

Participation of the Intestinal Epithelium and Mast Cells in Local Mucosal Immune Responses in Commercial Poultry

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ABSTRACT The intestinal mucosa of commercial poultry is continually subjected to invasion or colonization by a wide array of potentially hostile enteric pathogens. Although, recent investigations have focused on lymphocyte involvement in immune responses in the intestine, lymphocyte-mediated immunity alone will not explain the barrier nature of mucosal membranes associated with rejection of many enteric pathogens upon secondary homologous challenge. Our laboratories have focused on nontraditional elements of mucosal immunity in poultry to better understand host-pathogen interactions in the intestine. Following classical and novel immunization procedures, we have identified an antigen-specific mechanism of immediate responsiveness of the mucosal epithelium characterized by epithelial chloride secretion. This mechanism, characteristic of intestinal anaphylaxis, is mediated by local immune elements. Similar mecha-

nisms in mammals contribute to the barrier nature of mucosal membranes during pathogen challenge. To identify cells participating in these and similar responses, additional studies have described a role for mast cells in acute phase responses in the intestines of chickens experimentally challenged with *Eimeria*. To a more practical end, other experiments in our laboratories have characterized drinking water administration of BSA for elicitation of local and systemic antibody responses. These experiments have shown ad libitum drinking water administration of BSA to be as effective as i.p. administration of BSA; they present a novel approach to immunization of commercial poultry with protein vaccines. These investigations support continued research on host-pathogen interactions within the intestine of commercial poultry to better understand and control enteric pathogens through vaccination or immunomodulation.

(*Key words:* mucosal immunity, intestinal epithelium, mast cell, antibody titer)

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INTRODUCTION

Because ingestion and inhalation represent the most common routes of entry for pathogens into the body of commercially reared poultry, the intestinal and respiratory mucosa represent a critical link between the internal and external environment and define the barrier that pathogens must either colonize or invade for subsequent establishment in the body. Gut-associated lymphoid tissues (GALT) of the intestine are part of the common mucosal immune system of commercial poultry. As with the GALT of mammals, the GALT of commercial poultry are integral sights for induction of local inflammatory responses. As such, they have many cells and tissues of innate and adaptive (acquired) arms of the immune system. Further, given the likely heightened importance

of GALT in birds, considering birds lack classic well-defined lymph nodes, they represent essential sites for innate and adaptive immune system cooperativity where cells and mechanisms of innate immunity act to induce cells and mechanisms of adaptive, lymphocyte-mediated immunity. The objective of this manuscript was not to provide a review or overview of intestinal immunity or characteristics of GALT of commercial poultry on a comprehensive basis. Such reviews exist for commercial poultry (Schat and Myers, 1991; Jeurissen et al., 1994; Muir, 1998; Muir et al., 2000; Yun et al., 2000; Bar-Shira et al., 2003). Instead, we have chosen to focus upon 2 largely overlooked components of mucosal immunity in commercial poultry, the mucosal epithelium as a potential effector tissue of integrated host responses and mast cells as potential effector cells or mediators of such responses.

Due to our interest in investigating host-pathogen interactions in the intestine of commercial poultry and the

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Abbreviation Key: ESS = Eastern Shore strain; GALT = gut-associated lymphoid tissues; HBSS = Hank's balanced salt solution; ΔI_{sc} = change in short-circuit current; SCWL = Single Comb White Leghorn.

limited amount of interest shown to date by avian researchers in investigating functional roles of the mucosal epithelium and mast cells in host-pathogen responses in the intestine of commercial poultry, we have developed the following working hypothesis. We hypothesize that the mucosal epithelium of the intestine of commercial poultry is immunologically responsive to antigenic challenge and participates in integrated host responses to enteric pathogen invasion or colonization. To test this hypothesis, we have taken the following experimental approaches: 1) demonstrate epithelial responsiveness to antigen challenge following immunization or sensitization of chickens by measuring electrophysiological correlates of intestinal anaphylaxis in excised intestinal tissue, 2) immunize or sensitize intestinal tissues for electrophysiological epithelial responsiveness with routes of administration suitable for vaccine administration in commercial settings, 3) compare electrophysiological responses in sensitized intestinal epithelium with systemic and intestinal antigen-specific antibody responses, 4) immunize or sensitize intestinal epithelium with vaccine or antigen derived from an enteric pathogen of commercial poultry, and 5) characterize the involvement of mast cells as putative effector cells involved in observed epithelial responsiveness.

Epithelial Responsiveness to Antigen Challenge: Intestinal Anaphylaxis

Immediate responsiveness to antigenic challenge in mammalian intestine, expressed as gut hypersensitivity or intestinal anaphylaxis, has been described by many investigators (Field et al., 1984; Brunsson, 1987; Harari et al., 1987; Castro and Arntzen, 1993). A detailed description of the functional significance of this model as a mechanism of mucosal immunity has been provided by Castro (1989). In addition to regulation by paracrine, neurocrine, and endocrine factors, fluid and electrolyte transport by intestinal epithelial cells is governed by actions of the local mucosal immune system. To associate these phenomena with enteric disease resistance, it becomes necessary to recall that, following an initial infection, the intestine commonly remains refractory to subsequent re-infection by the same organism. These findings, which are not unique to the intestine of commercial poultry, are not uniformly explained by classical immune mechanisms. Examples of such nontraditional mechanisms of enteric immunity have been reported for many years in association with refractoriness to secondary coccidial challenge in commercial poultry (Rose et al., 1979; Danforth, 1998). Although not described to date in commercial poultry, this observed refractoriness in mammalian intestine is due in part to the ubiquitous expression of intestinal anaphylaxis or gut hypersensitivity (Castro, 1989). Resulting changes in epithelial function are regulated by elements of the local immune system. Thus, basic physiological responses of epithelial cells, including ion transport and subsequent fluid secretion, may be involved in functional immunity or may reflect involvement of other factors previously

unrecognized as mechanisms of immunity (Russell and Castro, 1985).

