A MORPHOLOGIC AND IMMUNOLOGIC STUDY

OF AMERICAN CANINE HEPATOZOONOSIS

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CHAPTER I

INTRODUCTION

Canine hepatozoonosis has a worldwide distribution. Bentley and James in India reported the first descriptions of the causative organism and disease in 1905.[11,51] In 1997, the etiologic agent for canine hepatozoonosis in North America was designated a new species, *Hepatozoon americanum*.[137] Before 1997, *H. americanum* was thought to be a particularly virulent strain of *Hepatozoon canis*, the organism responsible for canine hepatozoonosis in the Old World.[21,136] Reclassification of the North American disease was based on differences in clinical and pathological features of the disease, in the morphological characteristics of the parasite, and in the phylogenetic relationship of the organism to other related parasites.

The clinical course of hepatozoonosis in North America is chronic, progressive, and generally fatal for dogs.[5,8,21,104,136] The disease manifests with intermittent fever, stiffness, gait abnormalities, marked lethargy, depression, weight loss, muscle wasting, pain, and persistent neutrophilia. Parasitic stages accompanied by intense inflammation are found predominantly in skeletal and cardiac muscle. In addition to lesions in muscle, dogs develop generalized periosteal bone proliferation. This particular lesion has similarities to hypertrophic osteopathy, a poorly understood syndrome affecting the bones of man and animals. Inflammatory mediators such as cytokines generated as a result of the immune response to *Hepatozoon americanum* clearly play a role in the pathogenesis of the disease and possibly in the stimulation of periosteal bone proliferation. Using immunohistochemistry, transmission electron microscopy, and

cytokine bioassays, the focus of this research is to expand our understanding of the host:parasite relationship by clearly defining the role between the dog's immune response and the parasitic life cycle.

A. Literature Review

Hepatozoonosis

Taxonomy

Hepatozoon americanum is presumptively classified in the Phylum Protozoa, Subphylum Apicomplexa, Class Sporozoasida, Subclass Coccidiasina, Order Eucoccidiorida, Suborder Adeleorina, and Family Haemogregarina.[59,68,135] A major distinguishing feature of apicomplexans is the apical complex, consisting of a polar ring, micronemes, rhoptries, subpellicular tubules, and a conoid. Reproduction of apicomplexans involves both asexual (schizogony and sporogony) and sexual (gametogony) phases.[2,68,118,135,140,142]

The genus *Hepatozoon* is one of six genera of blood parasites called hemogregarines. All hemogregarines have heteroxeneous life cycles that require an intermediate vertebrate host and a blood-feeding, definitive invertebrate host. More than 300 different species of Hepatozoon found worldwide infect a variety of vertebrate hosts including mammals, birds, crocodilians, snakes, lizards, turtles, and anurans.[87,123,124,125,126,127] An even wider array of invertebrates serve as vectors or definitive hosts for *Hepatozoon* species. Invertebrates such as ticks, mites, reduviid bugs, sandflies, culicine, and anopheline mosquitoes, tsetse flies, and fleas have been

reported to serve as hosts.[123] Members of the genus *Hepatozoon* are often characterized by the location of gamonts in erythrocytes or leukocytes of vertebrate hosts and by the subsequent location of sporogony that occurs in the gut or haemocoel of invertebrate hosts.[80,125] Other key features include the method of transmission to vertebrates via ingestion of infected invertebrates, the development of monozoic or diazoic cysts in tissues of the vertebrate host, and most strikingly, the formation of large multisporocystic oocysts in the invertebrate host.[80,124]

General Life Cycle

Despite the diversity of vertebrate and invertebrate hosts, the basic life cycle for all *Hepatozoon* species is similar, at least for the few that have been thoroughly described.[125] The complexity of the life cycles is manifested by the unusual formation of latent cysts in the internal organs of some vertebrate hosts.[61,123]

The basic life cycle for a *Hepatozoon* species includes sporogonic development and oocyst formation in a hematophagous invertebrate, and merogonic and gametogonic development in a vertebrate.[123] Briefly, gamonts circulating in the peripheral blood of infected vertebrates are ingested by the hematophagous invertebrate host. Sexual reproduction occurs in the invertebrate host.[80] The zygote undergoes sporogonic development either in the gut wall or in the hemocoel.[123] Sporogony is completed with the formation of numerous large polysporocystic oocysts. The vertebrate host ingests the invertebrate containing oocysts and sporozoites are released into the intestinal tract. The sporozoites then undergo merogony in a variety of tissues and internal organs.

Following merogony, gamonts are formed and enter the peripheral circulation within erythrocytes or leukocytes.

