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Abstract Approved:__
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Staphylococcal tenosynovitis and osteomyelitis are world-wide problems of broilers and broiler breeders caused by staphylococci. Pathogenesis of the disease is ill defined. Avian heterophils are analogous to mammalian neutrophils but the granules appear to be different.

The first chemotactic study was done on heterophils from chickens having natural staphylococcal infection brought from a commercial broiler flock and on the heterophils obtained from healthy 6-8 weeks old chickens brought from a local hatchery as one day old chicks. In the second study, a chemotactic study was done with three different staphylococci on heterophils obtained from healthy 6-8 weeks old chickens brought from a local hatchery as one day old chicks.

Results for the first study showed a decreased chemotactic response in the heterophils of chickens naturally infected with staphylococcus compared to healthy chicken heterophils in response to minimum essential medium, pooled normal

chicken serum and *E. coli* endotoxin with normal chicken serum used as chemoattractants. Second study results showed that pathogenic capsule type 5 and type 8 *Staphylococcus aureus* isolates both induced chemotaxis in heterophils from healthy chickens to a significantly greater degree than did a non-pathogenic *Staphylococcus xylosus*. The *Staphylococcus aureus* isolate with capsule type 5 induced heterophil chemotaxis more than the capsule type 8 isolate.

CHEMOTACTIC EFFECT OF DIFFERENT TREATMENTS ON HETEROPHILS FROM HEALTHY CHICKENS AND CHICKENS WITH STAPHYLOCOCCAL INFECTION

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INTRODUCTION

Staphylococcal tenosynovitis is a major cause of leg weakness in poultry which has been reported in many countries. *Staphylococcus aureus* is considered a major pathogen in the development of clinical tenosynovitis and mortality (Kibenge, et al.,1982). For controlling staphylococcal tenosynovitis, immunization does not seem to be effective. A major difficulty in dealing with staphylococcal tenosynovitis is that the pathogenesis of the disease is not known (Mutalib, et al., 1982a; Mutalib, et al., 1982b; Jensen, et al., 1987). Cell function studies have been used to elucidate pathogenesis of many human and animal diseases (Andreasen, 1990). Avian heterophils are analogous to human neutrophils. Unfortunately we do not know much about heterophils, so functions like chemotaxis and phagocytosis should be investigated. In this study we investigated chemotaxis. In our first study, we compared cell functions in healthy and staphylococcal infected chickens. In the second study we exposed healthy chicken heterophils to three different strains of staphylococci and compared chemotactic effect of those organisms.

LITERATURE REVIEW

HETEROPHIL

Heterophil morphology

Heterophils are analogous to neutrophils. With Romanowski stain, avian heterophil granules appear different (heteros= different) from the mammalian neutrophil granules (neutro= neutral). Heterophils stain intensely eosinophilic when stained with Romanowski stain (Latimer, et al.,1988). Heterophils are rounded cells with small pseudopods (Maxwell and Trejo, 1970). The acidophilic granules of heterophil are found in various species like rabbits, guinea-pigs, birds and squamata like lizards, and snakes. In Wright-stained blood smears of most avian species, the heterophil has a basophilic nucleus with one or more lobes and prominent spindle-shaped cytoplasmic granules (Maxwell, 1985 in Montali, 1988). Andreasen stated about heterophils that "this may be contrasted to the eosinophil which contains fairly uniform orange-red round granules, basophilic lobulated nucleus, and lightly basophilic cytoplasm" (Andreasen, 1990). In blood smears, the morphology of the heterophil varies with different species, inflammation, or during the staining process due to the granule instability (Latimer, et al., 1988; Natt and Herrick,1954). Different stains are used for the avian leukocytes including phloxine B stain (Ferris and Bacha, 1984) and methyl violet 2B stain (Natt and Herrick, 1952). With Phloxine B stain, eosinophils and heterophils can be distinguished easily from each

other based on localization of stain within the cytoplasm. Heterophils always stain completely, appearing as a perfect red sphere while eosinophils stain partially.

Heterophil production

In avian species, erythropoiesis occurs within the vascular sinuses. The immature cells in the erythroid series are associated with the sinus wall, while mature ones are found in the center of the vascular lumen, while granulopoiesis occurs outside of the vascular sinuses. On the basis of the abundance of surface microvilli, both mature and immature granuloid cells can be differentiated by electron microscopy (Dieterlen-Lievre, 1988).

Heterophil ultrastructure

Heterophils show a similarity to fat cells with both granules and nucleus lying near the periphery of the cell and the remainder is filled with large vacuoles (Maxwell and Trejo, 1970). In birds, two populations of granules are consistently observed by electron microscopy and sometimes a third population can also be observed (Montali, 1988). The three populations of granules are: a) a large electron dense type (specific granule) corresponding to the spindle-shaped granule, approximately 1.5 *u*m long and 0.6 *u*m wide and accounts for 48.3% of the granules. They have a small circular and centrally situated inner dense structure which is delineated by a narrow clear space (Montali, 1988; Maxwell and Trejo, 1970). b) a smaller oval or shorter electron dense rod shaped granule 0.5 *u*m in diameter, accounting for 24.7% of the granules, and c) a small to intermediate, round electron-lucent granule having a diameter of 0.1

*u*m accounting for 27% of the granules (Brune and Spitznagel, 1973; Montali, 1988; Trowell and Brewer, 1976; Daimon and Caxton-Martin, 1977).

Heterophil cytochemistry and enzymatic contents

Cytochemical staining can be used to differentiate different types of cells and to identify enzymes, cytoplasmic and granular contents. Avian acidophilic leukocytes i.e., heterophils and eosinophils can be differentiated by cytochemical staining (Montali,1988). Morphological differentiation can be done for acidic leukocytes but sometimes it becomes difficult due to species variation (Campbell, 1988), cellular changes due to inflammation i.e., degranulation and degeneration (Montali, 1988; Latimer, et al; 1988). In this situation heterophil granules may resemble eosinophilic granules. Immature heterophil granules may be confused with eosinophilic granules because they may have the same staining affinity but are spherical (Dieterlen-Lievre, 1988).

The major difference between avian and mammalian granulocytes is that avian leukocytes lack myeloperoxidase and alkaline phosphatase (Brune, et al, 1972; Brune and Spitznagel, 1973). Heterophils from different species vary in their enzyme contents and cytochemical reactions. Rabbit heterophils are both functionally and biochemically like human neutrophils, so contain peroxidase (Montali, 1988).

Peroxidase and alkaline phosphatase activity are absent in chicken heterophils while chicken eosinophils are strongly positive for peroxidase (Brune and Spitznagel, 1973; Brune, et al, 1972; Daimon and Caxton-Martin, 1977). Alligator heterophils have positive cytochemical activity for acid phosphatase and alkaline phosphatase

(Mateo, et al., 1984). Acid phosphatase activity is seen in all of the large dense granules of heterophils. Turkey heterophils are positive for acid phosphatase. Turkey thrombocytes also show acid phosphatase activity (Topp and Carlson, 1972b). Most granules in chicken eosinophils show high activity for acid phosphatase (Daimon and Caxton-Martins, 1977; Caxton-Martins and Daimon, 1976). It has been shown that specific (spindle-shaped) granules of avian heterophil are lysosomes, based on a number of hydrolytic enzymes. These granules contain acid hydrolases, cationic proteins and lysozyme (Brune and Spitznagel, 1973). In one study, the chicken heterophil did not stain with peroxidase, Sudan black B, Periodic acid Schiff (PAS), alkaline phosphatase, or acid phosphatase (Caxton-Martins and Daimon, 1976).

HETEROPHIL ISOLATION FROM BLOOD

Isolation of pure cells is necessary for evaluating cell functions and investigating different diseases. A lot of work has been done on neutrophil cell separation (Gallin, et al., 1978; Latimer, et al., 1983). Latimer separated dog neutrophils with 99% purity using Ficoll-Hypaque gradients (Latimer, et al., 1989b). Due to not having good procedure availability for heterophil cell isolation, studies on heterophil functions have been limited. In the past, people were using different Ficoll gradients and the procedure was really time consuming (Noble and Cutts, 1968). People also used glass column with siliconized glass beads. Investigators obtained 90% viable heterophils but had problems with thrombocyte contamination (Topp and Carlson, 1972a). Glick used anticoagulated peripheral blood for chicken heterophil

separation with Ficoll-Hypaque double density gradients (Glick, et al., 1985). This procedure gave contamination of different cells in different bands of cells formed by the density gradients. Chicken heterophils were separated by Ficoll gradients with 99% purity and 99.8% viability (Andreasen and Latimer 1989). By using a similar technique, turkey heterophils have been separated with 96% purity in about three hours (Latimer, et al., 1989a).

LEUKOCYTE FUNCTION

Adherence, chemotaxis and phagocytosis of avian heterophils are considered analogous to human neutrophils (Topp and Carlson, 1972c; Andreasen, et al 1991). Neutrophils and heterophils must adhere to the endothelium, must move from the circulation to tissue (locomotion and chemotaxis), and engulf foreign material (phagocytosis). If the agent is bacteria, then bacterial killing is required. Phagocytosis in heterophils is followed by granule fusion, rapid granule lysis into the phagolysosome and finally degradation of the causative agent (Trowell and Brewer, 1976). As already mentioned, the heterophil does not contain peroxidase (a microbicidal enzyme), microbicidal activity is carried out by lysozyme, cationic proteins and acid hydrolases (Brune, et al., 1972). Ochratoxicosis and aflatoxicosis can impair chicken heterophil phagocytic activity (Chang and Hamilton, 1979; Chang and Hamilton, 1980).

