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Heterophil Chemotaxis in Chickens with Natural Staphylococcal Infections

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SUMMARY. Heterophil chemotaxis using heterophils isolated from the peripheral blood of five commercial broiler chickens naturally infected with staphylococcal bacteria was compared by the modified Boyden-chamber technique with chemotaxis of heterophils from two chickens from the same flock not infected with Staphylococcus (field controls) and from four healthy laboratory control broiler chickens. The infected chickens had gross and histologic lesions of staphylococcal tenosynovitis and osteomyelitis. Staphylococci were isolated from the lesions. Hematologic parameters and histologic lesions of infected chickens also were examined. Compared with field and laboratory controls, Staphylococcus-infected chickens had heterophilic leukocytosis. The heterophils of Staphylococcus-infected chickens had significantly lower chemotactic activity than both control groups in terms of random movement and directed chemotactic movement in response to stimulus. Toxic changes were observed in heterophils of some of the Staphylococcus-infected broilers.

RESUMEN. Quimiotaxia de los heterófilos en pollos con infección natural por Staphylococcus.

Se comparó la quimiotaxia de los heterófilos aislados de la sangre periférica de cinco pollos de engorde infectados naturalmente con Staphylococcus. La quimiotaxia se comparó mediante la técnica modificada de la cámara de Boyden, con la quimiotaxia de heterófilos de dos pollos del mismo lote no infectados con Staphylococcus (controles de campo) y con cuatro pollos de engorde sanos mantenidos en el laboratorio. Los pollos infectados mostraron lesiones macroscópicas e histológicas de tenosinovitis y osteomielitis por Staphylococcus. El Staphylococcus se aisló de las lesiones. Se examinaron los parámetros hematológicos y las lesiones histológicas de los pollos infectados. Comparados con los controles de campo y del laboratorio, los pollos infectados con Staphylococcus tenían leucocitosis heterófila. Los heterófilos de este grupo de pollos tenían una actividad quimiotáctica significativamente menor que ambos grupos controles, en lo relacionado con el movimiento al azar y el movimiento quimiotáctico dirigido como respuesta a un estímulo. Se observaron cambios tóxicos en los heterófilos de algunos de los pollos infectados con Staphylococcus.

Staphylococcal infections constitute a common and economically important disease of broiler breeders, broilers, and turkeys. Losses due to staphylococcosis result from decreased weight gain, decreased egg production, and condemnations. Staphylococcus aureus is normally present on the skin and mucous membranes of poultry and in the lower gastrointestinal tract (14). Proposed routes of infection include the gastrointestinal tract, respiratory tract, wounds (trauma, insect bites), and contaminated vaccination needles. Experimentally, both staphylococcal tenosynovitis and osteomyelitis may be produced in chickens and turkeys. The lesions of osteomyelitis are usually produced in conjunction with tenosinovitis at higher intravenous doses than are needed to produce only tenosinovitis (4,6,12). In natural cases of staphylococcal tenosinovitis, it is proposed that resident staphylococci invade the vascular system, resist phagocytosis, multiply rapidly, and produce toxins (11), but route of organism entry, pathogenesis, and host response are ill-defined. Because clinical disease
occurs despite increased heterophil counts and marked heterophil infiltration of tendons and synovial membranes, the role of the heterophil in the inflammatory process, bacteremia, and formation of lesions deserves better definition.

The heterophil is the avian functional analog of the mammalian neutrophil and is thus a major component of the avian defense against bacterial infections (16). Avian heterophils are phagocytic cells that utilize cationic proteins, lysozyme, and acid hydrolases contained in the heterophil granules for microbial killing (5). However, before heterophils can be active in fighting bacterial infections, they must reach the site of infection. Heterophils leave the circulation and approach a site of inflammation in the process of directed movement known as chemotaxis, in response to chemoattractant substances present in nanomolar concentrations. With human neutrophils, S. aureus has been reported to directly induce chemotaxis via structural components or metabolic products that act as chemotactic agents, to indirectly induce chemotaxis by causing the generation of chemotactic factors in serum, and also to produce factors that inhibit chemotaxis (1). Most chemoattractant activity of Staphylococcus spp. for neutrophils is reported to be serum-mediated via complement activation (17), although certain bacterial fractions and bacterial culture filtrates also possess neutrophil chemotactic activity (13,17). Heterophil chemotaxis has been little studied compared with the amount of work done with neutrophils, but one report (9) showed that, when exposed to pooled turkey serum in vitro, Pasteurella multocida was capable of generating factors chemotactic for turkey heterophils. A second report (4) demonstrated higher in vitro heterophil chemotaxis in chickens experimentally inoculated with S. aureus than in uninfected control chickens. Both studies reported using the modified Boyden chamber for heterophil chemotactic evaluation. Under-agarose techniques also have been used to evaluate heterophil migration (2,15).

