Hyporesponsiveness of the Systemic and Mucosal Humoral Immune Systems in Chickens Infected with *Salmonella enterica* serovar *enteritidis* at One Day of Age

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ABSTRACT Newly hatched chicks lack immunological maturity, which could compromise their ability to respond to infection by pathogens such as *Salmonella enterica* serovar *enteritidis* (*S. enteritidis*; SE). A study was conducted in which chicks were infected with a sublethal dose of SE at 1 d posthatch, and the systemic and intestinal immune responses to the challenge were followed over time. Birds infected at this age experienced difficulty in clearing the infection, and 50% of the individual birds remained persistently infected until 23 wk of age. These birds exhibited only a marginal systemic and mucosal humoral immune response to the infection. No response or little response was observed 1 wk postchallenge; responses increased somewhat over time. On many of the sampling times, 50% or more of the culture-positive birds lacked a detectable plasma or intestinal response. Levels of 10^3 to 10^5 SE/g of feces could be found in the intestines of birds eliciting a good IgA response, indicating that, when these birds did respond mucosally, the IgA produced was incapable of clearing the organism once the infection was established. Birds infected during this time also experienced reduced ability to respond to vaccination. Compared with uninfected controls, depressed responsiveness to an *S. enteritidis* bacterin was observed in infected birds 1 and 2 wk after administration, whereas those individuals receiving an inactivated Newcastle disease vaccine (NDV) experienced a reduced response 4 and 6 wk postvaccination, indicating that the persistent infection affected the ability of the immune system to respond to homologous and heterologous antigens. These results demonstrate that exposure of chickens to SE early in life interferes with the ability of these individuals to respond humorally to the infection and to other antigenic stimuli; such effects can be observed for at least 23 wk.

(*Key words*: humoral immunity, tolerance, immunoglobulin A, persistent infection, *Salmonella enteritidis*)

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INTRODUCTION

Newly hatched chicks are highly susceptible to infection by salmonellae, requiring fewer than 100 cells to become infected (Cox *et al.*, 1990a). This heightened susceptibility can result in increased severity of infection and rapid spread of the organism throughout the hatchery. High percentages of fluff, egg shells, and belting materials can be contaminated with *Salmonella* (Cox *et al.*, 1990b), which may serve as fomites for *Salmonella* transmission within a hatchery environment. *Salmonella enterica* serovar *enteritidis* (*S. enteritidis*; SE), because of its innate ability to disseminate extraintestinally and invade numerous tissues, including the ovary (Gast and Beard, 1990), can become deposited inside the egg, infect the embryo, and be transmitted vertically to the progeny. Once hatched, these chicks can serve as amplifiers of the SE numbers and the source for horizontal transmission of the organism within the hatching cabinet. Cason *et al.* (1994) showed that *S. typhimurium* could be recovered from 80% of chicks hatched in the same cabinet as chicks infected *in ovo* with the organism, and 44% of these birds became intestinally colonized. Therefore, the hatching cabinet can become an important site for amplification and dissemination of *Salmonella*.

Once infection with SE occurs at this young age, what are the consequences and progression of the infection? One-day-old chicks exposed to high levels of paratyphoid salmonellae often experience significant mortality over the first few weeks postexposure (Smith and Tucker, 1980). Lower exposure doses of salmonellae generally result in less mortality, and more birds are infected long-term (Nakamura *et al.*, 1993; Phillips and Opitz,

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Abbreviation Key: SE = *Salmonella enteritidis*; NDV = Newcastle disease virus.

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1995; Gast and Holt, 1998). The first week posthatch, therefore, poses the greatest threat to the chick with regard to *Salmonella* exposure. Thereafter, the individuals become progressively more resistant (Gast and Beard, 1989).

The immune system of the chick is marginally developed at hatch. Serum IgM can be detected as early as 4 d posthatch (Martin and Leslie, 1973) but does not achieve maximum levels until Day 40. Similarly, serum IgY (IgG) and IgA levels require 56 and 87 d, respectively, to reach their maximum. Lymphocyte populations in the intestinal tract are sparse the first week posthatch, and high numbers of antibody-producing cells are not found in intestinal tissues until 2 to 6 wk posthatch (Yamamoto *et al.*, 1977; Jeurissen *et al.*, 1989). Plasma cell deposition in gut lymphoid tissues is not oberved until 2 wk posthatch (Jeurissen *et al.*, 1989), and maximal development of these tissues requires 6 wk (Befus *et al.*, 1980; Jeurissen *et al.*, 1989; Schat and Myers, 1991). Consequently, during the first week posthatch, the ability of the chick to respond to infections by intestinal pathogens such as SE is limited. Therefore, the current study was undertaken to determine how effectively 1-d-old chicks could generate an intestinal and systemic antibody response against a sublethal dose of SE.

