Inc., New York, N.Y.). All intra-air-sac infections involved the posterior caudal air sac and were administered by a 5/8 in. 25-gauge needle attached to a 1-ml syringe. The needle was inserted at the anteriocaudal section of the intercostal space of the last two ribs for effective delivery of the inoculum.

Experimental design. In the first experiment, a group of 40 1-day-old chicks was inoculated orally with 10^8 CFU of *S. typhimurium* χ3761 while another group of 20 1-day-old chicks was infected orally with 10^8 CFU of χ3985; a noninfected group of 20 1-day-old chicks was used as the control. (Although we observed atrophy of lymphoid organs at low doses [10^3 and 10^5 CFU] of χ3761 in orally infected 1-day-old chicks [data not presented], variation due to edema of the lymphoid organs and the outbred nature of specific-pathogen-free chickens masked the significance of the results for these low doses. We therefore decided to use 10^8 CFU of χ3761 to enhance our understanding of the relationship between *Salmonella* infection and lymphocyte depletion in 1-day-old chicks; 10^8 CFU of χ3985 is also the established oral vaccination dose for chickens [21, 23]). Samples were taken from the bursa of Fabricius, thymus, and spleen at 2, 5, and 7 days after infection and fixed in 10% buffered formalin solution for histological analysis and also for *Salmonella* quantification. Samples were also taken from ileal and cecal contents for *Salmonella* quantification.

In the second experiment, two groups of 20 4-week-old chickens with a narrow weight range were inoculated orally with 10^{10} CFU of χ3761 or χ3985. A group of 40 chickens was infected intra-air-sac with 10^9 CFU of χ3761, while three groups of 20 chickens were infected intra-air-sac with 10^7 or 10^5 CFU of *S. typhimurium* χ3761 or 10^7 CFU of χ3985. A group of 20 4-week-old chickens was used as noninfected controls. (The LD_{50} of both χ3761 and χ3985 in 4-week-old chickens infected intra-air-sac is 10^8 CFU). Whole organs (i.e., thymus, spleen, and bursa of Fabricius) were collected, weighed, and processed for *Salmonella* quantification at 2, 5, and 7 days after infection. The ileal and cecal contents were also sampled for bacterial quantification. Sera and intestinal fluids were collected from chickens infected with 10^7 CFU of χ3761 or χ3985 and noninfected chickens and used to determine the levels of humoral response to *Salmonella* OMPs in an enzyme-linked immunosorbent assay (ELISA).

In the third experiment, two groups of 40 chickens were infected intra-air-sac with 10^8 CFU of *S. typhimurium* χ3761 or χ3985 at 4 weeks of age. Six chickens from each group were challenged intradermally in the foot web with 50 μg of *S. typhimurium* OMPs to determine the delayed-type hypersensitivity response (DTH) or 100 μg of concanavalin A (ConA) to determine the cutaneous leukocyte response at 7 and 5 days after infection, respectively. Two groups of 10 noninfected 5-week-old birds were used as controls.

In the fourth experiment, two groups of 50 chickens infected intra-air-sac with 10^8 CFU of χ3761 or χ3985 at 4 weeks of age were monitored at 5, 8, and 10 weeks of age for fecal excretion of salmonellae. Ten chickens from each group were euthanized when 10 weeks old, and cecal samples were taken for *Salmonella* quantification.

In the fifth experiment, two groups of 20 chickens were infected intra-air-sac with 10^8 CFU of χ3761 or χ3985 at 4 weeks of age. At 5 days after infection, 7 chickens from each group and 10 33-day-old control chickens were challenged intraocularly with a 0.1-ml suspension of 10^7 CFU of tetracycline-resistant *B. avium* per ml in BSG applied dropwise into the eye orbit. The aim was to deliver *B. avium* into the oculonasal duct, thereby delivering *B. avium* to the nares, trachea, and lungs. One week later, blood was collected for serum, and samples from the thymus, nares, spleen, and bursa of Fabricius and cecal contents were collected for *B. avium* quantification. This experiment was conducted to determine if the effect of salmonellae on the chicken immune system was specific to salmonellae.

In all the experiments in which chickens were infected with doses equal to or higher than the LD_{50}, at least two times the needed number of chickens were used and most of the experiments were conducted within 7 days to avoid sample shortage because of death of infected chickens. Control chickens were left untreated.

Sample collection. Blood was collected for serum by cardiac puncture, and chickens were euthanized by CO_2 asphyxiation. 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, bone shears were used to cut the clavicle, and the rib cage was reflected to one side to expose the organs in the abdominal and chest cavities. One milliliter of intestinal fluid was aspirated from the small intestine into a 3-ml syringe containing aprotinin by using a 16-gauge needle. All samples were collected under strict aseptic conditions, and all visceral organs were