A previous study compared heterophil function in healthy chickens with that in chickens with experimentally produced staphylococcal tenosynovitis (4). The purpose of the present study was to investigate heterophil chemotaxis in chickens with naturally occurring staphylococcal tenosynovitis or osteomyelitis.

**MATERIALS AND METHODS**

**Chickens.** Eight 46-day-old Arbor Acres × Petersen broiler chickens suspected of having naturally occurring staphylococcal infections were selected by the serviceman from a commercial broiler flock and donated for the present study. It was estimated that 2% of the chickens in the flock were lame, were reluctant to stand, did not eat and drink regularly, and died. Chickens in this flock had previously been diagnosed at our laboratory with purulent arthritis of hock and stifle joints and osteomyelitis from which S. aureus had been isolated. The eight chickens appeared depressed and dehydrated. Each bird was subcutaneously injected with 50 ml of 0.9% NaCl solution followed by 40 ml 5% dextrose in water. Birds were wing-banded and given easy access to feed and fresh water for 1 to 3 days until blood samples were collected for heterophil isolation. After blood collection, they were euthanized and necropsied.

One-day-old Arbor Acres × Petersen broiler chicks were obtained from a commercial source to serve as laboratory controls. The chicks were raised in floor pens with wood-shavings litter, given fresh water and standard broiler starter and finishing feeds (Oregon State University) ad libitum, and maintained under 24-hour lighting. The chickens were used as donors of blood for heterophil isolation between 6 and 8 weeks of age. At 8 weeks of age, the chickens were euthanized and necropsied.

**Blood samples.** Blood samples of 3.2 ml were collected from the ulnar vein of chickens into sterile plastic syringes containing 0.3 ml of 10% disodium ethylenediaminetetraacetic acid (EDTA). Approximately 3.0 ml of the blood sample were used for isolation of heterophils. The remaining 0.2 ml of blood was used for determination of blood parameters. Thin-film blood smears were stained with Wright-Giemsa stain and examined under a light microscope. Estimates and hemocytometer counts of total leukocyte numbers and differential counts of leukocytes were made by one clinical pathologist. The packed cell volume (PCV) was determined using microhematocrit capillary tubes. After determination of PCV, plasma was transferred from the microhematocrit tube to a hand-held refractometer (American Optical, Keene, N.H.) for determination of total plasma protein levels.

**Necropsy.** Broilers from the commercial flock were necropsied to identify Staphylococcus-infected individuals and distinguish them from uninfected flockmates (field controls). Swabs taken at necropsy from joints, tendon sheaths, or bone were streaked onto tryptose-blood agar plates (5% sheep blood) and incubated overnight at 37°C. Organisms were identified using standard techniques by diagnostic laboratory technicians trained in identification of microbial organisms.
Sections of tendon and bursa of Fabricius were fixed in 10% neutral buffered formalin solution, routinely processed, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. Sections of bone were decalcified by the formic acid method and processed as described above. Tissue sections were examined by light microscopy.

**Heterophil isolation.** Heterophils were isolated from blood samples using a described technique (4). Duplicate 1.5-ml samples were used from each chicken. Briefly, 1.5 ml of EDTA-anticoagulated blood mixed 1:5:1 with 1% methylcellulose (25 centipoises) was centrifuged at 25 × g for 5 minutes. The plasma and extended buffy coat layers were removed, diluted in 0.5 ml Hanks’ balanced salt solution (HBSS) without calcium and magnesium, and layered onto a discontinuous Ficoll-Hypaque gradient (specific gravity 1.077, 3 ml; over 1.119, 3 ml). Following centrifugation at 250 × g for 20 minutes, the plasma and mononuclear cells (at the plasma–1.077 interface) were discarded. The remaining gradient (partial 1.077 and 1.119) containing heterophils was collected. The cells were washed twice with HBSS (250 × g, 10 minutes for the first centrifugation followed by 7 minutes for the second) and resuspended in sufficient minimum essential medium (MEM) (M 0268, Sigma Chemical Co., St. Louis, Missouri) to achieve a concentration of 1.5 × 10^6 heterophils/ml.