MATERIALS AND METHODS

Chickens

In two replicate trials (Trials 1 and 2), 144 1-d-old Single Comb White Leghorn chicks from the laboratory's specific-pathogen-free flock were distributed evenly into 12 Horsfall-Bauer isolation cabinets. Chicks in 11 of the 12 cabinets were administered a 10^{-2} dilution of an overnight tryptic soy broth[3] culture of a nalidixic acid-resistant phage type 13 SE (7.5×10^6 SE). The chicks in the remaining cabinet were left uninfected to serve as controls. At 4 wk of age, the infected chicks were transferred to battery cages and, at 12 wk of age, were transferred to laying cages. Standard starter-grower feed was consumed *ad libitum* by the birds until 18 wk of age, at which time the ration was changed to a layer feed. The chicks were exposed to continuous light until Week 4 when the photoperiod was changed to 10 h daily. At 18 wk, the light exposure was increased to 16 h daily and remained at that level until the termination of the experiment at 23 wk.

For Trial 3, 70 1-d-old chicks were distributed evenly into the isolation cabinets and were administered the SE (1.2×10^6 SE). In a separate room, 60 chicks were distributed to isolation cabinets and left uninfected. Feed and light conditions were the same as those in the previous trials. The experiment was terminated after wk 16.

In a separate experiment, 10 15-wk-old hens were infected with 1 mL of an overnight broth culture of SE (7×10^8 SE). Blood and fecal samples were collected weekly for 4 wk, processed, and assayed for antibody responses as described subsequently. One gram feces was also added to 9 mL tetrathionate brilliant green broth[1]. The samples were incubated for 24 h at 37 C and plated onto BGNN (brilliant green agar[4] containing 20 μg/mL novobiocin[5] and 20 μg/mL naladixic acid[3]). Following a further 24-h incubation, the plates were examined for the presence of SE.

Sampling

At Weeks 1, 4, 8, 12, and 16 postchallenge in Trials 1 and 2, 10 infected and two uninfected chicks were randomly selected and placed into individual cubicles set up in battery cages. Feces from the individual birds were collected over the next 90 min on feed-grade polystyrene trays. Blood was collected into heparinized syringes from the wing veins of the 4 to 16-wk-old birds, whereas the 1-wk-old chicks were decapitated, and the blood was collected into tubes containing heparin. The birds were euthanized, and liver, spleen, and one cecum were aseptically collected from each individual. The SE levels were determined (Gast and Holt, 1998). In Trial 3, 10 infected and two control birds were sampled at Week 1, 4, and 8. Blood and feces collection were performed as previously mentioned. Organs for bacteriological sampling were the liver and cecum. Portions of spleen, thymus, bursa of Fabricius, cecum, cecal tonsil, and ileum were collected, immersed in neutral buffered formalin, embedded in paraffin, sectioned at five microns, mounted on glass slides, and stained with hematoxylin and eosin. The tissue sections were then examined by light microscopy. At Week 7, 10 infected and 10 uninfected birds were injected subcutaneously with a killed SE vaccine preparation (Gast *et al.*, 1993) and a second group of 10 infected and uninfected birds were injected subcutaneously with an inactivated Newcastle disease virus (NDV) vaccine (Stone, 1988). A second inoculation was administered 6 wk later. The birds were bled weekly from the wing veins into heparinized syringes.