A model used to illustrate the above scenario is murine trichinosis. Secondary challenge of rat intestine with viable *Trichinella spiralis* larvae results in immediate expulsion of the nematode within minutes of exposure to the intestinal epithelium. This intestinal mechanism of rapid parasite rejection is characterized by a physiological correlate of intestinal anaphylaxis, concurrent transepithelial Cl^- secretion (Harari et al., 1987). Such responses are mediated by mast cell-derived biogenic amines and arachidonic acid metabolites. Based on evidence obtained during these investigations, it was hypothesized that the mucosal epithelium of the small intestine was the effector tissue mediating this local immune response in rat intestine, and functional changes in the epithelium induced by an anaphylactic reaction were responsible for the rejection of the invading pathogen (Russell and Castro, 1985). The overall premise for this experimental model of mucosal immunity is based upon changes in the normal physiologic environment of the small intestine occurring so rapidly that the invading organism fails to establish itself in the epithelial tissue, its normal habitat (Harari et al., 1987).

To test the phylogenetic applicability of this model of mucosal immunity in chicken intestine, during 2 replicate experiments conducted over time, Single Comb White Leghorn (SCWL) chicks were obtained on day of hatch and randomly divided into 2 experimental groups, immunized and non-immunized. Chickens were housed in electrically heated brooder batteries and provided ad libitum water and an unmedicated starter ration that met or exceeded NRC guidelines (National Research Council, 1994) for poultry. To hypersensitize chickens to the readily available protein antigen BSA, 3 successive i.p. injections consisting of 10 μg of precipitated BSA in 10 mg of alum ($\text{AlK}(\text{SO}_4)_2$) were administered to immunized chickens on d 1, 7, and 14 post-hatch. Non-immunized chickens received i.p. injections of 10 mg of alum only at the same time points of each experiment.

All procedures below have previously been described by our laboratory (Caldwell et al., 2001). Briefly, to evaluate antigen-induced changes in epithelial function in chicken small intestine, we used classic Ussing-type chambers (Ussing and Zerahn, 1951). Ion transport in ileal segments was evaluated according to previously published methods for mammalian tissues (Harari et al., 1987). Following cervical dislocation of chickens, segments from the distal ileum were removed and rinsed free of luminal contents with Kreb's ringer solution. Segments were mounted as flat sheets between the 2 halves of a Lucite Ussing chamber with 1.13-cm² apertures. The mucosal and the serosal surfaces of each segment were bathed with 10 mL of Kreb's ringer solution, which was gassed continuously with 5% CO_2 and 95% O_2 and maintained at 37°C. After the tissue was mounted in Ussing chambers, intestinal segments were voltage clamped at zero transepithelial potential, and a continuous record of short-circuit current with respect to time was obtained

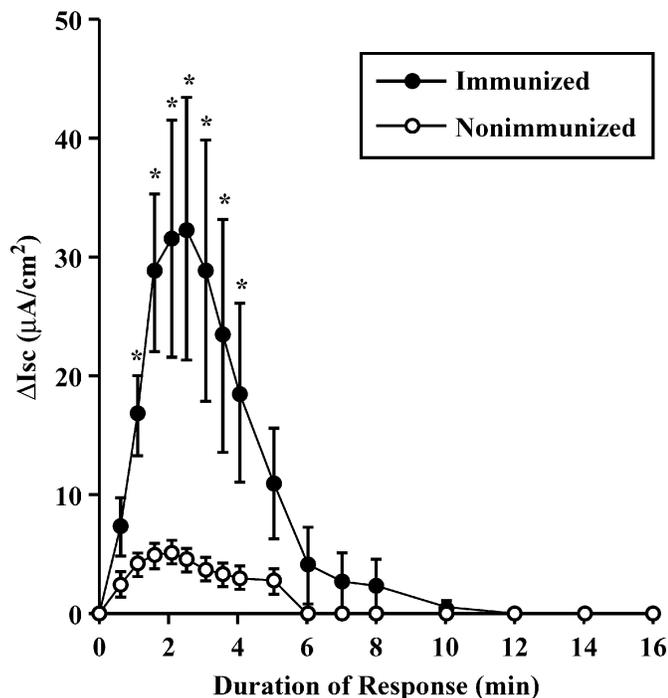


FIGURE 1. Electrophysiological responses evoked by BSA (100 $\mu\text{g}/\text{mL}$) in distal ileal segments from immunized and nonimmunized chickens as a function of time. Each point is the mean change in short-circuit current (ΔIsc) \pm SE for 7 chickens in each of 2 experiments. ΔIsc represents the difference between basal values (time 0) and the value after stimulation with BSA. *Represents statistically different mean ($P < 0.05$) ΔIsc when ileal segments from immunized and nonimmunized chickens were compared at specific time points. Reproduced with permission from Caldwell et al. (2001).

and recorded on a standard flatbed recorder. Changes in short circuit current (ΔIsc) induced by antigen are presented as maximal elevation (expressed as $\mu\text{A}/\text{cm}^2$). Steady-state transmural short-circuit current was established 20 to 30 min following tissue mounting. Tissues mounted in Ussing chambers were exposed to BSA at a final concentration of 100 $\mu\text{g}/\text{mL}$. We use ΔIsc in the present manuscript to describe responsiveness of the intestinal epithelium to *in vitro* antigen challenge as expressed by antigen-induced effects on ion transport.

