Comparison of the Effect of Different Opsonins on the Phagocytosis of Fluorescein-labeled Staphylococcal Bacteria by Chicken Heterophils

Claire B. Andreasen, *Oregon State University*
James R. Andreasen, Jr., *Oregon State University*
Anita E. Sonn, *Oregon State University*
Julie A. Oughton, *Oregon State University*
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Claire B. Andreasen,^ James R. Andreasen, Jr.,^ Anita E. Sonn,^ and Julie A. Oughton^b

^College of Veterinary Medicine  
^bEnvironmental Health Science Center, Oregon State University, Corvallis, Oregon 97331

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SUMMARY. Heterophil phagocytosis of fluorescein-labeled staphylococcal bacteria was analyzed by flow cytometry. Opsonization with two types of normal pooled sera and staphylococcal antisera significantly increased bacterial phagocytosis compared to samples without an opsonin. The staphylococcal antisera did not significantly increase bacterial phagocytosis compared to the normal pooled sera. Opsonization appears to increase bacterial phagocytosis but specific antisera may not increase phagocytosis beyond that caused by pooled normal sera.

RESUMEN. Comparación del efecto de diferentes opsoninas sobre la fagocitosis del Staphylococcus marcado con fluoresceína, por los heterófilos de pollo.

Mediante citometría de flujo, se analizó la fagocitosis del Staphylococcus marcado con fluoresceína, por los heterófilos. La opsonización con dos tipos de suero normal y antisuero contra Staphylococcus aumentó significativamente la fagocitosis bacteriana comparada con las muestras sin opsonina. El antisuero contra Staphylococcus no aumentó significativamente la fagocitosis bacteriana comparado con el suero normal. Parece que la opsonización aumenta la fagocitosis bacteriana pero el antisuero específico puede no aumentar la fagocitosis a niveles mayores de los causados por el suero normal.

Key words: heterophil phagocytosis, flow cytometry, Staphylococcus aureus, opsonization

Abbreviations: BSA = bovine serum albumin; CFU = colony-forming units; EDTA = ethylenediaminetetraacetic acid; FITC = fluorescein isothiocyanate; HBSS = Hank's balanced salt solution; MEM = minimum essential medium; PBS = phosphate-buffered saline

Staphylococcal tenosynovitis, usually caused by infection with Staphylococcus aureus, is a worldwide problem in broiler-breeder and broiler chickens, causing economic losses due to decreased weight gain, decreased egg production, and condemnation of carcasses at slaughter (9). Studies of the interaction between heterophils and staphylococcal bacteria have shown differences in cell function in experimental and field cases. In field cases of staphylococcal arthritis, heterophil chemotaxis was decreased, whereas in experimental cases, heterophil functions were increased (1,2). Subsequent studies of experimental induction of broiler staphylococcal arthritis/tenosynovitis failed to define a statistically significant point in time at which heterophil chemotaxis decreased (unpubl. data).

Staphylococcal arthritis/tenosynovitis is typically a chronic disease with a poor response to antimicrobial therapy or immunization. Controversy exists over the efficacy of staphylococcal antisera or vaccination preventing staphylococcal infections (5,6,11,15). Also, capsule type may contribute to staphylococcal virulence, since the majority of pathologic strains in human beings and chickens have capsular types 5 and 8 (4). Complement, in non-heat inactivated serum, is an important component of opsonization for S. aureus (10). Some studies have indicated that complement and antibodies in immune sera are better opsonins for S. aureus than complement alone (10). There is a need to study opsonins that may interact during in vivo staphylococcal infections.
Flow cytometry can evaluate more phagocytic cells than can light microscopy. The heterophil population is defined by granularity and size in a boundary map (bitmap). Flow cytometry can determine the number of heterophils that have phagocytosed fluorescein-labeled bacteria (frequency) and provide an estimate of how many fluorescein-labeled bacteria were phagocytosed (degree of fluorescence) by determining the mean channel number, defined as the peak fluorescence (13). Channel numbers, indicating peak fluorescence, are felt to be a more accurate comparison of phagocytic ability than examining only the percent of cells that have phagocytosed bacteria, because multiple bacteria within cells contribute to the fluorescent signal.

The present study was undertaken to characterize differences in fluorescein-labeled bacterial phagocytosis by heterophils with no opsonization, opsonization with pooled normal serum, and staphylococcal antisera, and to determine whether staphylococcal antisera increase bacterial phagocytosis.