Antibody Assays

Blood samples were centrifuged, and the plasma was diluted 1:250 (vol/vol) in PTB buffer (PBS, pH 7.2, containing 0.05% Tween 20[3] and 1% BSA). The samples were added in duplicate to separate ELISA trays coated with 1 μg/mL SE flagella and blocked with PTB. Fecal samples were processed as described by deVos and Dick (1991). A 1:10 dilution (wt/vol) of the feces was made in PBS, and the sample was vortexed vigorously and allowed to stand at room temperature for 1 h. After centrifugation at 15,000 $\times$ *g* for 10 min, the supernatant was serially diluted (two-fold), and the dilutions and neat samples were added to the ELISA plate. A positive and negative

[3]Oxoid, Ogdensburg, NY 13669.
[5]Sigma Chemical Co., St. Louis, MO 63178-9916.

plasma (diluted 1:250) and fecal sample (the extract used neat) were used on each plate. The samples were assayed for the presence of anti-SE flagella antibodies as described previously (Holt and Porter, 1993). Plasma IgG and IgM and intestinal IgA anti-SE flagella antibodies were assayed in Trial 1. In Trial 2, plasma IgG and intestinal IgA and IgG were examined, whereas, in Trial 3, plasma IgG, IgM, and IgA and intestinal IgA and IgG levels were determined. A sample was considered positive if the sample:negative control ratio was two or greater.

Antibody responses to vaccination in Trial 3 were analyzed by microagglutination for the SE-vaccinated birds (Williams and Whittemore, 1971) and by hemagglutination inhibition (Beard, 1989) for the NDV-vaccinated birds.

Comparison of Immunoglobulin A Levels and Salmonella enteritidis *Numbers*

On Week 23 of Trial 2, fecal samples were collected from seven birds, and a fecal extract for IgA anti-SE flagella was made. The extract was serially diluted (two-fold), and end-point titers (2× the negative control) were determined for the dilutions. A portion of the original 1:10 fecal extract was serially diluted (10-fold) and 100 μL were spread-plated onto BGNN. The plates were incubated for 24 h at 37 C, and SE counts/g feces were determined the following day.

Statistics

Significant differences between antibody responses in the infected *vs* noninfected vaccinates in Trial 3 were determined using the pooled-variance *t* test (Shott, 1990) at $P < 0.05$.

RESULTS

In all three trials, liver, spleen, and cecum samples were all culture-positive for SE at 1 wk postchallenge, and a high percentage was positive at Week 4 (Gast and Holt, 1998). No SE was detectable in livers or spleens after this time. In Trials 1 and 2, 30 to 50% of cecal or fecal samples remained positive from Weeks 8 to 23, when the experiments were terminated. In Trial 3, 100% of the cecal samples were positive at Week 8. One bird in Trial 2 produced eggs contaminated with SE.

The ELISA results for Trials 1 and 2 were combined and are presented in Figure 1 (A and B). Few IgM or IgA anti-SE antibodies were detected in plasma in the trials, whereas IgA was the predominant isotype in intestinal samples. No plasma IgG titers were detectable at 1 wk postchallenge (Figure 1A), but, by Week 4, 50% of the samples contained detectable antibody levels. The percentage of samples with antibody titers increased slightly at 8 wk, and these percentages never surpassed 50% over the next 8 wk. Intestinal immunoglobulin levels increased from 15% positive samples at 1 wk post-