Results of these experiments demonstrated an immediate *in vitro* responsiveness to antigen challenge in ileal tissue obtained from immunized chickens as compared with that in non-immunized chickens (Caldwell et al., 2001). This immediate responsiveness was expressed within 1 min of BSA challenge in Ussing chambers as an increase ($P < 0.05$) in ΔIsc (Figure 1) in immunized tissues and was minimally expressed in nonimmunized tissues. The observed hypersensitization of distal ileum in immunized chickens was consistent in all respects with the expression of intestinal anaphylaxis as described in mammals (Harari et al., 1987). Further investigation into these phenomena is needed to better understand the involvement of the observed findings in mechanisms of functional immunity in the intestine of commercial poultry.

Immunization of Chickens by Oral Antigen Administration: Measurement of Epithelial Ion Secretion and Systemic or Local Antigen-Specific Antibody Titer

The above investigation described the ability to hypersensitize distal ileal tissue of chicken small intestine against BSA. To extend these findings to a practical level of significance that may have impact on vaccine development or design for commercial poultry, immunization or sensitization by a commercially relevant route of antigen administration is necessary. Several recent published reports suggest that oral administration of protein antigen in solution may be a viable route for immunizing or sensitizing intestinal tissues. Miller and Cook (1994) failed to induce immunological tolerance in chickens when BSA in water, casein in feed, or an SRBC suspension was administered orally in a regimen very similar to one that effectively induced oral tolerance in rats in previous experiments. Antigen-specific antibody titers obtained in this study suggested that oral antigen administration prior to *i.p.* injection of the same antigen is likely to be immunostimulatory and not induce oral tolerance (Miller and Cook, 1994). Subsequent investigations (Klipper et al., 2000; Klipper et al., 2001) were associated with greater immunostimulation following oral BSA administration to chickens. When chickens older than 10 d were fed 25 of mg BSA daily for 6 consecutive d, very robust anti-BSA IgG responses were measured in the serum of immunized chickens (Klipper et al., 2000). Furthermore, although 25 mg BSA fed orally was determined to be consistently immunostimulatory, as little as 2 mg BSA fed daily was fully immunogenic. Additionally, it was determined that antigen fed on consecutive days was more immunogenic than single administrations (Klipper et al., 2000).

Data from this group of investigators in a follow-up investigation extended these initial findings. When BSA was fed orally in a dissolved form to chickens at 14 d, via a feeding needle, encapsulated in gelatin, or in the drinking water, robust anti-BSA titers were measured in serum (Klipper et al., 2001). When BSA was fed in powdered form, either in gelatin capsules or in the feed ration, tolerance was induced. Furthermore, oral administration of dissolved BSA to newly hatched chicks between 1 and 6 d similarly resulted in the induction of tolerance (Klipper et al., 2001). Collectively, these investigations demonstrate stimulation of humoral immunity in chickens when BSA was fed orally, but age of the chicken at time of first immunization and the chemical form of BSA administered were both very important with respect to immunostimulation or the induction of oral tolerance. Nonetheless, oral administration of antigen to immunize commercial poultry represents a potentially viable route for mass vaccination and was of distinct interest to our group for sensitizing mucosal tissues.

To investigate the ability of oral BSA administration to sensitize distal ileal tissues, as measured by ability to evoke electrophysiological responses with antigen in Ussing chambers, we obtained SCWL chicks on day of hatch

and randomly divided them into the following experimental groups: 1) nonimmunized control, 2) oral BSA, 3) i.p. vehicle (alum), and 4) i.p.BSA. Chickens were housed in electrically heated brooder batteries and provided water and an unmedicated starter ration that met or exceeded NRC guidelines (1994) for poultry ad libitum. Nonimmunized control chickens did not receive antigen by any route of administration. Chickens in the oral BSA group received 25 mg of BSA in water via a feeding needle for 6 consecutive d beginning on d 10 posthatch. Chickens in the i.p.BSA group received successive i.p. injections consisting of 10 μ g of precipitated BSA in 10 mg of alum ($\text{AlK}(\text{SO}_4)_2$) on d 10 and 16 posthatch. Chickens in the i.p.vehicle group received i.p. injections of 10 mg of alum only on d 10 and 16 posthatch. Evaluation of hypersensitization of chicken intestine by in vitro challenge of distal ileal segments in Ussing chambers was conducted according to previously published methods (Caldwell et al., 2001), as described above. Data presented are from 3 separate experiments conducted over time.

In a subsequent series of experiments, the ability of oral or i.p. BSA administration to evoke systemic (serum) or local (intestinal) antibody responses was also investigated. In 2 separate experiments, SCWL chicks were obtained on day of hatch and randomly divided into the following experimental groups: 1) nonimmunized control, 2) BSA water, 3) BSA gavage, 4) i.p. vehicle (alum), and 5) i.p.BSA. Chickens were housed in electrically heated brooder batteries and provided water and an unmedicated starter ration that met or exceeded NRC guidelines (1994) for poultry ad libitum. Nonimmunized control chickens did not receive antigen by any route of administration. Chickens in the BSA water group received BSA ad libitum in the drinking water at 1.4 mg/mL between 12 and d 17 posthatch. Based upon water consumption values for SCWL chickens of this age, it was calculated that providing chickens ad libitum access to drinking water containing 1.4 mg of BSA/mL would result in consumption of approximately 25 mg of protein over a 24-h period. Experimental animals in the BSA gavage group received 25 mg of BSA in water via a feeding needle for 6 consecutive d beginning on d 12 posthatch. Chickens in the i.p.BSA group received 2 separate i.p. injections consisting of 10 μ g of precipitated BSA in 10 mg of alum ($\text{AlK}(\text{SO}_4)_2$) on d 12 and 18 posthatch. Chickens in the i.p.-vehicle group received i.p. injections of 10 mg alum only on 12 d and 18 d post-hatch. On d 25 of each experiment, blood samples were obtained from all experimental animals by jugular venipuncture.