**MATERIALS AND METHODS**

**Experimental design.** Flow cytometry was used to analyze heterophil phagocytosis of fluorescein-labeled *S. aureus* bacteria. Each comparison of opsonins was performed in duplicate using heterophils from an individual broiler chicken. Ten individual chickens were used, yielding 10 trials that were analyzed statistically. In each trial, fluorescein-labeled and unlabeled (controls for background fluorescence) bacteria and heterophils were incubated with control media and three different opsonins.

**Heterophil separation.** Heterophils were isolated by slight modification of a described method (1). A total volume of 6 ml of ethylenediaminetetraacetic acid (EDTA) anticoagulated blood was collected and divided into four 13 × 100 mm glass tubes coated with Sigmacoat (#SL-2, Sigma Chemical Co., St. Louis, Mo.) in aliquots of 1.5 ml. Each aliquot was mixed with 1.0 ml of 1% methylcellulose (25 cps, #M-6385, Sigma) and centrifuged at 25 × g for 5 minutes. The serum and extended buffy coat layers were retained, 1.0 ml of Hank’s balanced salt solution (HBSS) without calcium or magnesium was added, and each of the four aliquots was placed on a Ficoll-Hypaque gradient (Sigma) (specific gravity 1.077, 3 ml over specific gravity 1.119, 3 ml). After centrifugation at 300 × g for 20 minutes, the 1.077/1.119 interface and 1.119 band were retained. After two washes in HBSS, the heterophils were suspended in minimum essential medium (MEM, #M-0268, Sigma) at a final concentration of 1 × 10⁶/ml (1).

**Bacterial fluorescein-labeling.** A characterized *S. aureus* strain #921-1 was used (3). Bacteria were labeled with fluorescein isothiocyanate (FITC) isomer I (#F-7250, Sigma) using a described method with slight modifications (6). After washing, a bacterial aliquot was removed and plate counts were performed to verify bacterial titers. Bacteria were heat-killed (60 C for 60 min) prior to labeling. After labeling and before each use, bacteria were examined by fluorescent microscopy to verify labeling. The bacteria were stored at −70 C in aliquots of 1 × 10⁶/ml in gelatin vernal-buffered saline buffer (6). Prior to use, fluorescein-labeled bacteria were thawed, diluted in HBSS, pH-adjusted to 7.2, and adjusted to a final concentration of 2 × 10⁷ colony-forming units (CFU) per ml. Unlabeled control bacteria were prepared similarly.

**Avian sera.** Staphylococcal antisera were created by intravenous inoculation of 0.1 ml containing *S. aureus* #921-1, 2.9 × 10⁶ CFU/ml, into 5-week-old Arbor Acres × Peterson broiler chickens (1). Sera were collected 5 weeks postinoculation and lesions consistent with staphylococcal arthritis were confirmed by necropsy and culture. Nonimmune chicken sera were collected from broiler chickens in Georgia and Oregon and pooled separately. Serum was filtered and stored at −70 C until used. Serum titers against *S. aureus* were determined by microtiter plate agglutination as follows: Oregon serum 1:32; Georgia serum 1:32; and staphylococcal antisera 1:320. Additionally, indirect immunofluorescent antibody titers were performed. Dilutions of staphylococcal antisera from 1:5 to 1:4000 were tested against *S. aureus* #921-1 and Streptococcus sp. (negative control) using fluorescein-labeled anti-chicken immunoglobulin G. Fluorescent endpoints, defined as the greatest dilution of staphylococcal antisera at which specific fluorescence could be observed, were 1:10 for the Oregon and Georgia sera and 1:500 for the staphylococcal antisera.