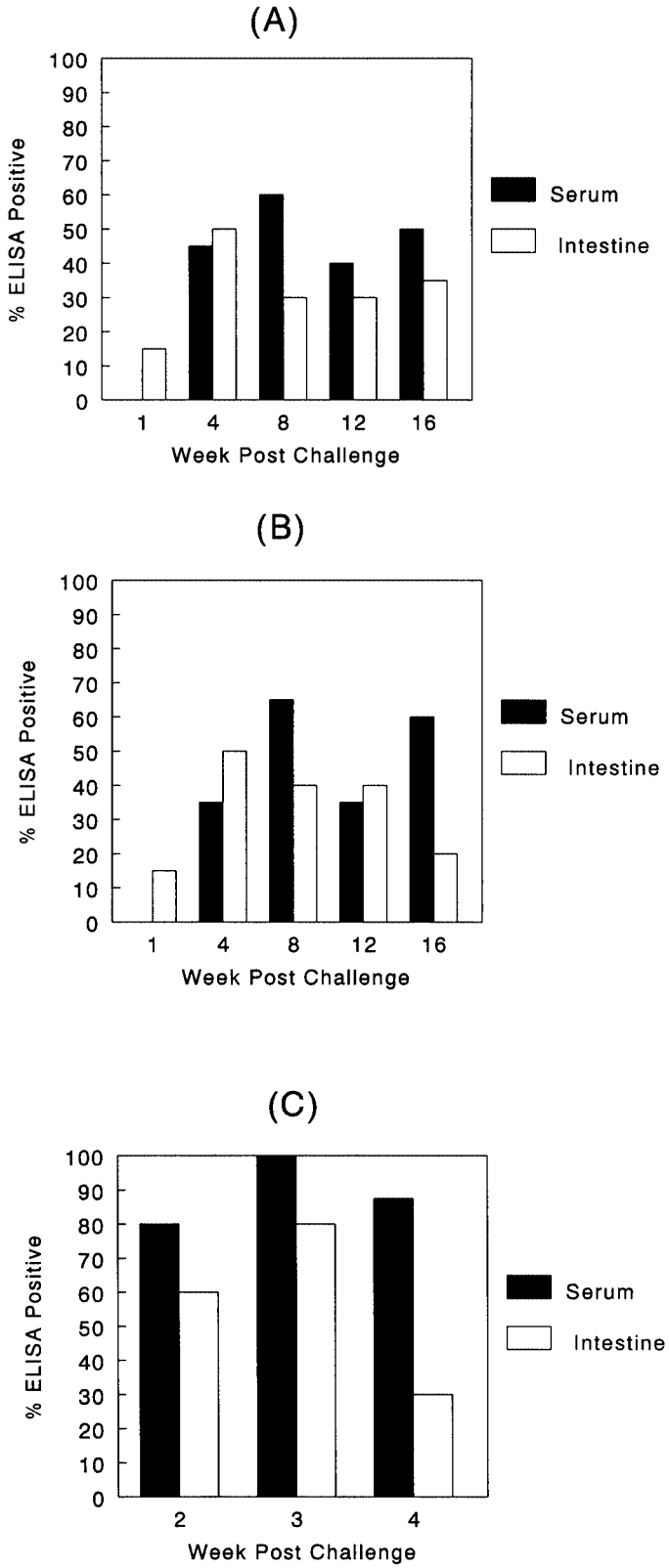

FIGURE 1. Serological detection of anti-*Salmonella enteritidis* antibodies in plasma (IgG) and intestinal (IgA) samples at various times from birds infected at 1 d of age (A and B; Trials 1 and 2 combined) and at 15 wk of age (C). A) Percentage of ELISA + plasma and intestinal samples from all individuals. B) Percentage of ELISA + plasma and intestinal samples from only individuals that were organ culture positive. C) Percentage of ELISA + plasma and intestinal samples from all individuals infected at 15 wk of age – all individuals.

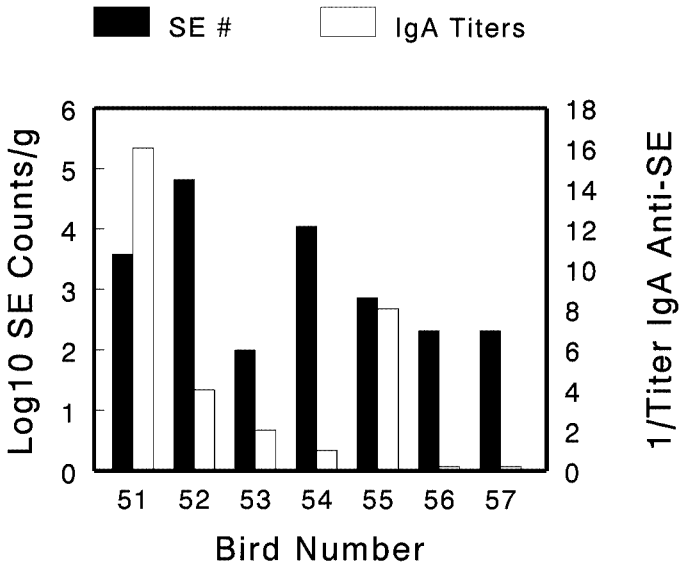

FIGURE 2. Comparison of *Salmonella enteritidis* (SE) levels and IgA anti-SE titers in intestinal samples from persistently infected birds. Fecal samples were obtained from birds in Trial 2, 23 wk after challenge with SE at 1 d of age, and were processed for SE levels and antibody titers.

challenge to 50% positive at Week 4. The intestinal immune response dropped dramatically at Week 8, and no more than 50% of the samples were positive at any point over the next 8 wk. No plasma or intestinal responses were detected in any of the uninfected control birds. In contrast to what was observed in the young birds challenged with SE, a vigorous immune response was observed in birds infected with SE at 15 wk of age (Figure 1C). Eighty percent of the plasma samples were positive at Week 2 postchallenge and remained at these high levels through Weeks 3 and 4. A similar trend was observed with intestinal samples, although a decreased response was observed at Week 4. Fifty percent of the fecal samples were culture negative for SE by Week 4 (data not shown).