CHEMOTAXIS

Chemotaxis can be defined as "a bias in the direction of movement of a cell or organism along the axis of a chemical gradient" (Zigmond and Sullivan, 1981). Due to little information on chicken heterophil activity, the following discussion focuses mostly on mammalian neutrophils.

One comes across several terms when talking about neutrophil movement, i.e., "random locomotion", "chemokinesis", and "chemotaxis". The term "random locomotion" refers to "locomotion in which the axis of the moving cell is not oriented in relation to any stimulus and in which the cell shows no preference for, or avoidance of, a particular direction". Chemokinesis is defined as "a reaction by which the speed or frequency of locomotion and/ or the frequency and magnitude of turns of cells is determined by substances in their environment". The definition of chemotaxis is "a reaction by which the direction of locomotion of cells is determined by substances in their environment" (Wilkinson and Alan, 1978).

The ability to migrate toward a site of infection is fundamental to the role of the neutrophil in host defense. This function requires some means to recognize chemotactic signals, adherence to the surface, and rearrangement of the cytoskeleton system to produce a chemotactic response (Styrt, 1989). Motility is important for the migration of the cells from bone marrow into the circulation. Phagocytic leukocytes are very motile (Territo, 1981). Chemotaxis involves both extracellular and intracellular factors. Human neutrophils have receptors for C5a, formyl-methionyl-leucyl-phenylalanine (FMLP), IgA, the Fc portion of immunoglobulins, and a

glycoprotein urate crystal induced chemotactic factor (CCF) (Territo, 1981). FMLP has reserve receptors in the granules of neutrophils (Styrt, 1989). Substances like complement fragment C5a, fibrinopeptides, prostaglandins, CCF, casein, certain oligopeptides like FMLP, and factors produced by bacteria which attract neutrophils are called cytotaxins (Territo, 1981; Styrt, 1989). Substances such as antigen-antibody complexes and endotoxin, which are not directly chemotactic but can act as chemotactic agents secondarily by inducing the generation of chemotactic factor (C5a) in serum are referred to as cytotaxigens (Territo, 1981). FMLP, initially thought of as a synthetic compound, is produced by bacteria such as *E. coli*. Neutrophils from different species like the guinea pig, rabbit, and mouse also are effected by FMLP. Activation of human neutrophils by this chemotactic peptide lead to chemotaxis, degranulation, and oxidative metabolism (Styrt, 1989). Neutrophils from species like dog, cat, and cow are not affected by FMLP, while equine neutrophils have secretory but no chemotactic effect (Styrt, 1989; Sedgwick et al., 1987). Equine mononuclear cells have chemotactic activity in response to FMLP (Sedgwick, et al., 1987).

In neutrophils, a complex system of microtubules and microfilaments are present which is necessary for cell movement (Territo, 1981). Substances like colchicine and cytochalasin (fungal product) inhibit chemotaxis (Gallin, et al., 1978; Territo, 1981). Cytochalasin has the property of disaggregating microfilaments and blocking the cellular motility (Vandenbroucke-Grauls, et al., 1984). Colchicine does not interfere with random movement of the neutrophils but it does block the responses of neutrophils to chemotactic agents. Colchicine prevents degranulation of neutrophils

by preventing polymerization of microtubule subunits into functional tubules (Murphy, 1976). Neutrophil movement probably resembles muscle contraction. The actin and myosin-like microfilament proteins in the neutrophil act as a contractile element, controlling changes in cell shape, phagocytosis, secretion, and movement (Territo, 1981). During the membrane contact of neutrophil and chemoattractant, a small depolarization occurs caused by influx of calcium and sodium and efflux of potassium (Gallin, et al., 1978). It is said that calcium influx or the release of bound calcium from the intracellular compartment activates the contractile process leading to the polarized contraction (Territo, 1981). Variation in the free intracellular concentration of calcium which is released to the cytoplasmic side of the neutrophil cytoplasmic membrane mediates chemotactic factors on neutrophils (Styrt, 1989).

Physiological agents which suppress chemotaxis may modulate inflammatory response. These agents are immunoglobulin (IgA), leukocyte inhibitory factor (LIF) derived from human lymphocytes by stimulation with concanavalin A, inhibitors of complement, and anaphylatoxin inactivators which inactivate C5a and C3b (Andreasen, 1990).

LIF acts in two ways: a) directly on the neutrophil to inhibit neutrophil migration, and b) production of a low molecular weight chemotactic inhibitor (Goetzl and Rocklin, 1978). LIF is beneficial in the sense that it maintains neutrophils in the area of inflammation (Andreasen, 1990).

Different procedures have been adopted to measure chemotaxis. These procedures include Boyden chamber micropore filter system (Malech, et al., 1977;

Latimer, et al., 1983; Goetzl and Rocklin, 1978; Chang and Hamilton, 1980, Hill, et al., 1974; Latimer, et al., 1990; Althaus, et al., 1980), migration under agarose (Thies, et al., 1983; Repo, et al., 1979; Nagaraja, et al., 1982), skin windows, and recently, cotton pellet implants (Onyia, 1986).

In the skin window technique performed *in vivo*, inflammatory cells migrate in the exposed area of skin. A glass coverslip is placed on the exposed area to collect the adherent cells that have migrated to the exposed skin area (Territo, 1981).

To measure chemotaxis *in vitro*, many methods are available. In all these techniques, leukocytes are physically separated from the chemoattractant. Assessment of the cells migrating through the gradient towards a chemoattractant is done by either microscopically counting the number of cells passing through the filter, by radiolabeling the cells and determining the radioactivity of the migrating cells, or by measuring the distance into the filter over which cells migrate (Territo, 1981).

The Boyden chamber system has played an important role in chemotactic study. In the Boyden chamber technique, chemoattractant and leukocytes are physically separated from each other. The cell movement may be measured by the distance cells have moved or by the number of cells that have traversed the filter (Zigmond, 1978).

Very limited heterophil migration studies have been performed in the turkey and chicken. In one experiment under agarose, turkey heterophil migration was influenced by zymosan-activated serum. Activated serum (25%) had greater chemotactic activity than 10% activated serum. Increasing incubation time also had

more chemotactic effect (Thies, et al., 1983). Some researchers did a study using cells from turkeys infected with *Alcaligenes faecalis* (Mc Corkle and Simmons, 1983). They found that capillary tube leukocyte migration was significantly increased in infected birds compared to uninfected birds. Leukocytes from chickens infected with *Salmonella typhimurium* showed a significant decrease of leukocyte migration under agarose (Nagaraja, et al., 1982). Infection with ochratoxicosis and aflatoxicosis had decreasing effect on both random locomotion as well as on chemotaxis of chicken heterophils when using the Boyden chamber technique (Chang and Hamilton, 1979; Chang and Hamilton, 1980). In one study, turkey heterophils were exposed to three different strains of *Pasteurella multocida*. It was found that heterophils showed more chemotactic activity to the most pathogenic strain of *Pasteurella multocida* by using Boyden chamber technique (Latimer, et al., 1990).

STAPHYLOCOCCUS

Staphylococci are in the family Micrococcaceae (Skeeles, 1991, Adlam and Easmon, 1983, Sheagren, 1984). The genus contains approximately 20 species. The term staphylococcus indicates morphology of the organism, i.e., grape-like clusters in stained smears. *Staphylococcus aureus* is the only species pathogenic to poultry. All strains of *Staphylococcus aureus* are gram-positive, coccoid in shape, and on solid media found in clusters (Skeeles, 1991). Depending on their ability or inability to clot rabbit plasma, staphylococci are categorized as coagulase-positive and coagulase-negative, respectively. The majority of the coagulase-positive staphylococci are

pathogenic and are more resistant to serum killing (Adlam and Easmon, 1983). Protein A is present in most of the human strains of staphylococci and is often present in bovine bacterial staphylococcal strains, but is usually absent in other animal strains (Oeding, 1983). Protein A has high affinity for the Fc fragment of immunoglobulins, primarily IgG, and for complement (Sheagren, 1984). Due to Fc fragment binding to bacteria, neutrophil binding to the same Fc fragment is blocked which probably inhibits opsonization by IgG antibodies (Andreasen, 1990).

Capsule

Approximately 98% of *Staphylococcus aureus* from blood isolates are encapsulated. The predominant type 5 and 8 (70% of the blood isolates) capsular polysaccharides (CPS) have been visualized on the surface of the capsulated organism by electron microscopy (Karakawa, et al., 1988). Eleven capsular serotypes of *Staphylococcus aureus* have been distinguished by using polyclonal antibodies or monoclonal antibodies (Albus, et al., 1991). Staphylococcal CPS covers the complex, rigid cell-wall matrix which consists of peptidoglycan and teichoic acid. The capsule is antiphagocytic and interferes with interaction between the teichoic acid-peptidoglycan complex and complement (Sheagren, 1984). The capsule prevent phagocytosis by blocking classical and alternative complement attack and anti-peptidoglycan antibodies (Adlam and Easmon, 1983). Karakawa used an *in vitro* phagocytic assay to assess whether *Staphylococcus aureus* microcapsules were antiphagocytic. They reported that type 5 and 8 *Staphylococcus aureus* strains were opsonized by phagocytosis only in the presence of specific capsular antibodies

(Karakawa, et al, 1988). The encapsulated strains produce extracellular polysaccharides different from teichoic acid (Karakawa, et al., 1985). One researcher said that bacterial capsules are classic virulence determinants, and animal models of infection have confirmed the premise that most encapsulated bacteria are more virulent than strains lacking capsule (Albus, et al., 1991).