**Phagocytosis.** Heterophils from an individual chicken were used in each phagocytosis comparison (n = 10). Samples were run in duplicate. Bacterial-to-heterophil ratios were optimal for detection at 20:1. Preparation of samples was similar to described methods (1, 14). Samples consisted of fluorescein-labeled bacteria and heterophils (20:1) with 5% Oregon serum, Georgia serum, staphylococcal antisera, or MEM (no opsonization). Evaluation of background fluorescence was performed by comparison of phagocytosis with unlabeled control bacteria. Microscopic evaluation verified that 5% sera concentrations did not cause bacterial clumping. Samples were lightly vortexed, then centrifuged for 10 minutes at 25 × g to maximize contact between bacteria and heterophils. Samples were incubated in a shaker water bath
at 41°C for 55 minutes; then 40 mM EDTA was added to each tube to stop phagocytosis. Samples were washed twice with sterile 0.9% saline at 150 × g for 15 minutes. Supernatants containing nonphagocyted bacteria were verified microscopically and discarded. The remaining cell pellets were resuspended to their original volume (0.5 ml) in 1% paraformaldehyde, incubated in the dark for 20 minutes, and washed with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 15 minutes at 150 × g. The supernatant was removed and the sample was reconstituted to the original volume with BSA-PBS. Tubes were wrapped in foil, stored at 4°C, and analyzed by flow cytometry within 48 hours.

**Flow cytometry.** Heterophil phagocytosis was analyzed on an EPICS V flow cytometer (Coulter Electronics, Hialeah, Fla.) equipped with an argon laser operated at an excitation wavelength of 488 nm and 300 mW. Heterophils were identified by both forward-angle and 90-degree light scatter measurements and were confirmed by FITC fluorescence and cell sorting. FITC fluorescence was measured using 515 nm long-pass interference and absorbance filters. Data were collected from 50,000 cells and subsequently analyzed using Cyclops Software (Cytomation, Fort Collins, Colo.). Using gating bitmaps (boundaries) around the heterophil population, fluorescence was collected on 10,000 heterophils. Negative controls, consisting of untreated heterophils and heterophils that had phagocyted unlabeled bacteria, were analyzed to determine the minimum fluorescence (<3.6%) above which positive fluorescence was identified. Fluorescein-labeled bacteria were analyzed to ensure that free bacteria were excluded from the heterophil bitmap. Parameters measured included the percentage of heterophils that contained fluorescein-labeled bacteria and the fluorescence of the heterophil populations defined as channel number.

**Statistical analysis.** The percentages of heterophils phagocytosing FITC-labeled bacteria were converted to arcsines (12) and the means and standard deviations determined. The means were then converted and expressed as percentages. In separate calculations, the numerical peaks of fluorescence (mean channel numbers) for each treatment group were compared using multifactor analysis of variance. Analysis identified significant differences among opsonization treatment groups (F[0.05; 3,41] = 16.3). Significant differences between pairs of mean channel numbers were located by the Scheffé method (Statgraphics version 5, STSC, Inc., Rockville, Md.) (Table 1).

**RESULTS**

Heterophils had a low level of nonspecific fluorescence, possibly due to the cytoplasmic granules. The mean percent of heterophils in MEM exhibiting detectable fluorescence was 2.2%. The heterophil population exhibiting fluorescence was within the gating bitmap (boundaries) that were established by identifying purified heterophils with forward-angle and 90-degree angle light scatter (Fig. 1). Prolonged exposure to 1% paraformaldehyde resulted in decreased fluorescence of labeled bacteria, necessitating the rinsing and storage of cells in BSA-PBS. This effect is not as marked when using the larger fluorescein-labeled beads, but decreased fluorescence is detected (unpubl. data).

The mean percentage of heterophil fluorescence after incubation with unlabeled bacteria and opsonins for Oregon serum was 2.5%; for Georgia serum, 3.2%; and for staphylococcal antiserum, 3.6%. These values were considered to be background fluorescence due to heterophil granule alterations during phagocytosis. The mean percentages with standard deviations of heterophils that phagocyted fluorescein-labeled bacteria were as follows: FITC bacteria + MEM, 8.6%; FITC bacteria + staphylococcal antiserum, 23.7%; FITC bacteria + Georgia pooled serum, 26.8%; and FITC bacteria + Oregon pooled serum, 38.9%, indicating increased phagocytosis of FITC-labeled bacteria with opsonization.