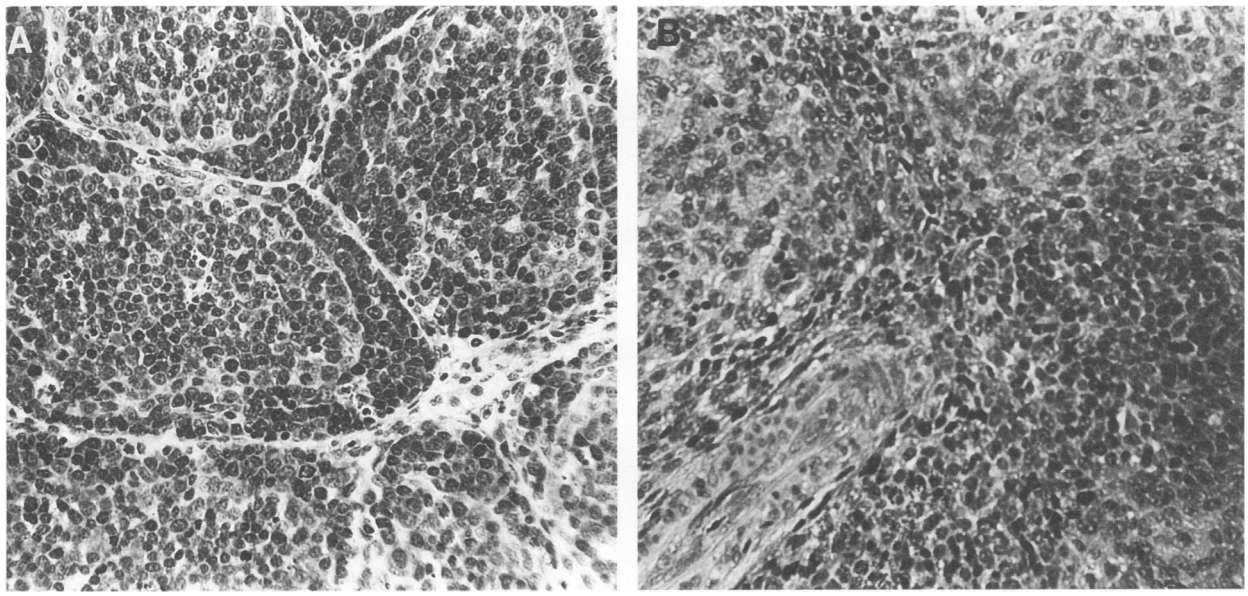


FIG. 1. Normal histology of the bursa of Fabricius (A) and spleen (B) taken from control chicks at 3 days of age, showing developed follicles.

removed before the incision of the gastrointestinal tract. The spleen was removed by detaching the posteriolateral end of the gizzard from the omentum and fascia and reflecting the gizzard ventrally to expose the spleen. A gentle displacement of the gizzard and the intestine along with the mesentery exposed the oviduct for its removal. The colon, cecum, and rectum were retracted posteriorly to expose the bursa of Fabricius; special care was taken in separating the bursa of Fabricius from the posterior end of the rectum at the junction with the cloaca to avoid fecal contamination during the surgical removal of the bursa of Fabricius. The mesentery was teased to free the ileum, and the ileum was opened longitudinally to obtain the ileal contents. The cecum was freed and cut at the middle after ligation, and the contents were milked into sterile sample tubes. Feces were collected from the cloaca. All samples were collected into sterile preweighed disposable polypropylene culture tubes with snap caps and kept on ice after sample collection.

Histological analysis. Fixed visceral organs in 10% buffered formalin solution were processed according to standard paraffin techniques. The tissues were dehydrated in a gradual series of increasing concentrations of ethanol, cleaned with xylene, processed in paraffin at 60°C, and embedded in paraffin. Sections 4 μm thick were cut with a microtome and attached to clean glass slides. Sections of tissues were deparaffinized in xylene, rehydrated through a graded series of alcohol, and finally placed in distilled water. Sectioned tissues mounted on glass slides were stained with hematoxylin-eosin for histological analysis of lymphocytes.

TABLE 1. Lymphocyte depletion in 1-day-old chicks infected orally with 10^8 CFU of *S. typhimurium* χ3761 or its Δ*cya* Δ*crp* derivative χ3985

Strain	Days after infection	Lymphocyte depletion[a]		
		Bursa of Fabricius	Thymus	Spleen
χ3761	2	+++	−	+++
	5	++	−	++
	7	+	−	+
χ3985	2	−	−	−
	5	−	−	−
	7	−	−	−

[a] Depletion of lymphocytes in lymphoreticular organs of chickens infected with salmonellae: −, no depletion; +, mild depletion; ++, moderate depletion; +++, severe depletion. Each result represents our observations of three histological slides per organ from each of five infected chickens. The observed results for chickens infected with χ3985 are the same as those observed for the noninfected control chickens.

Enumeration of viable *S. typhimurium*. Collected samples from the thymus, spleen, bursa of Fabricius, ileal and cecal contents, and feces were weighed, and nine times their weight of BSG was added. Samples were homogenized in a Brinkman tissue homogenizer (Brinkman Instruments, Westbury, N.Y.) and analyzed by using decimal dilutions of the sample in BSG, plating diluted samples on salmonella-shigella agar, and incubating them at 37°C for 24 h (permitting detection of 10^2 CFU/g). Presence or absence of salmonellae in the samples was confirmed by adding an equal volume of 2×-concentrated selenite-cysteine broth to the sample and incubating 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.

Enumeration of viable *B. avium*. Collected samples from the nares, lungs, spleen, bursa of Fabricius, thymus, and cecal contents were processed as described above, and diluted samples were plated on MacConkey agar containing 25 μg of tetracycline hydrochloride (Sigma, St. Louis, Mo.) per ml and incubated for 48 h at 37°C.

Preparation of OMPs. The 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)] (21). OMPs were prepared and characterized by a modified method of Newell et al. (37). *S. typhimurium* χ4172 grown in Luria broth at 37°C was harvested and sonicated as previously described (20). Crude membranes were recovered from the sonicated cell supernatant (25,000 rpm, 100,000 × *g*, 30 min). The membrane pellet was suspended in 2% (wt/vol) *N*-laurylsarcosine, sodium salt (Sarkosyl; Sigma) (45 ml of suspended pellet in PBS plus 5 ml of 20% [wt/vol] Sarkosyl) in phosphate-buffered saline (PBS)