**Chemoattractant preparation.** Pooled normal chicken serum was obtained from 10-week-old broiler chickens with no known exposure to staphylococcal organisms and stored at −70°C until use. Chemoattractant solutions were prepared using described techniques (4). Briefly, the endotoxin chemoattractant consisted of 3000 μl HBSS; 150 μl filtered, purified 0111:B4 *Escherichia coli* endotoxin solution (300 μg/ml) (L 4130, Sigma); and 250 μl pooled normal chicken serum, which were mixed.

An additional chemoattractant solution, used to evaluate the chemoattractant quality of the pooled chicken serum, was prepared by combining 900 μl HBSS and 100 μl pooled normal chicken serum. A negative control chemoattractant solution consisting of MEM was used to evaluate the normal random movement of heterophils. Each chemoattractant solution was incubated 1 hour at 37°C in a 5% CO₂ atmosphere and inactivated at 56°C for 30 minutes.

**Chemoattractant assay.** Heterophil chemotaxis was performed using a described technique (4). Briefly, the modified Boyden technique was used to quantitate random and total (random plus chemoatactic) heterophil movement using heterophil suspensions (1.5 × 10^6 heterophils/ml), blind wells, and 3-μm average-pore-diameter polycarbonate filters (Nucleopore Corp., Pleasanton, Calif.). Chemoattractant solutions were placed in the lower blind well chamber, and the heterophil suspension was placed in the upper chamber. Duplicate blind wells with each chemoattractant solution were prepared and incubated at 41°C for 45 minutes in a humid 5% CO₂ atmosphere. The filters were removed, air dried, stained with a quick Romanowsky-type stain (Diff-Quik Stain Set; Baxter Health Care Corp., Miami, Fla.), and permanently mounted on glass slides. Random and total heterophil movement were quantified by examining filter membranes microscopically using the oil immersion objective (100×) and a calibrated grid (Fig. 1). The numbers of heterophils that traversed the filter in response to MEM constituted random movement. Heterophils traversing the filter in response to either of the two chemoattractant solutions constituted total cell movement. Values were expressed as the mean number of heterophils/0.25 mm² of membrane surface area.

**Statistical analysis.** Heterophil counts from chemotaxis filters were transformed by taking the square roots. The transformed data were analyzed by two-way analysis of variance using a repeated-measures, completely randomized design with unequal numbers of subjects. This analysis was used to locate significant differences between means of control and infected chicken groups and chemoattractant treatment groups. Peripheral leukocyte estimates were subjected to square root and PCVs to arcsin transformation. Blood data were analyzed by one-way analysis of variance. Significant differences in mean values were located using Fisher’s least significant difference test.

**RESULTS**

**Necropsy, bacteriology, and histopathology.** Positive bacteriological and histopathological evidence of staphylococcal tenosynovitis or osteomyelitis identified five infected chickens from the commercial broiler flock. Lack of such evidence identified the remaining three commercial broilers as uninfected field controls. One uninfected field control was dropped from the study because of technical problems with the chemotaxis assay.

The naturally infected chickens had necropsy lesions, including purulent exudate within the stifle or hock joints and tendon sheaths (2/5), bilateral (4/5) or unilateral (1/5) necrotic foci within the proximal tibia consistent with osteomyelitis, and a ruptured gastrocnemius tendon (1/5). Tibial dyschondroplasia with necrosis of cartilage was observed in one chicken with bilateral osteomyelitis and purulent tenosynovitis. Bursae were smaller than expected in 46-day-old broilers, but other organs and tissues appeared unremarkable. Staphylococcal organisms were isolated in low to heavy numbers from joint and bone lesions in all five chickens;
S. aureus organisms were isolated from four of the five, and a coagulase-negative Staphylococcus was isolated from the other.

