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Chicken Heterophil Chemotaxis Using Staphylococcus-Generated Chemoattractants

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SUMMARY. Heterophil chemotaxis, in response to chemotactic factors generated by three different strains of staphylococcal bacteria, was measured using the modified Boyden-chamber technique. Heterophils were obtained from healthy 6-to-8-week-old broiler chickens. Each bacterial strain generated factors that were chemotactic for chicken heterophils. Factors generated by two pathogenic isolates of *Staphylococcus aureus,* however, induced significantly greater chemotaxis in chicken heterophils than those generated by a nonpathogenic *Staphylococcus* isolate.

RESUMEN. Quimiotaxis de los heterófilos de pollos, usando quimioatrayentes generados por Staphylococcus.

Se estudió la quimiotaxis heterofílica en respuesta a los factores quimiotácticos generados por tres diferentes cepas de *Staphylococcus,* usando la técnica de la cámara modificada de Boyden. Los heterófilos fueron obtenidos de pollos de engorde sanos de 6 a 8 semanas de edad. Cada cepa bacterial generó factores que fueron quimiotácticos para los heterófilos de pollo. Los factores generados por dos cepas patógenas de *Staphylococcus aureus* indujeron significantemente mayor quimiotaxis en los heterófilos de pollos que las cepas no patógenas de *Staphylococcus.*

The primary function of the heterophil, the avian analog of the mammalian neutrophil, is phagocytosis and destruction of foreign material, including bacteria (13). Heterophils come into contact with bacteria by moving to the site of bacterial infection in the process of directed movement known as chemotaxis. Chemotaxis is possible because the heterophil can detect minute gradations in the concentration of certain chemoattractants and respond by migrating toward the source of the chemoattractant agent (13). Staphylococcal bacteria are known to produce chemotactic substances that act both directly and indirectly on mammalian neutrophils to induce chemotaxis and also to produce substances that inhibit chemotaxis (1).

Considerable variation in virulence can occur among various strains or isolates of most microorganisms. The virulence factors elaborated by staphylococcal bacteria include a variety of toxins and enzymes. The role of *Staphylococcus aureus* capsules as a virulence factor has been studied recently. There are 11 capsular types known for *S. aureus* (12), but capsular types 5 and 8 make up the majority of isolates from humans (3,4,12), from cases of bovine, ovine, and caprine mastitis (8), and from chickens (Dr. Robert Daum, The University of Chicago Wyler Children's Hospital, personal communication). With *Pasteurella multocida,* a positive correlation has been demonstrated between strain pathogenicity and the magnitude of heterophil chemotaxis induced by the strains (6). The present study was undertaken to determine whether pathogenic isolates of *Staphylococcus aureus,* bearing capsular types 5 and 8, and a nonpathogenic *Staphylococcus* isolate can induce different degrees of heterophil chemotaxis.

MATERIALS AND METHODS

Chickens. One-day-old Arbor Acres × Peterson broiler chicks were obtained from a commercial source. The chicks were raised in floor pens with wood-shavings litter, given free access to fresh water

Table 1. Biochemical characterization and identification of three *Staphylococcus* isolates used to generate chemoattractants.

	Staphylococcus isolate		
Biochemical test	921-1	5658A	6969B
Coagulase	+	+	−
Phosphatase	+	+	−
Urease	−	+	+
β-glucosidase	+	+	+
β-glucuronidase	−	−	+
β-galactosidase	−	−	+
Mannose	+	+	−
Mannitol	+	+	−
Trehalose	+	+	−
Salicin	−	−	−
Arginine	−	+	−
Identification	*S. aureus*	*S. aureus*	*S. xylosus*
Capsular type	8	5	Not done

and standard broiler starter and finishing feeds (Oregon State University), and maintained under 24-hour lighting. Twelve chickens were used as donors of blood for heterophil isolation between 6 and 8 weeks of age. Blood samples of 6.0 ml were collected from the ulnar vein of chickens into two sterile plastic syringes, each containing 0.3 ml of 10% disodium ethylenediaminetetraacetic acid (EDTA) and used for heterophil isolation.