Karakawa reported a method of capsular typing by using capsular typing antisera. The proposed typing scheme relies upon the cultivation of encapsulated isolates of *Staphylococcus aureus*, the preparation of specific capsular antisera, and capsular antigen free of contaminating teichoic acid (Karakawa, et al., 1985).

AVIAN STAPHYLOCOCCAL TENOSYNOVITIS

A major cause of leg weakness in poultry is tenosynovitis, which occurs in several countries. Avian tenosynovitis can result from viral, bacterial, or mycoplasmal infections. *Staphylococcus aureus* is considered a major pathogen in the development of clinical synovitis and mortality (Kibenge, et al., 1982). The most frequent sites of infection are bones, tendons, tendon sheaths, and leg joints. Other sites for staphylococcal infection are skin, sternal bursa, yolk sac, heart, vertebrae, and eyelid (Skeeles, 1991). First researchers thought reovirus was a primary etiological agent causing tenosynovitis, but it is not a primary etiological agent for tenosynovitis (Kibenge, et al., 1983).

Osteomyelitis produced by *Staphylococcus aureus* and *E. coli* produce similar lesions in bones and joints, but hock joint involvement is very common with

Staphylococcus aureus infection. A diagnostic difference between *Staphylococcus aureus* and *E.coli* infection is that sternal bursae are frequently involved with *Staphylococcus aureus* infection while not with *E. coli*. Osteomyelitis starts in the terminal vessels of the cartilaginous growth plates of the long bones (Nairn, 1973). Gross lesions of osteomyelitis consist of yellow areas of caseous exudate or lytic areas, and bones become fragile. In tenosynovitis, joints and tendon sheaths are swollen and filled with purulent exudate (Skeeles, 1991).

In one study, microscopic tendon lesions were less common with staphylococcal infection than with reovirus infection (Hill, et al., 1989). Staphylococcal infection begins in vascular bundles, and causes cartilage necrosis and abcesses in bones. Histologically, staphylococcal lesions of acute tenosynovitis in chickens consists of hyperplasia and hypertrophy of synoviocytes, and heavy infiltration of heterophils (Hill, et al., 1989).

Staphylococcal tenosynovitis and osteomyelitis both can be produced experimentally in chickens and turkeys. A high number of bacteria (5×10^{11}) when given intratracheally, produced osteomyelitis in few chickens, suggesting that the oral route of infection is not common in chickens (Mutalib, et al., 1982b). Researchers have also tried to produce disease through the oral route in turkeys but with poor results. No histological lesions were reported in aerosol-inoculated chickens although staphylococcal organisms were isolated. Since the route of inoculation was oral, one could expect recovery of staphylococcal organism from the lungs and trachea (Jensen, et al., 1987).

Pathogenesis of osteomyelitis in chickens probably requires bacteremia (Miner, et al., 1968, Mutalib, et al., 1982a), but the route of bacterial entry into the blood is not clear. It has been suggested that staphylococci invade the vascular system, resist phagocytosis, and produce various kinds of toxins (Miner, et al., 1968). It has been suggested that entry into the blood might be via wounds, or mosquito bites. When the organism is given intravenously, lesions develop. *Staphylococcus aureus* was reisolated from bones of all chickens with lesions of osteomyelitis and from the liver, spleen, and heart blood of some chickens. Due to severe feed restriction, debeaking, and corticosteroid therapy, appearance of disease was delayed and severity of the disease was also decreased as well (Mutalib, et al., 1982a).

Control of staphylococcal tenosynovitis and osteomyelitis is still made difficult by lack of knowledge of the pathogenesis of the disease. Chemotherapy and immunization are not yet successful. Bacterial interference is said to be a possible means of controlling staphylococcal infection. Strain 115 (non pathogenic) of *Staphylococcus epidermidis* was used as an interfering agent (Nicoll and Jensen, 1987a; Nicoll and Jensen, 1987b). This bacterium both physically interfered with the attachment of virulent *Staphylococcus aureus* strain 24 to turkey cells and growth of some *Staphylococcus aureus* strains were also inhibited by bacteriocin. In an *in vitro* experiment, strain 115 interfered with the colonization of chicken cells by *Staphylococcus aureus* strain 24. Nicoll also stated that chicken and turkey cells possess similar sites for attachment of staphylococci (Nicoll and Jensen, 1987b, Wilkinson and Jensen, 1987). In one trial in Utah in 1985, a control study was

carried out to quantitate the level of reduction of staphylococcosis with immunization program. Out of 3 million turkeys, only 1 million turkeys got vaccinated for staphylococcosis. The gross mortality rate was 2.7% lower than that of unvaccinated turkeys (Nicoll and Jensen, 1987a).

STAPHYLOCOCCAL INDUCED CHEMOTAXIS

Staphylococci possess both positive and negative chemotactic substances. Staphylococcal cell wall and opsonization in the presence of an intact classical complement pathway contribute to chemotactic property of staphylococcus organism. Splitting C5 to C5a by peptidoglycan and teichoic acid is thought to be chemoattractant (Adlam and Easmon, 1983; Oeding, 1983). It has been proved that most of the chemotactic activity of staphylococci is serum mediated, probably through complement activation (Wilkinson, 1982). Culture filtrates from *Staphylococcus aureus* have a tetrapeptide that is chemotactic for monocytes (Rot, et al., 1989). *E. coli* filtrates contain lipids having chemotactic activity (Wilkinson, 1982). Protease production either from *in vivo* staphylococcal growth or from tissue damage can attack both C3 and C5 components of the complement to produce chemotactic C3a and C5a and thus promoting chemotaxis. In the same way, the process of clotting leads to the formation of tissue derived chemotactic factors (Adlam and Easmon, 1983).

Researchers described a cell wall mucopeptide from *Staphylococcus aureus* which inhibited the locomotion of neutrophils. It has been shown that cell wall

peptidoglycan fragments can also act as negative chemotactic substances. Sphingomyelinase C (B-toxin) of *Staphylococcus aureus* has an effect on locomotion of human leukocytes specially monocytes (Wilkinson, 1982; Adlam and Easmon, 1983). Log phase cultures of *Staphylococcus aureus* produce a cell wall deoxycholate residue which consists of protein and peptidoglycan and inhibits neutrophil chemotaxis. Alpha hemolysin production by staphylococci prevents neutrophil migration due to vasoconstriction. Leucocidin has a lot of inhibitory effect on PMN migration (Adlam and Easmon, 1983).

In summary, staphylococcal tenosynovitis is a world wide problem in broilers and broiler breeder chickens. This problem causes economic losses due to decreased weight gain, decreased egg production, and carcass condemnation. The pathogenesis of the disease is not well defined. The disease occurs in spite of the fact that heterophil infiltration occurs during clinical disease. Heterophil function studies can help elucidate the role of heterophils during inflammatory process of staphylococcal tenosynovitis.

CHAPTER I

COMPARATIVE HETEROPHIL CHEMOTAXIS IN HEALTHY CHICKENS AND CHICKENS WITH STAPHYLOCOCCAL TENOSYNOVITIS AND OSTEOMYELITIS.

ABSTRACT

Staphylococcal tenosynovitis and osteomyelitis are world-wide problems of broilers and broiler breeders. Avian heterophils are analogous to mammalian neutrophils but the granules appear to be different. The main objective of the study was to compare heterophil chemotaxis using heterophil obtained from healthy and staphylococcal infected chickens. Commercial broiler chickens were obtained to serve as healthy controls. The staphylococcal infected chickens were brought from a commercial broiler flock. Necropsy findings and culture tests were positive for staphylococcal bacteria in the commercial chickens. Chemotaxis of heterophils isolated from peripheral blood was measured using the Boyden chamber technique. Heterophil counts were made microscopically from membrane filters. A significantly reduced chemotactic response was observed in staphylococcal-infected chickens compared to healthy control chickens using repeated measures C.R.D. analysis of variance.

INTRODUCTION

Staphylococcal tenosynovitis is an ubiquitous problem in breeder and broiler chickens and can lead to economic losses like decrease in weight gain, decrease egg production, and carcass condemnation at the time of slaughter (Mutalib, et al., 1982b). The pathogenesis of the disease is poorly defined (Mutalib, et al., 1982a; Mutalib, et al., 1982b; Jensen, et al., 1987). Oral route does not seem to play a role in the pathogenesis as $5X10^{11}$ staphylococci when given intratracheally produced disease in some chickens. The disease was produced when $1X10^{5}$ or more organisms were given intravenously. The pathogenesis of septic arthritis in chickens may be due to presence of transphyseal blood vessels in young chickens which allow bacteria to reach to the synovial surface during septicemia (Alderson, et al., 1986). In staphylococcal tenosynovitis, heterophilic infiltration of tendons and synovial membranes of the hock joint occurs. The disease is usually chronic and response to antibiotics and immunization is not well defined.

Avian heterophils are analogous to human neutrophils in their action as tissue phagocyte and play a role in the host defense mechanism during bacterial infection. Recently it was found that heterophil chemotaxis was increased in chickens with experimentally-induced staphylococcal tenosynovitis as compared to chemotaxis of heterophils from healthy chickens (Andreasen, 1991).