Histologic lesions in the infected chickens varied in severity. All five chickens with staphylococcal infections had osteomyelitis, and three of the five also had tenosynovitis. Tendon lesions consisted of synovial hyperplasia (3/3), heterophil infiltration of tendons (2/3), and staphylococcal bacterial colonies (2/3). Necrotic cartilage was seen in the growth plate (4/5) and articular cartilage (3/5). Heterophils were associated with areas of cartilage necrosis and marrow necrosis. Staphylococcal emboli were present in vessels of cartilage and bone marrow in all five Staphylococcus-infected chickens. Chickens were ranked according to severity of the histologic lesions before results were known from bacterial cultures, heterophil chemotaxis, and peripheral hemograms. The two chickens ranked as having the most severe histologic lesions also had the heaviest growth of S. aureus when cultured. Cultures from the three other chickens yielding moderate growth of S. aureus, low growth of coagulase-negative Staphylococcus spp., and low growth of S. aureus corresponded to a decreasing order of histologic severity of lesions.

Field and laboratory control chickens lacked gross lesions of tenosynovitis or osteomyelitis. No staphylococci were isolated from joints of field controls, and no histological evidence of infection was seen in their bones, tendons, or synovial membranes. Synovial fluid from the hock and stifle joints of laboratory controls was examined using air-dried smears stained with Wright-Giemsa stain. No evidence of inflammation was seen cytologically. No histopathological examination of tissues or bacteriological culturing was performed on laboratory controls.

**Blood parameters.** Table 1 shows mean values of peripheral leukocyte estimates. Total leukocytes and heterophils were significantly greater in the infected birds than in either control group ($P < 0.05$). Heterophils were the predominant type of leukocyte in peripheral blood of Staphylococcus-infected chickens.

Toxic changes in heterophils consisting of increased cytoplasmic basophilia, granule rounding, and vacuolization were seen in three of the five birds with staphylococcal infection. One-way analysis of variance of monocyte estimates was barely significant ($P = 0.049$), but pairs of group means did not differ significantly. Estimates of lymphocytes, eosinophils, and basophils did not differ significantly. Packed-cell volume and total plasma protein levels were not significantly different.

**Chemotaxis.** Table 2 shows mean chemotactic counts of all treatments of infected chickens ($n = 5$) compared with field controls ($n = 2$) and laboratory controls ($n = 4$). No interaction was present between treatment groups. Significant differences ($P < 0.001$) were present between all chemotactic agent treatment groups. Over all treatment groups, the mean chemotactic count for infected chickens was 100.20 heterophils/0.25 mm² of filter area, which was less than 57% of the value for field controls and only half of the mean value for laboratory controls (Table 2). Heterophil response in the Staphylococcus-infected chickens was significantly ($P < 0.05$) lower for MEM, serum, and endotoxin than the heterophil response of both field and laboratory control chickens, but heterophil response did not differ significantly between the field and laboratory control groups for any chemotactic agent.
Table 1. Mean values* of peripheral leukocyte estimates, packed cell volume (PCV), and total plasma protein levels for uninfected laboratory and field control chickens and for *Staphylococcus*infected chickens.

<table>
<thead>
<tr>
<th>Peripheral blood parameter</th>
<th>Laboratory controls</th>
<th>Field controls</th>
<th>Infected chickens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total leukocytes/μl</td>
<td>11,015^a</td>
<td>8094^a</td>
<td>29,947^b</td>
</tr>
<tr>
<td>Heterophils/μl</td>
<td>3396^a</td>
<td>4019^a</td>
<td>17,336^b</td>
</tr>
<tr>
<td>Lymphocytes/μl</td>
<td>5994</td>
<td>3228</td>
<td>5104</td>
</tr>
<tr>
<td>Monocytes/μl</td>
<td>589</td>
<td>470</td>
<td>5005</td>
</tr>
<tr>
<td>Eosinophils/μl</td>
<td>169</td>
<td>15</td>
<td>29</td>
</tr>
<tr>
<td>Basophils/μl</td>
<td>403</td>
<td>225</td>
<td>346</td>
</tr>
<tr>
<td>PCV (%)</td>
<td>25.5</td>
<td>32.7</td>
<td>25.2</td>
</tr>
<tr>
<td>Plasma protein (g/dl)</td>
<td>4.4</td>
<td>4.8</td>
<td>5.3</td>
</tr>
</tbody>
</table>

*Within a row, values followed by different lowercase superscripts are significantly different (P < 0.05).