Canine Hepatozoonosis

The first descriptions of a *Hepatozoon* spp. in canids were by Bentley and James in 1905 and were of an organism found in the peripheral blood of dogs in India.[11,51] The organism was classified as *Leukocytozoon canis* but later reclassified as *Hepatozoon canis*.[140,141] In 1906, Christophers published the mode of schizogony in canine bone marrow and sporogony in the common brown dog tick, *Rhipicephalus sanguineus*.[17] Since the first descriptions of canine hepatozoonosis, the disease has been reported in dogs in Africa, Southern Europe, the Middle East, Asia, Brazil, the Philippines, and the United States.[9,6,22,38,43,44,52,55,63,72,83,93,94,95,101,103,136] The first documented case of canine hepatozoonosis in North America was described by Craig in 1978.[22] Recently the causative agent of American canine hepatozoonosis (ACH) was designated a new species, *Hepatozoon americanum*.[137] Prior to 1997, the causative agent of ACH was considered to be *H. canis*.[22,136,103]

A single report describing successful transmission of *H. americanum,* previously called *H. canis*, by *R. sanguineus* exists; yet, attempts by other researcher to establish *R. sanguineus* as the definitive host have failed.[79,100] On the other hand, *Amblyomma maculatum* has been shown to be an excellent definitive host.[79] The dog ingests *A. maculatum* ticks containing *H. americanum* oocysts. Following ingestion, the oocysts rupture, sporozoites are released in the gut, and presumably, the sporozoites penetrate the gut wall and are distributed in various internal organs and tissues. The earliest

evidence of the parasite's existence in the dog is the presence of modified host cells containing parasites.[105] The predominant locations for these tissue stages of the parasite are in skeletal and cardiac muscle.[23,28,100,137] Similar stages have also been found in liver, spleen, pancreas, lymph node and adipose tissue.[42,104] A prominent early lesion, referred to as the "onion skin cyst", is so named for its multilamellar, poorly or non-sulfated acid mucopolysaccharide material that surrounds a centrally located host cell that contains developing asexual stages of *H. americanum*.[104,107] The mucopolysaccharide is theorized to be produced by the host cell in response to the developing parasite.

The parasite undergoes merogony within the modified host cell. As merogony proceeds, the nuclear material of the meront infolds, and later gives rise to individual merozoites. Eventually the integrity of either the host cell wall or another element not yet described is lost releasing merozoites into the surrounding tissue. The release of merozoites activates the dog's immune system resulting in a localized acute inflammatory reaction consisting predominantly of neutrophils. Later the acute inflammatory reaction matures into a chronic inflammatory reaction with the formation of discrete granulomas. Many cells within the granuloma contain parasites. The cells harboring parasites are present in both extravascular and intravascular compartments of the granuloma. The parasite enters the peripheral blood through these vascular compartments. A dog kept in tick-free isolation for over four years has remained positive for ACH. Infection was confirmed based on periodic muscle biopsies. This finding has prompted researchers to hypothesize that once the parasite reaches the peripheral blood, it may either recirculate and undergo merogony again or undergo

gametogony that completes the parasite's asexual cycle.[104,105] Once the parasite becomes a circulating gamont, the tick acquires the organism during a blood meal. Inside the tick's hemocoel, the gametocytes undergo sexual reproduction that will result in the formation of numerous oocysts composed of sporozoite-containing sporocysts.[80]

Transmission of Hepatozoon americanum

Transmission of *H. canis,* the causative agent of hepatozoonosis in the Old World, has been experimentally documented using the brown dog tick, *R. sanguineus.*[17,143] Likewise, *R sanguineus* was assumed and later documented by workers in Texas to be a host of *H americanum*, originally believed to be *H. canis.*[100] However, three unsuccessful trials done by Alabama workers and four trials by Oklahoma workers to transmit *H. americanum* with *R. sanguineus* suggests that *R. sanguineus* may be only a marginal host for *H. americanum* or that an error was made in interpreting the results of the earlier study.[79,137] Other workers have documented that other tick species may be the vector for *H. canis* suggesting that the same may be true for *H. americanum.*[96] In 1997, workers in Alabama observed Hepatozoon-like oocysts, sporocysts, and sporozoites in *A. maculatum* ticks removed from a dog showing clinical signs of hepatozoonosis.[137] Thereafter in 1998, Oklahoma researchers experimentally demonstrated successful transmission of *H. americanum* to dogs using *Amblyomma maculatum.*[79] Successful transmission was achieved by allowing *A. maculatum* nymphs to feed on an infected dog; the nymphs were collected and allowed to molt to adults. Dissected adults contained *H. americanum* oocysts and whole adult ticks from the same group fed to experimental dogs resulted in successful experimental transmission.[79]

Confirmation of transmission in the dog was established by demonstration of gametocytes in peripheral blood smears and by histologic demonstration of tissue stages of the parasite in skeletal muscle.