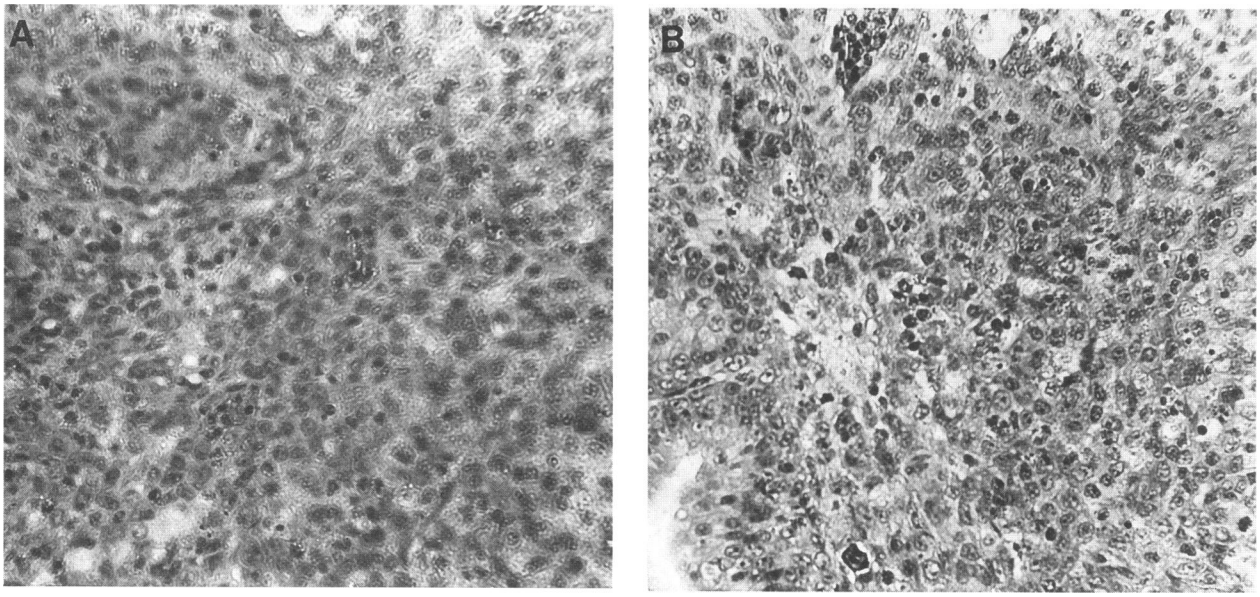


FIG. 2. Histology of the bursa of Fabricius (A) and spleen (B) taken from 3-day-old chicks 2 days after oral infection with 10^8 CFU of *S. typhimurium* wild-type strain χ3761, showing absence of follicles.

at room temperature and centrifuged at 25,000 rpm (100,000 × *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 (20). OMPs, immunogenic surface antigens of *S. typhimurium* (19), were used as the test antigen. Serum and intestinal fluid samples from infected or control chickens were diluted in PBS–0.5% Tween 20 at 1:200 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. Mouse anti-chicken immunoglobulin M (IgM), IgA, and IgG monoclonal antibodies (M11, P9, and G3, respectively) (33) were used to detect chicken IgM, IgA, and IgG, respectively, in sera or intestinal fluids from infected and noninfected chickens bound to *S. typhimurium* OMPs coated on ELISA plates (Dynatech Laboratories Inc., Alexandria, Va.). A rabbit anti-mouse IgG-alkaline phosphatase conjugate (Sigma) 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 using an automated microplate reader (Bio-tek Instrument Inc., Burlington, Vt.). Two times the standard deviation of absorbance for diluted samples from

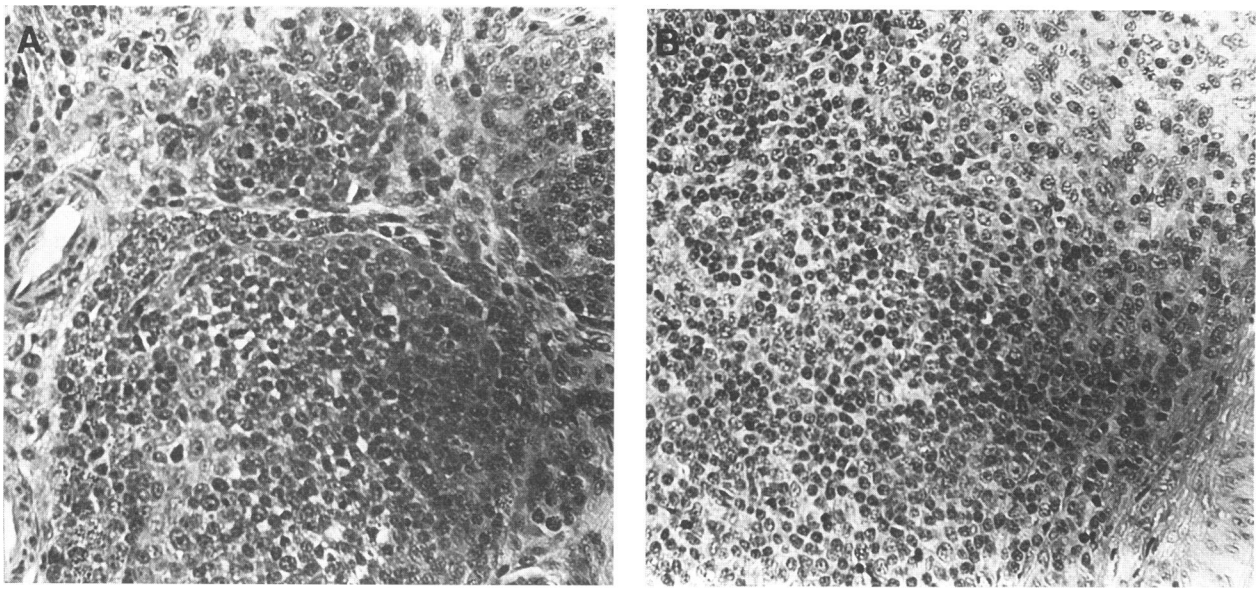


FIG. 3. Histology of the bursa of Fabricius (A) and spleen (B) taken 2 days after oral infection of 1-day-old-chicks with 10^8 CFU of *S. typhimurium* vaccine strain χ3985, showing follicles with heterophilic infiltration.

noninfected chickens was subtracted from the absorbance values of diluted samples from infected chickens. The above method was modified for detecting *B. avium*-specific immunoglobulin isotypes. The supernatant fluid obtained after centrifugation of sonicated *B. avium* cells was used as the coating antigen, and 0.5 N NaCl–1% BSA–0.1% Tween 20 was used as the diluent.

Cellular responses. Purified *S. typhimurium* OMPs or BSG was used as the eliciting antigen for DTH. The DTH tests were done as described by Kita et al. (27). Interdigital injection of lectins into the foot web of chickens has been used for the assessment of cellular immunocompetence in chickens (7). After a comparative study of phytohemagglutinin and ConA, we decided to use ConA to determine the degree of cellular responses in infected and noninfected chickens. The interdigital skin of the right foot was injected intradermally with 100 μg of ConA (Sigma) or 50 μg of *S. typhimurium* OMPs in 100 μl of BSG. The interdigital skin of the left foot was injected with 100 μl of BSG as a control. The thickness of the interdigital skin was determined before injection and at 24 and 48 h after injection. The cellular reaction was calculated as the response in the right foot minus the response in the left foot.

