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Heterophils are associated with resistance to systemic *Salmonella enteritidis* infections in genetically distinct chicken lines

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Abstract

Heterophils mediate acute protection against *Salmonella* in young poultry. We evaluated susceptibility of genetically distinct lines of broilers to systemic *Salmonella enteritidis* (SE) infections. SE was administered into the abdomen of day-old chickens (parental lines [A and B]; F1 reciprocal crosses [C and D]) to assess modulation of leukocytes and survivability of chickens. Line A was more resistant to SE than line B; likewise cross D was more resistant than cross C. Significantly more heterophils migrated to the abdominal cavity post-infection in the resistant lines. These data indicate that increased heterophil influx to the infection site contributes to increased resistance against systemic SE infections in neonatal chickens.

Keywords: Chicken; Heterophil; Innate immunity; Intra-abdominal; Resistance; *Salmonella*

1. Introduction

Salmonella-related infections originating from poultry and/or poultry products are one of the major causes of human food-borne disease. There are approximately 40,000 cases of salmonellosis reported in the US each year; however, many milder cases are not diagnosed or reported, so the actual number of infections may be 30 or more times greater (www.cdc.gov/ncidod/dbmd/diseaseinfo/salmonellosis_g.htm). To this end, the poultry industry has sought to identify *Salmonella*-resistant chickens and turkeys.

There is pressure on breeders to produce poultry that grow rapidly and have efficient feed conversions. However, selecting poultry based on growth characteristics can adversely affect the ability of the bird to respond to pathogens and leave them more susceptible to infections and disease [1]. Instead of identifying birds resistant to a single pathogen, our laboratory is interested in identifying immune indicators for poultry breeders that would reveal which line(s) has the potential to mount the most effective immune response against multiple microorganisms.

Heterophils, the primary polymorphonuclear (PMN) leukocyte in chickens, are the avian counterpart to mammalian neutrophils [2,3]. Functionally, heterophils modulate the acute innate host response through the rapid phagocytosis of invading microbes and foreign

Abbreviations: cfu, colony forming units; IA, intra-abdominal; PAMP, pathogen associated molecular pattern; PBS, phosphate buffered saline; PMN, polymorphonuclear cells; SE, Salmonella enteritidis

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particles, the production of oxygen intermediates, and the release of proteolytic enzymes [4–7]. In chickens, invasion of the intestine by *Salmonella enteritidis* (SE) and other salmonellae is known to initiate an inflammatory response [8] characterized by a large influx of heterophils to the site of infection [9].

We recently showed differences in in vitro heterophil functional efficiency between two distinct parental lines (A > B) of broilers and between the F1 reciprocal crosses (D > C) [7]. We found that the lines with an increased heterophil functional efficiency also had an increased in vivo resistance to oral challenge with SE [10]. However, an oral challenge with SE is generally not associated with disease in chickens, while intra-abdominal (IA) infections result in a systemic infection characterized by disease and mortality [11,12]. Additionally, the chicken abdomen is an immunologically privileged site with few resident inflammatory cells thus facilitating the study of host-defense mechanisms that protect against systemic SE infections [11]. The objectives of this study were: (1) to determine whether there were differences in susceptibility of four genetically distinct lines of broilers (two parent lines [A and B] and the F1 reciprocal crosses (C = A hen × B rooster; D = B hen × A rooster)) to a systemic infection following an IA injection of SE and (2) to evaluate the acute cellular response in the abdominal lavage fluid collected following the SE abdominal challenge between the four lines of broilers.