Although all of the birds were initially infected and the infection appeared to disseminate extraintestinally, not all of the birds were capable of mounting a response to this challenge. Figure 1B presents the results delineating the percentage of the SE culture-positive birds that were either antibody positive in the plasma or intestinal tract. As shown in Figure 1, the percentage of culture-positive birds with detectable plasma or intestinal antibody responses was lower than might be expected. The percentage of plasma samples that were ELISA+ increased from no positives at Week 1 to 40% positive at Week 4. The maximum percentage of the organ+ birds, which were ELISA+, was 60% at Week 8 and decreased thereafter. Similar results were observed for intestinal samples, although only a maximum of 50% of the organ+ birds were also intestinal antibody positive.

To examine how the intestinal immune response correlated with the levels of SE in the intestinal tract, feces collected from 23-wk-old persistently infected hens in Trial 2 were processed for IgA levels and for the numbers of SE per gram of feces. As shown in Figure 2, the organism could be found in birds that were unable to mount a detectable IgA response (Birds 56 and 57) against it, similar to the results described in Figure 1B. In several of the birds, SE could be found at high levels, between 10^3 to 10^5 SE/g of feces, and two of the birds (Birds 51 and 55) achieved these levels in the face of a reasonably high mucosal antibody response against it.

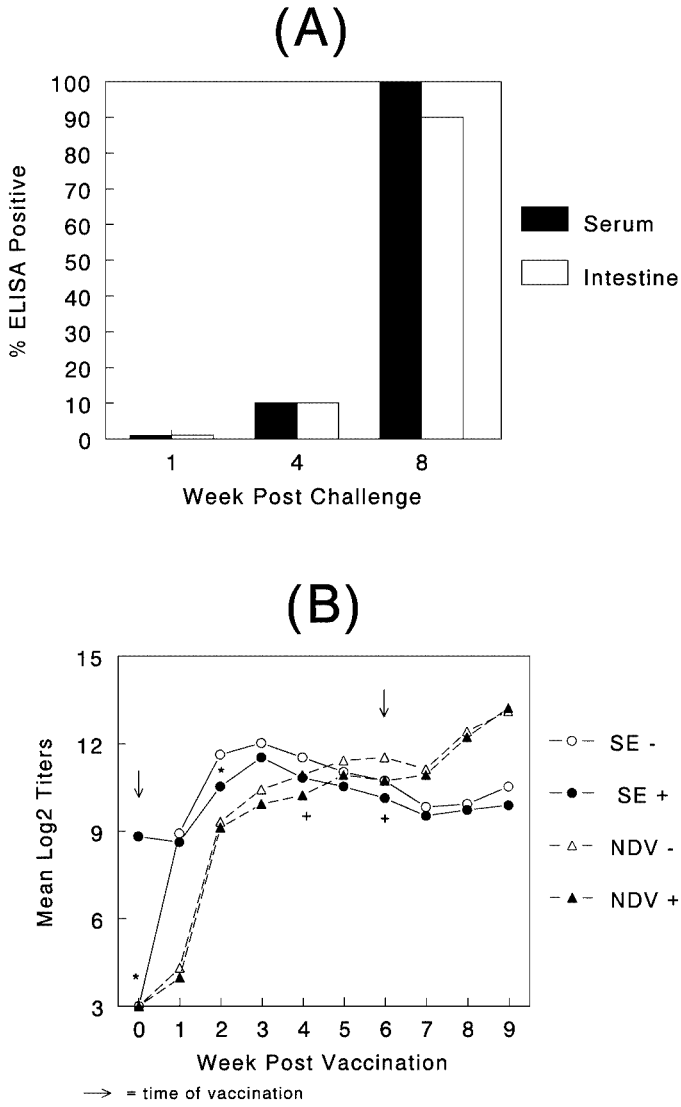

FIGURE 3. Serological detection of anti-*Salmonella enteritidis* (SE) antibodies in plasma and intestinal samples at various times from Trial 3 birds infected at 1 d of age and the effect of infection in these birds on response to vaccination. A) Percentage of ELISA + plasma and intestinal samples from all individuals. B) Response of infected (+) *vs* uninfected (–) birds immunized with either SE bacterin or Newcastle disease virus vaccine (NDV). * = significantly different from uninfected birds in responding to the SE bacterin ($P < 0.05$). + = significantly different from uninfected birds in responding to the NDV vaccine ($P < 0.05$).