The main objective of this study was to compare heterophil chemotaxis in healthy chickens and chickens with natural staphylococcal tenosynovitis and osteomyelitis.

MATERIALS AND METHODS

CHICKENS

Eight 46-day-old Arbor Acres X Peterson commercial broilers suspected of having staphylococcal tenosynovitis were obtained and wing banded. They were used as heterophil donors, then euthanized and necropsied.

CONTROLS

Day-old Arbor Acres X Peterson broiler chicks were obtained and raised with growth ration and water ad libitum. They were wing banded for individual identification. The chickens were used as heterophil donors between 6 to 8 weeks of age.

NECROPSY OF CHICKENS

Swabs from tendons, joints, and bones were cultured onto tryptose- blood agar plates. Isolated bacterial organisms were identified by standard techniques.

Tendons and bones were fixed in 10% neutral buffered formalin, routinely processed, embedded in paraffin, sectioned at 5*u*, and stained with hematoxylin and eosin. Bones were decalcified by the formic acid method and then processed.

BLOOD SAMPLES

Three ml of blood was collected from ulnar vein into sterile syringes containing 0.3 ml 10% disodium ethylenediamine tetraacetic acid (EDTA). Blood (0.5 ml) was kept for determination of white blood cell (WBC) count, packed cell volume (PCV), and total protein.

HETEROPHIL ISOLATION

Reagents for heterophil separation were filter sterilized and stored at 4°C. Before using, reagents were brought to room temperature. All glassware was siliconized by Sigmacote, (Sigma Chemical Co,. St. Louis, Missouri) to minimize adherence of heterophils to the walls of the glassware.

Anticoagulated blood (2.5 ml) was divided equally into two Borosilicate disposable culture tubes, size 13 X 100 mm (Fisher Scientific, Pittsburgh, PA 15219, U.S.A.), then 1 ml of 1% methyl cellulose was added to each tube. After mixing, the blood and methyl cellulose were centrifuged (Beckman, Model TJ6) for five minutes at 25 x g. All centrifugation steps were performed at room temperature. During all centrifugation processes, the brake of the centrifuge was turned off to minimize disturbance to the gradients.

The plasma supernatant along with the buffy coat was removed gently, mixed with 0.5 ml Hank's Balanced Salt Solution (HBSS) and placed on preconstructed discontinuous Ficoll-Hypaque gradients made by placing 3 ml of Ficoll-Hypaque (Histopaque-1119 and -1077, Sigma) with specific gravity 1.119 in the bottom of the

tube overlayered by 3 ml of Ficoll-Hypaque with specific gravity 1.077. Gradient tubes were centrifuged for 20 minutes at 250 x g.

Following centrifugation, the top plasma layer and mononuclear cells at the plasma Ficoll-Hypaque 1.077 interface containing a) methyl cellulose residual plasma, b) thrombocytes, mononuclear white blood cells, eosinophils, and basophils, respectively were discarded (Figure 1). The remaining layers (partial 1.077 and 1.199) were washed twice with HBSS having pH 7.2, and centrifuged at 250 x g for 10 and 7 minutes, respectively. On each washing with HBSS, the supernatant was discarded. Finally, cells were resuspended in minimum essential medium (MEM), a chemically-defined nutritional source providing certain physiological factors required for the culture of cells *in vitro*, to a total volume 2 ml.

Counting of the cells was done with a Thoma pipette (Pfeiffer Glass Inc., U.S.A.). Natt and Harrick stain was used for heterophil counting. Before counting, the cells were gently vortexed to equally distribute the cells concentration. The cell solution was drawn into the Thoma pipette up to 1 mark and rest of the Thoma pipette i.e., up to 101 mark was filled with Natt and Harrick stain. The cells were mixed with the stain on a rotator (American Rotator V, American Dade, Division of American Hospital Supply Corp. Miami, FL 33152, U.S.A.) at 90 RPM for 2 minutes, then gently vortexed (Rotator Mixer, Scientific Industries, Inc. Bohemia, N.Y. 11716, U.S.A.) three times at low speed for 3 seconds each time. The counting was done on a hemocytometer (VWR Scientific Counting Chamber) by placing a drop from Thoma pipette on the edge of the coverslip. An average count of cells from

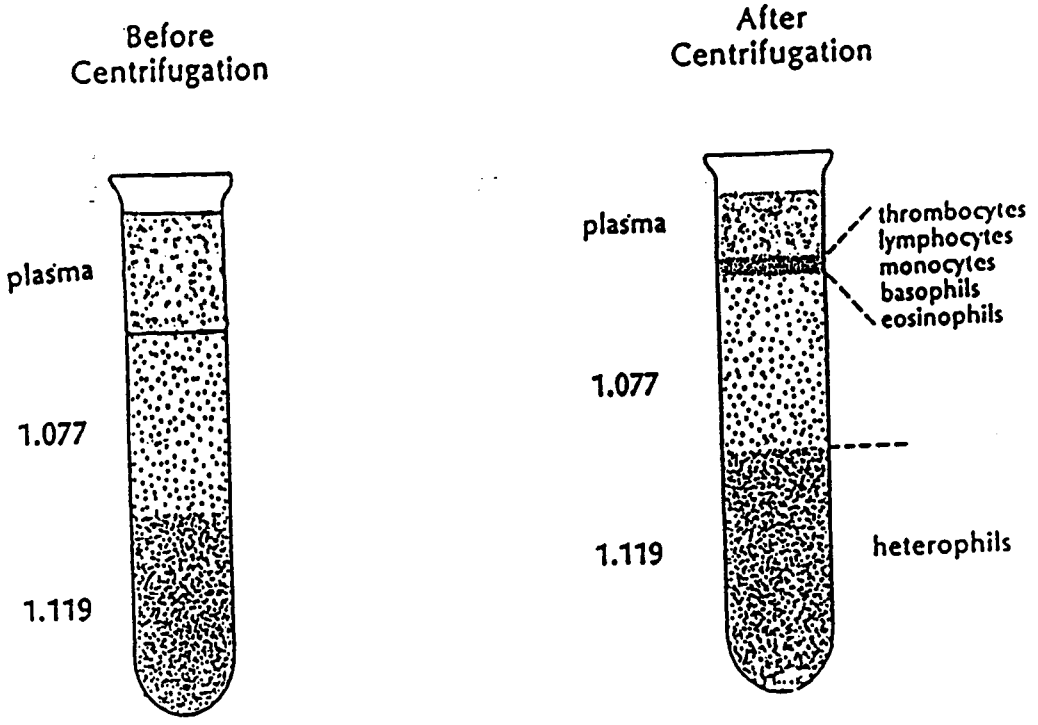

Figure 1: Ficoll-Hypaque discontinuous gradient before and after centrifugation.

both chambers of the hemocytometer was taken. Cells were resuspended in MEM to achieve a concentration of 1.5 $X10^6$ heterophils/ml.

CHEMOATTRACTANT SOLUTIONS

Three chemoattractant solutions were used in this study:

1) MEM (Negative control)

2) Pooled normal chicken serum (50 *u*l serum + 450 *u*l HBSS)

3) Endotoxin with pooled normal chicken serum as chemoattractant (125 *u*l serum +75 *u*l endotoxin solution +1500 *u*l HBSS). Endotoxin (lipopolysaccharide [LPS]) 0.001g from *E. coli* serotype 0111:B4 (Sigma Chemicals) was dissolved in 3.3 ml 0.9% normal saline making final concentration 60,606 U/ml.

All solutions for chemotaxis were filter sterilized.

CHEMOTAXIS ASSAY

By using the modified Boyden chamber technique, heterophil random (heterophils that traversed the filter in response to MEM) and total (heterophils that traversed in response to chemoattractants) movements were calculated. For each treatment, 2 blind wells were used (Andreasen, et al., 1991).

The chemoattractant solutions were incubated at 37°C for 60 minutes in a CO_2 incubator (VWR 6000, Sheldon Manufacturing Inc., Cornelius, Oregon, U.S.A.) and were also heat inactivated for 30 minutes at 56°C in water bath. Chemoattractant solutions (185 *ul*) were placed in the lower portion of the blind well, while 185 *ul* (1.5 X 10^6 heterophils/ml) of heterophil suspension were placed in the upper chamber, separated by 3 *um* average pore diameter polycarbonate filter (Costar Nucleopore Filtration Prod., Pleasanton, California 94588-8008, U.S.A.). These wells were incubated at 41°C for 45 minutes in a humid 5% CO_2 incubator. Following incubation, the filters were removed and placed on glass slides, air dried, stained with Diff Quik stain (Baxter Health-care Corporation, Scientific Products Division, Mc. Gaw Park, IL 60085-6787,U.S.A.) and then permanently mounted. Random and total heterophil movement on filter membrane were quantitated microscopically using the 100X oil immersion objective and a calibrated grid. Values were expressed as the mean number of heterophils/0.25 mm^2 membrane surface area, and all the values were averaged for statistical anaysis.

STATISTICAL ANALYSIS

For statistical analysis a square root transformation was used to transform the cell counts. The transformation was necessary to satisfy the normality assumption associated with the F-tests of the analysis of variance (ANOVA). The analysis of variance was carried out using a general linear model in the SAS GLM procedure (SAS Institute, 1988). Fischer LSD was used for mean cell separation.