In experiment 2 only, intestinal specific IgG titer to BSA was determined according to a previously published *in vivo* tissue culture procedure (Zigterman et al., 1993; Girard et al., 1997) with slight modification. Briefly, after cervical dislocation was performed on experimental birds, intestinal tissue comprising the distal ileum, cecal

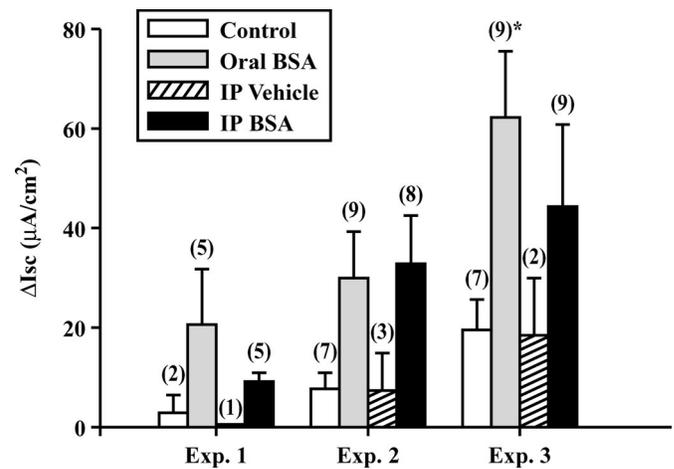


FIGURE 2. Electrophysiological responses evoked by BSA (200 μ g/mL) in distal ileal segments from immunized and nonimmunized chickens as a function of maximal responsiveness. Each bar represents the mean change in short-circuit current (Δ Isc) \pm SE for each experimental group within 3 independent experiments. Δ Isc represents the difference between basal values and values obtained following stimulation with BSA. *Represents a statistically different mean Δ Isc when the oral BSA group was compared with the nonimmunized group in experiment 3. Statistical analysis was performed by comparing Δ Isc obtained from oral BSA chickens with Δ Isc from nonimmunized chickens and Δ Isc from i.p. BSA chickens with Δ Isc from i.p. vehicle chickens by one-way ANOVA. The number of birds (n) evaluated per individual group within each experiment is identified above each bar. Exp. = experiment.

tonsil, and the distal cecum were removed and immersed in Hank's balanced salt solution (HBSS) containing 500 IU/mL penicillin and 500 μ g/mL streptomycin. Gut contents were removed by flushing with HBSS. The tissues were then cut into segments weighing 0.3 g each. Each intestinal tissue segment was cut into 3 smaller pieces and further washed with HBSS penicillin-streptomycin. Segments were then suspended in 6 mL of RPMI 1640² supplemented with 100 IU/mL penicillin, 100 μ g/mL streptomycin, 100 μ g/mL gentamicin, and 40 mM HEPES buffer. The suspensions were centrifuged (5 min, 300 \times g), and 800- μ L samples of supernatant (specific for tissue type and respective to experimental group) were removed and labeled as time zero ($t = 0$) samples. All tissues were resuspended in fresh RPMI medium and incubated at 41°C in an environment of saturated humidity and 5% CO₂ in 12-well culture plates for 16 h. Following 16-h tissue culture, aliquots were removed as described above and marked as time 16 ($t = 16$) samples. IgG titers for serum and intestinal tissue culture samples were determined by indirect ELISA. Titer for intestinal specific IgG was determined by subtracting the $t = 0$ absorbance values from $t = 16$ values.

During evaluation of the ability of the described routes of BSA immunization to induce intestinal anaphylaxis, although suggestive in experiments 1 and 2, only experiment 3 showed a difference in Δ Isc evoked by antigen in distal ileal tissue obtained from oral-BSA-immunized birds as compared with nonimmunized control ileal tissues; the oral-BSA tissues responded in greater fashion ($P < 0.05$) than did non-immunized control tissues (Figure 2). Similar results were obtained when comparing distal

²Sigma Chemical Co., St. Louis, MO.

TABLE 1. Serum and intestinal antigen-specific IgG titer in chickens immunized with BSA by i.p. or oral routes of administration¹

Experimental group ²	Experiment 1				Experiment 2			
	Serum	Ileum	Cecal tonsils	Distal cecum	Serum	Ileum	Cecal tonsils	Distal cecum
Control (n = 4,5)	0.07 ± 0.10 ^B	ND ³	ND	ND	0.16 ± 0.08 ^B	0.02 ± 0.01 ^b	0.03 ± 0.01 ^b	0.06 ± 0.03 ^b
BSA water (n = 10,5)	0.47 ± 0.07 ^A	ND	ND	ND	0.55 ± 0.07 ^A	0.13 ± 0.03 ^a	0.12 ± 0.03 ^a	0.28 ± 0.13 ^a
BSA gavage (n = 9,5)	0.15 ± 0.07 ^B	ND	ND	ND	0.20 ± 0.07 ^B	0.03 ± 0.01 ^b	0.05 ± 0.02 ^b	0.11 ± 0.07 ^b
i.p. vehicle (n = 3,5)	0.02 ± 0.12 ^B	ND	ND	ND	0.14 ± 0.08 ^B	0.01 ± 0.01 ^b	0.01 ± 0.01 ^b	0.04 ± 0.03 ^b
i.p. BSA (n = 9,5)	0.59 ± 0.07 ^A	ND	ND	ND	0.45 ± 0.07 ^A	0.18 ± 0.10 ^a	0.12 ± 0.04 ^a	0.38 ± 0.16 ^a

^{A,B,a,b}Means ± SE with no common superscript differ significantly at the following levels of significance: ^a*P* < 0.05 or ^A*P* < 0.001. Statistical significance among groups per sample type within each experiment was determined by the GLM procedure for ANOVA and statistical differences were further separated by Duncan's multiple range test.

