Avian Heterophils in Inflammation and Disease Resistance

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ABSTRACT Heterophils are the predominate granulocytic leukocyte in the acute inflammatory response in gallinaceous birds. Heterophils are highly phagocytic and are capable of a broad spectrum of antimicrobial activity. They accumulate in inflamed tissue, causing tissue damage and forming heterophil granulomas that are morphologically similar to inflammatory lesions in reptiles. The avian heterophil lacks myeloperoxidase and depends primarily on nonoxidative mechanisms for antimicrobial activity. The beta-defensins found in heterophil granules can kill a wide variety of bacterial pathogens and are a major component of the heterophil antimicrobial arsenal. Heterophils form the first line of cellular defense against invading microbial pathogens in the lungs and air sacs where resident macrophages are lacking.

(Key words: heterophils, avians, phagocytes, inflammation, beta-defensins)

INTRODUCTION

The avian heterophil has been declared a counterpart to the neutrophil in mammals. Although there are many similarities between these two granulocytes, there also are important differences. The heterophilic inflammatory response in avian species more closely resembles the reptilian response than the mammalian response (Montali, 1988). The few studies that have been conducted with avian heterophils demonstrate a functional role that is similar to that of neutrophils in mammals, but differences exist in granule components and in the pathogenesis of inflammatory lesions to which heterophils contribute (Carlson, 1982; Montali, 1988). When compared to the experimental work on neutrophils and the inflammatory response in mammals, similar work in birds and reptiles is rather meager. Therefore, much about avian and reptilian heterophils has been inferred from neutrophil studies in mammals. The focus of this presentation is to summarize and update the available information on avian heterophils and their role in inflammation and disease resistance.

THE AVIAN HETEROPHIL IN ACUTE INFLAMMATION

The hematologic response to inflammatory stimuli has been studied in chickens and turkeys using irritants, lipopolysaccharide (LPS), and various infectious agents.
The hematologic response of turkeys to intravenous administration of LPS has recently been characterized (Carmichael et al., unpublished data). Immediately following LPS injection, there is a transient leukopenia due to lymphopenia (2 to 4 h after injection) followed by a rebound leukocytosis due to heterophils (between 8 and 24 h after injection). This response differs from the mammalian response to endotoxin in that the leukopenia in mammals is primarily due to neutropenia instead of lymphopenia (Morrison and Ulevitch, 1978; Olson et al., 1988). However, the rebound leukocytosis with heterophilia is consistent with responses to endotoxin in mammals, where the rebound leukocytosis or neutrophilia is due to the release of granulocyte-colony-stimulating factor (CSF) and granulocyte-macrophage-CSF from macrophages (Morrison and Ulevitch, 1978; Olson et al., 1988). It is not clear why turkeys become profoundly lymphopenic after LPS injection. The mild to moderate depression in circulating heterophils, prior to the rebound, is probably due to sequestration of heterophils in the microvasculature of the lung and in hepatic sinusoids (Carmichael et al., unpublished data).

The morphology of the acute inflammatory response in avian tissues has been described in several experimental models of inflammation. Using artificial irritants and infectious agents, investigators have demonstrated a predominance of heterophils in the acute exudative phase of inflammation (Jortner and Adams, 1971; Carlson, 1972; Awadhya et al., 1980; Toth et al., 1987; Ficken and Barnes, 1989; Prantner et al., 1990a,b; Fulton et al., 1993; Kogut et al., 1993; Mendes et al., 1994). Some researchers have observed that lymphocytes and basophils also are part of the early inflammatory infiltrate (Katiyar et al., 1992; Mendes et al., 1994). This influx of heterophils is the first line of cellular defense in the avian respiratory tract because there is no resident population of pulmonary macrophages (Toth and Siegel, 1986; Ficken and Barnes, 1989).

Based on histopathology, we assume that the sequence of events for avian granulocyte emigration into tissue is similar to that in mammals. Granulocytes in mammals marginate in the vessels, interact with endothelial receptors, emigrate through the vascular wall, and follow chemotactic gradients to accumulate at the site of inflammation (Edwards, 1994). Changes in hemodynamics are responsible for early neutrophil margination, and cytokines cause increased expression of leukocyte and endothelial adhesion molecules. These molecules (selectins and integrins) mediate leukocyte adhesion to the vascular wall (Edwards, 1994).

