Morphology and Function of Toxic Neutrophils in the Dog.

Kent Alan Gossett
Louisiana State University and Agricultural & Mechanical College

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MORPHOLOGY AND FUNCTION OF TOXIC NEUTROPHILS IN THE DOG

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Interdepartmental Program in Veterinary Medical Sciences

by

Kent Alan Gossett
D.V.M., Purdue University, 1978
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ABSTRACT

Turpentine-induced inflammation was used as a model for the study of toxic neutrophils in the dog. Changes in the number of circulating toxic neutrophils paralleled changes in clinical signs, body temperature, total leukocyte count, and neutrophil count. Light microscopic appearance of neutrophils from turpentine-injected dogs was similar to toxic neutrophils seen clinically in dogs with severe bacterial or other inflammatory diseases. Ultrastructural studies showed that foamy vacuolation of the cytoplasm was due to irregular, electron-lucent areas which often contained membrane remnants and myelin figures. Increased cytoplasmic basophilia was due to the retention of rough endoplasmic reticulum and polyribosomes in mature toxic neutrophils. Bone marrow studies indicate that dilation of rough endoplasmic reticulum, followed by membrane disruption and degradation led to the formation of electron-lucent areas in mature toxic neutrophils. Morphologic evidence obtained from this study suggests that cytoplasmic immaturity, increased cellular activity, and cell degeneration may be involved in the formation of toxic neutrophils in the dog.

In vitro function tests on neutrophils from turpentine-injected dogs showed normal phagocytosis of Staphylococcus aureus, but diminished bactericidal activity after 30 minutes
of incubation. The biological significance of this
functional defect is uncertain due to the lack of correlation
between diminished bactericidal activity and toxic change,
body temperature, neutrophil number, or clinical signs.
CHAPTER I

Introduction

The major function of the neutrophil is to protect against pyogenic infection. Following bacterial invasion or tissue necrosis, neutrophils respond to chemotactic stimuli and migrate to the lesion. In tissue, they are capable of phagocytosis, bactericidal activity, and augmenting the inflammatory process. Production of neutrophils in the bone marrow is a dynamic process which increases in response to inflammation.

Granulopoiesis

Experimental evidence supports the existance of a stem cell, colony forming units-lymphoid-myeloid (CFU-L-M), capable of differentiating into erythrocytes, granulocytes, monocytes, megakaryocytes, and lymphoid cells.1 Studies in lethally irradiated mice demonstrate the existence of a more differentiated stem cell, colony forming units-spleen (CFU-S), capable of producing erythrocytic, granulocytic, monocytic, and megakaryocytic cells. CFU-S differentiate under the influence of their microenvironment, factors from helper lymphocytes, cell to cell interaction, and undefined humoral factors.3 Colony forming units-culture (CFU-C) are the first stem cells committed to granulopoiesis. These
cells have been cultured in vitro and differentiate into monocytes and granulocytes.

Adequate numbers of circulating neutrophils are maintained by a series of positive and negative feedback mechanisms. Colony stimulating activity (CSA), which is required for in vitro growth, is analogous to erythropoietin. CSA has been extracted from macrophages, monocytes, activated lymphocytes, and endothelial cells. Increased demand for monocytes or granulocytes is associated with elevated levels of CSA in serum and tissue. Humoral substances produced by monocytic cells in inflammatory lesions not only stimulate CFU-C into granulopoiesis and monocytopoiesis, but also induce production of additional CFU-C from pluripotent stem cells (CFU-L-M).

Neutrophil-releasing activity (NRA) has been found in animals given vinblastine sulfate, nitrogen mustard, or endotoxin. NRA induces neutrophilia by increasing the release of mature neutrophils from the bone marrow storage pool. In response to endotoxin, circulating leukocytes produce NRA while CSA is released from monocytic cells in tissue. Extracts from mature granulocytes specifically inhibit granulopoiesis. Granulocyte chalone is a polypeptide that inhibits DNA synthesis. A glycoprotein (possibly lactoferrin) derived from neutrophil granules blocks production or release of CSA from macrophages.
Neutrophil Kinetics

Factors influencing the number of circulating neutrophils are: 1) bone marrow production, 2) margination of blood neutrophils, 3) egress of cells from blood to tissue, and 4) senescence and removal of cells from the blood stream.

Bone Marrow Production

Total granulocytic mass in canine bone marrow has been estimated using radioiron dilution. This method quantitates the red cell compartment. Granulocytic mass as derived from granulocyte:erythrocyte ratio was $6.6 + 0.59 \times 10^9$ cells/kg body weight. Of this total, 21% were promyelocytes and myelocytes, 35% were metamyelocytes and bands, and 44% were segmented neutrophils. Myeloblasts, promyelocytes, and myelocytes are capable of mitosis and are referred to collectively as the "mitotic pool". Metamyelocytes, bands, and segmented neutrophils cannot divide and comprise the "post-mitotic pool". The "storage pool" is composed of band and segmented neutrophils which are released to the blood upon demand. Thirty percent of the marrow storage compartment is released into circulation daily. At a normal rate of neutrophil turnover, the marrow storage pool contains a 3–4 day supply of neutrophils. Experimental studies in dogs and man have elucidated a model for normal granulopoiesis. Myeloblasts and progranulocytes divide once with cell cycle times of 18 and 24 hours, respectively. Two divisions (and possibly four or five) occur at the
myelocyte stage with a cycle time of 51 hours. The minimum
time for transition from myelocyte to metamyelocyte is 3 hours. It takes 48 hours from the last division at the
myelocyte stage until the cell matures into a segmented
neutrophil. Neutrophils are stored in the marrow
approximately 50 hours prior to release into circulation.
The more mature cells leave the marrow first. Increased
deformability and motility associated with maturation are
important factors in marrow release. 17

Radioactive labeling experiments indicate an average
transit time of 4–5 days between the last myelocyte division
and the appearance of mature neutrophils in the bloodstream.
14,18–20 In response to severe inflammation, tissue
demand for neutrophils rises and marrow transit time
decreases. Marsh, et al. measured a transit time of 2.3 days
in dogs with experimental pneumonia. 21 Turpentine-
induced metritis reduced marrow transit time between
metamyelocytes and neutrophils to 24 hours (normal 60–70
hours). 22 These studies prove that maturation of
post-mitotic neutrophils is accelerated to meet increased
tissue demand. A rise in mitotic activity at the myelocyte
stage leads to an increase in the number and rate of entry of
neutrophils in the post-mitotic pool. The number of
granulocyte precursors undergoing mitosis as well as the rate
of DNA synthesis are both increased. The authors concluded
that augmented mitotic activity was due to either a reduction
in the length of G1 or diminished DNA synthesis time. 22
Additional myelocyte generations and diminished cell cycle time may contribute to the expansion of the post-mitotic pool.\textsuperscript{22} Attrition of granulocytes occurs at the myelocyte stage in dogs.\textsuperscript{23,24} Reduced myelocyte attrition has been proposed as a mechanism of increasing neutrophil production in response to need. Although the mechanism is not fully understood, increased committal of pluripotent stem cells to granulopoiesis is an important factor in prolonged inflammatory diseases.\textsuperscript{22,25}

**Margination of Blood Neutrophils**

Blood neutrophils are divided into circulating and marginated compartments. Neutrophil counts in peripheral blood enumerate cells in the circulating compartment. The remaining neutrophils adhere to endothelial surfaces and form the marginated compartment.\textsuperscript{26} In normal dogs, 1/3 to 1/2 of the total blood neutrophils are marginated.\textsuperscript{14,27} Adrenergic drugs cause neutrophilia by increasing blood flow and flushing out marginated cells.\textsuperscript{28} The neutropenic effect of endotoxin results in part from increased margination at the expense of the circulating pool.\textsuperscript{29} Neutrophils must marginate prior to tissue migration. Margination is especially pronounced during inflammation when tissue migration is accelerated. As a consequence, the neutrophilia of inflammation can be "masked" by an increase in the ratio of marginated to circulating cells. Therefore, the absolute neutrophil count may not accurately reflect the marrow response.\textsuperscript{21}
Egress From Blood to Tissue

Canine neutrophils circulate briefly before migrating into tissue. The half-life of canine neutrophils in circulation is 5.6 to 7.6 hours, depending on whether diisopropylfluorophosphate-P³² (DFP) or tritiated thymidine is used as the radioactive cell marker.¹⁴,²¹,²²,²⁷ Deubelbeiss, et al. showed by simultaneous labeling with both markers that lower values obtained with DFP result from elution of the marker from the neutrophil surface.¹⁴ Therefore an estimate of approximately seven hours is probably more accurate and correlates well with studies in man.¹⁴,²² The disappearance of neutrophils from blood is a simple exponential function, suggesting random migration. Labeled neutrophils are detectable in oral lavages shortly after their release from the bone marrow.³⁰ This supports random emigration from blood which is unrelated to age or length of time in circulation.

Senescence of Circulating Cells

Morphologic changes in neutrophils associated with senescence are a loss of nuclear segmentation and increased homogeneity of the chromatin pattern; the cytoplasm remains relatively unchanged. Cells with multiple, round, homogeneous, deeply basophilic nuclear remnants are called pyknotic neutrophils. These degenerative changes are noted 24–36 hours after appearance of neutrophils in the blood. Senescent neutrophils are probably removed by the monocyte-macrophage system.³⁰
**Tissue Phase**

In normal animals, most neutrophils are lost through the gastrointestinal mucosa. Commensal organisms at this portal of entry release chemotactic factors which induce migration to this area. During inflammation, chemotactic factors derived from bacterial, viral, and tissue products cause neutrophils to accumulate at the inflammatory site. Migration of neutrophils into tissue is primarily a unidirectional process, however a recent study indicates that limited recirculation is possible. Once in tissue, neutrophil lifespan is probably short since mature neutrophils live only 2-3 days in cell culture.

**Neutrophil Morphology**

Neutrophil maturation at the ultrastructural level has been examined in several species, but not in the dog. Individual stages of granulopoiesis are not easily identified at the ultrastructural level. Consequently, it is more realistic to divide neutrophil development into three stages rather than the five discernable at the light microscopic level.

"Early neutrophils" (myeloblast, promyelocyte) have a round to oval nucleus with a slight invagination in the region of the Golgi apparatus. One or more prominent nucleoli are present and euchromatin predominates. Polyribosomes are abundant and rough endoplasmic reticulum increases in the promyelocyte. Mitochondria are prominent.
A variable but small number of large (azurophilic) granules are present. They are primarily seen in the region of the Golgi and may not be discernable by light microscopy.

"Intermediate neutrophils" (myelocytes, metamyelocytes) have a flattened and indented nucleus. Chromatin begins to condense and nucleoli are less prominent. Polyribosomes and rough endoplasmic reticulum are decreased in number. Mitochondria are reduced in size and number, and the Golgi is less prominent. Smaller, specific granules, some with a crystalline structure, appear first at the myelocyte stage and increase in number as maturation progresses. The larger granules (azurophilic granules), which are not produced after the promyelocyte stage, decrease in number with each cell division.