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

RESULTS

Lymphocyte depletion. Histologically, the bursa of Fabricius and spleen of 2-day-old noninfected chickens showed developed follicles (Fig. 1), while the bursa of Fabricius and spleen taken 2 days after infection of 1-day-old chicks with 10^8 CFU of χ3761 in experiment 1 revealed marked lymphocyte depletion and absence of follicles (Table 1) (Fig. 2). The spleen and bursa of Fabricius taken 7 days after infection with χ3761 were partially repopulated with lymphocytes (Table 1). Lymphocyte depletion and partial repopulation were reflected by atrophy of the lymphoid organ at 2 days postinfection and an increase in the weight of affected organs by 7 days postinfection, respectively. Oral infection of 1-day-old chicks with 10^8 CFU of Δ*cya* Δ*crp* *S. typhimurium* vaccine strain χ3985 did not cause lymphocyte depletion in the spleen or bursa of Fabricius (Table 1) (Fig. 3). Histological analysis of the spleen and bursa of Fabricius from chickens infected with χ3985 revealed infiltration by granulated mononuclear cells called heterophils in chickens (Fig. 3). These cells are comparable to neutrophils in mammals. In our preliminary studies oral infection of chickens with low doses (10^2 to 10^5 CFU) of χ3761 was found to be associated with edema of the lymphoid organs. Edema in the organ occluded the losses in organ weight that are well defined with high doses of χ3761. No edema was observed during histological analysis of samples from chickens infected with low or high doses of χ3985. Twenty-one chicks died within 7 days in the group of 40 chicks infected with 10^8 CFU of χ3761, while no death was observed in the group of 20 chicks infected with 10^8 CFU of χ3985 within the same period.

Macroscopic observations. Gross observation showed that the bursa of Fabricius and the thymus from chickens infected intra-air-sac with χ3761 at 4 weeks of age were markedly reduced in size while the spleen increased in size when compared with similar organs from chickens infected intra-air-sac with χ3985 or from the noninfected control chickens (Fig. 4). The posterior caudal air sac, which is the site of infection, was thickly covered with a milky paste of cell debris in chickens infected with χ3761, whereas the air sac membranes of chickens infected with χ3985 and the noninfected control group were clear.

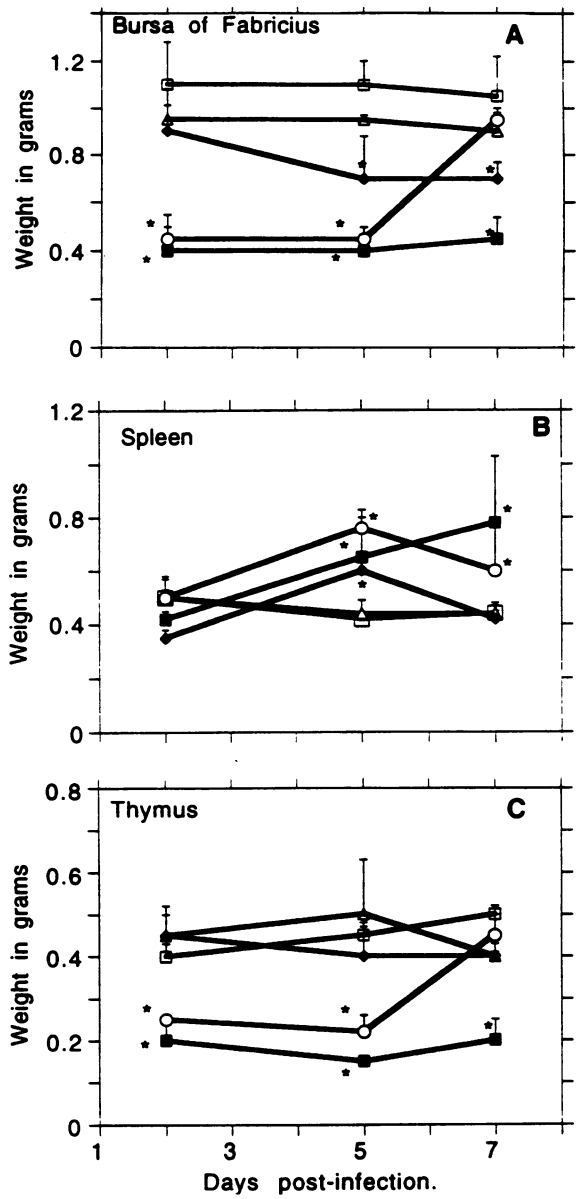
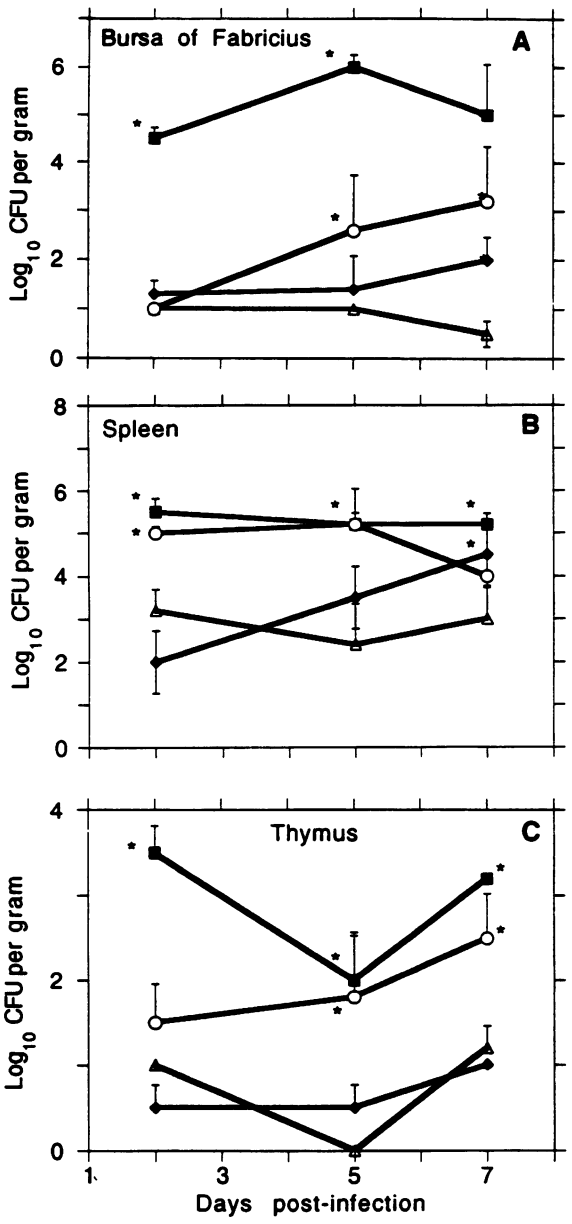