2. Materials and methods

2.1. Experimental chickens

Broiler chickens and the F1 reciprocal crosses used in this study were obtained from a commercial breeder. To maintain confidentiality, the lines were designated A, B, C, and D where lines A and B are parental lines and C and D are F1 reciprocal crosses of the two parent lines (C = A hen × B rooster; D = B hen × A rooster). Fertilized eggs were set in incubators (G.Q.F. Manufacturing Company, Savannah, GA; Jamesway Incubator Company, Inc., Ontario, Canada; or Petersime Incubator Co., Gettysburg, PA) and maintained at wet and dry bulb temperatures of 32.2 and 37.8 °C, respectively. After 10 days of incubation, the eggs were candled; non-fertile and non-viable eggs were discarded. The viable eggs were returned to the incubator until day 18 when they were transferred to hatchers (Humidaire Incubator Company, New Madison, OH or Petersime Incubator Co.) and maintained under the same temperature and humidity conditions until hatch. At hatch, straight-run chickens (not separated by sex) were placed in their respective floor pens (4 feet × 4 feet) containing wood shavings, provided supplemental heat, water, and a balanced, un-medicated corn and soybean meal based chick starter diet ad libitum. The feed was calculated to contain 23% protein and 3200 kcal of metabolizable energy/kg of diet, and all other nutrient rations met or exceeded the standards established by the National Research Council [13].

2.2. Bacteria

A poultry isolate of *Salmonella enterica* serovar *enteritidis* (SE) (# 97-11771) was obtained from the National Veterinary Services Laboratory (Ames, IA) and approved by the US Department of Agriculture Animal and Plant Health Inspection Service for use in our facilities. SE was selected for resistance to novobiocin and carbenicillin and was maintained in tryptic soy broth (Difco Laboratories, Becton Dickinson Co., Sparks, MD) containing antibiotics (25 μg/ml novobiocin and 100 μg/ml carbenicillin; Sigma Chemical Co., St. Louis, MO). A stock culture of SE was prepared in sterile phosphate buffered saline (PBS) and adjusted to a concentration of 1×10^9 colony forming units per ml (cfu/ml) using a spectrophotometer at a reference wavelength of 625 nm (Spectronic 20D, Milton Roy, Co., Golden, CO). The viable cell concentration of the challenge dose was determined by colony counts on brilliant green agar (Difco Laboratories, Detroit, MI) plates containing carbenicillin and novobiocin.

2.3. Systemic SE infection model

Administration of SE via the IA route has been shown to be an acceptable model for inducing a systemic infection in young chickens [11]. Day-old chickens from each line were randomly placed into either control (20–25 per experiment) or infected (30–35 per experiment) groups and maintained in floor pens housed in separate isolation rooms under the same conditions described in Section 2.1. Chickens were administered either 0.1 ml sterile PBS (controls) or 5×10^4 cfu/ml SE IA (5×10^3 cfu/chick). Any chickens found dead within 4 h of the injection were not included in the study as the death was likely due to internal trauma at the injection site and not a result of an SE infection. The chickens were then monitored for an additional 72 h for SE-induced mortality. Extremely moribund chickens were humanely euthanized and regarded as a fatality. The study was conducted in triplicate with chickens from different hatches and the data were pooled for presentation and statistical analyses.

2.4. Collection of abdominal lavage fluids and inflammatory cells

Administration of SE via the IA route is a good model for evaluating cellular influx into the abdominal cavity of young chickens [11]. Day-old chickens from each line were randomly placed into control or infected groups

and maintained in floor pens housed in separate isolation rooms (10 chickens per group). Chickens were administered either 0.1 ml sterile PBS (controls) or 5×10^4 cfu/ml SE IA (5×10^3 cfu/chick). Four hour post-challenge chickens were euthanized by CO_2 asphyxiation and their abdominal cavities lavaged three times with Ca^{2+}–Mg^{2+}-free Hanks' balanced salt solution containing 0.1 M disodium ethylene diamine tetraacetic acid and 0.25% bovine serum albumin (2 ml per lavage) (Sigma Chemical Co.) [11]. Previous studies determined that peak cellular influx into the abdominal cavity occurred at 4 h post-injection [11]. The abdominal exudates from each chick within a line were pooled (control and infected were maintained separately). The recovered total leukocyte numbers were counted on a hemacytometer. Three separate samples (300 μl) were removed from each abdominal cell suspension and cytospin smears were prepared for differential cell counts (Shandon cytospin3; Shandon Inc., Pittsburgh, PA), stained with Hematology three-step stain (Biochemical Sciences, Inc., Swedesboro, NJ), and examined by light microscopy with an oil immersion objective (100×). At least 100 cells on each slide were examined microscopically and the proportions of macrophages, PMN, and lymphocytes were determined. Because of the low number of eosinophils and basophils in young chickens, all PMNs counted were considered to be heterophils [14]. The number of inflammatory heterophils and macrophages recovered from each chick (10 chickens per group in three separate experiments) was determined.