¹Data represent mean ± SE of peak absorbance values determined by indirect ELISA. Absorbance values were measured at 450 nm on an automated multiwell plate reader. Serum samples were diluted 1:640, and media from intestinal culture were diluted 1:10.

²n = number of birds in experiment 1, number of birds in experiment 2.

³ND:= not determined.

ileal tissues from i.p. BSA and i.p. vehicle experimental birds. Although suggestive of a trend of increased responsiveness in i.p. BSA immunized intestinal segments, statistical differences were not observed between these experimental groups (Figure 2). During evaluation of BSA-specific IgG titer in serum from chickens in all experimental groups of 2 consecutive experiments, an elevation (*P* < 0.001) in anti-BSA titer was observed in i.p. BSA and BSA water experimental birds, as compared with all other experimental groups. These groups were not different from one another in either experiment, indicating an equivalent level of effectiveness in promoting a systemic humoral response against BSA when immunization was performed by either route of administration (Table 1). In experiment 2, when intestinal specific BSA IgG titer was determined by ex vivo culture of distal ileum, cecal tonsil, or distal cecum from experimental birds in all experimental groups, a very similar trend was observed. Similar to systemic antibody responses, local intestinal-specific IgG responses were greater (*P* < 0.05) in birds from BSA water and i.p. BSA groups within each intestinal tissue type as compared with all other experimental groups. These findings further supported the effectiveness of either route of immunization for promoting local humoral antibody responses within the intestine of the chicken.

Collectively these data confirm and extend findings by other investigators (Miller and Cook, 1994; 2000; Klipper et al., 2001) and support the effectiveness of oral antigen administration for both local intestinal and systemic immunostimulation of commercial poultry. The fact that these data clearly demonstrate that drinking water administration of protein antigen in chickens is immunostimulatory as opposed to promoting the induction of peripheral tolerance warrants further investigation into the observed phenomenon as a potential method of mass vaccination of poultry through the use of purified or recombinant protein-based vaccines.

Immunization of Chicken Intestine Against Coccidia

Major economic losses in the poultry industry occur due to clinical coccidial infection. Presently these losses

are incurred through anticoccidial drug costs and mortality or morbidity leading to decreases in weight gain and increases in feed-to-gain ratios (Henken et al., 1994; McDougald and Reid, 1997; Danforth, 1998; Yun et al., 2000). Vaccination against coccidiosis through the use of live oocyst vaccines has been viewed as a viable alternative to present anticoccidial therapies and can elicit a protective immune response to parasite infection through use of low-level coccidial exposure to stimulate immunity (Danforth, 1998). Although commercially available live oocyst vaccines will provide solid immunity when applied carefully under good rearing conditions (Danforth, 1998), the problem of incomplete protection due to immunovariability between coccidial species present in the vaccine and those found in poultry rearing facilities has emerged as a potential complicating factor associated with vaccination (Martin et al., 1997). Reductions in intestinal lesions and decreases of loss in body weight gain after parasite infection upon subsequent challenge have been the principal means for measuring live oocyst vaccine immunization efficacy. The difficulties in determining the immune function of chickens toward parasitic infection has preempted the need for other evaluative methods that may provide insight into the immune function elicited by intestinal responses.

Many laboratories have investigated intestinal immune system responsiveness to coccidial challenge, and several comprehensive reviews on coccidial immunity have been published to date (Jeurissen et al., 1996; Lillehoj and Lillehoj, 2000; Yun et al., 2000). Although conclusions differ on the establishment of complete immunity or refractoriness to infection in chickens to coccidial challenge, and although more research into the host-parasite relationship is clearly needed, evidence does exist for dual participation of both humoral (Rose, 1971; Smith et al., 1994a,b; Wallach et al., 1995) and cell-mediated (Vervelde and Jeurissen, 1995; Vervelde et al., 1996; Lillehoj, 1998; Choi et al., 1999; Choi and Lillehoj, 2000) mechanisms of immunity in combating coccidial infection in the intestine of commercial poultry.

Electrophysiological Responses Evoked by E. maxima Antigen Challenge Following Live Oocyst Vaccination. Immunity to secondary coccidial infection in

chickens is accompanied by the rapid onset of intestinal permeability to serum proteins (Rose et al., 1975), an event in mammals characterized by intestinal anaphylaxis. By measuring intestinal mucosal dye leakage in response to sporozoite inoculation directly into the intestinal lumen of chickens immunized against *Eimeria acervulina*, these investigators (Rose et al., 1975) were the first to propose a model of gut hypersensitivity as a mechanism involved in the establishment of immunity to coccidia in the intestine of chickens. Based upon our ability to demonstrate the expression of intestinal anaphylaxis in distal ileal tissue in chickens immunized against BSA (described above; Caldwell et al., 2001), the objective of the present series of experiments was to immunize the intestine of commercial strain broiler chickens against 2 commercially available live oocyst coccidial vaccines (Immucox³ and Coccivac-B⁴) and elicit electrophysiological responses in midintestinal segments from immunized chickens to *Eimeria maxima* antigen challenge in Ussing chambers.