RESULTS

Analysis of data (Appendix Table A.1) showed a highly significant effect of chemoattractants on chicken heterophils. All the three treatments i.e., a) Minimum Essential Medium, b) Serum, and c) Endotoxin were significantly different from each other (Table 1). Heterophil random migration and chemotactic movement for MEM, serum and endotoxin activated serum were all significantly greater in healthy chickens as compared to heterophils from staphylococcal infected chickens (Figure 2). Endotoxin-activated chicken serum appeared to be a stronger chemoattractant than non-activated serum for heterophils from both healthy chickens and chickens with staphylococcal tenosynovitis.

Necropsy and histologic examination confirmed lesions of purulent tenosynovitis and osteomyelitis in infected chickens. The bacterial cultures from field cases of tenosynovitis and osteomyelitis were positive for *Staphylococcus aureus*. Control chickens appeared normal and contained no lesions of joint disease.

We measured WBC, PCV, and plasma protein for uninfected and infected chickens. For uninfected chickens, values averaged 10,500/*ul* for WBC, 25.8% for PCV, and 4.4gm/dl for plasma protein, where as the values for infected chickens were 28,500/*ul* for WBC, 25.8% for PCV, and 5.3gm/dl for plasma protein. The averaged heterophil count was 3,800 and 14,880 in uninfected and infected chickens, respectively. The WBC and heterophil counts were significantly different in

Table 1: Effect of chemoattractants on heterophil chemotaxis

Treatments	Average[A] Cell Count
MEM	20.25[c]
Serum	185.23[b]
Endotoxin	304.85[a]
SED[B]	0.60

[A]Average of mean counts of heterophils/0.25mm^2 membrane surface area. Means within column with different superscripts differ significantly ($P < .05$).

[B]Standard error for difference.

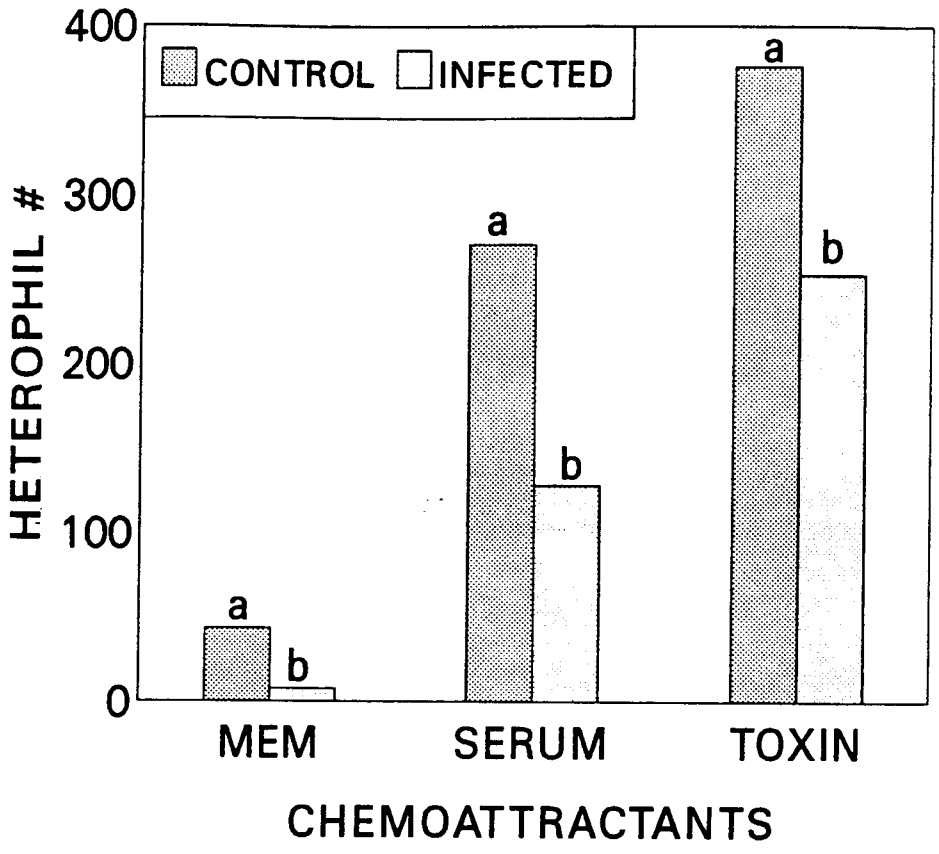

Figure 2: Chemotactic response of staphylococcal infected and healthy control chickens heterophils to three different treatments. Bars with different letters are significantly different from each other at a=0.05 (Fisher's protected LSD).

uninfected and infected chickens, while PCV and plasma protein were not significantly different in uninfected and infected chickens.

DISCUSSION

Values for heterophil random migration and chemotactic movement for serum and endotoxin activated serum were all greater in healthy chickens as compared to heterophils from staphylococcal infected chickens (Table 2). In our results, functional defects in heterophil function were demonstrated in staphylococcal infected chickens by showing decreased chemotaxis.

Even though heterophil chemotaxis was decreased in infected chickens *in vitro*, histologic sections showed that some heterophils were capable of adhering to the endothelial lining, and arriving at the site of infection/inflammation.

Our results differ from the results of Andreasen, 1991 in which heterophil chemotaxis was increased in experimental staphylococcal joint infections. The decrease in chemotactic response might be due to certain changes in the heterophils, as there might not be any initiation of cytokines. During bacterial infection in humans, increase in chemotactic response occurs which might be mediated by the cytokines (Hill, et al., 1974). In Andreasen's experiment, they infected the chickens experimentally, knowing the course of the disease, while in our case the chickens were naturally infected and we do not know the course of the disease. Kibenge, et al., 1983, reported that pathogenesis of the disease observed in the experimental infections was different from that considered to occur in the natural cases of tenosynovitis. In experimentally infected chickens, the onset of the lameness was

Table 2: Effect of bird condition on heterophil chemotaxis

Condition	Average Cell Count[A]
Control	200.56[b]
Infected	100.14[a]
SED[B]	0.99

[A]Average of mean counts of heterophils/0.25mm^2 membane surface area. Means within column with different superscripts differ significantly ($P < .05$).
[B]Standard error for difference.

preceded by acute depression, fever and other signs of septicemia, while in naturally occurring cases of tenosynovitis, lameness was the initial sign.

Staphylococcal alpha hemolysin decreases neutrophil chemotaxis effect on neutrophils (Adlam and Easmon,1983). It is possible that naturally infected chickens remained infected over a longer period of time than experimentally infected chickens and had more exposure to alpha hemolysin which might have resulted in functional defects. Also possibly, early infection of 6-7 days duration, as in Andreasen's experimental model, induces peripheral heterophils to actively migrate to the site of infection. A longer chronic infection may produce circulatory factors which influence heterophils to have decreased chemotaxis and therefore remain in the area of infection. The chickens were raised in the broiler house under a totally different environment in Andreasen's experiment, while in this study chickens were raised under field conditions.

Numerous heterophils were present in the tendons and bones on histologic sections, however it is unknown what percentage of available heterophils this represents.

Even though heterophils arrive at the site of infection by chemotaxis, bacterial infections such as staphlococcosis may persist if decreased phagocytosis or bacterial killing occur. These functions were not evaluated in this study.

Additional research could be done to try to clarify the reason for a difference in results of heterophil chemotactic response between chickens naturally and experimentally infected with staphylococcosis.

CHAPTER 2

CHEMOTACTIC EFFECT OF THREE DIFFERENT STRAINS OF STAPHYLOCOCCI ON HEALTHY CHICKEN HETEROPHILS

ABSTRACT

Staphylococci are a major cause of tenosynovitis and osteomyelitis in broilers and broiler breeders throughout the world. The pathogenesis of the disease is not known. Avian heterophils are analogous to mammalian neutrophils but the granules appear to be different. Commercial broiler chickens were obtained to serve as heterophil donors. Chemotaxis of heterophils isolated from the peripheral blood was performed using Boyden chamber technique. As chemoattractants, three different isolates of staphylococci were used. Isolate 921 and isolate 5658a are *Staphylococcus aureus* and have capsule type 8 and 5, respectively and are coagulase positive, while strain 6969b is *Staphylococcus xylosus* which is coagulase negative and capsule is not typed. As a measure of their chemotactic response heterophil counts were made microscopically from membrane filters. The three staphylococcal organisms caused chemotaxis to different degrees.

INTRODUCTION

Staphylococcal infections are national and international problem in the poultry industry. Staphylococcosis in poultry and other avian species has been recognized for nearly one hundred years. This problem causes economic losses due to decrease in weight gain, egg production, and carcass condemnation (Mutalib, et al., 1982b).

The pathogenesis of the disease is not well defined. Infection, in most cases, involves skin wound or damaged mucous membrane (Mutalib, et al., 1982a; Mutalib, et al., 1982b, Skeeles, 1991). The most frequent sites for infection are bones, tendon sheaths, and leg joints. This infection has also been reported in other locations including skin, sternal bursa, yolk sac, heart, and eyelids (Skeeles, 1991). The disease is usually chronic and gives a poor response to antibiotics and immunization.

The avian heterophils are analogous to human neutrophils in their action as tissue phagocytes and in host defense against bacterial infections (Topp and Carlson, 1972c). The avian heterophil does not contain myeloperoxidase as do mammalian neutrophils. Despite lacking myeloperoxidase, the heterophil's capability for phagocytosis and bacterial killing has been shown by previous investigations (Brune, et al., 1972; Brune, et al., 1973). Increased heterophilic infiltration occurs in tendons and synovial membranes during staphylococcal infection.