The nucleus of "late neutrophils" (bands, segmented neutrophils) is lobulated with condensed and margined chromatin. Nucleoli are absent. The Golgi apparatus is atrophic; mitochondria and rough endoplasmic reticulum are rare. Specific granules predominate and all granules are smaller than in earlier neutrophils. Dumbbell-shaped or oblong dense granules and granules with a crystalloid appearance are present. Glycogen granules accumulate as the cell matures.

Canine blood neutrophils have been studied ultrastructurally. The nucleus has two to three round or oval lobes. Chromatin is clumped and margined. A pair of centrioles is occasionally seen near an atrophic Golgi
apparatus in an area otherwise free of organelles. Mitochondria, polyribosomes, and segments of rough endoplasmic reticulum are infrequent.

Appearance of the canine cytoplasmic granules is greatly affected by the method of fixation and processing. Three types of granules are detectable with osmium (OsO₄) fixation.39 One type is homogeneous, electron-dense and either round (0.1 - 0.24 μm) or rod-shaped (0.34 - 0.84 μm long). A second is similar in size to the first, but has a granular or lattice-like appearance. The third is round or slightly irregular, smaller than the other two, and has a low electron density.

With glutaraldehyde or potassium permanganate (KMnO₄) fixation, canine neutrophil granules vary from spherical to narrow and elongate.38 Granules are less easily classified as to type with glutaraldehyde fixation. KMnO₄ fixed neutrophils contain three types of granules similar to those described in man.40 One type is spherical to elliptical, 0.8 μm in diameter, and is frequently extracted. A second is rod- to dumbbell-shaped, less than 0.2 μm in length, and has a density between that of the first type of granules and the cytoplasm. The third type of granule is cylindrical with hemispherical ends (1.0 μm x 0.3 μm), and has a crystalline structure.38

Cells fixed in formaldehyde-glutaraldehyde mixture have a higher percentage of elongated or dumbbell-shaped granules than those fixed in glutaraldehyde alone. Fixation in
glutaraldehyde-OsO\textsubscript{4} mixture gives similar results to glutaraldehyde followed by OsO\textsubscript{4}. When propylene oxide is used in processing, the otherwise dense granules of the promyelocyte stage (azurophils) are irregularly extracted. After uranyl acetate staining, the smaller specific granules appear darker.\textsuperscript{41}

In spite of this morphologic heterogeneity of neutrophil granules, there appears to be only two distinct biochemical groups: azurophilic and specific granules. Azurophils arise from the concave or proximal surface of the Golgi during the progranulocyte stage\textsuperscript{42} and contain acid phosphatase,\textsuperscript{43,46} peroxidase, and lysosomal hydrolases.\textsuperscript{43,44,46} Specific granules originate from the distal or convex surface of the Golgi beginning at the myelocyte stage.\textsuperscript{42} They contain lysozyme,\textsuperscript{47} lactoferrin,\textsuperscript{47,48} and several cationic proteins.\textsuperscript{43,44} Alkaline phosphatase is present in specific granules of many species,\textsuperscript{43-45,47} but is absent in the dog.\textsuperscript{49}

**Toxic neutrophils**

In toxemias associated with bacterial infection or other severe inflammatory states, morphological changes in blood neutrophils can be detected with Romanowski stains.\textsuperscript{50} In man, these changes include: 1) "shift to the left" or the presence of immature cells in the blood (bands, metamyelocytes and rarely myelocytes), 2) blue-gray amorphous inclusions in the cytoplasm (Döhle bodies), 3) toxic granulation or increased prominence of cytoplasmic granules,
and 4) cytoplasmic vacuolation. Cells exhibiting these characteristics are designated "toxic neutrophils". McCall, et al. (1969) studied the ultrastructural appearance of human toxic neutrophils in patients with severe bacterial infections. They concluded that Döhle bodies were lamellar aggregates of rough endoplasmic reticulum. Prominence of cytoplasmic granules could not be explained ultrastructurally. However, by using a variety of staining techniques, similar light microscopic changes could be produced in normal cells. Toxic granulation apparently represents altered affinity of granule contents for Wright's stain. Patients treated with chloroquine had similar granules. These appeared ultrastructurally as large myelin figures, suggesting a defect in formation of organelles or autophagy. Clear vacuoles were identified as electron-lucent, homogeneous, membrane bound structures.

Morphologic changes in toxic neutrophils may be due to cytoplasmic immaturity, increased cellular activity, or cell degeneration. Cytoplasmic immaturity is supported by the nuclear immaturity and increased amount of rough endoplasmic reticulum. However, toxic changes are seen in cells with mature nuclei with equal frequency. This may imply asynchronous development. Human toxic neutrophils have increased rough endoplasmic reticulum, azurophilic granules, and cytoplasmic basophilia. Similar changes are seen in stimulated lymphocytes and monocytes in certain chronic
infections. Perhaps these changes in toxic neutrophils are caused by increased cell activity secondary to inflammatory stimuli.

Toxic change in dog neutrophils is most commonly expressed by cytoplasmic basophilia and vacuolation, and the occasional Döhle body. Toxic granulation is seldom seen.51

Neutrophil Function

Antibacterial activity of neutrophils can be divided into four phases: chemotaxis, opsonization, ingestion, and microbicidal mechanisms.

Chemotaxis

Chemotaxis can be defined as cell migration in response to a concentration gradient of chemical mediators. Chemotactic factors are produced in response to microbial invasion or tissue damage and guide the movement of the phagocyte toward the site of inflammation.56 Fragments of C3, C5, and C567 of the complement system are perhaps the most potent chemotactic factors for neutrophils.57 Bacterial, viral, or tissue products are chemotactic both directly and indirectly by cleaving C3 and C5 into active fragments.58 Kallikrein and plasminogen activator are also chemotactic for neutrophils.58

Intrinsic neutrophil defects or defective production of chemotactic factors result in impaired chemotaxis.58

Opsonization

Opsonization is the process by which serum factors alter
the surface of a particle to facilitate phagocytosis. Ingestion proceeds at a slow rate in the absence of opsonins, but is greatly enhanced by their presence. Opsonins may be either heat labile or heat stable. IgG and IgM antibodies are heat stable and bind to specific antigens on the bacterial surface. The F(ab)_2 portion of the immunoglobulin molecule binds to the microbial surface while the Fc portion binds to receptors on the phagocyte plasma membrane. Antibody bridging between microbe and phagocyte facilitates recognition and ingestion of the invader. In many instances, optimal phagocytosis is achieved only after fixation of complement by antigen-antibody complexes. Normal non-immune serum has the capacity to enhance phagocytosis, a property which is lost after heating at 56°C for 30 minutes. This heat labile fraction is part of the complement system. Johnston (1969) showed that sequential addition of C1, C2, C4, and C3 is required for optimal phagocytosis. The direct pathway of complement activation begins by the formation of C124 or C3 convertase. C3 convertase splits C3 into two active products, C3a which is both chemotactic and anaphylotoxic, and the opsonin C3b. Complement can opsonize by adsorbing to the bacterial surface or by fixing to the Fc portion of an antibody attached to the microbe. Phagocytosis of opsonized bacteria is enhanced by attachment to specific C3 receptors on the phagocyte surface. Properdin is a serum protein which activates complement without the presence
of specific antibody, and is probably an important source of opsonic activity early in the course of infection. As antibody levels increase, IgG and IgM become important complement activators to enhance phagocytosis.

**Ingestion**

Upon contact with a particle, a phagosome forms when neutrophil pseudopodia encircle the invader. The phagosome then buds from the plasma membrane and migrates centrally. Studies of microfilament inhibitors (e.g., cytochalasin B) and microtubule inhibitors (e.g., colchicine) show the importance of these two structures in phagocytosis. The presence of receptors for C3b and IgG Fc fragment on the plasma membrane of phagocytes suggests that initiation of phagocytosis is a membrane phenomenon. Receptor function requires divalent cations and can be destroyed by trypsin. Phagocytic rate is affected by pH, osmotic pressure, and surface charge of both cell membrane and particle surface. Morphine analogs cause membrane alterations resulting in decreased phagocytic rate. Influenza virus binds to the phagocyte surface and inhibits phagocytosis. Prior exposure of neutrophils to *S. aureus* antigens enhances phagocytosis of both *S. aureus* and *E. coli*.

**Microbicidal Mechanisms**

Bacterial killing is initiated by two cellular events: degranulation and the respiratory burst.

Bainton (1973) used cytochemical markers to study the
process of degranulation. Thirty seconds after phagocytosis of bacteria, half of the phagosomes contained alkaline phosphatase indicating fusion of specific granules with phagosomes. By one minute, peroxidase, from azurophilic granules was present in only a few phagosomes. However, by three minutes, peroxidase was found in nearly every phagosome. These observations indicate that specific granules and azurophilic granules sequentially fuse and discharge their contents into the phagosome. The sequential nature of this process becomes more important when these results are evaluated in conjunction with pH changes in the phagocytic vacuole. Intravacuolar pH decreases to 6.5 within 3 minutes and to 4.0 within 7-15 minutes. Alkaline phosphatase, lysozyme, and lactoferrin within specific granules function optimally at neutral or alkaline pH, whereas the contents of azurophilic granules (peroxidase, acid phosphatase, and lysosomal enzymes) function optimally at an acid pH.

Azurophilic and specific granules contain many substances with bactericidal or bacteriostatic activity. Lysozyme hydrolyzes the bacterial cell wall. Lactoferrin chelates iron which is necessary for bacterial growth. Cationic proteins have enzyme activity and bind to the surface of the microorganism. Killing activity of these proteins is related to their ionic interaction with the microbial surface.

The respiratory burst is a set of biochemical events which accompany phagocytosis and is characterized by:
1) increased O\textsubscript{2} uptake, 2) superoxide anion (O\textsubscript{2}^-) generation, 3) H\textsubscript{2}O\textsubscript{2} production, and 4) increased hexose monophosphate shunt (HMS) activity.