For these experiments, commercial-strain broiler chicks were obtained from a local hatchery on day of hatch. From day of hatch through 30 d of age, all chickens were housed in electrically heated brooder batteries. On d 30 of each experiment, chickens were placed in floor pens with unused pine shavings as litter material, where they were reared until the termination of each experiment. A commercial broiler starter ration that met or exceeded NRC guidelines (1994) for poultry and water were provided to chickens ad libitum.

On day of hatch, chicks were vaccinated with 1 of 2 commercially available coccidial vaccines. Experimental groups consisted of a nonvaccinated control group, an Immucox³-vaccinated group, and a Coccivac-B⁴-vaccinated group. For the initial vaccination, chickens respective to experimental group received a single manufacturer's recommended dose of each vaccine, and nonvaccinated control chickens received a same volume per os gavage of physiologic saline. In an attempt to hypersensitize intestinal tissue against coccidial antigens, on d 10 and 20 posthatch of each experiment, the immunization dosage of each vaccine was increased to 10 \times and 50 \times the label-recommended doses, respectively. Nonvaccinated control chickens similarly received a per os gavage of saline on d 10 and 20 posthatch of each experiment. Tissues were challenged in Ussing chambers, according to methods described above, with antigen preparations created by probe sonication of suspensions of viable oocysts of 2 field strains of *E. maxima* (Eastern Shore strain (ESS) or Guelph strains).

Ion secretion by chicken intestinal epithelium, as an electrophysiological correlate of intestinal anaphylaxis in chickens (Caldwell et al., 2001), in response to in vitro challenge with *E. maxima* antigen was measured between d 30 and 35 of each experiment in classic Ussing-type chambers. In experiment 1, challenge of ileal segments

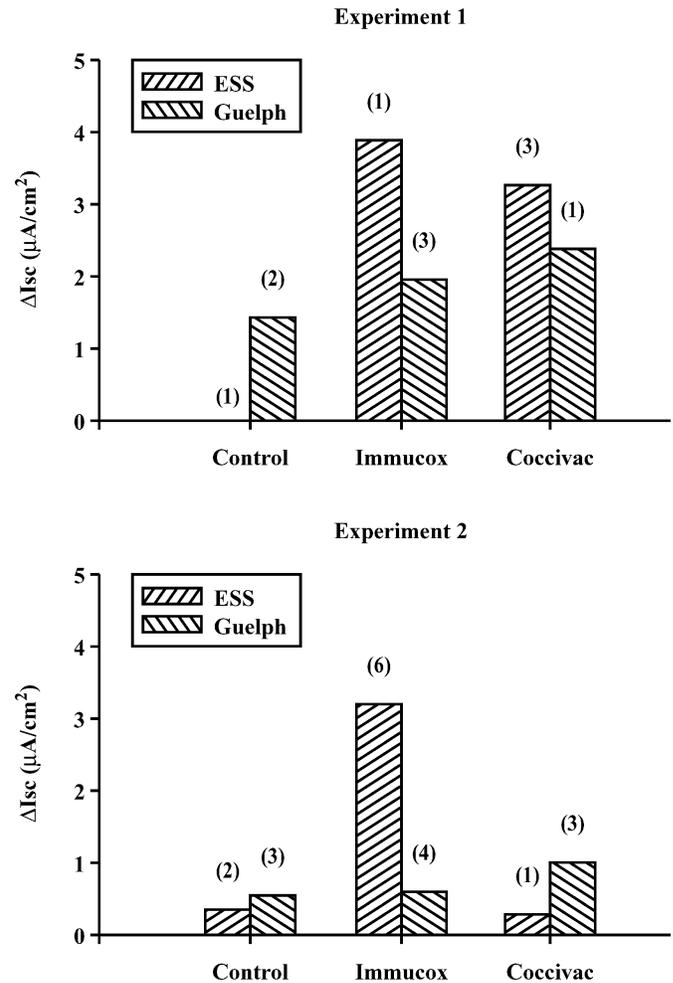


FIGURE 3. Electrophysiological responses evoked by *Eimeria maxima* antigen (100 $\mu g/mL$) in midileal segments from nonimmunized chickens and chickens immunized with Immucox or Coccivac-B as a function of maximal responsiveness. Each bar represents the mean change in short-circuit current (ΔI_{sc}) \pm SE for each experimental group within 2 independent experiments. ΔI_{sc} represents the difference between basal values and values obtained following stimulation with crude antigen preparations isolated from oocysts of the Eastern Shore (ESS) or Guelph field strains of *E. maxima*. Significant differences ($P > 0.05$) were not observed among experimental groups in either experiment. The number of birds (n) evaluated per individual group within each experiment is identified above each bar.

with Guelph or ESS strain *E. maxima* antigen in vitro initially appeared to result in increases in ΔI_{sc} in Immucox-vaccinated and Coccivac-B-vaccinated chickens when compared with nonvaccinated control chickens (Figure 3); however, these data were not different ($P > 0.05$) following statistical analysis. Similarly, in Experiment 2, ΔI_{sc} in response to Guelph or ESS strain *E. maxima* antigen challenge in vitro was apparently increased in intestinal tissue derived from Immucox-vaccinated birds when compared with nonvaccinated control chickens (Figure 3); however, these responses were not statistically distinguishable ($P > 0.05$). In experiment 2, ΔI_{sc} between Coccivac-B-vaccinated chickens and nonvaccinated controls were similar and not statistically different ($P > 0.05$; Figure 3). Because statistical evaluation of data in the present experiments was affected by limited numbers of birds

³Vetech Laboratories, Inc., Buffalo, NY.

⁴American Scientific Laboratories, Inc., Millsboro, DE.

per each experimental group, the present experiments require replication with larger numbers of animals per each experimental group. Nonetheless, they do warrant further investigation into the potential involvement of ion secretion by the intestinal epithelium in response to *Eimeria* antigen challenge as an indicator of coccidial immunity in chickens.