Chemoattractiveness of bacteria for leukocytes varies with the bacterial strain. In one study, turkey heterophils were exposed to different strains of *Pasteurella multocida* using Boyden chamber technique and it was found that chemotactic factors

produced by the more pathogenic bacterial isolates induced greater heterophil migration *in vitro*. It is not known if infiltration of heterophils at the site of inflammation (the joint) is due to the staphylococcal organism itself, or the products elaborated by the bacteria or a combination of these factors. To investigate, we did a chemotactic study using Boyden chamber technique.

MATERIALS AND METHODS

CHICKENS

Day-old Arbor Acres X Peterson broiler chicks were obtained and raised with growth ration and water *ad libitum*. They were wing banded for individual identification. The chickens were used as heterophil donors between 6 to 8 weeks of age.

HETEROPHIL ISOLATION

Heterophil isolation was performed as described in chapter 1, except that larger samples (5 ml) of anticoagulated blood were used, which were split among 4 Borosilicate disposable culture tubes.

PREPARATION OF BACTERIA

Two of the staphylococcal bacteria were isolated from clinical cases of staphylococcal tenosynovitis and osteomyelitis in chickens from Georgia (921), and Oregon (5658a). Bacteria 6969b was a coagulase negative, non pathogenic bacteria which was isolated from a chicken's non-purulent hock joint and was considered to be a contaminant. Certain characteristics of these bacteria are shown in table 3.

The bacteria were stored at -70°C in Brucella glycerol broths. The bacteria were streaked on tryptose blood agar (5% sheep blood) and incubated at 37°C for 24 hours. Following incubation, a single colony was picked from each isolate and

TABLE 3: Characteristics of staphylococcal isolates

ISOLATE	COAGULASE	HEMOLYSIS	CAPSULE[A]	IDENTIFICATION[B]
921	+	B,narrow zone	8	*S. aureus*
5658A	+	B,wide zone	5	*S. aureus*
6969B	-	B,narrow zone	Not typed	*S. xylosus*

[A]Staphylococcal capsular types identified by three independent researchers using quanitative precipitin analysis. Double immunodiffusion was performed in 0.7% agarose (Karakawa, et al., 1988).
[B]Identification of bacteria was done using a commercial kit "Staph-Ident System" by Analytab Products, Plainview, NY, U.S.A.

inoculated into Brain Heart Infusion (BHI) broth (Oxoid). Inoculated BHI broths were incubated on a rotator at 37°C for 20 hours. After incubation, the bacteria were washed and resuspended in phosphate buffered saline without calcium or magnesium (PBS) two times at 900 X g for 15 minutes. Spectrophotometric determination of optical densities of bacterial suspensions was performed (Bausch and Lomb, Spectronic 20, U.S.A.) at 595 nm. Suspensions were adjusted to an optical density of 0.2. Titrations of the adjusted suspensions were performed. Isolate 921 had a range of 2.4 X 10^8 CFU/ml to 1.12 X 10^9 CFU/ml. Isolate 5658A had a range of 3.0 X 10^8 CFU/ml to 7.1 X 10^8 CFU/ml and isolate 6969b had a range of 2.4 X 10^8 CFU/ml to 7.3 X 10^8 CFU/ml bacteria/ml.

CHEMOATTRACTANT SOLUTIONS

Six chemoattractant solutions were used:

1) MEM (Negative control)

2) Pooled normal chicken serum (50 *u*l serum + 450 *u*l HBSS)

3) Endotoxin with chicken pooled serum as chemoattractant (125 *u*l serum +75 *u*l endotoxin solution +1500 *u*l HBSS). Endotoxin (lipopolysaccharide [LPS]) (0.001 g) from *E. coli* serotype 0111:B4 (Sigma Chemicals) was dissolved in 3.3 ml 0.9% normal saline making final concentration 60,606 U/ml.

4) Staph #921

5) Staph #5658a

6) Staph 6969b

The solutions i.e., MEM, serum, and endotoxin were filter sterilized. The bacterial suspensions (optical density 0.2) were filtered through low protein binding filters (0.2 *u*m pore diameter) (Gelman Sciences, 600 S. Wagner Road, Ann Arbor, MI 48106, U.S.A.). All the solutions were incubated at 37°C for 60 minutes in a CO_2 incubator (VWR 6000, Sheldon Manufacturing Inc., Cornelius, Oregon, U.S.A.) and were also heat inactivated for 30 minutes at 56°C in water bath.

CHEMOTAXIS ASSAY

By using the modified Boyden chamber technique, heterophil random (heterophil that traversed the filter in response to MEM), and total (heterophil that traversed in response to chemoattractants) movements were calculated. For each treatment, 2 blind wells were used (Andreasen, et al., 1991).

Chemoattractant solutions (185 *u*l) were placed in the lower portion of the blind wells, while 185 *u*l (1.5 X 10^6 heterophils/ml) of heterophil suspension were placed in the upper chambers, separated by 3 *u*m average pore diameter polycarbonate filter (Costar Nucleopore Filtration Prod., Pleasanton, California 94588-8008, U.S.A.). These wells were incubated at 41°C for 45 minutes in a humid 5% CO_2 incubator. Following incubation, the filters were removed and placed on glass slides, air dried, stained with Diff Quik stain (Baxter Healthcare Corporation, Scientific Products Division, Mc. Gaw Park, Il 60085-6787, U.S.A.) and then permanently mounted. Random and total heterophil movement on filter membrane

were quantitated microscopically using the 100X oil immersion objective and a calibrated grid. Values were expressed as the mean number of heterophils/0.25 mm^2 membrane surface area.

STATISTICAL ANALYSIS

A square root transformation was used to transform the cell counts. The transformation was necessary to satisfy the normality assumption associated with the F-tests of the analysis of variance (ANOVA). The SAS General Linear Model (GLM) procedure was used to fit the model to the data (SAS Institute, 1982). Fischer's Protected Least Significant Difference (FPLSD) was used for mean separation.

RESULTS

Analysis of data (Appendix table A.2) showed a highly significant effect (Table 4) of chemoattractants on chicken heterophils. Results obtained with control chemoattractants MEM, normal chicken serum, and endotoxin are similar to those obtained previously (see chapter 1) indicating that chemotaxis is reproducible. Multiple mean comparison suggested that isolate 5658a had the highest chemotactic activity and was significantly different from all other treatments (Figure 3). Isolates 921 and 6969b were significantly different from each other. Isolate 921 and Endotoxin were not significantly different from each other, but were significantly different from other treatments. Isolate 6969b and serum treatments were not significantly different from each other, but were significantly different from other treatments. Minimum essential medium had the lowest chemotactic activity of all the treatments and was significantly different from all other treatments.

Table 4: Chemotactic effect of six treatments on chicken[A] heterophils

Treatments	Average cell count[B]
MEM	36.24[d]
Serum	149.82[c]
Endotoxin	267.94[b]
Isolate #921	283.79[b]
Isolate #5658a	363.55[a]
Isolate #6969b	167.18[c]
SED[C]	0.60

[A]Healthy/Non-infected chicken were used.
[B]Average of mean counts of heterophils/0.25mm^2 membrane surface area. Means within column with different superscripts differ significantly ($P < .05$).
[C]Standard error for difference.

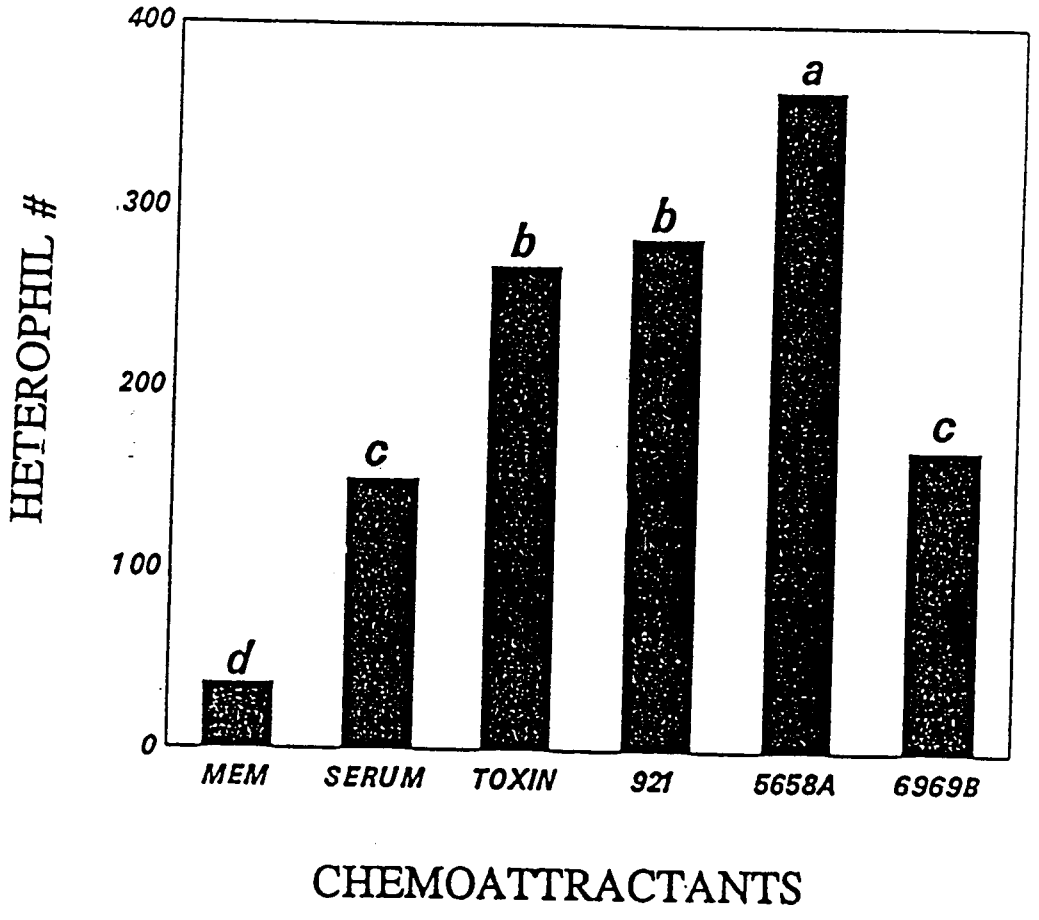

Figure 3: Chemotactic response of healthy chicken heterophils to six different chemoattractants. Bars with different letters are significantly different from each other at a=0.05 (Fisher's protected LSD).