Normal neutrophils consume small amounts of oxygen, but uptake is increased during phagocytosis.\textsuperscript{68,82,83} Glycolysis provides energy necessary for phagocytosis, which proceeds in the absence of O\textsubscript{2} or in the presence of mitochondrial inhibitors.\textsuperscript{68} Therefore phagocytosis is not responsible for enhanced O\textsubscript{2} uptake during the respiratory burst. A connection between the respiratory burst and bactericidal activity was established when Iyer (1961) proved that oxygen was converted to H\textsubscript{2}O\textsubscript{2}.\textsuperscript{82}

HMS activity also increases during phagocytosis.\textsuperscript{68} This pathway oxidizes glucose to CO\textsubscript{2} and a five carbon sugar using nicotinamide adenine dinucleotide phosphate (NADP\textsuperscript{+}) as an electron acceptor.\textsuperscript{84} Stimulation of HMS by NADP\textsuperscript{+} suggests that NADP\textsuperscript{+} is produced during the respiratory burst.\textsuperscript{85}

Initiation of the respiratory burst is a membrane phenomenon which requires neither phagocytosis nor lysosomal degranulation.\textsuperscript{86} Oxygen reduction by an enzyme on the plasma membrane is the initial step and requires NADPH or NADH as the electron donor. Most data indicate that NADPH and NADPH oxidase are involved in this reaction which produces superoxide anion (O\textsubscript{2}^-).\textsuperscript{85-91}
\[ 20_2 + \text{NADPH} \xrightarrow{\text{NADPH oxidase}} 20_2^- + \text{NADP}^+ + H^+ \]

Essentially all \( O_2 \) consumed is converted to \( O_2^- \) and 80% of this is enzymatically converted to \( H_2O_2 \) by superoxide dismutase (SOD):\(^{86,87,92} \)

\[ 20_2^- + 2H^+ \xrightarrow{\text{SOD}} O_2 + H_2O_2 \]

Respiratory burst activity is important because it produces oxidizing agents with bactericidal activity. Several mechanisms of oxidative killing have been proposed for the neutrophil. Bactericidal activity has been demonstrated for these agents in vitro, but their relative in vivo importance is unproven.\(^{85} \)

Superoxide anion (\( O_2^- \)) is produced by neutrophils and is thought to be an important bactericidal agent.\(^{87,93} \) Recent in vitro experiments cast doubt on its importance,\(^{94-98} \) but these studies used lower levels of \( O_2^- \) than are produced within phagocytes.\(^{98} \)

Myeloperoxidase (MPO) is a hemoprotein with a molecular weight of approximately 150,000.\(^{99} \) It is found in abundance in azurophilic granules.\(^{44} \) This enzyme in the presence of \( H_2O_2 \) and chloride ion (\( Cl^- \)) has bactericidal activity.\(^{100} \) Recent studies indicate that MPO attaches to the bacterial wall and forms a complex with \( H_2O_2 \) and \( Cl^- \). A series of intramolecular rearrangements result in
the production of an "activated chlorine" (Cl₂-, Cl⁺, OCl⁻). This complex reacts with proteins on the bacterial cell wall to produce bactericidal agents (chloramines, aldehydes) and breaks peptide bonds. Sbarra (1979) suggested that activated chlorine itself is the most important bactericidal agent.

Recent experiments suggest that neither H₂O₂ nor O₂⁻ is the important bactericidal agent. Hydroxyl radical (·OH), a substance formed by the reaction between these two agents, has gained support as an important bactericidal agent.94,95,97,98,105,106

Singlet oxygen (¹O₂) differs from atmospheric oxygen only in the configuration of the electrons around the two oxygen nuclei. This difference greatly enhances the reactivity of singlet oxygen such that the two molecules are considered distinct chemical entities.107 Several substances produced during the respiratory burst are capable of participating in reactions which form singlet oxygen. Direct proof of the production of ¹O₂ or its importance in bacterial killing, however, has not been found.87,89

Recurrent bacterial or fungal infections occasionally occur in patients with normal serum immunoglobulin levels and delayed hypersensitivity reactions, and adequate numbers of blood neutrophils.67 Defective neutrophil function is responsible for impaired resistance in these patients. Disorders of neutrophil function in man have been recently reviewed.77,108 Impairments of chemotaxis,
adherence, phagocytosis, or microbial killing have all been demonstrated. In many instances, more than one of these functions is compromised in the same patient. These dysfunctions may be either inherited or acquired. Several drugs have been associated with diminished neutrophil activity.\textsuperscript{109,110} Metabolic disturbances such as diabetes mellitus, uremia, alcoholism, severe burns, and shock induce reversible impairment of neutrophil function.\textsuperscript{56,111-116} Blood neutrophils from humans with systemic lupus erythematosus\textsuperscript{117} and synovial fluid neutrophils from rheumatoid arthritis patients\textsuperscript{118} have decreased phagocytic ability. Patients with untreated bacterial endocarditis have diminished bactericidal activity.\textsuperscript{119} Reversible chemotactic defects are seen in some children with periodontal disease due to \textit{Capnocytophaga}.\textsuperscript{120}

Persons with severe bacterial infections and toxic neutrophils can exhibit transient defects in bactericidal capacity.\textsuperscript{121-125} Phagocytic activity is generally not impaired. Function is not restored by addition of control serum and patient serum does not induce the defects in normal cells. Weinstein (1976) found no intrinsic defect in neutrophils from patients with Gram negative septicemia and concluded that inadequate opsonization, rather than an intrinsic neutrophil defect, was a more important cause of neutrophil dysfunction.\textsuperscript{126} Variations in these results are likely due to the stage and severity of disease along with differences in assay technique.
McCall (1971) measured metabolic activity of toxic neutrophils. Oxygen uptake and hexose monophosphate shunt activity were elevated slightly. Quantitative NBT reduction was normal, but an increased number of cells reduced the dye. Metabolic changes were unrelated to degree of leukocytosis, cellular immaturity, presence of fever, azotemia, hypotension, or antibiotic therapy.

Lysosomal depletion and cell immaturity have been proposed as causes for decreased function in toxic neutrophils. Increased lysosomal permeability and spontaneous degranulation are noted in toxic neutrophils. Features of cytoplasmic immaturity (basophilia, increased rough endoplasmic reticulum) suggest functional immaturity.

List of Objectives
The objectives of this dissertation were:

1) To develop an experimental model to produce toxic neutrophils in the dog.

2) To study morphologic changes of toxic neutrophils in blood at the light and electron microscopic levels.

3) To study the morphogenesis of toxic neutrophils in bone marrow at the light and electron microscopic levels.

4) To quantitate in vitro function of normal and toxic neutrophils.

Experimental Design
Data presented in Chapters 2-4 were derived from a
research project involving a total of 14 mongrel dogs. Animals were divided into two treatment groups (3 males, 4 females per group). The first group of dogs was injected intramuscularly with 1.0 ml turpentine. Dogs in the second group (controls) were injected similarly with 1.0 ml 0.9% sterile saline. Chapter 2 includes clinical signs, body temperature, hematology and bone marrow data from these 14 dogs. The ultrastructure of blood neutrophils and granulocyte precursors in the bone marrow of 8 of these dogs (2 males, 2 females from each group) is described in Chapter 3. Chapter 4 contains the results of neutrophil function studies from 10 of these dogs (2 males, 3 females per group).

Statistical evaluation of the data was performed by analysis of variance.
CHAPTER II

Toxic Neutrophils in the Dog:
Hematologic and Bone Marrow Response to
Turpentine-induced Inflammation

Introduction

Acute inflammation causes changes in the number and morphology of circulating neutrophils, as well as their bone marrow production and release. One hour after induction of inflammation, circulating neutrophils are decreased due to margination in small vessels and tissue migration.22 During the next several hours, accelerated release of segmented and band neutrophils from the bone marrow causes a steady rise in circulating neutrophils and a shift to the left. Bone marrow production of neutrophils increases by accelerated maturation of post-mitotic precursors, increased rate and number of myelocyte mitoses, and addition of committed stem cells to granulopoiesis.21,22

Toxic change or toxic neutrophils are terms used to describe alterations in neutrophil morphology associated with severe bacterial infections or other severe inflammatory disorders. In the dog, cytoplasmic basophilia, vacuolation, and an occasional Döhle body are features of toxic neutrophils.51

The purpose of this study was to develop a reliable model for the production and study of toxic neutrophils in
the dog. Subcutaneous or intramuscular turpentine injection is a classical model of the acute inflammatory process. Acute, local inflammation was induced in dogs by injecting turpentine intramuscularly. Clinical signs, complete blood counts, neutrophil morphology and bone marrow cytology were compared in control and turpentine-injected dogs.

Materials and Methods

Fourteen mongrel dogs were vaccinated, treated for intestinal parasites, and pre-conditioned for two weeks in an animal holding unit. Seven dogs (3 male, 4 female) were injected intramuscularly with 1.0 ml turpentine in the left gluteal region. Seven control dogs (3 male, 4 female) were injected similarly with 1.0 ml sterile 0.9% saline.

Clinical signs, rectal temperatures, and gross appearance of the lesion were recorded daily. Venous blood samples were collected in potassium EDTA on three days immediately prior to injection. Post-injection samples were collected at 8, 16, and 24 hours, and then daily for nine days. Standard laboratory techniques were used to measure packed cell volume and total plasma protein concentration.

\[\text{Vanguard DA}_2\text{PL} + \text{Bordetella, Nordon Laboratories, Lincoln, NE.}\]
\[\text{Strongid T, Pfizer Inc., New York, NY.}\]
\[\text{Medi-Kay Pharmaceutical Co., Brookfield, MD.}\]
\[\text{Vacutainer, Becton-Dickinson, Rutherford, NJ.}\]
\[\text{Coulter Model Zf, Coulter Electronics, Hialeah, FL.}\]
\[\text{Uni-Smear Spinner, Coleman Instruments Division of Perkin-Elmer, Oak Brook, IL.}\]
Leukocyte counts were determined by an electronic cell counter. Blood films were prepared and stained with Wright's-Giemsa for differential leukocyte counts and quantitation of toxic change.

Bone marrow samples were aspirated from the ilium or humerus of four dogs from each group using an 18 gauge Rosenthal needle. Samples were collected immediately prior to injection and on days 1, 2, 3, 4, and 9 post-injection. Marrow smears were stained with Wright's-Giemsa to assess granulocyte morphology. Five hundred cells were identified to determine myeloid:erythroid ratio.

The hematology and bone marrow data were evaluated by 2 x 14 and 2 x 6 analyses of variance respectively.

Results

Turpentine-injected dogs were depressed within 8 hours and became increasingly anorectic and lethargic by days 2-3. The dogs were lame on the turpentine-injected limb by 16 hours but regained use of the limb by day 7. Most dogs were clinically normal by day 7, however two animals were depressed through day 9. Injection sites were swollen, reddened, warm, but not severely painful by 24 hours. The size of the lesion increased over the first three to five days into a firm mass involving the entire left gluteal region. Control dogs remained clinically normal throughout the course of the experiment.

Average rectal temperatures in turpentine-injected
animals rose to 103.7 F at 24 hours, peaked at 104.2 F on day 2, and gradually declined to baseline by day 7 (Fig. 1). Body temperatures in the control group did not vary significantly during the experiment.

Changes in neutrophil numbers paralleled the total leukocyte count in turpentine-injected dogs. Leukocytosis and an absolute neutrophilia were observed at 8 hours post-injection (x = 29,300/µl and 24,500/µl respectively) and remained relatively constant through day 5 (Fig. 2 and 3). By day 7, neutrophil counts had returned to pre-injection levels.

Immature forms of neutrophils were seen rarely in control and pre-injection blood samples. Band neutrophils increased abruptly between 8 and 16 hours post-injection to a mean value of 2400/µl (Fig. 4) and were significantly increased through day 4.

Increased cytoplasmic basophilia and foamy or vacuolated cytoplasm were common in the neutrophils of post-injection blood samples (Fig. 5). Blue-gray, amorphous, cytoplasmic inclusions (Döhle bodies) were seen occasionally. Morphologic features of toxic change were seldom seen in control blood samples. Toxic neutrophils were first observed at 8 hours and were significantly elevated by 16 hours (Fig. 6). On day 2, the number of toxic neutrophils in test dogs peaked at an average of 15,000/µl and declined gradually to baseline levels by day 7.