Mast cell-mediated vasodilation and increased vascular permeability has been described in mesenteric vessels of chickens given intraperitoneal turpentine (Awadhya et al., 1980). Heterophil accumulation follows these vascular changes. Heterophil-endothelial cell interactions at the receptor level have not been studied in birds, but heterophils are commonly observed adhered to and migrating through vascular walls in microscopic sections of inflamed tissue. Heterophil chemotaxis has been demonstrated for chicken and turkey heterophils (Thies et al., 1983; Latimer et al., 1990; Andreasen et al., 1993). Avian heterophils are chemotactic for endotoxin and zymosan activated plasma (probably due to plasma derived complement components-C5a or C5a des-arg). Similarly, chemotactic factors are generated by mixing whole bacteria (Staphylococcus aureus or Pasteurella multocida) with serum or plasma (Latimer et al., 1990; Andreasen et al., 1993).

The pathogenesis of lesions as a result of heterophil infiltration in birds and reptiles differs markedly from that created by neutrophils in mammals (Montali, 1988). In mammals, neutrophil accumulation commonly progresses to liquefaction and abscess formation, suppurative exudate that migrates along tissue planes, or exudates that make their way out via clearance pathways such as the muco-ciliary apparatus in the respiratory tract. In birds and reptiles, the pathway to resolution involves inspissation of the necrotic heterophils into a caseous mass rather than liquefaction (Carlson, 1982; Montali, 1988; Klasing, 1991). These necrotic heterophils are walled-off by epithelioid macrophages and fibrous connective tissue to form heterophil granulomas.

Typically, heterophil infiltrates predominate in the first 6 to 12 h of the inflammatory response, but macrophages, lymphocytes, and even giant cells are present at 48 h and giant cells are numerous at 72 h (Jortner and Adams, 1971); epithelioid macrophages are then surrounded by fibroblasts. This method of isolating pathogens or irritants is advantageous, except when the caseous mass interferes with organ function, such as is the case in the lungs and digestive tract. In these locations, a suppurrative exudate might be more readily and completely resolved, whereas a caseous mass is likely to persist indefinitely. Currently, it is not known why heterophilic exudates do not liquefy or why there is an acute massive epithelioid macrophage response to heterophil exudates. As in mammals, a massive influx of granulocytes into tissue results in direct tissue damage from release of granule contents into surrounding tissue (Ficken and Barnes, 1989; Fulton et al., 1993). Leukocyte-mediated tissue damage can be the primary cause of organ failure even though the disease was initiated by an infectious agent.

**ANTIMICROBIAL ACTIVITY OF AVIAN HETEROPHILS**

Once heterophils reach the offending microbial agents in tissue, clearance depends on phagocytosis and the microbicidal mechanisms of the heterophils. Heterophils readily phagocytose a variety of microbial agents *in vitro* (Topp and Carlson, 1972; Harmon *et al*., 1991; Stabler *et al*., 1994). It is apparent that as part of the inflammatory response, heterophils are activated by cytokines and possible chemokines to increase their phagocytic and
microbicidal activity (Andreasen et al., 1991; Kogut et al., 1994). Circulating heterophils from chickens with staphylococcal tenosynovitis have greater phagocytic activity than heterophils from healthy chickens (Andreasen et al., 1991). In vitro, heterophils seem to be more phagocytic for *P. multocida* and for *Salmonella enteritidis* than are macrophages or monocytes (Harmon et al., 1991; Stabler et al., 1994). In addition, heterophil phagocytosis and bactericidal activity for some bacteria appears to be less dependent on opsonization than that for macrophages or monocytes (Harmon et al., 1992; Stabler et al., 1994).

Avian heterophils depend primarily on nonoxidative microbicidal mechanisms. The measurable oxidative response in heterophils is quite meager when compared to the oxidative response to phagocytosis in mammalian neutrophils. When exposed to phagocytic stimuli, chicken heterophils undergo a respiratory burst and oxidize glucose, but fail to produce increased amounts of hydrogen peroxide or superoxide anion (Penniall and Spitznagel, 1975; Stabler et al., 1994). When challenged with phagocytic stimuli in vitro, chicken heterophils produced little hydrogen peroxide when compared directly to neutrophils from dogs and humans. (D. Bounous, University of Georgia, Athens, GA 30602, personal communication.) Avian heterophils do not contain catalase (Breton-Gorius et al., 1978). Furthermore, avian heterophils lack myeloperoxidase, which potentiates microbicidal capacity of neutrophils by utilizing hydrogen peroxide and chloride ion for hypochlorite-mediated halogenation (Brune et al., 1972; Brune and Spitznagel, 1973; Penniall and Spitznagel, 1975; Styrt, 1989). Despite the absence of an efficient oxidative microbicidal mechanism, heterophils are quite effective in killing bacteria (Brune et al., 1972, Brune and Spitznagel, 1973; Andreasen et al., 1991; Harmon et al., 1992; Stabler et al., 1994). Therefore, it is likely that heterophils rely primarily on oxygen-independent mechanisms for antimicrobial activity (Stabler et al., 1994).