In Trial 3, no plasma samples from chicks infected with SE at 1 d of age were positive at Week 1, and only 10% were positive at Week 4 (Figure 3A). By Week 8, 100% of the plasma samples were positive, and a similar trend was observed for intestinal samples. To determine whether the hyporesponsiveness was antigen-specific or was a generalized phenomenon, infected birds and their

uninfected hatchmates in Trial 3 were immunized with either an SE bacterin or inactivated NDV vaccine, and plasma antibody titers were determined weekly. As shown in Figure 3B, previously infected birds possessed an anti-SE titer prior to vaccination, which was unaffected by bacterin administration 1 wk postvaccination, and only a slight elevation of titer was observed at Week 2. This result is contrasted with bacterin vaccination in birds not previously exposed to SE in which a vigorous response to the immunization at Weeks 1 and 2 was observed, with the titer at Week 2 higher ($P < 0.05$) than that observed for their infected hatchmates. No significant differences in responses were observed between the two groups at later sampling times, although titers in the previously infected birds were always lower. For the groups of birds receiving the NDV vaccine, the responses at Weeks 1 and 2 were identical, but responses in the prior-infected birds began to lag behind their uninfected hatchmates and were significantly lower ($P < 0.05$) at Weeks 4 and 6. No significant differences were observed in response to either vaccine following a re-immunization at Week 6. No anti-SE responses were observed in the group of birds not previously infected and vaccinated with the NDV vaccine.

Histologic evaluation of tissues from infected chickens revealed a relative lymphoid depletion in the spleen of infected chickens at Week 1 when compared with noninfected chickens. The spleens of infected chickens (Week 1) had moderate to marked hyperplasia of macrophages surrounding penicilliform capillaries (ellipsoids), accompanied by a decrease in lymphocytes (Figure 4). This change was associated with multifocal deposits of fibrin and rare necrotic foci in the white pulp (splenitis). The spleens of infected chickens at Week 4 had less extensive changes, consisting of mild to moderate hyperplasia of macrophages around penicilliform capillaries and mild depletion of lymphocytes relative to the spleens of noninfected chickens. No overt differences between lymphocyte populations in the bursa of Fabricius, thymus, and cecal tonsil were observed in any group. Seven of 10 infected chickens at Week 1 had typhlitis (cecal inflammation), varying from heterophils infiltrating the lamina propria of mucosal folds in mild cases, to a luminal exudate of heterophils, sloughed epithelial cells, and bacteria in severe cases. This inflammation occasionally involved the ileum.

DISCUSSION

Many factors can affect the development of an immune response against a pathogen. Disease (Phillips and Opitz, 1995), stress (Holt, 1992), and age (Williams and Whittemore, 1975) all can play a role in directing how well an immune response will progress. Age especially can be a major determinant because the immune system in birds shortly after hatch is immature and only partially developed (Yamamoto *et al.*, 1977; Jeurissen *et al.*, 1989; Schat and Myers, 1991). For this reason, the current study was conducted that examined the capacity of birds infected with SE at 1 d posthatch to elicit a humoral immune response in the plasma and intestinal tract.

The results of the study indicate that immunological immaturity hampers the ability of the immune system to respond to, and ultimately protect against, the SE infection. Few systemic or intestinal immunological responses were observed 1 wk postchallenge, but the situation improved by Week 4. However, although the bulk of internal organs and ceca were culture-positive for SE, such could not be said for the immunity to the challenge, for which the average responsiveness for the three trials was less than 50%. This result is in contrast to infections in more mature birds, in which the bulk of the individuals responded strongly at Week 4 post-SE challenge, resulting in a 50% reduction in the number of infected birds at this time period.