FIG. 4. Mean gross weights of the bursa of Fabricius (A), spleen (B), and thymus (C) from 4-week-old chickens infected intra-air-sac with 10^7 CFU of Δ*cya* Δ*crp* *S. typhimurium* vaccine strain χ3985 (△) or 10^9 (■), 10^7 (○), or 10^5 (◆) CFU of *S. typhimurium* wild-type χ3761 and from uninfected control chickens (□). Values represent the means of five samples, and vertical bars indicate standard errors of the means. *, significant difference between samples from infected chickens and those from noninfected chickens, $P < 0.05$.

Oral infection of 4-week-old chickens with 10^{10} CFU of χ3761 or χ3985 in experiment 2 failed to cause atrophy or hypertrophy of the spleen and bursa of Fabricius (data not shown). After an extensive preliminary investigation of the effect of dose and route of infection on lymphocyte depletion, we established that intra-air-sac infection with salmonellae is the best method that can allow us to compare and analyze the effects of infection with wild-type and avirulent *S. typhimurium* in 4-week-old chickens at a comparable dose level. The LD_{50} of χ3761 or χ3985 in 4-week-old chickens infected by the air



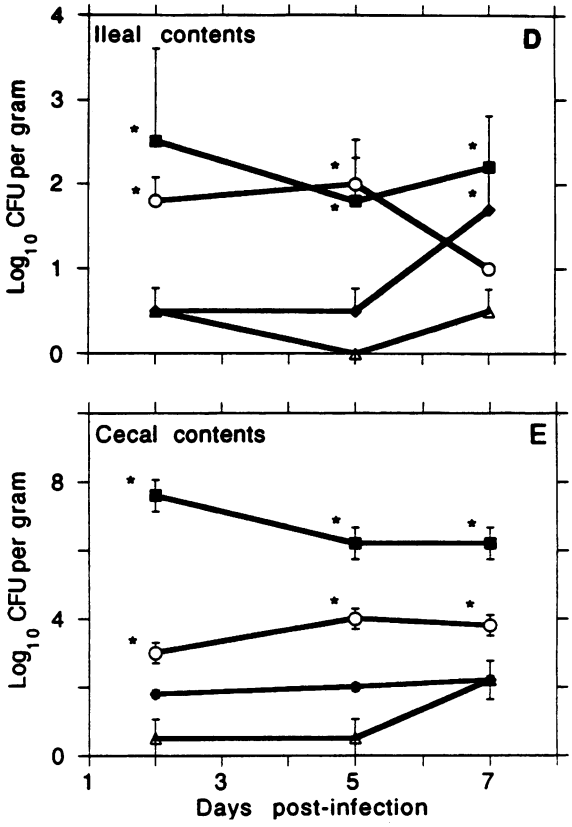


FIG. 5. Recovery of salmonellae from the spleen, thymus, bursa of Fabricius, ileal contents, and cecal contents of 4-week-old chickens infected intra-air-sac with 10^7 CFU of *Δcya Δcrp S. typhimurium* vaccine strain χ3985 (△) or 10^9 (■), 10^7 (○), or 10^5 (◆) CFU of *S. typhimurium* wild-type strain χ3761. Values are means ± standard errors of the means of five samples. Each value represents the $\log_{10}$ CFU of salmonellae isolated per gram of sample. *, significant difference between samples from chickens infected with various doses of χ3761 and those infected with χ3985, $P < 0.05$.

sac route is 10^8 CFU. In experiment 2, intra-air-sac infection of 4-week-old chickens with χ3761 caused significant atrophy of the bursa of Fabricius and thymus and hypertrophy of the spleen (Fig. 4). The degree of atrophy or hypertrophy was highest after an infective dose of 10^9 CFU. Most of the birds infected with 10^7 CFU of χ3761 showed an increase in the weight of the thymus and bursa of Fabricius by 7 days after infection. Infection with 10^5 CFU of χ3761 induced significant atrophy of the bursa of Fabricius (Fig. 4A) and a reduction in the weight of thymus (Fig. 4C) at 5 and 7 days postinfection. A significant hypertrophy of the spleen was also observed 5 days after infection (Fig. 4B). The 18 and 11 chickens that died within 7 days of intra-air-sac infection with 10^9 or 10^8 CFU of χ3761 at 4 weeks of age in experiments 2 and 3, respectively, showed marked atrophy of lymphorecticular organs. The weights of lymphoid organs from eight birds that died within 7 days of intra-air-sac infection with 10^8 CFU of χ3985 in experiment 3 were similar to those of the control noninfected chickens. No significant atrophy of the thymus or bursa of Fabricius or hypertrophy of the spleen was observed in samples collected from 4-week-old chickens infected intra-air-sac with 10^7 CFU of χ3985 (Fig. 4).

Intra-air-sac infection of 4-week-old chickens with χ3761 or χ3985 led to *Salmonella* invasion and colonization of the thymus, spleen, bursa of Fabricius, ileum, and cecum (Fig. 5). χ3761 was isolated at significantly higher numbers than χ3985. In most cases, infection with 10^5 CFU of χ3761 led to equal or higher organ titers than infection with 10^7 CFU of χ3985 (Fig. 5).