Investigations into the oxygen-independent microbicidal mechanisms of heterophils have focused on granule contents. Avian heterophil granule contents differ from those found in mammalian neutrophils (Table 1). There appear to be at least two major types of heterophil granules and possibly three. The largest granules are elliptical (0.8 to 3 μm) and contain cationic peptides, lysozyme, and acid phosphatase, but lack peroxidase and alkaline phosphatase, which are common components of mammalian neutrophil primary granules (Brune and Spitznagel, 1973; Rausch and Moore, 1975; Daimon and Caxton-Martins, 1977; Fujimori and Yamada, 1978; Maxwell, 1984; Styrt, 1989). Smaller spherical granules (0.2 to 1 μm) contain the acid hydrolases such as β-glucuronidase (Brune and Spitznagel, 1973). Cathepsin and α-glucosidase activities are present in heterophils, but have not been assigned to either of the two types of granules (Brune and Spitznagel, 1973; Fujimori and Yamada, 1978). It should not be surprising that the granule contents of avian heterophils differ from those of mammalian neutrophils. After all, there is plenty of heterogeneity in neutrophil granule types and granule contents among mammalian species (Styrt, 1989).

The known microbicidal components of avian heterophils include the cationic peptides and lysozyme. The presence of cationic proteins with bactericidal activity in heterophil granules was demonstrated more than 20 yr ago, but the proteins were not purified or characterized (Zeya and Spitznagel, 1969, 1971; Brune and Spitznagel, 1973). These cationic proteins are found in the large elliptical granules (MacRae and Spitznagel, 1975; MacRae and Powell, 1979). Crude extracts from heterophil granules kill *E. coli*, *Serratia marcescens*, and *Staphylococcus albus* in vitro at concentrations of 20 to 35 μg/mL (Brune et al., 1973). Lysed heterophils are capable of killing *E. coli* and *S. aureus* in vitro, but are not effective in killing *P. multocida*; however, intact heterophils kill all three bacteria (Harmon et al., 1992). These findings may indicate that heterophils can kill some bacteria by releasing granule contents extracellularly and that intact heterophils are required to kill other microbes.

Members of one group of avian heterophil-granule antimicrobial peptide have been purified and characterized. These peptides are called gallinacins (Gal 1-α, Gal 1, Gal 2), chicken heterophil antimicrobial peptides (CHP 1, CHP 2) and turkey heterophil antimicrobial peptides (THP 1, THP 2, THP 3) (Harwig et al., 1994; Evans et al., 1994). All eight of these peptides belong to the β-defensin class of antimicrobial peptides (Table 2). These β-defensins are larger than classical defensins and have three cysteine-based intramolecular disulfide bonds that differ from the classical defensins found in humans, rabbits, rats, and guinea pigs (Harwig et al., 1994; Martin et al., 1995). β-Defensins have 38 to 42 amino acid residues, six invariant cysteines, two invariant glycines and one invariant proline (Harwig et al., 1994). Defensins are primarily synthesized as pre-pro-peptides during maturation in the bone marrow

### Table 1. Comparison of avian heterophil granule contents with human neutrophil granule contents

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<thead>
<tr>
<th>Granule product</th>
<th>Human neutrophil</th>
<th>Avian heterophil</th>
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<tbody>
<tr>
<td>Myeloperoxidase</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>Defensins</td>
<td>++</td>
<td>–</td>
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<tr>
<td>β-Defensins</td>
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<td>–</td>
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<tr>
<td>Cathepsin</td>
<td>+</td>
<td>+</td>
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<td>Lysozyme</td>
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<tr>
<td>Catalase</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Acid phosphatase</td>
<td>+</td>
<td>±</td>
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<tr>
<td>Alkaline phosphatase</td>
<td>+</td>
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</tr>
<tr>
<td>β-Glucuronidase</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>α-Glucosidase</td>
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<td>+</td>
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1+ = present; – = absent.
(Michaelson et al., 1992). However, treatment of mature human neutrophils with granulocyte-macrophage CSF results in a twofold increase in recovery of cationic proteins and enhanced killing of Salmonella typhimurium (Waksman et al., 1990). Thirteen β-defensins have been isolated from bovine neutrophils, one from bovine respiratory mucosa, and one from the plasma of human blood (Selsted et al., 1993; Diamond et al., 1991; Bensch et al., 1995) (Table 2). Bovine neutrophil β-defensins are located in large granules that are unique to bovine neutrophils (Selsted et al., 1993).