**DISCUSSION**

The results of the present study involving chickens infected naturally with staphylococcal organisms were similar in some ways to the results of a previous study (4) involving *Staphylococcus*infected chickens. *Staphylococcus*infected chickens in both studies had significantly higher peripheral heterophil counts than did controls. The lesions of tenosynovitis and osteomyelitis were purulent in both cases, indicating egress of heterophils from the bloodstream and migration to the site of infection. There also were important differences between naturally infected chickens and the experimentally *Staphylococcus*infected chickens in the present study. The heterophils of the naturally infected chickens had lower chemotactic activity than the uninfected field and laboratory controls. This reduction in activity was evident in each treatment group, indicating that the heterophils of the naturally infected chickens were less active in terms of random movement and in total directed chemotactic movement in response to a stimulus. This finding contrasts with the previous observation (4) that heterophils of experimental *Staphylococcus*infected chickens were significantly more active than those of uninfected controls, both in random movement and in directed chemotactic movement. The naturally infected chickens tended to have more severe bone lesions than the experimentally infected chickens. Although the duration of infection of the naturally infected chickens is not known, it is presumably longer than the 6 to 9 days in the case of the experimentally infected chickens.

Toxic changes were observed in the heterophils of the three naturally infected chickens with the most severe gross and microscopic lesions. Toxic changes in neutrophils or heterophils are usually the result of severe inflammation or bacterial infection. Toxic changes are due to alterations in granule content and persistence of ribosomes that contribute to cytoplasmic basophilia (8). Toxic changes in heterophils have been documented in acute inflammation and found to have similarities to toxic changes in mammalian neutrophils (10).

Although the number of peripheral monocytes in the naturally infected chickens was not

Table 2. Mean values* of chemotactic heterophil counts in response to different chemotactic agents for *Staphylococcus*infected chickens (n = 5), uninfected field control chickens (n = 2), and laboratory control chickens (n = 4).

<table>
<thead>
<tr>
<th>Type of chicken</th>
<th>Chemotactic agent</th>
<th></th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MEM</td>
<td>Serum</td>
<td>Endotoxin</td>
</tr>
<tr>
<td>Staph-infected</td>
<td>7.78^b</td>
<td>128.14^c</td>
<td>253.45^a</td>
</tr>
<tr>
<td>Field control</td>
<td>33.99^b</td>
<td>207.94^b</td>
<td>387.70^b</td>
</tr>
<tr>
<td>Laboratory control</td>
<td>43.96^b</td>
<td>271.26^b</td>
<td>375.97^b</td>
</tr>
<tr>
<td>Average</td>
<td>25.84</td>
<td>197.96</td>
<td>336.11</td>
</tr>
</tbody>
</table>

*Within columns, means followed by the same lower-case superscript are not significantly different (P > 0.05). Within rows, all means are significantly different (P < 0.05).

*Heterophil counts are given as number of heterophils/0.25 mm² of filter area. Two filters were counted per chicken.

*See text for descriptions of chemotactic agents.
significantly greater than the number in uninfected controls, the peripheral monocyte count was increased in naturally infected chickens. Monocytes are often increased because of chronic infection but also may be increased in acute inflammation (10). Lymphocytes were the predominant cell type in the uninfected laboratory control chickens (11), but heterophils slightly outnumbered lymphocytes in the field control chickens.

Both increased and decreased neutrophil chemotactic movement have been associated with bacterial infections. Initial increased leukocyte movement may be cytokine-directed; this phase also is thought to be transient by some researchers (3,7). As the disease progresses, heterophils or neutrophils may exhibit decreased in vitro chemotactic movement due to circulating cytokotins that decrease the chemotactic gradient between blood and the tissues (3). In vitro, human neutrophil locomotion is impaired in staphyloccocal bacterial infections but returns to normal upon patient recovery (3). Natural staphylococcal infections in chickens may have several factors that contribute to decreased heterophil chemotaxis. More work is required to elucidate reasons for the observed differences in heterophil function between experimental Staphylococcus-infected chickens and naturally infected chickens.

REFERENCES


ACKNOWLEDGMENTS

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