Infection of very young individuals with *Salmonella* can have a dramatic effect on immunity. Williams and Whittemore (1975) noted the poor response of young birds exposed to paratyphoid *Salmonella* infection. Hassan and Curtiss (1994) demonstrated that chicks infected with high doses of *S. typhimurium* exhibited significant lymphocyte depletion of lymphoid tissues; similar lymphocyte decreases were observed in the current study. However, comparable with the effects reported by Hassan and Curtiss (1994), the diminished lymphocyte numbers were only observed over a short time span, whereas the immune hyporesponsiveness was more of a chronic occurrence. Interestingly, a study by Hassan *et al.* showed that chicks infected with *S. typhimurium* at 4 d of age mounted a significant serum and intestinal antibody response to infection. Similarly, a recent study by Sasai *et al.* (1997) showed that splenic IgA+ and IgM+ lymphocytes were increased in 16-d-old chicks infected with SE. These results indicate that a certain degree of maturation of immunity occurs rapidly post hatch, which allows the birds to respond to infection. A delineation of lymphocyte populations affected in chicks infected shortly after hatch may provide more insight into the poor immune responses observed longterm.

Animals exposed to antigens very early in life can be rendered tolerant to those antigens and remain unresponsive for extended periods (Golub and Green, 1991). Traub (1938) demonstrated that mice infected *in utero* with lymphocytic choriomeningitis virus remained persistently infected with the virus throughout their lives and mounted a minimal antibody response to the pathogen. Mice infected as adults elicited a strong antiviral response to the challenge organism. Similarly, in the current study, birds were actively infected with SE but were unable to mount an antibody response to the organism. Responses to vaccination in these birds to an SE bacterin, but not to NDV, were delayed by a couple of weeks, indicating a certain degree of antigen specificity to the hyporesponsiveness. The response to vaccination recovered by Week 3, indicating that the antigenic stimulation provided by the vaccine emulsion was sufficient to break through the hyporesponsiveness. Interestingly, responses to the NDV vaccine were also affected in the