Circulating lymphocytes were significantly decreased by
Fig. 1 Body temperatures in control (-----) and turpentine-injected dogs (——). Each point represents $X \pm$ SEM, $n = 7$. Arrow signifies time of injection. Means are statistically different ($P < .05$) from 24 hours to day 6 post-injection.
Fig. 2  Total leukocyte counts in control (---) and turpentine-injected dogs (—). Each point represents $\bar{x} \pm$ SEM, n = 7. Arrow signifies time of injection. Means are statistically different ($P<.01$) from 8 hours to day 6 post-injection.
Fig. 3  Blood neutrophil counts in control (---) and turpentine-injected dogs (----). Each point represents X + SEM, n = 7. Arrow signifies time of injection. Means are statistically different (P<.01) from 8 hours to day 6 post-injection.
Fig. 4  Circulating band neutrophil counts in turpentine-injected dogs (---). Each point represents $X \pm$ SEM, $n = 7$. Arrow signifies time of injection. Means are statistically different ($P < .05$) from 16 hours to day 4 post-injection.
Fig. 5  A) Normal canine neutrophil (640x). B) Canine toxic neutrophil with foamy vacuolation of the cytoplasm and Döhle bodies (640x). C) Canine toxic neutrophil with increased cytoplasmic basophilia and foamy vacuolation (640x).
Fig. 6  Circulating toxic neutrophil counts in turpentine-injected dogs (---). Each point represents $x \pm$ SEM, $n = 7$. Arrow signifies time of injection. Means are statistically different ($P < .01$) from 8 hours to day 4 post-injection.
8 hours and remained below control levels through day 9 (Fig. 7). Eosinopenia began at 16 hours and persisted through day 5 (Fig. 8). A mild monocytosis occurred at 8 and 16 hours after injection, but regressed to baseline by 24 hours (Fig. 9). A second, more pronounced monocytosis began on day 2, peaked at a mean value of 4600/μl on day 3, and declined gradually to pre-injection levels by day 7.

A mild but statistically significant decrease in packed cell volume occurred in the test dogs. Average PCV values declined from a pre-injection level of 42.6% to 38.6% by day 9. Comparable changes in total plasma protein concentration did not occur.

Myeloid:erythroid ratios of pre-injection bone marrow samples from control and test animals were not significantly different (X = 1.4:1 and 1.2:1 respectively) (Fig. 10). The myeloid cell series of control and pre-injection bone marrow samples contained an average of 88.4% post-mitotic neutrophils (metamyelocytes, bands, and segmented neutrophils) and 11.6% mitotic granulocytes (myeloblasts, progranulocytes, and myelocytes) (Fig. 11).

At 24 hours post-injection with turpentine, the M:E ratio had not changed. However, the storage compartment (bands and segmented neutrophils) was depleted and the mitotic pool had increased (X = 27.6%). Mitotic figures in myelocytes were numerous (Fig. 12). Several myeloblasts and progranulocytes had irregular (monocytoid) nuclei, suggesting premature lobulation or nuclear blebbing (Fig. 13). A few
Fig. 7  Blood lymphocyte counts in control (----) and turpentine-injected dogs (—). Each point represents $\bar{x} \pm$ SEM, $n = 7$. Arrow signifies time of injection. With the exceptions of days 2, 3, and 7, means were statistically different ($P < .05$) from 8 hours to day 9 post-injection.
Blood eosinophil counts in control (---) and turpentine-injected dogs (——). Each point represents $X \pm$ SEM, $n=7$. Arrow signifies time of injection. Means in turpentine-injected dogs were significantly less than control ($P<.05$) from 16 hours to day 5 post-injection.
Fig. 9  Blood monocyte counts in control (---) and turpentine-injected dogs (—). Each point represents $x \pm$ SEM, $n = 7$. Arrow signifies time of injection. Means were statistically different ($P<.01$) from day 2 to day 6 post-injection.
Fig. 10 Myeloid:erythroid ratios from control (---) and turpentine-injected dogs (—). Points represent $x \pm$ SEM, $n = 4$. Arrow signifies time of injection. Means are statistically different ($P<.01$) on days 2, 3, and 4 post-injection.
Fig. 11 Mitotic pool as a percentage of total myeloid mass in control (---) and turpentine-injected dogs (-----). Points represent X ± SEM, n = 4. Arrow signifies time of injection. Means are statistically different (P < .01) on days 1, 2, and 3 post-injection.
Fig. 12  Two granulocyte precursors undergoing mitosis (320x).
Fig. 13  Two progranulocytes with monocytoïd nuclei and distinct, clear, cytoplasmic vacuoles (640x).
neutrophils had doughnut-shaped nuclei. Distinct, clear, circular, intracytoplasmic vacuoles were present in myeloblasts and progranulocytes (Fig. 14). Increased cytoplasmic basophilia and foaminess were noted in myelocytes, but were more prominent in more mature cells (Fig. 15).

By day 2, granulocytic hyperplasia was obvious; the M:E ratio averaged 21.1:1. The average percentage of neutrophils in the mitotic pool had increased to 34.0% and mitotic figures were numerous. Morphologic changes in neutrophil precursors were similar to day 1, except that round, clear, distinct cytoplasmic vacuoles were also observed in myelocytes and metamyelocytes (Fig. 16).

On day 3, M:E ratios remained high (X = 20.0:1), but the percentage of cells in the mitotic pool had declined (X = 21.2%). Cytoplasmic vacuolation was less prominent and was seen mainly in myelocytes and metamyelocytes. The numbers of toxic neutrophils and early precursors with monocytoid nuclei were decreased.

On day 4, granulocytic hyperplasia was still pronounced, but the percentage of cells in the mitotic pool had returned to baseline (X = 14.9%). Morphologic abnormalities of the granulocytic series were minimal.

M:E ratios were approaching control values by day 9. The percentage of neutrophils in the mitotic pool and neutrophil morphology were similar to control dogs.

Changes in M:E ratios, percentage of cells in the mitotic pool, and neutrophil morphology in control bone
Fig. 14 Several progranulocytes with distinct, clear, cytoplasmic vacuoles (320x).
Fig. 15 Mature toxic neutrophils with increased basophilia and foamy vacuolation in the bone marrow 24 hours post-injection (320x).
Fig. 16 Distinct cytoplasmic vacuolation from the progranulocyte through band neutrophil stage (320x).
marrow samples were not significant.

Discussion

Intensity of clinical signs paralleled changes in rectal temperature, neutrophil count, total leukocyte count, and number of toxic neutrophils. Body temperatures were significantly elevated by 24 hours and peaked on day 2. Clinical signs were most severe on days 2 and 3. By day 7, rectal temperatures were normal and clinical signs had abated. There was a strong correlation between neutrophil and total leukocyte numbers throughout this experiment (P<.01). Both variables reached a stable maximum between 8 hours and day 5, and declined to baseline by day 7. The number of toxic neutrophils paralleled body temperature and was unrelated to the number of band neutrophils. Morphologic appearance of toxic neutrophils was similar to neutrophils seen clinically in dogs with severe inflammatory or infectious processes.51

The relative change in mitotic and post-mitotic compartments on day 1 indicates that neutrophilia during the first 24 hours was due to release of neutrophils from the storage pool. Cronkite reported a 6-7 fold increase in neutrophil release from bone marrow 7 hours after induction of sterile inflammation.22 Band neutrophils peaked 8 hours after total neutrophils reached maximum and declined rapidly through day 3. Marrow release of neutrophils is age-related; mature cells are released preferentially in
response to demand. When the reserve of mature neutrophils is depleted, band cells are released. As granulocytic hyperplasia replenishes the storage pool, the release of mature cells precludes entry of bands into circulation and the left shift subsides.

Bone marrow samples from 24 hours post-injection revealed increased numbers of myeloblasts and progranulocytes, and brisk mitotic activity at the myelocyte stage. Cronkite observed increased tritiated thymidine uptake and mitotic rate in granulocyte precursors within hours after induction of turpentine-induced metritis in dogs. He concluded that the granulocyte response was amplified by: 1) addition of committed stem cells to granulopoiesis, 2) increased mitotic activity primarily at the myelocyte stage, and 3) accelerated maturation of post-mitotic neutrophils.

Enlargement of the mitotic pool did not produce an increase in M:E ratio at 24 hours because of concurrent storage pool depletion. Differentiation of the mitotic pool with repopulation of the post-mitotic pool produced intense granulocytic hyperplasia and high M:E ratios on days 2-4.

The initial monocytosis, along with persistent eosinopenia and lymphopenia is likely due to endogenous glucocorticoid release. The second monocytosis (days 2-6) is probably inflammatory in origin. Due to minimal storage of monocytes in the bone marrow, the monocytosis of inflammation is due to enhanced monocytopoiesis. On days 2 and
3, progranulocytes with irregular, monocytoiud nuclei were observed, suggesting intensified monocytopoiesis.

Sequential leukograms in turpentine-injected dogs indicate that the increased M:E ratio was due to granulocytic activity. The mild, but statistically significant decrease in packed cell volume suggests that decreased erythroid activity may play a minor role.
CHAPTER III

Ultrastructure of Canine Toxic Neutrophils in Blood and Bone Marrow

Introduction

Toxic neutrophils are frequently seen on Wright's-Giemsa stained blood smears of animals and people with severe bacterial infections or other inflammatory disorders. Features of toxic neutrophils include basophilia and vacuolation of the cytoplasm, blue-gray, amorphous, intracytoplasmic inclusions or Döhle bodies, and prominent cytoplasmic granules. McCall described the ultrastructure of human toxic neutrophils. Döhle bodies corresponded to lamellar aggregates of rough endoplasmic reticulum. Cytoplasmic vacuoles were electron-lucent, membrane bound structures. Granules in toxic neutrophils were azurophilic granules which were visible because of increased stain affinity. The ultrastructure of toxic neutrophils in the dog has not been described. Furthermore, electron microscopic studies of granulopoiesis in animals with circulating toxic neutrophils have not been reported.

The purpose of this study was to describe the ultrastructure of: 1) normal canine neutrophils, 2) granulopoiesis in the normal dog, 3) canine toxic neutrophils, and 4) granulopoiesis in dogs with circulating toxic neutrophils.
Intramuscular turpentine injection is a predictable model for acute inflammation and generation of toxic neutrophils (see Chapter II). In the present study, the ultrastructure of blood neutrophils and neutrophil precursors in the bone marrow was compared in control and turpentine-injected dogs.

Materials and Methods

Eight mongrel dogs were vaccinated, treated for intestinal parasites, and preconditioned for two weeks. Four dogs (2 male, 2 female) were injected intramuscularly with 1.0 ml turpentine in the left gluteal region. Four control dogs (2 male, 2 female) were injected similarly with 1.0 ml sterile 0.9% saline solution.

Hematologic and bone marrow responses of this experimental model, along with the clinical course have been described in Chapter II.