Mast Cell Responsiveness to *E. acervulina* or *E. tenella* Challenge. In light of the classically described role of mast cells for promoting local inflammatory responses, coupled with mast cells being the pivotal cells in mediating reactions of immediate hypersensitivity (Abbas et al., 2000), our laboratories have a fundamental interest in investigating the role of mast cells in host-pathogen interactions in the intestines of commercial poultry. Mast cell-derived paracrine factors mediate inflammatory and immediate hypersensitivity responses by acting directly on epithelial, endothelial, and smooth muscle surfaces, as well as by recruiting granulocytes, monocytes or macrophages, and lymphocytes to locations of antigen encounter (Lin and Befus, 1999). Several characteristics of mast cell function illustrate the importance of these cells as integral mediators of mucosal immune responses. These characteristics include 1) the location of intestinal mucosal mast cells at the interface between the external and internal environments of the animal, 2) the capacity of mast cells to release a myriad of pro-inflammatory and anaphylactic mediators, and 3) the capacity of the cells to undergo multiple cycles of mediator release during prolonged responses in the intestine (Abraham and Arock, 1998). Mast cell-derived mediators can be separated into 3 distinct categories, preformed mediators, newly synthesized mediators, and cytokines (Lin and Befus, 1999).

Mastocytophilia and mast cell hyperactivity have been observed following *Eimeria* sporozoite challenge of intestinal tissue in immunized chickens (Rose et al., 1980). Because mast cells have been identified in mammalian parasitic infection as mediators of intestinal anaphylaxis and parasite immunity (Harari et al., 1987), these findings support the previous characterization of gut hypersensitivity in secondary coccidial infection in immunized chicken intestine by this group of investigators (Rose et al., 1975). Of all avian species studied to date, mast cells have been identified in greatest numbers in chickens and ducks where they are most prevalent in the lamina propria of the small intestine and in the ovary (Swayne and Wisebrode, 1990). Mast cells present in chicken intestine have the same preformed mediators and capacity to synthesize arachadonic acid metabolites that have been described in mammalian mast cells (Rose et al., 1980). Furthermore, a homocytotropic antibody analogous to mammalian IgE, and likely avian IgG(Y), required for classical reactions of type I hypersensitivity, has been identified in chickens following hyperimmunization with horse serum (Burns and Maxwell, 1981).

To investigate mast cell responsiveness in host-pathogen interactions in chicken intestine, several experiments involving *Eimeria* and *Salmonella* challenge were con-

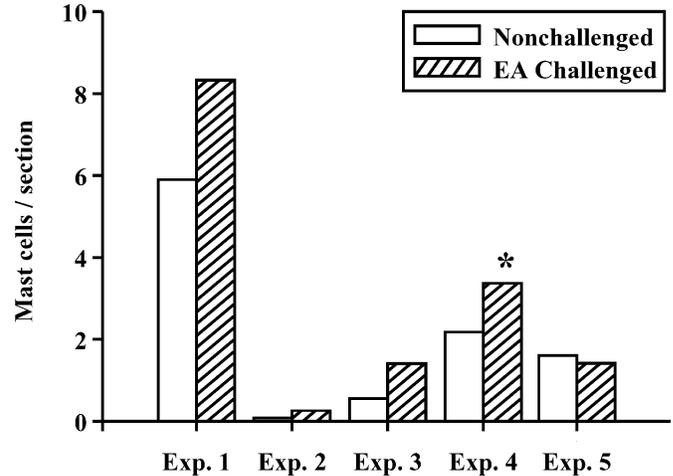


FIGURE 4. Identification of intestinal mucosal mast cells responding to *Eimeria acervulina* (EA) challenge in 14-d-old broiler chickens over 5 independent experiments. Mast cells were enumerated 6 d postchallenge within the lamina propria of the villus of descending duodenal tissue using standard light microscopy and an ocular micrometer following standard tissue processing and staining in toluidine blue. Statistical analysis was performed by comparing nonchallenged and EA-challenged mast cell counts within each experiment by one-way ANOVA. *Represents a statistically higher mean count in the EA-challenged group when compared with the nonchallenged group in experiment (Exp.) 4 only.

ducted. In a series of 5 independent experiments conducted over time, broiler chicks were obtained on day of hatch from a local commercial hatchery, randomly assigned to challenged or nonchallenged experimental groups, and placed in floor pens with supplemental heating, a ration that met or exceeded NRC guidelines (1994) for poultry, and water ad libitum. On d 14 posthatch, chickens, respective to the experimental group, were challenged with *E. acervulina*, and descending samples of duodenum were taken for mast cell identification and enumeration at 6 d postchallenge.

In a separate experiment, broiler chicks were similarly obtained on day of hatch from a local supplier, randomized into experimental groups, and placed in floor pens for rearing as described above. On day of placement, chicks were challenged with 1×10^6 cfu of *Salmonella enteritidis* or 1×10^3 sporulated oocysts of *Eimeria tenella*, and distal ileal and cecal tissue were taken 6 d postchallenge for mast cell identification and enumeration. In all experiments, each intestinal tissue section was fixed in neutral buffered formalin (10%) and cut into 5 smaller sections for standard histologic processing. Tissues were stained in toluidine blue for specific mast cell staining. Mast cell counts were made in the lamina propria region of a villus using an intraocular 5×5 eyepiece grid (total area $156.25 \mu\text{m}^2$) with the $40\times$ objective of a bright-field microscope. A total of 3 complete tissues per slide and 15 grids per tissue were counted to enumerate mast cells in intestinal tissue derived from each experimental chicken.