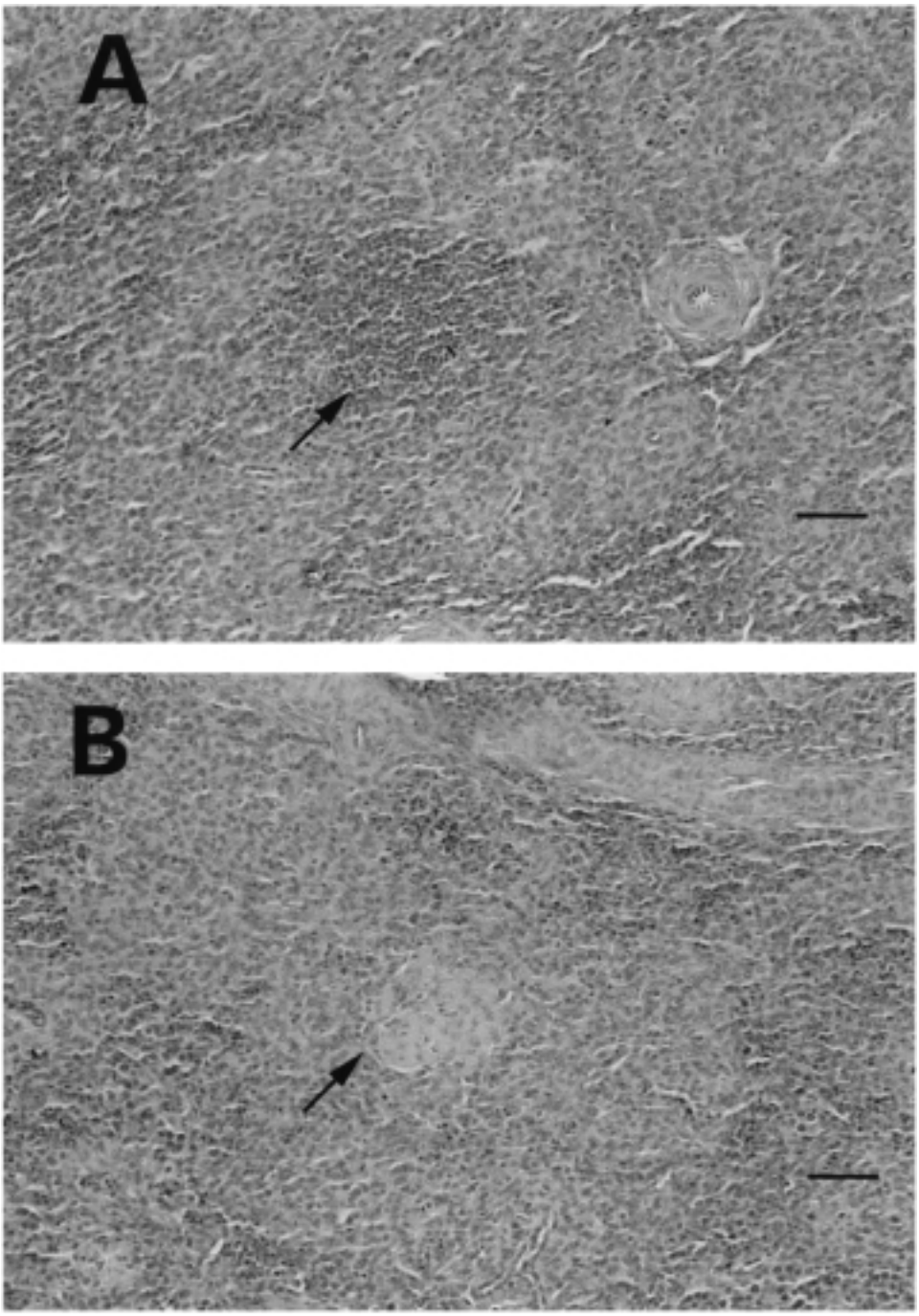

FIGURE 4. Sections of spleens from control and infected birds 1 wk post challenge. A) Spleen from an uninfected control chick showing an intact lymphoid follicle in the center of an ellipsoid (arrow). B) Spleen from infected chick showing an ellipsoid (arrow) with a central aggregate of fibrin, an absence of lymphocytes, and a marked hyperplasia of macrophages. Hematoxylin and eosin 20×. Bar = 33 μm.

SE-infected birds. Contrary to what was observed for the bacterin, the anti-NDV responses were diminished at Week 4 and 6 in the vaccination schedule. This diminished response may be a reflection that damage to lymphoid tissue might have occurred.

The primary immunoglobulin at the mucosal surfaces is IgA, although other isotypes may also be found (Schat and Myers, 1991). Protection by IgA is mediated primarily through binding to the microorganism, which inhibits adherence and colonization (Majumdar and Ghose, 1981; McGhee *et al.*, 1992). Antibody-mediated cellular cytotoxicity directed by specific IgA has also been described (Tagliabue *et al.*, 1983). The results shown in Figure 1 indicate that many of the birds lacked any sort of intestinal antibody response, and that protection in the intestinal tract had broken down. Further, as shown in Figure 2, high levels of SE existed in the presence of reasonable titers of IgA to the organism. It is possible that 1) the SE had become so entrenched in the tissue that the IgA response was unable to eliminate it or 2) the SE was

sequestered in a tissue site that was out of reach of the immune response. Considering that protection by IgA is mediated primarily at mucosal surfaces, immune protection within tissues must be effected through other mechanisms. Cell-mediated immunity has been shown to be very important in mammals (Nauciel, 1990) and in chickens (Lee *et al.*, 1981) for protecting the individual against *Salmonella* infections. A third possibility, and one not addressed in the study, is that cell-mediated immunity was compromised along with the humoral response. Further investigation into this area is warranted.

The implications of the current study are severalfold. First, birds infected at a young age are able to mount only a modest immune response against the SE challenge, and this does not improve with age. A certain percentage of the birds remain persistently infected and can go on to produce eggs contaminated with SE (Gast and Holt, 1998). These flocks can become a serious food safety concern. Second, because the birds continue to respond poorly to SE, the question arises as to whether humoral immunity is damaged. As a result, the ability of the immune system to respond to other invading organisms may be compromised, increasing the risk of flock infections by various microbial pathogens. Responses to vaccination are decreased (Figure 3B), and similar effects may also occur for resistance to other disease-causing agents. Further studies evaluating other vaccination regimens and challenge organisms are necessary before the extent of the immune problems in these young chicks can be ascertained. Third, serological surveillance is an important tool for many egg industry quality assurance programs. A portion of birds infected at 1 d of age carry active SE infections and yet do not elicit an immune response to this infection. This reduced immunity will compromise the effectiveness of serological surveillance and may allow infection in some flocks to evade detection. The impact of infections by SE in the hatchery can potentially be far-reaching. Special attention needs to be paid to reducing contact of the chicks with the pathogen at this important stage of their lives.

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