Despite the differences in length, amino acid sequence and spacing of the cysteines, the tertiary structure of the β-defensins is similar to that of the classical defensins (Zimmermann et al., 1995). β-Defensins consist of a triple-stranded, antiparallel β-sheet (Zimmerman et al., 1995). The molecule has a cationic/hydrophobic amphiphilic character that is consistent with all defensins (Zimmerman et al., 1995). This structure allows defensins to bind to the negatively charged sites on microbial membranes and create lethal voltage gated ion channels in the membrane (Kagan et al., 1990). Once bound to anionic sites on the microbial membrane, defensins are pulled into the membrane by negative charges on the inner membrane leaflet. Dimer formation creates a channel between the amino-terminal β strands of the two defensin monomers (Hill et al., 1991).

As a group, defensins have a broad spectrum of activity against Gram-positive and Gram-negative bacteria, protozoa, fungi, and even some enveloped viruses (Lehrer et al., 1993; Aley et al., 1994). Chicken Gal 1, 1-α, and 2 have been shown to kill E. coli and Listeria monocytogenes in vitro. Gallinacins 1 and 1-α kill Candida albicans, but Gal 2 does not (Harwig et al., 1994). Chicken heterophil peptide-1, CHP-2, and THP-1 have antimicrobial activity against C. albicans, Bordetella avium, E. coli, S. enteritidis, Salmonella typhimurium, Campylobacter jejuni, P. multocida, and Mycoplasma gallisepticum (Evans et al., 1995). Turkey heterophil peptide-3 is less effective against all Gram-negative bacteria at the concentrations tested and higher concentrations of all the peptides are required to kill P. multocida (Evans et al., 1995). Based on these findings, heterophils, having a full complement of these antimicrobial peptides, should be able to kill these pathogens in vivo.

In Sprague Dawley rats, four distinct defensin phenotypes are identified (Eisenhauer et al., 1990). If such phenotypes exist in commercial poultry, these defensin phenotypes could vary in their disease resistance capability. Investigations comparing the susceptibility of pathogenic and nonpathogenic bacteria to bactericidal activity of avian defensins are needed to determine whether resistance to these antimicrobial peptides is associated with virulence for avian pathogens. Resistance of S. typhimurium to killing by defensins does appear to be associated with virulence in a mouse model (Groisman et al., 1992; Parra-Lopes et al., 1993). The discovery that mouse intestinal defensins (cryptdins) can kill Giardia lamblia trophozoites may have implications for defense mechanisms against coccidia in poultry (Aley et al., 1994). It will be interesting to test avian antimicrobial peptides against pathogenic Eimeria sp. The avian β-defensins are probably just one group of many heterophil antimicrobial mechanisms that await discovery. In the process of purifying these β-defensins, other antimicrobial fractions from heterophil granules have been detected, but have not yet been purified or characterized (Harmon, unpublished data).

Because preventive medicine is emphasized in poultry medicine and therapies to modify or ameliorate inflammation are not very practical in poultry species, studies to understand the heterophil and the acute inflammatory response in avian species have lagged behind those in humans and other mammals. More recently, it has become apparent that the heterophil and other first-line defense mechanisms activated during the acute inflammatory response have an important role in innate disease resistance. Therefore, it is important to understand these early defense mechanisms so that we do not inadvertently compromise their effectiveness by management practices. Furthermore, we may find ways in the future to manipulate these mechanisms to better protect birds from infectious diseases.

**REFERENCES**


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<table>
<thead>
<tr>
<th>β-Defensin</th>
<th>Species</th>
<th>Source</th>
<th>Reference</th>
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<tr>
<td>Bovine neutrophil β-defensins (BNBD 1–13)</td>
<td>Bovine</td>
<td>Neutrophil</td>
<td>Selsted et al., 1993</td>
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<td>Tracheal mucosa</td>
<td>Diamond et al., 1991</td>
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<tr>
<td>Gallinacins (Gal 1, Gal 1-α, Gal 2)</td>
<td>Chicken</td>
<td>Heterophil</td>
<td>Harwig et al., 1994</td>
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<td>Turkey heterophil antimicrobial peptides (THP1, 2, and 3)</td>
<td>Turkey</td>
<td>Heterophil</td>
<td>Evans et al., 1995</td>
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<tr>
<td>Chicken antimicrobial peptides (CHP 1 and 2)</td>
<td>Chicken</td>
<td>Heterophil</td>
<td>Evans et al., 1995</td>
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<tr>
<td>Human β-defensin 1 (bBD-1)</td>
<td>Human</td>
<td>Plasma</td>
<td>Bensch et al., 1995</td>
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