Mast cell responsiveness evoked by *E. acervulina* challenge of 14-d-old broilers over 5 independent experiments is presented in Figure 4. Differences in mast cell counts

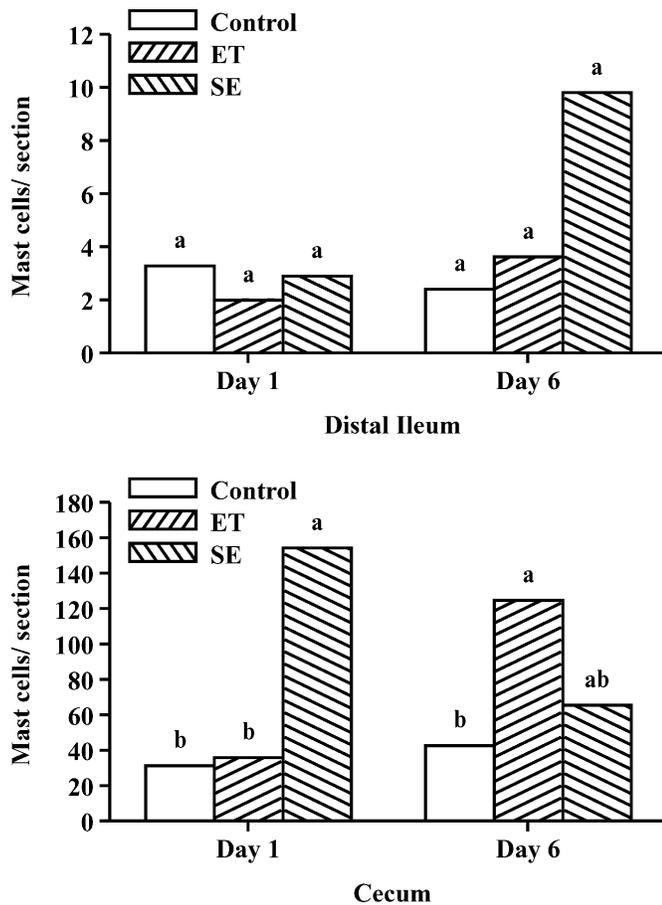


FIGURE 5. Identification of intestinal mucosal mast cells responding to day of hatch *Eimeria tenella* or *Salmonella enteritidis* challenge in broiler chickens. Mast cells were enumerated on d 1 or 6 postchallenge within the lamina propria of the villus of distal ileal or cecal tissue using standard light microscopy and an ocular micrometer following standard tissue processing and staining in toluidine blue. Statistical analysis was performed by comparing nonchallenged and challenged mast cell counts within each experiment using the GLM procedure for ANOVA. ^{a,b}Mean mast cell counts \pm SE with no common superscript differ significantly within day for each respective tissue type at $P < 0.05$.

in duodenal tissue isolated from challenged or nonchallenged chickens occurred only in experiment 4. In a subsequent experiment when day of hatch broilers were challenged with *E. tenella* or *S. enteritidis*, differences ($P < 0.05$) in mast cell counts were observed in cecal tissue, but not distal ileal tissue, when challenged and nonchallenged experimental groups were compared (Figure 5). Specifically, 1-d-postchallenge mast cell counts in cecal tissue were greater ($P < 0.05$) in cecal tissue of *S. enteritidis*-challenged experimental birds when compared with other experimental groups. These data are likely reflective of the rapid degree of invasiveness associated with experimental challenge of neonatal chickens with this particular *S. enteritidis* isolate, which typically occurs within 24 h of challenge (data not shown). Interestingly, mast cell counts were greater ($P < 0.05$) in the cecum of *E. tenella* challenged chickens on d 6 postchallenge as compared with nonchallenged controls but not *S. enteritidis* challenged chickens. Temporally, d 6 postchallenge in this case would correspond to the state of infection when

stages of *Eimeria* development and replication would occur at maximum levels of *E. tenella* in challenged chickens.

CONCLUSIONS

The basis for the present body of research is that an improved knowledge of the cellular and humoral responses occurring within the intestine of commercial poultry is fundamental to our understanding the mechanisms of immunity that afford protection to the bird upon subsequent re-exposure to homologous pathogens. To date, aside from a limited number of reported investigations and the data as discussed and presented in this manuscript, the involvement of the intestinal epithelium and cells with tremendous potential for modulating normal physiological process of cells within the epithelium (i.e., intestinal mucosal mast cells) have received scant attention for investigation by avian researchers. This fact is somewhat perplexing when one considers the obligate involvement of the intestinal epithelium in the events associated with successful establishment or immune-mediated rejection of enteric pathogens in commercial poultry.

The present investigation has revealed several previously unreported findings related to antigen recognition and functional regulation of chicken GALT. First, the small intestine of the chicken may be hypersensitized to antigen by i.p. or oral routes of administration. Second, subsequent exposure of the intestinal epithelium to the immunizing antigen results in immediate change in the function of the mucosal epithelium of chicken small intestine. Collectively, these data suggest epithelial ion secretion is a potential mechanism of immunity associated with integrated host responses in the intestinal immune system of commercial poultry (Caldwell et al., 2001). The ability of an orally administered protein antigen to elicit mucosal and systemic humoral responses warrants further investigation into the potential for this application as a means of mass vaccination of commercial poultry flocks with purified or recombinant protein vaccines. Additionally, while not completely conclusive and in need of further investigation, these data support mast cell responsiveness and involvement in host-pathogen interactions in the intestinal immune system of chickens challenged with enteric pathogens, specifically organisms within the genera *Eimeria* and *Salmonella*.

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