Blood and bone marrow samples were collected prior to injection and 24 hours after injection. Blood for transmission electron microscopy was collected in potassium EDTA and maintained at 4°C throughout fixation. Two parts blood were added promptly to one part 6% dextran in 0.9% saline. After sedimentation for 10 minutes, leukocyte rich plasma was

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\(^a\)Vanguard DA\(_2\)PL + Bordetella. Nordon Laboratories, Lincoln, NE.
\(^b\)Strongid T, Pfizer, Inc., New York, NY.
\(^c\)Medi-Kay Pharmaceutical Co., Brookfield, MO.
\(^d\)Vacutainer, Becton-Dickinson, Rutherford, NJ.
\(^e\)100,000-200,000 MW, Sigma Chemical Co., St. Louis, MO.
harvested and dispersed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer. One hour of fixation was followed by centrifugation at 400 g for 5 minutes. The supernatant was discarded and the cellular button resuspended in two drops of canine plasma. Glutaraldehyde (2.5% in 0.1 M cacodylate buffer) was layered over the specimen and left for fifteen minutes.

Bone marrow samples were aspirated from the humerus or ilium using an 18 gauge Rosenthal needle. Aspirated marrow was placed on a glass slide, allowed to clot, transferred to 2.5% glutaraldehyde in 0.1 M cacodylate buffer, and fixed for one hour at 4 C.

After fixation, bone marrow and blood specimens were washed in 5% sucrose in 0.1 M cacodylate, post-fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer, and dehydrated through an ascending alcohol series (50-100%). Processing through increasing concentrations of propylene oxide (25-100%) was followed by infiltration and embedding in Epon 812. Thin sections were cut on an ultramicrotome, placed on 300 mesh grids, stained with uranyl acetate and lead citrate, and examined on an electron microscope.

Results

Granulopoiesis is divided into six stages at the light

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fErnest F. Fullam, Inc., Schenectady, NY.
gSorvall MT 2B, Ivan Sorvall, Inc., Norwalk, CT.
hZeiss EM 10, Carl Zeiss, Inc., New York, NY.
microscopic level: myeloblast, progranulocyte, myelocyte, metamyelocyte, band and segmented neutrophils. These stages cannot be distinguished clearly by transmission electron microscopy. Consequently, for ultrastructural studies, neutrophil maturation is generally divided into three stages: early, intermediate, and late neutrophils.\textsuperscript{33}

The ultrastructure of canine neutrophil precursors was similar to descriptions in man.\textsuperscript{33} Early neutrophils (myeloblasts and promyelocytes) in control dogs had oval nuclei, which were sometimes slightly indented near the Golgi (Fig. 1). Nucleoli were prominent and euchromatin predominated. The cytoplasm contained several large mitochondria, abundant rough endoplasmic reticulum, numerous polyribosomes and a few large, oval, granules with a moderately dense, homogeneous matrix which was variably extracted. These granules were analogous to azurophilic or primary granules. Activity of the Golgi, amount of rough endoplasmic reticulum, and number of cytoplasmic granules increased with maturity of the cell.

Nuclei of intermediate neutrophils (myelocytes and metamyelocytes) were elongated and indented (Fig. 2). The chromatin was condensed centrally around an indistinct nucleolus and along the inner surface of the nuclear membrane. Mitochondria were smaller and less numerous. The Golgi was active; polyribosomes and rough endoplasmic reticulum were abundant. Azurophilic granules, observed first in early neutrophils, were decreased in number. Small,
Fig. 1 Normal early neutrophil (10,000x).
Fig. 2  A) Normal intermediate neutrophil (myelocyte) (10,000x). B) Normal intermediate neutrophil (metamyelocyte) (12,000x).
oval or oblong, dense, specific granules, many with a crystalline inner structure, increased as the intermediate neutrophils matured.

Late neutrophils (bands and segmented neutrophils) had elongated, lobulated nuclei with pronounced condensation and margination of chromatin (Fig. 3). Nucleoli were no longer discernible. Mitochondria, polyribosomes, and rough endoplasmic reticulum were sparse and the Golgi was atrophic. Oval, oblong, and dumbbell-shaped specific granules were abundant and only a few large azurophilic granules remained. Otherwise, the cytoplasm was dense and granular.

Blood neutrophils were similar in structure to late neutrophils observed in the bone marrow. Nuclei were lobulated, with a predominance of marginated heterochromatin. The cytoplasm contained numerous specific granules and a few azurophilic granules in a finely granular background (Fig. 4).

Ultrastructural examination of circulating neutrophils from turpentine-injected dogs revealed irregular, variable sized, electron-lucent areas in the cytoplasm. These areas were not membrane bound but some had remnants of a limiting membrane (Fig. 5 and 6). Several lucent areas were perinuclear and associated with breaks in the perinuclear membrane. Dense myelin figures were observed in lucent areas, both perinuclear and cytoplasmic. Otherwise these areas were free of organelles. Polyribosomes, mitochondria, and segments of rough endoplasmic reticulum were increased,
Fig. 3 Normal late neutrophil (8,000x).
Fig. 4  Normal segmented neutrophil from blood (13,000x).
Fig. 5  A) Circulating segmented neutrophil with mild toxic change. Amount of rough endoplasmic reticulum is increased and there are many electron-lucent areas within the cytoplasm (16,000x). B) Enlargement of 5a with membrane remnants bordering several electron-lucent areas and a few electron-lucent areas which are confluent with a disrupted perinuclear membrane (32,000x).
Fig. 6 Circulating segmented neutrophil with severe toxic change. Mitochondria are numerous. Myelin figures are present within large electron-lucent areas; one area is confluent with perinuclear membrane (12,000x).
when compared to normal neutrophils. In severely affected neutrophils, the number of lysosomal granules and their electron density were diminished.

Ultrastructural evaluation of early and intermediate neutrophils in bone marrow from turpentine-injected dogs revealed dilation and fragmentation of rough endoplasmic reticulum associated with large, amorphous, electron-lucent areas in the cytoplasm. Some of these areas were confluent with breaks in the perinuclear membrane (Fig. 7 and 8). Myelin figures noted within lucent areas were similar to those seen in circulating blood neutrophils. Lipid droplets were observed occasionally in the cytoplasm (Fig. 9).

Discussion

Light microscopic changes in blood neutrophils of turpentine-injected dogs are similar to those observed clinically in dogs with severe inflammatory processes (see Chapter II). Cytoplasmic features of canine toxic neutrophils include increased basophilia, foamy vacuolation, and the occasional Döhle body. Toxic neutrophils in man are characterized by Döhle bodies and prominent cytoplasmic granulation along with cytoplasmic basophilia and mild vacuolation.50

Ultrastructurally, canine toxic neutrophils have increased amounts of rough endoplasmic reticulum, but lamellar aggregates corresponding to Döhle bodies were not observed. Vacuoles in human toxic neutrophils are
Fig. 7  Early granulocyte precursor with dilated rough endoplasmic reticulum (10,000x).
Fig. 8 Early granulocyte precursor with disrupted segments of rough endoplasmic reticulum associated with electron-lucent areas in the cytoplasm. A few lucent areas are confluent with breaks in the perinuclear membrane.
Fig. 9 Intermediate neutrophil with two lipid droplets (22,000x).
electron-lucent and membrane bound. In dogs, vacuolation of the cytoplasm is due to irregular, electron-lucent areas which are not membrane bound. These may be due to increased fluid in the cell sap. However, the presence of a few membrane remnants and myelin figures within lucent areas suggests that some of these areas were originally membrane bound. The decrease in number and electron-density of granules in toxic neutrophils may be due to decreased production or granule rupture. Granule rupture and autodigestion could be an alternate explanation for the presence of electron-lucent areas. Increased basophilia of the cytoplasm in canine toxic neutrophils is likely due to the persistence of rough endoplasmic reticulum and polyribosomes. These structures are essentially absent in normal neutrophils.

In the bone marrow, clear, round, distinct cytoplasmic vacuoles were observed in early granulocyte precursors at the light microscopic level (see Chapter II). These vacuoles probably represent accumulations of lipid which were noted ultrastructurally in early and intermediate neutrophils.

Reasons for ultrastructural lesions in toxic neutrophils are speculative. McCall proposed three mechanisms: 1) cytoplasmic immaturity, 2) increase cellular activity, or 3) cellular degeneration. In the present study, dilation of the cytocavitary network in early granulocyte precursors and accumulation of fluid in the cell sap are morphologic features of cell injury. Continued dilation of
the rough endoplasmic reticulum and perinuclear cisterna with subsequent rupture would produce large, irregular, electron-lucent areas. Degradation of these membranous structures could result in myelin figures and lipid accumulation. Alternatively, increased synthesis of lysosomal enzymes may cause the initial dilatation of the rough endoplasmic reticulum. Subsequent disruption of the membranes would lead to autodigestion. Immaturity of the cytoplasm is indicated by the persistence of rough endoplasmic reticulum, polyribosomes, and mitochondria in mature cells. These findings suggest that all three mechanisms are involved in the morphogenesis of toxic neutrophils in the dog.
CHAPTER IV

In Vitro Function of Canine Neutrophils
During Acute Inflammation

Introduction

Transient intrinsic defects in bactericidal capacity can occur in neutrophils from persons with severe bacterial infections. Phagocytosis is generally not impaired. Bactericidal activity is not restored by addition of normal serum and patient serum does not induce the defect in normal cells. Weinstein found no intrinsic killing defect in neutrophils from patients with Gram negative septicemia and concluded that inadequate opsonization, rather than an intrinsic neutrophil defect, was a significant cause of neutrophil dysfunction. Variations in these results are likely due to the stage and severity of disease along with differences in assay technique.

McCall measured metabolic activity of toxic neutrophils from human patients with severe bacterial infections. Oxygen uptake and hexose monophosphate shunt activity were elevated slightly. Quantitative NBT reduction was normal, but an increased number of cells reduced the dye. Metabolic changes were unrelated to degree of leukocytosis, cellular immaturity, presence of fever, azotemia, hypotension, or
antibiotic therapy.

Lysosomal depletion and cell immaturity have been proposed as causes for decreased function in toxic neutrophils. Increased lysosomal permeability and spontaneous degranulation are noted in toxic neutrophils. Features of cytoplasmic immaturity in toxic neutrophils (basophilia, increased rough endoplasmic reticulum) suggest functional immaturity.

Intramuscular injection of turpentine is a reliable model for the production of acute inflammation and toxic neutrophils in the dog (see Chapter II). In the present study, the ability of toxic neutrophils from turpentine-injected dogs to phagocytize and kill Staphylococcus aureus was compared to normal neutrophils.

Materials and Methods

Ten mongrel dogs (4 male, 6 female) were vaccinated, treated for intestinal parasites, and preconditioned for two weeks. Five dogs (2 male, 3 female) were injected intramuscularly with 1.0 ml turpentine in the left gluteal region. Five control dogs (2 male, 3 female) were injected similarly with 1.0 ml sterile 0.9% saline.

Hematology, bone marrow evaluation, neutrophil morphology, and clinical course of the experimental model

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aVanguard DA2PL + Bordetella, Nordon Laboratories, Lincoln, NE.
bStrongid T, Pfizer Inc., New York, NY.
cMedi-Kay Pharmaceutical Co., Brookfield, MD.
have been described in Chapter II.