Immune responses. One-day-old chicks infected orally with χ3985 in experiment 1 showed high serum and mucosal immune responses with significant IgM and IgA titers 7 days after infection compared with serum and mucosal immune responses in 1-day-old chicks infected orally with χ3761 (Table 2). Serum (IgG and IgM) and mucosal (IgA) immune responses in chickens infected intra-air-sac when 4 weeks old with χ3985 were significantly higher both 5 and 7 days after infection than responses observed in 4-week-old chickens infected intra-air-sac with χ3761 (Table 3).

Cellular immune responses. In experiment 3, chickens in-

TABLE 2. Systemic and mucosal immune responses to *S. typhimurium* OMPs in 1-day-old chicks infected orally with 10^8 CFU of *S. typhimurium*

Strain	Sampling time (wk)	Serum[a]		Intestinal fluid[a]	
		IgG	IgM	IgM	IgA
χ3761	1	0.6 ± 0.09	0.4 ± 0.05	0.1 ± 0.05	0.3 ± 0.05
	2	NS	NS	NS	NS
χ3985	1	0.8 ± 0.18	1.0* ± 0.14	0.1 ± 0.05	0.8* ± 0.09
	2	1.4 ± 0.27	1.3 ± 0.23	0.2 ± 0.05	1.0 ± 0.18

[a] Values are means ± standard errors of means of absorbance values. Each value represents the absorbance value of *Salmonella*-specific immunoglobulin measured in duplicates at 1:200 (sera) or 1:10 (intestinal fluid) dilutions from five samples. Two times the mean of the absorbance values of samples from noninfected chickens served as the baseline. NS, no sample. Chickens infected with χ3761 died within 10 days of infection. *, significant differences between values for chickens infected with χ3985 and those for chickens infected with χ3761, $P < 0.05$.

fected intra-air-sac with χ3985 at 4 weeks of age showed significantly higher DTH responses to *Salmonella* OMPs 7 days after infection than did chickens infected intra-air-sac with χ3761 (Table 4). No significant difference was observed in DTH to OMPs in chickens infected with virulent χ3761 when compared with that in noninfected control chickens (Table 4). Significantly higher cutaneous cellular responses to ConA were also detected 5 days after *Salmonella* infection with χ3985 compared with those in chickens that had been infected with χ3761. Thus, the cutaneous cellular response to ConA was suppressed in chickens infected intra-air-sac with virulent χ3761.

Virulence and *Salmonella* excretion. The effect of *Salmonella* virulence on continuous excretion of salmonellae (carrier status) after infection of 4-week-old chickens was assessed in experiment 4 by monitoring the excretion of salmonellae by chickens infected intra-air-sac with virulent χ3761 or avirulent χ3985 over a 6-week period. Compared with χ3761, significantly lower levels of χ3985 were isolated from fecal droppings postinfection (Table 5). At 6 weeks postinfection, no salmonellae were isolated from the ceca of chickens infected with χ3985, but 10^3 CFU of salmonellae per g was detected in the ceca of chickens infected with χ3761 (Table 5).

Salmonellae and secondary infection. In experiment 5, we investigated the effect of primary infection of chickens with salmonellae on secondary infection with other avian pathogens by challenging chickens infected intra-air-sac with salmonellae at 4 weeks of age with 10^6 CFU of *B. avium* 5 days after *Salmonella* infection. Infection with χ3985 before *B. avium* challenge induced protection against colonization of the spleen, bursa of Fabricius, and cecal contents by *B. avium* (Table 6). Higher titers of *B. avium* were found in the spleen and ceca of chickens infected with χ3761 before *B. avium* challenge than in the spleen and ceca of chickens exposed only to *B. avium*. However, infection with χ3761 or χ3985 reduced colonization of the thymus by *B. avium* but did not affect colonization of the nares by *B. avium* (Table 6).

The groups of chickens infected with *B. avium* with or without prior infection with salmonellae had similar IgM and IgG responses to *B. avium* (Table 7). A significantly higher *B. avium*-specific IgG response was detected in chickens infected with *S. typhimurium* χ3985 before *B. avium* challenge than in chickens infected with *B. avium* only (Table 7).

DISCUSSION

The effect of *Salmonella* infection in chickens varied with the route of infection, *Salmonella* strain, inoculation dose, and age at infection. One-day-old chicks are more susceptible to *Salmonella* infection than 4-week-old chickens. Depletion of lymphocytes in the spleen and bursa of Fabricius was marked in 1-day-old chicks orally infected with 10^8 CFU of χ3761 but not in chicks orally infected with 10^8 CFU of χ3985. The infective dose of χ3761 in experiment 1 was 10^8 CFU, i.e., 10^5 above the LD_{50} of 10^3 CFU in orally infected 1-day-old chicks. The infective dose of χ3985 was 10^8, i.e., at least 40 times below the LD_{50} of 4×10^9 CFU in 1-day-old chicks. The above bias was introduced because lymphocyte depletion in 1-day-old chicks infected with lower doses of χ3761 was masked by edema of the bursa of Fabricius. We therefore used 10^8 CFU of χ3761 to optimize our observations for orally infected 1-day-old chicks; 10^8 CFU of χ3985 is also the established oral vaccination dose for chickens (21, 23). *S. typhimurium* χ3761 is a virulent wild-type strain that induced lymphocyte depletion in orally infected 1-day-old chicks. Although 4-week-old chickens were resistant to oral infection with 10^{10} CFU of χ3761, they were susceptible to intra-air-sac infection with 10^8 CFU of χ3761. The similarity in the LD_{50}s of χ3761 and χ3985 in 4-week-old chickens infected by the air sac route allows us to use intra-air-sac infection of 4-week-old chickens with salmonellae as a model for investigating the effect of a virulent and an orally avirulent *S. typhimurium* strain on the chicken immune system. Intra-air-sac infection with 10^9 or 10^7 CFU of χ3761 caused atrophy of the thymus and bursa of Fabricius and

TABLE 3. Systemic and mucosal responses in chickens infected intra-air-sac with 10^7 CFU of *S. typhimurium* wild-type strain χ3761 or the Δ*cya* Δ*crp* vaccine strain χ3985 at 4 weeks of age

Strain	Sampling time (days)	Serum[a]		Intestinal fluid[a]	
		IgG	IgM	IgM	IgA
χ3761	5	0.5 ± 0.18	0.6 ± 0.23	0.3 ± 0.09	0.4 ± 0.15
	7	1.0 ± 0.32	0.8 ± 0.18	0.5 ± 0.15	0.7 ± 0.23
χ3985	5	1.9* ± 0.27	1.6* ± 0.41	0.6 ± 0.36	1.3* ± 0.32
	7	2.6* ± 0.23	2.0* ± 0.41	0.8 ± 0.18	2.1* ± 0.41

[a] The values represent means ± standard errors of means of absorbance values. Each value represents the mean of five samples. *, significant difference between values obtained for chickens infected with χ3985 and those for chickens infected with χ3761, $P < 0.05$.