DISCUSSION

A virulence factor for *Staphylococcus aureus* is resistance to phagocytosis due to bacterial capsules. When capsules are present, they may prevent phagocytosis by blocking classical and alternative complement attack and anti-peptidoglycan antibodies. Eleven antigenically distinct capsular polysaccharides are recognized for *Staphylococcus aureus*. Two of these, type 5 and type 8, comprise about 70% of the isolates from human patients having *Staphylococcus aureus* infection. Avian isolate 5658a has capsule type 5 and isolate 921 has capsule type 8. Capsule type 5 constitutes 22% of human isolates examined, while capsule type 8 constitutes 53% of human isolates examined (Albus, et al., 1991). *Staphylococcus aureus* isolates from cows with mastitis are predominantly serotypes 5 and 8 (Sutra and Poutrel, 1990). Recently, over 100 isolates of *Staphylococcus aureus* from invasive bacterial diseases (primarily joint, bone, and tendon) in chickens and turkeys were serotyped and found to be capsular type 5 and 8 (unpublished data, Dr. Robert Daum).

The data analysis shows that each bacterial strain or isolate was capable of generating chemotactic factors (Table 4). These findings suggest that staphylococcal infection in chickens can result in chemotactic factor generation with heterophil localization within infected tissues. *In vivo* studies using the chicken support this speculation in that inoculation of *Staphylococcus aureus* intravenously resulted in rapid localization of heterophils in the joints (Andreasen, et al., 1991).

A similar kind of chemotaxis experiment was done with different strains of *Pasteurella multocida* showing that the most pathogenic bacteria had high chemotactic

activity using Boyden chamber technique (Latimer, et al., 1990). The analysis shows that isolate 5658a has higher chemotactic activity, which indicates that it may be more a pathogenic bacteria. This may indicate that more pathogenic staphylococcal bacteria induce a stronger chemotactic reaction. The relation of the chemotactic activity of the staphylococcal organism to virulence is still unknown but we do know the capsule is a virulence factor. The data suggests that capsule type 5 staphylococci might possess high pathogenicity because of having high chemotactic activity. This might indicate that capsule type 8 staphylococci are less pathogenic compared to capsule type 5 staphylococci. The pathogenicity would have to be evaluated by experimental infections. Isolate 6969b had less chemotactic activity than 921 and 5658a. This bacterium isolated from a non-purulent hock joint is considered non-pathogenic and causes no lesions.

LITERATURE CITED

Adlam C, Easmon CSF: Immunity and hypersensitivity to staphylococcal infection. In: Staphylococci and Staphylococcal infections. Volume 1 - Clinical and epidemiological aspects. Easmon CSF, Adlam C, eds. Academic press, New York pp. 275-323, 1983.

Albus A, Arbeit RD, Lee JC: Virulence of *Staphylococcus aureus* mutants altered in type 5 capsule production. Infect Immun 59:1008-1014, 1991.

Alderson M, Speers D, Emslie K: Acute hematogenous osteomyelitis and septic arthritis -- a single disease. J Bone Joint Surg 68B:268-274, 1986.

Althaus D, Keller HU, Hess MW, Cottier H: Impaired neutrophil locomotion during acute bacterial infections. Int Archs Allergy Appl Immunol 61:321-328, 1980.

Anderson C: Veterinary aspects of staphylococci. In: Staphylococci and Staphylococcal infections. Volume 1 - Clinical and epidemiological aspects. Easmon CSF, Adlam C, eds Academic press, New York pp.194-241, 1983.

Andreasen CB: Heterophil function in healthy chickens and in chickens with staphylococcal tenosynovitis: Dissertation 1990.

Andreasen CB, Latimer KS: Separation of avian heterophils from blood using Ficoll-Hypaque discontinuous gradients. Avian Dis 33:163-167, 1989.

Andreasen CB, Latimer KS, Harmon BG, Glisson JR, Golden JM, Brown J: Heterophil function in healthy chickens and in chickens with experimentally induced staphylococcal tenosynovitis. Vet Pathol 28:419-427, 1991.

Brickman TJ, Kier AB, Collier LL: In vitro demonstration of defective neutrophil chemotaxis in Chediak-Higashi affected cats (Abstract). Fed Proc 43:390, 1984.

Brune K, Leffell MS, Spitznagel JK: Microbicidal activity of peroxidaseless chicken heterophil leukocytes. Infect Immun 5:283-287, 1972.

Brune K, Spitznagel JK: Peroxidaseless chicken leukocytes: Isolation and characterization of antibacterial granules. J Infec Dis 127:84-94, 1973.

Campbell TW: Avian Hematology and Cytology. Iowa State University Press, Ames, Iowa, pp.10-13, 1988.

Caxton-Martins AE, Daimon T: Histochemical observations on chicken blood and bone marrow cells. J Anat 122:553-558, 1976.

Chang C-F, Hamilton PB: Impaired phagocytosis by heterophils from chickens during aflatoxicosis. Tox Appl Pharm 48:459-466, 1979.

Chang C-F, Hamilton PB: Impairment of phagocytosis by heterophils from chickens during ochratoxicosis. Appl Environ Microbiol 39:572-575, 1980.

Daimon T, Caxton-Martins A: Electron microscopic and enzyme cytochemical studies on granules of mature chicken granular leukocytes. J Anat 123:533-562, 1977.

Dhingra LD, Parrish WB, Venzke WG: Electron microscopy of granular leukocytes of chicken (Gallus domesticus). Am J Vet Res 30:637-642, 1969.

Dieterlene-Lievre, F: Birds. In Vertebrate Blood Cells. Rowley, AF, Ratcliffe NA, ed. Cambridge University Press, New York, pp.296-298, 1988.

Ferris M, Bacha WJ: A new method for the identification and enumeration of chicken heterophils and eosinophils. Avian Dis 28:179-182, 1984.

Gallin JI, Gallin EK, Malech HL, Cramer EB: Structural and ionic events during leukocyte chemotaxis. In: Leukocyte Chemotaxis: Methods, Physiology, and Clinical Implicat ions. Gallin, JI, Quie PG, eds. Raven Press, New York pp.123-141, 1978.

Glick B, Madyastha P, Koger B, La Via MF: The use of Ficoll-Hypaque double density gradients in the separation of avian granulocytes from other cell types for the purpose of cell flow cytometric analysis. Devel Comp Immunol 9: 477-484, 1985.

Goetzl EJ, Rocklin RE: Amplification of the activity of human leukocyte inhibitory factor (LIF) by the generation of a low molecular weight inhibitor of PMN leukocyte chemotaxis. J Immunol 121:891-896, 1978.

Hill HR, Gerrard JM, Hogan NA, Quie PG: hyperactivity of neutrophil leukotactic responses during active bacterial infection. J Clin Invest 53:996-1002, 1974.

Hill JE, Rowland GN, Glisson JR, Villegas P: Comparative microscopic lesions in reoviral and staphylococcal tenosynovitis. Avian Dis 33:401-410, 1989.

Jain NC: Schalm's Veterinary Hematology, 4th ed., Lea & Febiger, Philadelphia, pp.737-738, 1986.

Jensen MM, Downs WC, Morrey JD, Nicoll SD, LeFevre SD, Meyers CM: Staphylococcosis of turkeys . 1. Portal of entry and tissue colonization. Avian Dis 31:64-69,1987.

Karakawa WW, Sutton A, Schneerson R, Karpas A, Vann WF: Capsular antibodies induce type specific phagocytosis of *Staphylococcus aureus* by human polymorphnuclear leukocytes. Infec. Immun 56:1090-1095, 1988.

Karakawa WW, Fournier JM, Vann, WF, Arbeit R, Schneerson RS, Robbins JB: Method for serological typing of the capsular polysaccharides of *Staphylococcus aureus*. J Clin Microb 22:445-447, 1985.

Kibenge FSB, Robertson MD, Wilcox GE: *Staphylococcus aureus* isolated from poultry in Australia, II. Epidemiology of strains associated with tenosynovitis. Vet Microbiol 7:485-491, 1982.

Kibenge FSB, Wilcox GE, Pass DA: Pathogenecity of four strains of staphylococci isolated from chickens with clinincal tenosynovitis. Avian Pathol 12:213-220, 1983.