Neutrophil collection and separation: Eight ml of venous blood were collected in potassium EDTA. The sample was mixed with 4 ml of 6% dextran in 0.9% saline, allowed to sediment for 30 minutes at room temperature. Leukocyte-rich plasma was transferred to a sterile, 13 x 100 mm tube, and centrifuged at 400 g for 5 minutes. The supernatant was discarded and the cellular button resuspended in 0.3 ml sterile 0.9% saline. This leukocyte rich suspension was layered over 1 ml 31% bovine serum albumin in RPMI (Roswell Park Memorial Institute Media 1640). After centrifugation at 400 g for 20 minutes, the supernatant was removed and the cells resuspended in 1.0 ml Hank's Balanced Salt Solution (HBSS). Total leukocyte and differential counts were determined and the sample diluted in HBSS to approximately 11 x 10^6 neutrophils/ml. Viability of the phagocytes was confirmed by trypan blue exclusion.

Preparation of bacteria: *Staphylococcus aureus* (ATCC 29213) was grown in tryptose broth at 37 C overnight. After twice washing in sterile 0.9% saline, the culture was resuspended in saline and adjusted to an optical density of 0.5 at 620 nm (1-2 x 10^9 bacteria/ml). The
suspension was diluted serially in HBSS until a concentration of $5 \times 10^7$ bacteria/ml was achieved.

Assay procedure: Nine-tenths ml of the bacterial suspension and an equal volume of neutrophil suspension were added to 0.2 ml pooled canine serum in a sterile 16 x 100 mm screw cap tube. The concentrations of bacteria and neutrophils were $20-40 \times 10^6$ bacteria/ml and $4-5 \times 10^6$ neutrophils/ml respectively. During incubation at 37 C, tubes were mixed on an aliquot mixer$^k$ to facilitate contact between phagocytes and bacteria. Aliquots were removed initially and at 30 and 60 minutes for quantitation of viable bacteria and extracellular bacteria. Immediately prior to plating on tryptose agar,$^1$ each aliquot was sonicated for 30 seconds$^m$ to disrupt neutrophils with minimal loss of bacterial viability. Viable bacteria were counted by withdrawing a 0.1 ml aliquot from the assay tube, diluting to approximately $10^4-10^5$ bacteria/ml, sonicating, and plating in triplicate on tryptose agar with a 0.001 ml calibrated platinum wire loop. Extracellular bacteria were enumerated by removing a second 0.1 ml aliquot from each assay tube, diluting to $10^4-10^5$ bacteria/ml, and centrifuging at 50 g for 3 minutes. The supernatant was sonicated and plated as before. Results were expressed as a percentage of the original bacteria phagocytized or killed.

$^k$ Ames Company, Elkhart, IN.
$^l$Difco Laboratories, Detroit, MI.
$^m$Virusonic (micro-tip, 35% maximum), The Virtis Co., Gardiner, NY.
initial count - count remaining  
____________________________ x 100 = % phagocytized or
initial count killed

Control assay tubes: For each dog, a tube containing
0.9 ml bacterial suspension, 0.9 ml cell suspension, and
0.2 ml pooled serum was incubated at 37°C for one hour without
mixing. In these tubes, the chance of contact between phago-
cyte and bacteria, a prerequisite of phagocytosis, is
minimized. Diminished bacterial counts in these tubes
represent bactericidal effects of extracellular factors. A
second control tube to detect killing effect of the media was
prepared (0.9 ml bacterial suspension, 0.9 ml HBSS without
neutrophils, 0.2 ml pooled serum). At sixty minutes, 0.1 ml
was removed from these tubes, diluted to 10^4-10^5
bacteria/ml, sonicated, and plated in duplicate with a
0.001 ml platinum wire loop. The amount of extracellular
trapping by centrifugation at 50 g for 3 minutes was
calculated as the difference between initial counts of the
suspension and supernatant. Data from phagocytosis and
killing assays were evaluated by a 2 x 7 analysis of
variance.

Results

Hematology and clinical signs of turpentine-induced
inflammation in dogs have been described in Chapter II. Body
temperatures were elevated and dogs were depressed within 24
hours of injection. Clinical signs and temperature were most
severe on days 2 and 3 post-injection and gradually returned to normal by day 7. Neutrophil numbers peaked at 8 hours post-injection (\(x = 29,300/\mu\text{l}\)) and remained elevated through day 6. Toxic neutrophils were first observed at 8 hours after injection and were significantly elevated at 16 hours (\(x = 8,500/\mu\text{l}\)). Toxic cells peaked at day 2 (\(x = 15,000/\mu\text{l}\)) and declined gradually to baseline by day 5.

Turpentine-induced inflammation did not affect in vitro neutrophil phagocytosis after 30 or 60 minutes of incubation (Fig. 1). Bactericidal activity at 60 minutes was unaffected, but at 30 minutes the bactericidal activity was significantly decreased in turpentine-injected dogs on days 1, 4, and 9 post-injection (Fig. 2). Significant differences in control dogs were not observed in either function test. The bactericidal effects of extracellular factors and trapping of bacteria during centrifugation were insignificant.

Averages for the function tests on the four days immediately following injection are listed in Table 1. The percentage of bacteria killed by neutrophils from turpentine-injected dogs at 30 minutes was significantly less than control values (\(P < .05\)). At 60 minutes, neutrophils from control and turpentine-injected dogs had similar bactericidal ability. Phagocytosis was not impaired in turpentine-injected dogs at 30 or 60 minutes.
Fig. 1 Percent of *Staphylococcus aureus* phagocytized after 30 and 60 minutes of incubation in control (---) and turpentine-injected dogs (——). Each point represents $\bar{x} \pm$ SEM. Arrow signifies time of injection.
Fig. 2  Percent of Staphylococcus aureus killed after 30 and 60 minutes of incubation in control (---) and turpentine-injected dogs (-----). Each point represents $X \pm$ SEM. Arrow signifies time of injection.
Table 1 - Average percent of *Staphylococcus aureus* killed or phagocytized in the four days immediately after injection (X ± SEM, n = 20). *Signifies that difference is statistically significant (P < .05).
Discussion

Neutrophils from some human patients with severe bacterial infections and toxic neutrophils had decreased bactericidal activity. Others reported normal neutrophil function in similar patients. Phagocytosis in turpentine-injected dogs is not impaired at 30 or 60 minutes incubation, which is consistent with previous reports in man.

Results of the bactericidal assays in turpentine-injected dogs are inconclusive. Bactericidal activity was normal at 60 minutes, while the average percentage of bacteria killed at 30 minutes was significantly less than control (P<.05). An analysis of daily results of bactericidal activity at 30 minutes shows little variation in the control group. However, bactericidal activity fluctuated daily in the test group during the post injection period. Killing was significantly diminished on days 1, 4, and 9. Rapid turnover of blood neutrophils (T 1/2 = 7 hours in normal dogs) means that the population of blood neutrophils changes every few hours. Therefore, a functional defect affecting only a few generations of neutrophils would cause a transient depression of in vitro function.

Diminished killing function in neutrophils from turpentine-injected dogs cannot be attributed to defects in opsonization. Pooled canine serum, rather than autologous serum was used in the assay system. In addition, defective opsonization should cause diminished phagocytic activity.
The pattern of functional change in the present study does not correlated with the degree of neutrophilia, number of toxic neutrophils, body temperature, or clinical signs. If results of the function assay were related to these variables, function should be at its lowest on days 1-3 post-injection and have returned to normal by day 9. Results of this study showed killing to be normal on days 2 and 3 and abnormal on day 9.

It is tempting to equate morphologic changes with abnormal function. The present data do not indicate a clear relationship. It is possible that cells which appear "toxic" still have considerable functional capabilities. On the other hand, cells which do not meet the criteria of "toxic neutrophils" may well have biochemical alterations which result in impaired function. It is possible that some factor not measured in this experiment, such as a tissue breakdown product, serum factor, or metabolite of turpentine may induce biochemical defects in neutrophils which do not result in morphologic alterations.

Another aspect to consider is the reliability of the assay procedure. In vitro phagocytosis and killing are affected by the ratio of bacteria to neutrophils. Repine found that many patients had defective neutrophil function when tested at a high bacteria to phagocyte ratio. When tested at lower ratios, neutrophil function was normal. If a higher bacteria to neutrophil ratio had been used, perhaps neutrophil function in
turpentine-injected dogs would have been more severely affected.

This is the first experiment reported where neutrophil function is monitored before and during experimentally induced acute inflammation. The obscure pattern of diminished bactericidal activity, and the lack of correlation between neutrophil function and other variables cast doubt on the biological importance of this observation. However, the decrease in bacterial killing (30 minutes), when all post-injection samples are averaged, is comparable with reports in man.121-125. These results point out the importance of repeated assays rather single determinations when working with a dynamic cell population.
CHAPTER V

Summary and Conclusions

Intramuscular injection of turpentine (1 ml) produced acute inflammation and clinical illness in dogs lasting an average of 7 days. Intensity of clinical signs paralleled changes in rectal temperature, neutrophil count, total leukocyte count, and number of toxic neutrophils.

Toxic neutrophils in turpentine-injected dogs were similar morphologically to those seen clinically in dogs with severe inflammatory or infectious processes. Basophilia and foamy vacuolation of the cytoplasm, along with the occasional Döhle body, were observed in neutrophils from all turpentine-injected dogs.

Bone marrow changes were dramatic during the course of the acute inflammation. Depletion of the bone marrow storage pool of neutrophils (bands, segmented neutrophils), was followed by marked increase in the number of myeloblasts and progranulocytes, and brisk mitotic activity at the myelocytic stage. Morphologic changes in the granulocytic series included: 1) irregular or monocytoid nuclear shape of myeloblasts and promyelocytes, 2) distinct vacuolation of the cytoplasm of early granulocytes, and 3) increased basophilia and foamy vacuolation of the cytoplasm of metamyelocytes, bands, and segmented neutrophils. This was followed by
intense granulocytic hyperplasia and a return to normal
granulocyte morphology within 4 days of injection.

Ultrastructure of the normal granulocytic series in the
dog was similar to descriptions in man. Explanation of light
microscopic changes in canine toxic neutrophils was obtained
from electron microscopic studies. Vacuolation of the
cytoplasm was due to irregular, electron-lucent areas which
were not membrane bound. However the presence of membrane
remnants and myelin figures within lucent areas suggests that
some of these areas were originally membrane bound.
Accumulation of fluid in the cell sap, independent of
membrane structures may also produce cytoplasmic vacuolation.
Increased cytoplasmic basophilia was attributed to the
persistence of rough endoplasmic reticulum and polyribosomes
in toxic neutrophils.

Bone marrow ultrastructure of turpentine-injected dogs
indicates that vacuolation in the cytoplasm in mature toxic
neutrophils may result from a lesion in the cytocavitary
network of early neutrophil precursors. Dilation of rough
endoplasmic reticulum may lead to disruption and degradation
of these membranous structures. These lesions produced
irregular, electron-lucent areas and myelin figures seen in
mature toxic neutrophils.