2.5. Statistical analyses

Statistical analyses (Student's t test) were performed using Microsoft® Excel 2000 version (Microsoft Corporation, 2000) with $p \leqslant 0.002$. All statistical analyses are based on comparisons between the parental pair (A and B) or between the F1 reciprocal crosses (C and D). No analyses were done between the parental lines and the crosses.

3. Results

3.1. 72-h evaluation following abdominal challenge

Chickens from each line were injected IA with SE (5×10^3 cfu/chick) or sterile PBS (controls) and morbidity and mortality was observed for 72 h (Table 1). One chicken (line D) was found dead within 4 h of the SE challenge and was not included in the data. The death was likely due to internal bleeding caused by the injection. None of the chickens administered PBS died. Of the chickens administered SE, fewer ($p \leqslant 0.002$) line A chickens died over 72 h when compared to line B chickens (1% and 33.7%, respectively). Also, fewer ($p \leqslant 0.002$) chickens from line D died compared to line C (1% and 10.8%, respectively). These data clearly demonstrate that lines A and D chickens are more resistant to a systemic SE infection compared to lines B and C chickens, respectively; again, showing a similar differential response between the lines that we have observed in our previous studies.

Table 1
Mortality of chickens challenged with SE via IA route

Line	Treatment	No. of dead/total chickens challenged	% Mortality
A	PBS	0/60	0
A	SE	1/101	1.0*
B	PBS	0/60	0
B	SE	34/101	33.7
C	PBS	0/60	0
C	SE	11/102	10.8
D	PBS	0/59	0
D	SE	1/98	1.0**

* Statistical difference ($p \leqslant 0.002$) between lines A and B SE-infected chickens.
** Statistical difference ($p \leqslant 0.002$) between lines D and C SE-infected chickens.

3.2. Cellular response to SE abdominal challenge

To determine the role of leukocytes in protecting neonatal chickens from a systemic SE infection, the number of heterophils and macrophages that migrated to the abdominal cavity of day-old chickens following an IA injection of PBS or SE (5×10^3 cfu/chick) was determined. An earlier report made by our laboratory found no differences in the number of abdominal macrophages in neonatal chickens following an IA injection of SE [11]. To confirm that the results are similar for neonatal broiler chickens, abdominal macrophages were also counted. There were no differences in the number of abdominal macrophages between the chickens administered PBS or SE in any of the lines (data not shown). In all four lines evaluated, the number of heterophils significantly increased within 4 h of receiving the SE injection compared to basal levels obtained from the PBS controls. Despite an increased influx of heterophils observed in all four lines, the numbers were not equivalent between the lines (Fig. 1). Chickens from line A had greater numbers of heterophils ($p \leqslant 0.001$) migrate to the abdominal cavity following an IA injection with SE compared to line B (9.77 ± 0.9 and $3.24 \pm 0.25 \times 10^5$, respectively) (Fig. 1(A)). Also, line D chickens had more ($p \leqslant 0.001$) heterophils migrate into the abdominal cavity compared to chickens from line C (10.11 ± 1.42 and $2.97 \pm 0.26 \times 10^5$, respectively) (Fig. 1(B)).

These data indicate that an increased number of heterophils at the primary site of infection contributes to increased resistance to a systemic SE infection.