TABLE 4. Cellular immune responses in chickens infected intra-air-sac at 4 weeks of age with 10^8 CFU of *S. typhimurium* wild-type strain χ3761 or the Δ*cya* Δ*crp* vaccine strain χ3985

Strain	Days after infection	Foot web thickness (mm)[a]			
		Response to ConA		DTH response	
		24 h	48 h	24 h	48 h
χ3761	5	0.7 ± 0.18	0.8 ± 0.14	ND	ND
	7	ND[b]	ND	0.3 ± 0.09	0.7 ± 0.05
χ3985	5	1.9* ± 0.27	1.9* ± 0.23	ND	ND
	7	ND	ND	2.5* ± 0.32	3.0* ± 0.41
Control	5	1.6* ± 0.18	1.3* ± 0.14	ND	ND
	7	ND	ND	0.8 ± 0.14	0.8 ± 0.18

[a] Values are means ± standard errors of means. Each value represents measurements from five chickens. *, significant difference between values for chickens infected with χ3985 or for the noninfected control group and those for chickens infected with χ3761, $P < 0.05$.
[b] ND, foot web thickness not determined.

hypertrophy of the spleen in 4-week-old chickens. The Δ*cya* Δ*crp* vaccine strain χ3985 did not cause atrophy or hypertrophy of lymphoid organs of 1-day-old chicks infected orally or 4-week-old chickens infected intra-air-sac. This indicates that the use of χ3985 as a vaccine strain at a dose of 10^8 CFU will not cause a deleterious effect on the chicken immune system.

Lymphocyte depletion and atrophy of lymphoreticular organs have been reported in chickens exposed to various pathogens or chemicals: Newcastle disease virus (26), infectious bursa disease (5, 17), Marek's disease virus (15), *Escherichia coli* (35), cyclophosphamide (18, 24), mycotoxin (25), or dexamethasone and cyclosporin (13, 29, 38). However, there has been no report on lymphocyte depletion in chicken lymphoid organs due to infection with *S. typhimurium*. In our studies, histological samples of the bursa of Fabricius and spleen taken 2 days after oral infection of 1-day-old chicks with χ3761 did not reveal any lymphocyte follicles and the organ was depleted of lymphocytes. By the fifth day after infection, there were patches of lymphocyte populations in the bursa of Fabricius and spleen, and partial follicles could be seen in some slides by 7 days postinfection. Samples from chickens infected with χ3985 showed well-developed follicles similar to the follicles in samples from a noninfected control group. Heterophilic infiltration of the spleen and bursa was observed in samples from chickens infected with χ3985 when compared with samples from the spleen and bursa of noninfected control chickens. The ability of chickens infected with χ3761 to partially repopulate their lymphoid organs with lymphocytes 1 week after infection suggests that the observed lymphocyte depletion is transient.

Intra-air-sac infection of 4-week-old chickens with salmonellae created a model that allowed the isolation of high levels of χ3761 from the visceral organs, which simulated the situation of oral infection of 1-day-old chicks with salmonellae. Intra-air-sac infection of 4-week-old chickens with 10^9 or 10^7 CFU of wild-type χ3761 produced significant atrophy of lymphoid organs. The degree of organ atrophy was directly related to the infective dose of salmonellae. The level of salmonellae isolated from chicken visceral organs also increased with dose. This suggests that lymphocyte depletion in chickens was directly related to proliferation of salmonellae. Air sacculitis was observed in the posterior air sac in chickens infected with 10^8 CFU of the wild-type χ3761 but not in chickens infected with 10^8 CFU of avirulent χ3985. The presence of massive cellular infiltration of the air sac suggests that leukocytes accumulated at the site of inoculation. We surmise that when lymphocytes encountered χ3985 or χ3761 in the chickens, χ3985 was less resistant to the invasive leukocytes. Therefore, the proliferation of χ3985 was balanced and well controlled. In contrast, the wild-type χ3761 was resistant to the action of the invasive leukocytes and therefore proliferated faster than χ3985. χ3761 can also multiply within chicken splenic macrophages, leading to macrophage killing (21a) and *Salmonella* dissemination.

The observed difference in the status of the spleen, thymus, and bursa of Fabricius in chickens infected with 10^5 and 10^7 CFU of χ3761 compared with that in chickens infected with 10^9 CFU of χ3761 indicates that high doses of χ3761 were able to nullify the defense efforts of the chicken immune system, leading to necrosis and accumulation of lymphocyte debris at the site of infection (air sacculitis associated with a milky air sac). The continuous presence of the virulent pathogen may induce excessive demand for leukocytes, which in turn may led to active lymphopoiesis associated with high levels of precursor cells in the spleen, manifested grossly by splenomegaly. The complex immunological scenario that results from establishing a balance between combating the pathogen, down-regulating the immune system, and generating a new leukocyte population may influence the depletion of lymphocytes. The effect of virulent salmonellae on chicken lymphoid organs as observed in this study revealed a new phenomenon in the pathogenesis of *S. typhimurium* in chickens.