Morphologic evidence obtained from this study suggests
that cytoplasmic immaturity, increased cellular activity, and
cell degeneration may be involved in the formation of toxic
neutrophils in the dog.
Results of neutrophil function tests in turpentine-injected dogs were inconclusive. Phagocytosis of *Staphylococcus aureus* was not impaired. Decreased bactericidal activity was present after 30 minutes but not 60 minutes of incubation. The pattern of decreased bactericidal activity did not correlate with severity of clinical signs, degree of neutrophilia, or number of toxic neutrophils. Further studies with *in vitro* function tests or other comparable methods are required before the biological significance of the bactericidal defect can be established.

Turpentine-induced inflammation is a predictable model for the production of toxic neutrophils in the dog. Since dramatic changes had already taken place by 24 hours, more intensive study of the first 24 hours post-injection is needed to better understand the morphogenesis of toxic change. Histochemical studies of bone marrow precursors to differentiate granulocytic and monocytic precursors would determine the significance of monocytopenesis in the model. Histochemical staining for lipid would confirm the composition of the clear, circular vacuoles in early granulocyte precursors. Localization of lysosomal enzymes which are being synthesized in the rough endoplasmic reticulum during granulocyte development would elucidate the role of rough endoplasmic reticulum in the formation of electron-lucent areas in the cytoplasm of mature toxic neutrophils.

Toxic neutrophils have long been associated with severe
inflammatory disease in animals. The present study shows a
definite relationship of toxic neutrophils and the clinical
course of the disease. Several reports have shown decreased
bactericidal activity of human toxic neutrophils and these
authors suggest that this defect may compromise patient
survival. Results of the present study, although
inconclusive, suggest that this may be true in the dog.
However, the differences in ultrastructural appearance of
human and canine toxic neutrophils suggest that the
biological behavior of these cells may be very different.
Further study of canine neutrophil function in clinical
disease is necessary before determining the effect of toxic
change and abnormal function on host survival.
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TABLE 1: Body Temperature (°F)

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<th>Control Group*</th>
<th>Turpentine-Injected Group*</th>
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<td></td>
<td>X ± SD</td>
<td>X ± SD</td>
</tr>
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<td>6</td>
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<td>102.1 ± 1.1 (P &lt; .05)</td>
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<td>7</td>
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<td>8</td>
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<td>101.4 ± 0.6 (NS)</td>
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<td>9</td>
<td>101.5 ± 0.4</td>
<td>101.8 ± 0.5 (NS)</td>
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*n = 7

**NS = Not significantly different. Data were evaluated by a 2 x 10 analysis of variance.
TABLE 2: Total Leukocyte Counts (x10^3/μl)

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<td>X ± SD</td>
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<td>13.0 ± 4.2</td>
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<tr>
<td>8 hrs.</td>
<td>17.0 ± 5.3</td>
<td>29.3 ± 11.0 (P &lt; .01)</td>
</tr>
<tr>
<td>16 hrs.</td>
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<td>4</td>
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<td>9</td>
<td>11.7 ± 3.2</td>
<td>10.7 ± 2.7 (NS)</td>
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*n = 7

**Not significantly different. Data were evaluated by a 2 x 14 analysis of variance.
### TABLE 3: Neutrophil Counts (x10³/µl)

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<td>8 hrs.</td>
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* n = 7

**Not significantly different. Data were evaluated by a 2 x 14 analysis of variance.
TABLE 4: Monocyte Counts (x10³/µl)

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*n = 7

**Not significantly different. Data were evaluated by a 2 x 14 analysis of variance.
TABLE 5: Lymphocyte Counts (x10³/µl)

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<td>( x \pm SD )</td>
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<td>3.3 ± 1.3</td>
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<td>8 hrs.</td>
<td>3.3 ± 0.9</td>
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<td>16 hrs.</td>
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<td>3</td>
<td>3.0 ± 1.3</td>
<td>2.6 ± 0.6 (NS)</td>
</tr>
<tr>
<td>4</td>
<td>3.2 ± 1.6</td>
<td>2.2 ± 0.7 (P &lt; .05)</td>
</tr>
<tr>
<td>5</td>
<td>3.6 ± 1.3</td>
<td>2.1 ± 0.5 (P &lt; .01)</td>
</tr>
<tr>
<td>6</td>
<td>2.7 ± 1.1</td>
<td>1.7 ± 0.6 (P &lt; .05)</td>
</tr>
<tr>
<td>7</td>
<td>2.7 ± 1.0</td>
<td>2.0 ± 0.5 (NS)</td>
</tr>
<tr>
<td>8</td>
<td>3.2 ± 1.5</td>
<td>2.3 ± 0.3 (P &lt; .05)</td>
</tr>
<tr>
<td>9</td>
<td>2.8 ± 1.3</td>
<td>2.0 ± 0.4 (P &lt; .05)</td>
</tr>
</tbody>
</table>

*n = 7

**Not significantly different. Data were evaluated by a 2 x 14 analysis of variance.
### TABLE 6: Eosinophil Counts (x10^3/μl)

<table>
<thead>
<tr>
<th>DAY</th>
<th>Control Group*</th>
<th>Turpentine-Injected Group*</th>
</tr>
</thead>
<tbody>
<tr>
<td>-2</td>
<td>0.8 ± 0.4</td>
<td>2.1 ± 1.2 (P &lt; .05)**</td>
</tr>
<tr>
<td>-1</td>
<td>1.3 ± 1.1</td>
<td>2.1 ± 1.5 (P &lt; .05)</td>
</tr>
<tr>
<td>0</td>
<td>1.4 ± 0.6</td>
<td>1.8 ± 1.2 (NS)§</td>
</tr>
<tr>
<td>8 hrs.</td>
<td>1.2 ± 0.6</td>
<td>0.9 ± 1.4 (NS)</td>
</tr>
<tr>
<td>16 hrs.</td>
<td>1.4 ± 1.0</td>
<td>0.0 ± 0.0 (P &lt; .01)</td>
</tr>
<tr>
<td>1</td>
<td>0.9 ± 0.7</td>
<td>0.0 ± 0.1 (P &lt; .01)</td>
</tr>
<tr>
<td>2</td>
<td>1.3 ± 0.8</td>
<td>0.6 ± 0.7 (P &lt; .05)</td>
</tr>
<tr>
<td>3</td>
<td>1.6 ± 0.8</td>
<td>0.3 ± 0.3 (P &lt; .01)</td>
</tr>
<tr>
<td>4</td>
<td>1.5 ± 0.6</td>
<td>0.3 ± 0.2 (P &lt; .01)</td>
</tr>
<tr>
<td>5</td>
<td>1.6 ± 1.3</td>
<td>0.2 ± 0.3 (P &lt; .01)</td>
</tr>
<tr>
<td>6</td>
<td>1.4 ± 0.8</td>
<td>0.7 ± 0.5 (NS)</td>
</tr>
<tr>
<td>7</td>
<td>1.4 ± 1.3</td>
<td>0.8 ± 0.7 (NS)</td>
</tr>
<tr>
<td>8</td>
<td>1.2 ± 0.6</td>
<td>1.0 ± 0.7 (NS)</td>
</tr>
<tr>
<td>9</td>
<td>1.4 ± 1.5</td>
<td>0.7 ± 0.5 (NS)</td>
</tr>
</tbody>
</table>

*n = 7

**Data were evaluated by a 2 x 14 analysis of variance.

§Not significantly different.
TABLE 7: Band Neutrophil Counts (x10^3/μl)

<table>
<thead>
<tr>
<th>DAY</th>
<th>Control Group*</th>
<th>Turpentine-Injected Group*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0  ±  0.0</td>
<td>0.0  ±  0.0 (NS)**</td>
</tr>
<tr>
<td>-2</td>
<td>0.0  ±  0.0</td>
<td>0.0  ±  0.0 (NS)</td>
</tr>
<tr>
<td>-1</td>
<td>0.0  ±  0.0</td>
<td>0.0  ±  0.0 (NS)</td>
</tr>
<tr>
<td>0</td>
<td>0.0  ±  0.1</td>
<td>0.0  ±  0.0 (NS)</td>
</tr>
<tr>
<td>8 hrs.</td>
<td>0.0  ±  0.0</td>
<td>0.3  ±  0.3 (NS)</td>
</tr>
<tr>
<td>16 hrs.</td>
<td>0.0  ±  0.1</td>
<td>2.4  ±  1.0 (P &lt;.01)</td>
</tr>
<tr>
<td>1</td>
<td>0.0  ±  0.0</td>
<td>2.2  ±  1.1 (P &lt;.01)</td>
</tr>
<tr>
<td>2</td>
<td>0.0  ±  0.0</td>
<td>1.2  ±  1.0 (P &lt;.01)</td>
</tr>
<tr>
<td>3</td>
<td>0.0  ±  0.0</td>
<td>0.5  ±  0.5 (P &lt;.05)</td>
</tr>
<tr>
<td>4</td>
<td>0.0  ±  0.0</td>
<td>0.4  ±  0.5 (NS)</td>
</tr>
<tr>
<td>5</td>
<td>0.1  ±  0.1</td>
<td>0.3  ±  0.6 (NS)</td>
</tr>
<tr>
<td>6</td>
<td>0.0  ±  0.1</td>
<td>0.2  ±  0.4 (NS)</td>
</tr>
<tr>
<td>7</td>
<td>0.0  ±  0.1</td>
<td>0.1  ±  0.1 (NS)</td>
</tr>
<tr>
<td>8</td>
<td>0.0  ±  0.0</td>
<td>0.0  ±  0.0 (NS)</td>
</tr>
<tr>
<td>9</td>
<td>0.0  ±  0.0</td>
<td>0.0  ±  0.0 (NS)</td>
</tr>
</tbody>
</table>

*n = 7

**Not significantly different. Data were evaluated by a 2 x 14 analysis of variance.
TABLE 8: Toxic Neutrophil Counts (x10³/µl)

<table>
<thead>
<tr>
<th>DAY</th>
<th>Control Group*</th>
<th>Turpentine-Injected Group*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X ± SD</td>
<td>X ± SD</td>
</tr>
<tr>
<td>-2</td>
<td>0.0 ± 0.1</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>-1</td>
<td>0.1 ± 0.1</td>
<td>0.0 ± 0.1</td>
</tr>
<tr>
<td>0</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>8 hrs.</td>
<td>0.1 ± 0.1</td>
<td>0.4 ± 0.9</td>
</tr>
<tr>
<td>16 hrs.</td>
<td>0.1 ± 0.1</td>
<td>8.5 ± 8.0</td>
</tr>
<tr>
<td>1</td>
<td>0.0 ± 0.0</td>
<td>12.6 ± 8.5</td>
</tr>
<tr>
<td>2</td>
<td>0.0 ± 0.1</td>
<td>15.0 ± 8.2</td>
</tr>
<tr>
<td>3</td>
<td>0.0 ± 0.0</td>
<td>13.8 ± 7.6</td>
</tr>
<tr>
<td>4</td>
<td>0.1 ± 0.2</td>
<td>8.9 ± 3.0</td>
</tr>
<tr>
<td>5</td>
<td>0.0 ± 0.0</td>
<td>8.8 ± 9.7</td>
</tr>
<tr>
<td>6</td>
<td>0.1 ± 0.1</td>
<td>2.5 ± 3.4</td>
</tr>
<tr>
<td>7</td>
<td>0.0 ± 0.0</td>
<td>1.4 ± 2.5</td>
</tr>
<tr>
<td>8</td>
<td>0.0 ± 0.1</td>
<td>0.6 ± 0.9</td>
</tr>
<tr>
<td>9</td>
<td>0.0 ± 0.0</td>
<td>0.1 ± 0.2</td>
</tr>
</tbody>
</table>