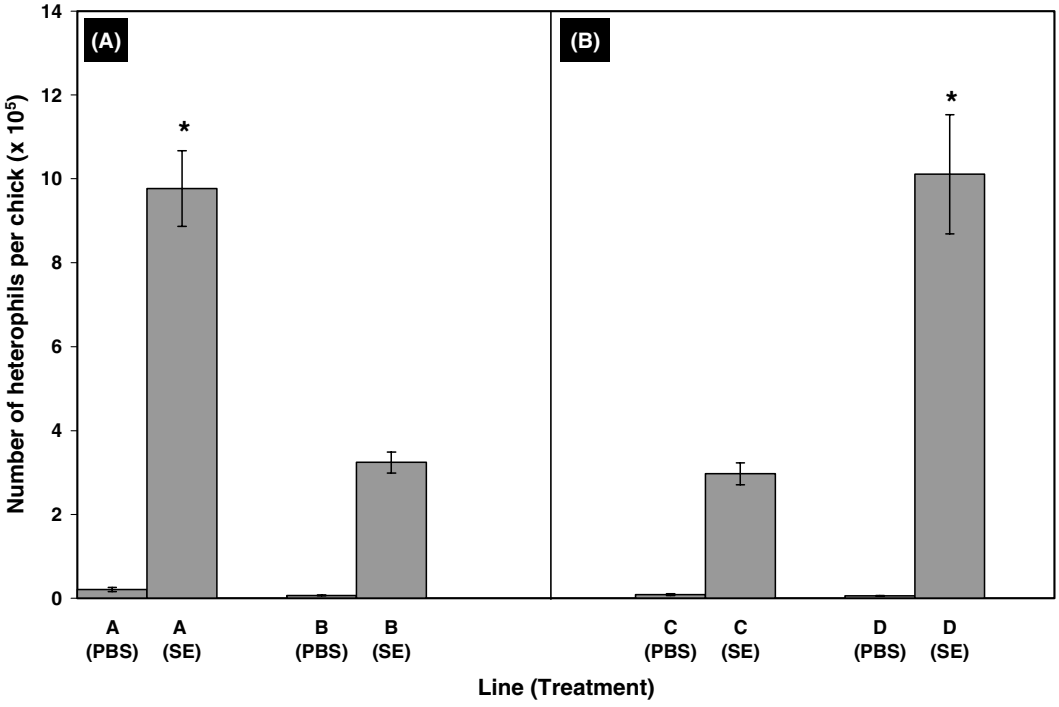

Fig. 1. Heterophil influx into the abdominal cavity of day-old chickens following an IA injection with SE or PBS. Chickens were administered 5×10^3 cfu/chick SE IA or sterile PBS and 4 h later abdominal lavage fluid was collected and the number of heterophils per chick was determined. (A) Comparison of lines A and B heterophil influx to the abdominal cavity. An "*" above the column indicates statistical differences between the SE-infected groups ($p \leqslant 0.002$). (B) Comparison of lines C and D heterophil influx to the abdominal cavity. An "*" above the column indicates statistical differences between the SE-infected groups ($p \leqslant 0.002$). All statistical analyses are based on comparisons between the SE-infected parental pair (A and B) or between the SE-infected F1 reciprocal crosses (C and D). No analyses were done between the parental lines and the crosses. There were no differences in the basal number of heterophils present in any of the control groups. Data presented are the average of three replicate experiments, error bars are the standard error mean. Data were pooled for presentation and statistical analyses.

4. Discussion

The present study compared susceptibility to a systemic SE infection and the acute cellular response in four genetically distinct lines of broiler chickens (parental lines [A and B] and the F1 reciprocal crosses [C = A hen × B rooster; D = B hen × A rooster]). We found significant differences in resistance to a systemic SE infection observed between the two parent lines (A > B) and the F1 reciprocal crosses (D > C) (Table 1). To date, chickens from lines A and D have been consistently immunologically more responsive and more resistant to SE infections than line B and C chickens, respectively [7,10,15]. Heterophils isolated from line A and D chickens have an increased in vitro responsiveness compared to heterophils isolated from line B and C chickens, respectively [7,15]. Additionally, heterophils from chickens more resistant to extraintestinal SE infection (A and D) had increased pro-inflammatory cytokine mRNA expression levels compared to heterophils isolated from susceptible chickens (B and C, respectively) [10]. To our knowledge, none of the lines were selected for increased resistance to *Salmonella* or any other pathogen.