Mean channel numbers are presented in Table 1. Increasing channel numbers indicate an increased total fluorescent signal, reflecting the number of cells that ingest fluorescent bacteria and the number of fluorescent bacteria within the cells. The example in Fig. 1 shows the mean peak fluorescence at channel 150, with fluorescence less than channel 75 identified as background fluorescence. There were significant differences between the FITC-labeled bacteria +

<table>
<thead>
<tr>
<th>Opsonin</th>
<th>Mean channel no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM</td>
<td>119.6</td>
</tr>
<tr>
<td>Staph antiserum</td>
<td>146.0</td>
</tr>
<tr>
<td>Oregon sera</td>
<td>148.0</td>
</tr>
<tr>
<td>Georgia sera</td>
<td>154.5</td>
</tr>
</tbody>
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*Mean channel numbers followed by an asterisk are not significantly different (P < 0.05).
Staph antiserum = staphylococcal antiserum.
Heterophil phagocytosis of staphylococcal bacteria

Fig. 1. Boundaries (bitmaps) are drawn around the heterophil population (top graph). Heterophils are represented by a dot plot histogram and located using forward-angle light scatter (FALS) on the y axis and 90-degree light scatter (LI90) and fluorescein labeling (FITC+) on the x axis (top graph). The bottom graph is a representative histogram of FITC fluorescence with the bracket including significant fluorescence (greater than 75 channel number, excluding nonspecific fluorescence) on the x axis (LIGFL). The mean peak fluorescence is located at channel number 143. The y axis corresponds to the frequency of events (number of particles or cells with fluorescence).

MEM and FITC-labeled bacteria incubated with other opsonins.

DISCUSSION

Optimal phagocytosis of bacteria in suspension assays requires opsonization, as little bacterial phagocytosis occurs without opsonization (8). This was true in the present study. Optimal phagocytosis usually requires immunoglobulin and the deposition of complement fragments, usually the third component of complement. It has been suggested that the major limitation for the human neutrophil to function in staphylococcal infections is the phagocytic capacity and not the bactericidal capacity (10).

Since staphylococci are ubiquitous in the environment and are present on the skin and within the intestine of the chicken, it is expected that normal pooled chicken sera would have a low-level staphylococcal titer. Additionally, titer levels to S. aureus in 1-day-old specific pathogen-free chickens are similar to those in our nonimmune pooled chicken sera (unpubl. data). The staphylococcal immune antiserum did not significantly increase phagocytosis compared to either pooled normal sera. It would be expected that specific antiserum would increase phagocytosis; however, several factors might prevent the increase. The titer of the staphylococcal antiserum may not have been sufficiently high to produce increased phagocytosis beyond the sera with natural immunity to S. aureus.

Also, normal pooled serum may contain enough antibodies to maximally opsonize S. aureus for heterophil phagocytosis compared to specific immune antiserum. Reviews of S. aureus state that it is unlikely that antibodies would generate more opsonization in vivo than that produced by the exposed complement-activating cell wall materials, including peptidoglycan and teichoic acid (11). An increase in phagocytosis may require specific antibodies against capsular antigens or cell surface proteins (5,7).

Conversely, increased phagocytosis using antibodies specific to bacterial membrane proteins has not always increased bacterial phagocytosis. Opsonization studies of Actinobacillus pleuropneumoniae found that the greatest increase in phagocytosis was seen with complement-replete convalescent antiserum (14). Pooled normal serum and antiserum to specific membrane proteins from A. pleuropneumoniae did not increase phagocytosis beyond the convalescent serum (14).

Fluorescein-labeling of the bacteria probably results in some loss of surface antigenicity because of stringent alkaline conditions and heat-killing of bacteria. Therefore, differences in bacterial strain antigenicity probably could not be evaluated using this technique.
The percentage of heterophils from clinically healthy chickens phagocytosing opsonized beads (37.8% and 38.7%) was similar to the percentage of phagocytosis when fluorescein-labeled bacteria were opsonized by Oregon pooled serum (39%) (1). Fluorescein-labeled staphylococcal bacteria more closely approximate the particle size normally ingested by heterophils than do fluorescent latex beads. Beads offer the advantages of a stronger fluorescent signal and the ability to quantify the number of beads per cell, and they are already fluorescein-labeled. Opsonization and phagocytosis are important in the in vivo defense against staphylococcal infections. For this reason, the study of antibodies against *S. aureus* needs further investigation. Since the majority of pathogenic poultry strains of *S. aureus* are capsule types 5 and 8, polyclonal or monoclonal capsular antibodies may facilitate greater type-specific opsonization and phagocytosis by heterophils than a nonspecific convalescent staphylococcal antiserum. Immunization studies utilizing *S. aureus* type 5 capsular polysaccharide are being evaluated for the prevention of *S. aureus* mastitis in dairy cattle (7, 15).

REFERENCES


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