Heterophil isolation. Heterophils were isolated from blood samples using a described technique (2). Briefly, 1.5 ml of EDTA-anticoagulated blood was placed in four siliconized test tubes, mixed 1.5:1 with 1% methylcellulose (25 centipoises), and centrifuged at 25 × *g* for 5 minutes. The plasma and extended buffy-coat layers, removed and diluted in 0.5 ml Hanks' balanced salt solution (HBSS) without calcium and magnesium, were layered onto a discontinuous Ficoll-Hypaque gradient (3 ml at specific gravity 1.077; 3 ml at 1.119). 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.

Staphylococcal bacteria. Three staphylococcal bacterial isolates—isolate 921-1, isolate 5658A, and isolate 6969B—were used as chemoattractants. Isolates 921-1 and 5658A were obtained from clinical cases of staphylococcal tenosynovitis from chickens in Georgia (921-1) and Oregon (5658A). They are considered to be virulent isolates, based on the clinical lesions. Isolate 6969B was isolated from the normal, non-purulent hock joint of a chicken and was considered to be a nonpathogenic contaminant. Each isolate was characterized and identified (Table 1) with the aid of a commercial kit (Staph-Ident; Analytab Products, Plainview, N.Y.). The two *S. aureus* isolates (921-1 and 5658A) were further characterized as to capsular type by two independent laboratories using quantitative precipitin analysis (5). Isolate 921-1 has capsule type 8, and isolate 5658A has capsule type 5. Bacteria were stored at −70 C in *Brucella* glycerol broth.

Chemoattractant preparation. Pooled normal chicken serum was obtained from 10-week-old broiler chickens with no known exposure to *Staphylococcus* organisms and stored at −70 C until use. Chemoattractant solutions were prepared using described techniques (2, 6). Six chemoattractant solutions were prepared, including supernatants from three strains of staphylococcal bacteria: 1) MEM; 2) pooled normal chicken serum; 3) endotoxin-activated serum; 4) pathogenic isolate 921-1; 5) pathogenic isolate 5658A; and 6) nonpathogenic isolate 6969B.

The negative control chemoattractant solution consisting of MEM was used to evaluate the normal random movement of heterophils. To evaluate the chemoattractant quality of the pooled normal chicken serum, a solution was prepared by combining 900 μl HBSS and 100 μl pooled normal chicken serum. The endotoxin-activated serum chemoattractant consisted of 1500 μl HBSS, 75 μl filtered, purified O111:B4 *Escherichia coli* endotoxin solution (300 μg/ml) (L 4130; Sigma), and 125 μl pooled normal chicken serum, which were mixed for a final concentration of 60,606 endotoxin U/ml.

Bacterial supernatants were prepared by growing each isolate in brain-heart infusion broth for 18 hours at 37 C and washing twice in phosphate-buffered saline at 900 × *g* for 15 minutes. Suspensions of each *Staphylococcus* isolate were adjusted to an optical density of 0.2 at 595 nm. This reading corresponded to a mean titer (± standard deviation) of 4.31 ± 1.96 × 10^7 colony-forming units (CFU)/ml for isolate 921-1, 5.61 ± 1.34 × 10^7 CFU/ml for isolate 5658A, and 4.59 ± 1.86 × 10^7 CFU/ml for isolate 6969B. The chemoattractant solutions were prepared by mixing 250 μl bacterial suspension, 250 μl pooled chicken serum, and 2000 μl HBSS. All chemoattractant solutions were incubated for 1 hour at 37 C in 5% CO_2, filtered using a 0.2-μm low-protein-binding filter, and heat-inactivated for 30 minutes at 56 C.

Chemotaxis assay. Heterophil chemotaxis was performed using a described technique (2). Briefly, the modified Boyden-chamber technique was used to quantitate random and total (random plus chemotactic) 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 (185 μl) were placed in the lower blind well chamber, and the heterophil suspension (185 μl) 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% CO_2 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. The numbers of heterophils that traversed the filter in response to MEM constituted random movement. Heterophils traversing the filter in response to other specific chemoattractant solutions constituted total cell movement. Values were expressed as the mean number of heterophils per 0.25 mm^2 of membrane surface area.

Statistical analysis. Heterophil counts from chemotaxis filters were transformed by taking the square roots. The transformed data were analyzed by one-way analysis of variance. Significant differences in mean values were located using Fisher's least significant difference test.