Latimer KS, Prasse KW, Mahaffey EA, Dawe DL, Lorenz MD, Duncan JR: Neutrophil movement in selected canine skin diseases. Am J Vet Res 44:601-605, 1983.

Latimer KS, Tang K-N, Goodwin MA, Steffens WL, Brown J: Leukocyte changes associated with acute inflammation in chickens. Avian Dis 32:760-772, 1988.

Latimer KS, Kircher IM, Andreasen CB: Separation of turkey heterophils from blood using two-step Ficoll-Hypaque discontinuous gradients. Avian Dis 33:571-573, 1989a.

Latimer KS, Kircher IM, Lindl PA, Dawe DL, Brown J: Leukocyte function in Pelger-Huet anomaly of dogs. J Leukocyte Biol 45:301-310, 1989b.

Latimer KS, Harmon BG, Glisson JR, Kircher IM, Brown J: Turkey heterophil chemotaxis to *Pasteurella multocida* (serotype 3,4)-generated chemotactic factors. Avian Dis 34:137-140, 1990.

LeFevre SD, Jensen MM: Staphylococcosis of turkeys. 2. Assay of protein A levels of staphylococci isolated from turkeys. Avian Dis 31:70-73, 1987.

Malech HL, Root RK, Gallin JI: Structural analysis of human neutrophil migration. Centriole, microtubule, and microfilament orientation and function during chemotaxis. J Cell Biol 75:666-693, 1977.

Mateo MR, Roberts ED, Enright FM: Morphologic, cytochemical, and functional studies of peripheral blood cells of young healthy American alligators (*Alligator Mississippiensis*) Am J Vet Res 45:1046-1053, 1984.

Maxwell MH, Trejo F: The ultrastructure of white blood cells and thrombocytes of the domestic fowl Br Vet J 126: 583-592, 1970.

McCorkle FM, Simmons DG: In vitro cellular migration of leukocytes from turkey poults infected with *Alcaligenes faecalis*. Avian Dis 28:853-857, 1983.

Meyers CM, Jensen MM: Staphylococcosis of turkeys. 3. Bacterial interference as a possible means of control. Avian Dis 31:74-79, 1987.

Miner ML, Smart RA, Olson AE: Pathogenesis of staphylococcal synovitis in turkeys: Pathologic changes. Avian Dis 12:46-60, 1968.

Murphy: The Neutrophil. Plenum Publishing Corporation, New York, pp.25-26, and 85-124, 1976

Mollby R: Isolation and properties of membrane damaging toxins. In: Staphylococcus and Staphylococci Infections. Volume 2 - The organism in vivo and in vitro. Easmon CSF, Adlam C, eds. Academic Press, New York pp. 619-669, 1983.

Montali RJ: Comparative pathology of inflammation in the higher vertebrates (reptiles, birds and mammals). J Comp Pathol 99:1-26, 1988.

Mutalib A, Riddel C, Osborne AD: Studies on the pathogenesis of staphylococcal osteomyelitis in chickens. 1. Effect of stress on experimentally induced osteomyelitis. Avian Dis 27:141-156, 1982a.

Mutalib A, Riddel C, Osborne AD: Studies on the pathogenesis of staphylococcal osteomyelitis in chickens. ll. Role of the respiratory tract as a route of infection. Avian Dis 27:157-160, 1982b.

Nagaraja KV, Newman JA, Pomeroy BS: Leukocyte migration inhibition in chickens inoculated with *Salmonella typhimurium*. Am J Vet Res 43:916-918, 1982.

Nairn ME: Bacterial osteomyelitis and synovitis of the turkey. Avian Dis 17:504-516, 1973.

Natt MP, Herrick CA: A new blood diluent for counting the erythrocytes and leukocytes of the chicken. Poult Sci 31:735-738, 1952.

Natt MP, Herrick CA: Variation in the shape of the rod-like granule of the chicken heterophil leukocyte and its possible significance. Poult Sci 33:828- 830, 1954.

Nicoll TR, Jensen MM: Staphylococcosis of turkeys. 5. Large scale control programs using bacterial interference. Avian Dis 31:85-88, 1987a.

Nicoll TR, Jensen MM: Preliminary studies on bacterial interference of Staphylococcosis of chickens. Avian Dis 31:140-144, 1987b.

Noble PB, Cutts JH: Isolation of individual leukocyte types from peripheral blood. J Lab Clin Med 72:533-538, 1968.

Oeding P: Taxonomy and identification. In: Staphylococci and Staphylococcal Infections. Volume 1 - Clinical and epidemiological aspects. Easmon CSF, Adlam C, eds. Academic Press, New York pp. 10-12, 1983.

Onyia KA: Leukocyte migration into cotton pellets: a new *in vivo* method for the study of chemotaxis and leukocyte migration. Comp Immunol Microbiol Infect Dis: 79-88, 1986.

Repo H, Vuopio P, Leirisalo M, Jansson SE, Kosunen TU: Impaired neutrophil chemotaxis in Pelger-Huet anomaly. Clin Exp Immunol 36:326-333, 1979.

Robertson GW, Maxwell MH: Modified stainig techniques for avian blood cells. British Poult Sci 31:881-886, 1990.

Rot A, Henderson LE, Sowder R, Leonard EJ: *Staphylococcus aureus* tetrapeptide with chemotactic potency and efficacy for human leukocytes. J Leukocyte Biol 45:114-120, 1989.

SAS Institute, SAS/STAT User's Guide. SAS Institute Inc., Cary, N.C., 1988.

Sedgwick AD, Dawson J, Lees P: Influence of chemotactic agents on the locomotion of equine polymorphonuclear and mononuclear leukocytes. Res Vet Sci 43:55-58, 1987.

Sheagren JN: *Staphylococcus aureus* - The Persistant Pathogen 1. New Engl J Med 310:1368-1373, 1984.

Skeeles: Staphylococcosis, In: Diseases of poultry. Calnek BW, Barnes HJ, Beard CM, Reid WM, Yoder HW eds. Iowa State University Press, Ames, Iowa pp. 293-299, 1991.

Styrt B: Species variation in neutrophil biochemistry and function. J Leukocyte Biol 46:63-74, 1989.

Sutra 1, Poutrel B: Detection of capsular polysaccharides in milk of cows with natural intramammary infection caused by *Staphylococcus aureus*. Am J Vet Res 51:1857-1859, 1990.

Territo, MC: Chemotaxis. In: Leukocyte Function. Cline MJ, ed Churchill Livingstone, New York, pp.39-51, 1981.

Thies ES, Nelson RD, Maheswaran SK: Isolation of the turkey heterophil and measurement of its migratory functions under agarose. Am J Vet Res 44:288-292, 1983.

Topp RC, Carlson HC: Studies on avian heterophils. l. cell separation. Avian Dis 16:364-368, 1972a.

Topp RC, Carlson HC: Studies on avian heterophils. ll. histochemistry. Avian Dis 16:369-373, 1972b.

Topp RC, Carlson HC: Studies on avian heterophils. 111. Phagocytic properties Avian Dis. 16:374-380, 1972c.

Trowell JE, Brewer DB: Degranulation of chicken heterophil leukocytes during phagocytosis, studied by phase contrast and interference microscopy. J Pathol 120:129-144, 1976.

Vandenbroucke-Grauls CMJE, Thijssen HMWM, Verhoef J: Interaction between human polymorphonuclear leucocytes and *Staphylococcus aureus* in the presence and absence of opsonins. Immunol 52:427-435, 1984.

Wilkinson PC, Allan RB: Assay systems for measuring leukocyte locomotion: An overview. In: Leukocyte chemotaxis: Methods, physiology, and clinical implications. Gallin, JI, Quie PG, eds. Raven Press, New York pp. 1-24, 1978.

Wilkinson DM, Jensen MM: Staphylococcosis of turkeys. 4. Characterization of a bacteriocin produced by an interfering staphylococcus. Avian Dis 31:80-84, 1987.

Wilkinson PC: Microorganisms and leukocyte locomotion: stimulatory and inhibitory effects. In: Chemotaxis and Inflammation. Wilkinson PC, ed. Churchill Livingstone, New York pp. 143-155, 1982.

Young S, Beswick PA: A comparison of the oxidative reactions of neutrophils from a variety of species when stimulated by opsonized zymosan and F-met-leu-phe. J Comp Pathol 96:189-196, 1986.

Zigmond SH: A model for understanding millipore filter assay systems. In: Leukocyte Chemotaxis: Methods, Physiology and Clinical Implications. Gallin JI, Quie PG, eds. Raven Press New York, pp.87-95, 1978.

Zigmond SH, Sullivan SJ: Receptor modulation and its consequences for the response to chemotactic peptides. In: Biology of the chemotactic response. Lackie JM, Wilkinson PC eds. Cambridge University Press, Cambridge pp.73-87, 1981.

APPENDICES

Table A.1: Analysis of Variance for chemotactic effect of three treatments on healthyandstaphylococcal infected chicken heterophils

Source	DF	SS	MS	F-value	P
Health	1	230.20	230.20	17.53	.0041
Trt[1]	2	1596.18	798.09	246.73	.0001
Health*Trt	2	6.93	3.46	1.07	.3691

[1]Treatments: MEM, Serum, Endotoxin.

Table A.2: Analysis of Variance table for chemotactic effect of six treatments on healthy chicken heterophils

Source	DF	SS	MS	F-value	P
Trt	5	2704.45	540.89	56.65	.0001