Recent studies indicate that innate immunity provides instruction for the acquired immune response [16–19] which begins with the recognition of host from pathogen by detecting molecules unique to invading organisms referred to as pathogen-associated molecular patterns (PAMPs) [16,20–23]. As the first cells to migrate to the site of infection, PMNs are vital cellular components of the innate immune response [24–27]. Therefore, heterophils are pivotal in initiating the innate immune response [3,5,6]. In this study, evaluation of the acute cellular influx to the abdominal cavity following a systemic SE infection revealed resistant chickens (A and D) had a higher number of heterophils at the site of infection compared to susceptible chickens (B and C). Combined with our previous studies, heterophils appear to be pivotal in the chicken innate immune response to Salmonellae infections.

The recruitment of peripheral blood heterophils to the site of infection is dependent on the local production and release of chemoattractant mediators [11,12]. Our laboratory recently reported, for the first time, that heterophils isolated from neonatal chickens expressed increased levels of mRNA for the pro-inflammatory cytokines IL-1β, IL-6, and IL-8 mRNA upon stimula-

tion with SE [28]. Further, an IL-8-like (chemoattractant) cytokine is involved in the recruitment of avian heterophils to the abdominal cavity of young chickens following an IA injection of SE [12]. Additionally, heterophils isolated from chickens more resistant to an extraintestinal SE infection (A and D) had the highest expression levels of pro-inflammatory cytokine mRNA [10]. This increased production of cytokine mRNA by heterophils may result in a population of heterophils that are primed, thereby more efficient in responding to a pathogen. Since peripheral blood heterophils from line A and D chickens produce a more effective pro-inflammatory cytokine and chemoattractant response, it is likely that abdominal heterophils from the same lines also produce increased levels of immune mediators including cytokines. Further study is necessary to determine if abdominal heterophils produce cytokines or other immune mediators, which may affect the resistance and/or susceptibility to a systemic infection. The importance of cytokine production in combating and limiting the effects of an SE infection has been shown in mice [29]. Based on these data, recruiting additional numbers of primed heterophils to the site of infection would likely enable lines A and D chickens to more efficiently resist extraintestinal infection by SE compared to chickens from lines B and C, respectively.

Barbour et al. [30] showed recruitment and activation of abdominal macrophages after an IA injection of SE in three-week-old broilers, and the highest mortality was observed in the chickens with the greatest macrophage influx. However, in day-old chickens the number of abdominal macrophages does not significantly change when chickens are administered PBS compared to SE [11]. In the current study, the only difference observed was the number of heterophils that migrated to the abdominal cavity, not the number of macrophages. This could be due, in part, because broilers typically have fewer numbers of mononuclear cells compared to other types of chickens [31]. It is also possible that the timing was not optimized for monitoring mononuclear migration into the abdominal cavity, since mononuclear cell migration does not peak until four days post-challenge [32] compared to 4 h post-challenge for heterophils. Henderson et al. [32] also reported that macrophages were less efficient at killing various *Salmonella* spp. compared to heterophils. Collectively, these data in addition to the findings of the present study strongly implicate the significance of the heterophil in conferring resistance to systemic *Salmonella* infections in neonatal chickens and to a lesser extent the mononuclear cells.

Previously, our laboratory showed heterophils isolated from line A chickens (resistant) killed significantly more SE compared to heterophils isolated from line B chickens (susceptible) [15]. An additional study showed that by eliminating circulating heterophils the chickens were more susceptible to SE infections and experienced an increased pathogenic effect(s) of the infection compared to control chickens with a normal population of heterophils [9]. Collectively, these data support and advance the earlier in vitro [7,15] and in vivo [10] findings and further implicate the significance of the avian heterophil in clearance of SE and its role in protecting neonatal poultry from *Salmonella* infections.

To our knowledge, this is the first report to follow parental broiler chickens and the F1 reciprocal crosses through extensive in vitro and in vivo studies. To date, we have shown that an increased in vitro heterophil function corresponds with an increased in vivo resistance to SE infections, and an increase in mRNA expression levels of pro-inflammatory cytokines [7,10,15]. Now, we have shown that all of our previous findings may be used as phenotypic markers to predict resistance and/or susceptibility of neonatal chickens to SE infections.

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