RESULTS

One-way analysis of variance demonstrated significant differences between mean heterophil counts induced by the six chemotactic agents ($F = 57.946$, $P < 0.0001$). Table 2 shows mean chemotactic counts of heterophils crossing the polycarbonate filter in response to the chemotactic agents. Random heterophil movement in response to MEM averaged 36.2 cells and was significantly less than heterophil movement in response to the five other groups of specific chemoattractants. There was no significant difference between heterophil chemotactic response to pooled normal chicken serum and to the nonpathogenic *Staphylococcus* isolate 6969B. Both *S. aureus* isolates stimulated significantly greater chemotactic response from the heterophils than did nonpathogenic isolate 6969B. Heterophil chemotaxis induced by *S. aureus* isolate 921-1 was not significantly different from that induced by the *E. coli* endotoxin positive control. *S. aureus* isolate 5658A, with capsule type 5, induced significantly greater heterophil chemotaxis than did *S. aureus* isolate 921-1, which has capsule type 8.

Table 2. Mean counts of normal broiler chicken heterophils crossing chemotaxis membranes in modified Boyden chambers in response to six chemotactic agents.

Chemotactic agent[A]	Mean heterophil count[B]
MEM	36.2[a]
Serum	149.8[b]
Endotoxin	267.9[c]
Isolate 921-1	283.8[c]
Isolate 5658A	360.2[d]
Isolate 6969B	167.2[b]

[A]See text for descriptions of chemotactic agents.

[B]Heterophil counts are given as number of heterophils per 0.25 mm^2 of filter area. Two filters were counted per chicken ($n = 12$ chickens). Means followed by the same superscript are not significantly different ($P > 0.05$).

DISCUSSION

Chemotactic factors were produced by each *Staphylococcus* isolate mixed *in vitro* with pooled normal chicken serum. This finding is in accord with the pathogenesis of staphylococcal infections of chickens *in vivo*, where heterophils infiltrate the infected tissues (11). Both pathogenic *S. aureus* isolates 921-1 and 5658A produced significantly higher chemotactic counts than did the nonpathogenic *Staphylococcus* isolate 6969B. Isolate 6969B failed to produce significantly greater heterophil chemotaxis than pooled normal chicken serum alone. These results resemble those obtained in a study of *P. multocida*-induced chemotaxis of turkey heterophils, in which the magnitude of heterophil chemotaxis was positively correlated with the pathogenicity of the bacterial isolate or strain (6). It can be expected that infections with isolate 921-1 or 5658A would result in a greater heterophil infiltration of tissues in response to infection. Isolates 921-1 and 5658A have not been compared for their *in vivo* pathogenicity in chickens.

There was no attempt to identify the chemoattractant substance that induced heterophil chemotaxis in the present study. *S. aureus* is known to release a tetrapeptide into culture media that is chemotactic for neutrophils (9,14). However, few strains of washed *S. aureus* have any direct chemotactic activity (10). A chloroform-soluble fraction of *S. aureus* is a direct neutrophil chemoattractant, but its role may be

minor in aqueous environments (14). Most of the chemoattractant activity of *S. aureus* appears to be serum-mediated via activation of the classical pathway or, more likely, the alternative complement pathway, which leads to production of C5a (6,10,14).

Given the primary role of the heterophil in fighting bacterial infections and that more pathogenic *Staphylococcus* isolates induce greater heterophil chemotaxis, other factors must enter into the pathogenesis of staphylococcal infections. Staphylococci are known to produce inhibitors of neutrophil chemotaxis, such as alpha-hemolysin, beta-hemolysin (sphingomyelinase-C), and leucocidin (1), which have anti-chemotactic effects at lower concentrations than those required to produce cytotoxicity. It has been suggested that chemoattractants may induce neutrophils to move to a focus of infection but that inhibitors of chemotaxis may act to protect the staphylococci (10). As was suggested with *P. multocida,* disease or mortality may not be due to failure to recruit heterophils to sites of infection (6). Indeed, tissue damage from the infection may be partly due to the effects of histotoxic substances such as acid hydrolases, trimetaphosphatase, and cationic proteins that may be released by heterophil degranulation at the site of infection (6,7). Besides chemotaxis, heterophils must phagocytose and kill the staphylococci to successfully control an infection. Pathogenic *Staphylococcus* strains may have other defenses that more successfully avoid heterophil phagocytosis and killing than do those of less pathogenic isolates.

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