*n = 7

**Not significantly different. Data were evaluated by a 2 x 14 analysis of variance.
TABLE 9: Myeloid:Erythroid Ratios

<table>
<thead>
<tr>
<th>DAY</th>
<th>Control Group*</th>
<th>Turpentine-Injected Group*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \bar{x} \pm SD )</td>
<td>( \bar{x} \pm SD )</td>
</tr>
<tr>
<td>0</td>
<td>1.4 ± 0.2</td>
<td>1.2 ± 0.2 (NS)**</td>
</tr>
<tr>
<td>1</td>
<td>1.9 ± 0.3</td>
<td>11.5 ± 0.5 (NS)</td>
</tr>
<tr>
<td>2</td>
<td>1.8 ± 0.3</td>
<td>21.1 ± 9.2 (P &lt; .01)</td>
</tr>
<tr>
<td>3</td>
<td>1.5 ± 0.3</td>
<td>20.0 ± 8.7 (P &lt; .01)</td>
</tr>
<tr>
<td>4</td>
<td>1.5 ± 0.3</td>
<td>18.2 ± 9.4 (P &lt; .01)</td>
</tr>
<tr>
<td>9</td>
<td>1.5 ± 0.3</td>
<td>4.2 ± 2.2 (NS)</td>
</tr>
</tbody>
</table>

\*n = 4

**Not significantly different. Data were evaluated by a 2 x 6 analysis of variance.
<table>
<thead>
<tr>
<th>DAY</th>
<th>Control Group*</th>
<th>Turpentine-Injected Group*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \bar{x} \pm SD )</td>
<td>( \bar{x} \pm SD )</td>
</tr>
<tr>
<td>0</td>
<td>13.4 ± 4.1</td>
<td>11.6 ± 2.4 (NS)**</td>
</tr>
<tr>
<td>1</td>
<td>11.7 ± 3.2</td>
<td>27.6 ± 5.2 (P &lt; .01)</td>
</tr>
<tr>
<td>2</td>
<td>8.0 ± 2.2</td>
<td>34.1 ± 7.2 (P &lt; .01)</td>
</tr>
<tr>
<td>3</td>
<td>11.2 ± 1.5</td>
<td>21.5 ± 5.2 (P &lt; .01)</td>
</tr>
<tr>
<td>4</td>
<td>9.2 ± 3.5</td>
<td>14.9 ± 5.1 (NS)</td>
</tr>
<tr>
<td>9</td>
<td>12.0 ± 3.0</td>
<td>8.3 ± 2.1 (NS)</td>
</tr>
</tbody>
</table>

*n = 4

**Not significantly different. Data were evaluated by a 2 x 6 analysis of variance.
TABLE 11: Percent of *Staphylococcus aureus* Phagocytized After 30 Minutes Incubation

<table>
<thead>
<tr>
<th>DAY</th>
<th>Control Group*</th>
<th>Turpentine-Injected Group*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X ± SD</td>
<td>X ± SD</td>
</tr>
<tr>
<td>-1</td>
<td>92.2 ± 3.5</td>
<td>93.4 ± 3.8 (NS)**</td>
</tr>
<tr>
<td>0</td>
<td>90.6 ± 3.0</td>
<td>92.9 ± 3.2 (NS)</td>
</tr>
<tr>
<td>1</td>
<td>90.1 ± 4.0</td>
<td>90.2 ± 5.2 (NS)</td>
</tr>
<tr>
<td>2</td>
<td>93.7 ± 3.9</td>
<td>87.6 ± 2.7 (P &lt; .01)</td>
</tr>
<tr>
<td>3</td>
<td>91.6 ± 2.7</td>
<td>94.2 ± 2.0 (NS)</td>
</tr>
<tr>
<td>4</td>
<td>93.6 ± 1.7</td>
<td>93.5 ± 2.2 (NS)</td>
</tr>
<tr>
<td>9</td>
<td>94.4 ± 3.0</td>
<td>93.0 ± 2.7 (NS)</td>
</tr>
</tbody>
</table>

*n = 5

**Not significantly different. Data were evaluated by a 2 x 7 analysis of variance.
TABLE 12: Percent of *Staphylococcus aureus* Phagocytized After 60 Minutes Incubation

<table>
<thead>
<tr>
<th>DAY</th>
<th>Control Group*</th>
<th>Turpentine-Injected Group*</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1</td>
<td>$94.7 \pm 2.6$</td>
<td>$95.8 \pm 2.8$ (NS)**</td>
</tr>
<tr>
<td>0</td>
<td>$95.7 \pm 1.4$</td>
<td>$96.8 \pm 0.6$ (NS)</td>
</tr>
<tr>
<td>1</td>
<td>$96.1 \pm 2.6$</td>
<td>$97.5 \pm 2.3$ (NS)</td>
</tr>
<tr>
<td>2</td>
<td>$96.7 \pm 2.2$</td>
<td>$96.3 \pm 1.7$ (NS)</td>
</tr>
<tr>
<td>3</td>
<td>$96.7 \pm 1.7$</td>
<td>$97.9 \pm 1.1$ (NS)</td>
</tr>
<tr>
<td>4</td>
<td>$96.2 \pm 1.6$</td>
<td>$98.1 \pm 1.4$ (NS)</td>
</tr>
<tr>
<td>9</td>
<td>$96.1 \pm 2.1$</td>
<td>$94.8 \pm 2.3$ (NS)</td>
</tr>
</tbody>
</table>

*n = 5*

**Not significantly different. Data were evaluated by a 2 x 7 analysis of variance.
TABLE 13: Percent of Staphylococcus aureus Phagocytized After 30 Minutes Incubation

<table>
<thead>
<tr>
<th>DAY</th>
<th>Control Group*</th>
<th>Turpentine-Injected Group*</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1</td>
<td>80.5 ± 4.9</td>
<td>82.8 ± 4.4 (NS)**</td>
</tr>
<tr>
<td>0</td>
<td>84.9 ± 3.1</td>
<td>81.4 ± 5.8 (NS)</td>
</tr>
<tr>
<td>1</td>
<td>83.7 ± 6.2</td>
<td>69.8 ± 5.9 (P &lt; .01)</td>
</tr>
<tr>
<td>2</td>
<td>84.4 ± 5.4</td>
<td>80.3 ± 8.7 (NS)</td>
</tr>
<tr>
<td>3</td>
<td>82.1 ± 6.1</td>
<td>81.4 ± 2.8 (NS)</td>
</tr>
<tr>
<td>4</td>
<td>81.6 ± 5.4</td>
<td>71.8 ± 6.5 (P &lt; .05)</td>
</tr>
<tr>
<td>9</td>
<td>85.4 ± 7.2</td>
<td>75.9 ± 7.7 (P &lt; .05)</td>
</tr>
</tbody>
</table>

*n = 5

**Not significantly different. Data were evaluated by a 2 x 7 analysis of variance.
TABLE 14: Percent of *Staphylococcus aureus* Killed After 60 Minutes Incubation

<table>
<thead>
<tr>
<th>DAY</th>
<th>Control Group*</th>
<th>Turpentine-Injected Group*</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1</td>
<td>90.7 ± 3.5</td>
<td>89.3 ± 3.7 (NS)**</td>
</tr>
<tr>
<td>0</td>
<td>89.7 ± 0.7</td>
<td>90.8 ± 3.0 (NS)</td>
</tr>
<tr>
<td>1</td>
<td>88.5 ± 5.7</td>
<td>86.4 ± 3.7 (NS)</td>
</tr>
<tr>
<td>2</td>
<td>93.3 ± 3.7</td>
<td>88.0 ± 1.6 (NS)</td>
</tr>
<tr>
<td>3</td>
<td>91.8 ± 3.2</td>
<td>91.9 ± 3.0 (NS)</td>
</tr>
<tr>
<td>4</td>
<td>91.1 ± 2.4</td>
<td>88.4 ± 3.3 (NS)</td>
</tr>
<tr>
<td>9</td>
<td>89.1 ± 3.1</td>
<td>88.5 ± 3.1 (NS)</td>
</tr>
</tbody>
</table>

*n = 5

**Not significantly different. Data were evaluated by a 2 x 7 analysis of variance.
CURRICULUM VITAE

KENT ALAN GOSSETT

BORN:
June 1, 1953, Greenfield, Indiana

HOME ADDRESS:
Box 19356
Baton Rouge, LA 70893
(504) 926-1240

OFFICE ADDRESS:
Department of Veterinary Pathology
School of Veterinary Medicine
Louisiana State University
Baton Rouge, LA 70803
(504) 346-3264

EDUCATION:
Ph.D. (Veterinary Clinical Pathology) Louisiana State University; May, 1981
M.S. (Veterinary Microbiology) Purdue University; 1978
DVM Purdue University; 1978

PROFESSIONAL ORGANIZATION:
American Veterinary Medical Association

RESEARCH EXPERIENCE:
1) 1974-1978: Purdue University, School of Veterinary Medicine; research assistant and graduate student in enteric microbiology.
2) 1979: Louisiana State University, Department of Veterinary Pathology; study of experimentally induced liver disease in the horse.
3) 1980-81: Louisiana State University, Department of Veterinary Pathology; study of neutrophil morphology and function in the dog.

WORK EXPERIENCE:
Microbiology research assistant, Purdue University, 1974-75.
Graduate student, microbiology, Purdue University, 1975-78.
Extern at the Greencastle Veterinary Hospital, Greencastle, Indiana, August-September, 1977.
Extern at the Gentilly Veterinary Hospital, New Orleans, Louisiana, February-March, 1978.
Extern at the Urban Practice Clinic, University of Tennessee, School of Veterinary Medicine, Knoxville, Tennessee, March-April, 1978.
Graduate student, teacher of clinical pathology in the fourth year veterinary curriculum, service work in the clinical pathology laboratory, Louisiana State University, School of Veterinary Medicine, Baton Rouge, Louisiana, 1978-81.
MANUSCRIPTS:
M.S. Effect of Fish Passage on the Virulence of Salmonellae in White Mice, K. A. Gossett, Master's Thesis, Purdue University, May 1978.

PUBLICATION:

PROFESSIONAL INTERESTS:
1) Morphology and function of hematopoietic cells.
2) Clinical chemistry.
3) Diagnostic clinical pathology.

CAREER GOALS:
1) Board certification in veterinary clinical pathology.
2) Develop research interests in the areas of clinical chemistry and hematopoietic cell structure and function.
EXAMINATION AND THESIS REPORT

Candidate: Kent Alan Gossett

Major Field: Veterinary Medical Sciences – Option: Veterinary Clinical Pathology

Title of Thesis: Morphology and Function of Toxic Neutrophils in the Dog

Approved:

Co-Major Professor

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:


Date of Examination:

April 27, 1981