TABLE 5. Fecal excretion and cecal isolation of *S. typhimurium* from chickens infected intra-air-sac at 4 weeks of age with 10^8 CFU of *S. typhimurium* wild-type strain χ3761 or the Δ*cya* Δ*crp* vaccine strain χ3985

Age (wk)	$\log_{10}$ CFU/g[a]			
	χ3761		χ3985	
	Feces	Ceca	Feces	Ceca
5	5.4* ± 1.05 (10/10)[c]	NS[b]	2.5 ± 0.64 (6/10)	NS
8	1.8* ± 0.68 (8/10)	NS	0.2 ± 0.36 (2/10)	NS
10	2.6* ± 0.6 (6/10)	3.6* ± 1.09 (8/10)	0 (0/10)	0 (0/10)

[a] Values are means ± standard errors of means. Each value represents the $\log_{10}$ CFU of salmonellae detected per gram of sample. Direct culture of samples on salmonella-shigella agar permit detection of 10^2 CFU/g. Absence or presence by the enrichment method (10 CFU/g) was confirmed by the inability or ability, respectively, to detect salmonellae after selective enrichment in selenite-cysteine broth. *, significant difference between *Salmonella* isolation from chickens infected with χ3761 and from those infected with χ3895, $P < 0.05$.
[b] NS, no sample collected.
[c] Number positive/total number.

TABLE 6. *B. avium* isolation from chickens infected intra-air-sac at 4 weeks of age with 10^8 CFU of *S. typhimurium* wild-type strain χ3761 or the Δ*cya* Δ*crp* vaccine strain χ3985 and challenged with 10^8 CFU of *B. avium* intraocularly 5 days later

Organism(s)	$\log_{10}$ CFU/g in[a]:				
	Thymus	Nares	Spleen	Bursa of Fabricius	Cecum
χ3761 and *B. avium*	<2	6.7 ± 1.18	4.2* ± 0.64	3.7* ± 0.41	3.1* ± 0.32
χ3985 and *B. avium*	<2	6.4 ± 0.86	<2	<2	<2
B. avium only	2.8	5.7 ± 1.05	2.2 ± 0.41	2.7 ± 0.55	2.6 ± 0.36

[a] Samples were collected 1 week after challenge with *B. avium*. Values are expressed as means ± standard errors of means. Each value represents the mean $\log_{10}$ CFU of *B. avium* isolated per gram of sample from five samples. The assay used can detect a minimum of 10^2 CFU of *B. avium*; thus, the inability to detect *B. avium* in samples is expressed as $<10^2$ CFU. *, significant difference between *B. avium* isolation from chickens infected with χ3761 or χ3985 and challenged with *B. avium* and those infected with *B. avium* only, $P < 0.05$.

Immunosuppression caused by *Salmonella* infection in mice was attributed to an active mechanism mediated by a soluble suppression factor(s) which was linked to the presence of macrophage precursors isolated from adult mouse spleens (1). The bursa of Fabricius and thymus are central lymphoid tissues of chickens (14) and are the major sites of B- and T-cell differentiation, respectively (6, 46, 47). The bursa also functions as a peripheral lymphoid organ (42). The lymphocyte population in each follicle in the bursa of Fabricius is derived from clonal expansion of a single B-lymphocyte precursor cell, making the lymphocyte population in each follicle monoclonal in origin (39). This may enhance the possibility of total depletion of lymphocytes in the follicles of day-of-hatch chicks, which have an immature lymphoid system, when infected with salmonellae in the hatchery. A major shift in lymphocyte population is reflected by the sizes of these organs.

Lower levels of humoral and cellular immune responses observed in chickens infected intra-air-sac with χ3761 than in chickens similarly infected with χ3985 or in noninfected control chickens showed that highly virulent salmonellae do have an immunosuppressive effect on experimentally infected chickens. Chickens infected intra-air-sac with χ3761 excreted salmonellae for a longer time than did chickens infected with χ3985. It can be inferred from these data that lymphocyte depletion and immunosuppression caused by virulent salmonellae contribute to the development of a *Salmonella* carrier status in chickens. The major difference in the pathoclinical observations of chickens infected intra-air-sac with virulent χ3761 or avirulent χ3985 is the depletion of lymphocytes and atrophy of lymphoid organs.

There is a similarity in the immunosuppression we observed in chickens and immunosuppression in mice. Immunosuppression in mice was associated with marked splenomegaly, impairment of response to ConA, diminished antibody production, and prolonged *Salmonella* proliferation (12, 28, 36). We agree with Lee et al. (28) that the observed suppression is an indication of a powerful immunomodulatory process induced by live salmonellae rather than an indicator of poor immune status of the host.

Intra-air-sac infection of chickens with χ3761 did not affect the development of humoral immune responses to *B. avium* but affected the clearance of *B. avium* from the bursa of Fabricius and spleen. This implies that virulent χ3761 impaired the induction of protection against *B. avium*.

We have described a phenomenon in which wild-type *S. typhimurium* compromised chicken immunocompetence. Our results suggest that lymphocyte depletion and immunosuppression observed during infection may play a critical role in the development of the *Salmonella* carrier status in chickens. We are currently investigating the role that maternal immunity might play in reducing the lethal effect of salmonellae in chicks. We also plan to identify and characterize genes produced by salmonellae in vivo and analyze the effects such genes may have on chicken lymphoid cells.

TABLE 7. Humoral responses to *B. avium* sonicates in chickens infected intra-air-sac at 4 weeks of age with 10^8 CFU of *S. typhimurium* wild-type strain χ3761 or the Δ*cya* Δ*crp* vaccine strain χ3985 and challenged with 10^8 CFU of *B. avium* intraocularly 5 days later

Organism(s)	A_{405}[a]	
	IgM	IgG
χ3761 and *B. avium*	1.3 ± 0.18	1.1 ± 0.09
χ3985 and *B. avium*	1.6 ± 0.23	1.5* ± 0.23
B. avium	1.8 ± 0.14	0.7 ± 0.14

[a] Samples were collected 1 week after challenge with *B. avium*. Values are means ± standard errors of means of absorbance values. Each value represents the mean absorbance value of *B. avium*-specific immunoglobulins measured in duplicate at a 1:200 dilution, using five samples. *, significant difference between values for chickens infected with χ3761 or χ3985 and challenged with *B. avium* and those for chickens infected with *B. avium* only, $P < 0.05$.

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