Successful transmission of *H. americanum* using *A. maculatum* introduces other questions relative to the vertebrate host and the endemic cycle. The natural cycle for *H. americanum* in the wild may involve other intermediate hosts.[24,56,79,111] In fact, many coyotes in Oklahoma have naturally acquired hepatozoon infections; they also have been experimentally infected with *H. americanum*.[57] This finding has led to the assumption that a susceptible wildlife reservoir is likely the natural vertebrate host for *H. americanum* rather than the domestic dog.[32,56,57,79] Speculation is that infection of domestic dogs with *H. americanum* is accidental and most likely to occur when the dog ingests an infected tick while grooming. Clearly, other studies are needed to further define the life cycle of *H. americanum* and to determine the natural/usual vertebrate host(s).

Clinical and Pathologic Findings of Hepatozoon americanum

One focus of this study is to further describe the disease syndrome in the dog as it relates to the parasitic stages of *H. americanum*. The disease is chronic, progressive, and often fatal. The disease presents with intermittent fever, stiffness and gait abnormalities, marked lethargy, depression, weight loss, muscle wasting and pain. A persistent leukocytosis with a mature neutrophilia (30 to 200 x $10^3/\mu l$) is observed and a mild to moderate normocytic, normochromic, nonregenerative anemia is typical.[22,36,39,136] There are mild elevations in serum alkaline phosphatase and a

decrease in creatine kinase and glucose. Most often albumin is low; however, this decrease has been attributed to decreased protein intake, chronic inflammation, or possibly renal dysfunction.[21,136]

Pathologic lesions associated with *H. americanum* infection in dogs occur predominantly in skeletal and cardiac muscle.[21,104,105] The disease causes a severe diffuse pyogranulomatous myositis. Dogs infected with *H. canis* in the Old World reportedly do not have muscle lesions. Other pathologic findings in dogs infected with *H. americanum* include glomerulonephritis, and amyloidosis has been reported in the spleen, lymph nodes, small intestine, kidney, and liver.[136] Another lesion occurring in dogs infected with *H. americanum* is periosteal bone proliferation that occurs in multiple bones including long bones, ribs, skull, and vertebra.[21,106,136] The bone lesion has many features similar to hypertrophic osteopathy, a disease of obscure pathogenesis that affects the bones of man and animals.[106]

Diagnosis and Treatment of Hepatozoon americanum

Canine hepatozoonosis can be diagnosed either by detecting gametocytes in circulating leukocytes on a blood smear or by finding parasitic stages in a muscle biopsy.[23,33,74,84] In the Old World, gametocytes of *H. canis* are readily detected on peripheral blood smears due to marked parasitemia. In North America, parasitemia in domestic dogs with *H. americanum* infection is low. Therefore, finding gametocytes in a peripheral blood smear is time consuming, and often nonproductive. For this reason, the definitive diagnosis of hepatozoonosis in North America is currently by muscle biopsy.[23] In the last year, a serologic method for diagnosing ACH has been developed

using an indirect enzyme-linked immunosorbent assay.[82] Preliminary results have shown that the test is sensitive and specific and as reliable as histopathologic examination.

Infected dogs have been treated with various drugs, including toltrazuril, trimethoprim-sulfadiazine, clindamycin, pyrimethamine, and decoquinate.[73] Treatment schemes that currently appear to extend survival times and provide excellent quality of life include trimethoprim-sulfadiazine, clindamycin, and pyrimethamine followed by long-term administration of decoquinate.[73] Other treatments that have been used include antiprotozoal agents and palliative NSAIDS (nonsteroidal anti-inflammatory drugs) that are useful in the control of pain and fever.[136] Nevertheless, neither the current regimen nor other treatments that have been used eliminate the infection.

Cytokines

Introduction

Inflammation occurs when cells of the immune system are activated.[47,97,139] The nature of the inflammatory response is determined by the inciting agent and by the type of cells stimulated. Organisms such as intracellular protozoal parasites are known for their ability to evade the host immune system thereby escaping an inflammatory reaction that would otherwise lead to their destruction.[12] Some common mechanisms used by protozoan parasites include avoiding digestion, interfering in the expression of certain cell receptors, inhibiting antigen presentation or T-cell stimulation, or transforming macrophages so as to interfere with their production/secretion of

cytokines.[12,98,114,132] Most often, these evasive mechanisms work well for the parasite but result in a chronically progressive disease state for the host.

The mechanism of immune evasion by *H. americanum* is theorized to be somewhat similar to other intracellular protozoal organisms.[12] During parasitic development, *H. americanum* is consistently observed within the cytoplasm of a "modified" host cell.[104,107] Early in the infection, a non-sulfated acid mucopolysaccharide material surrounds this cell. Thus, it appears that *H. americanum* evades the dog's immune system early in the infection by existing intracellularly and by presumably activating the cell to release a mucopolysaccharide material that will further shield the parasite from immune detection. Additionally, inflammation is not observed around early-encysted parasitic lesions; however, once merozoites are released, an inflammatory reaction occurs. This observation further supports the assumption that prior to the release of merozoites, the parasite is shielded from immune detection.

The complex immunological interaction between *H. americanum* and the dog has not been investigated, but most certainly, the interaction contributes to the outcome of the infection. Since *H. americanum* is an intracellular pathogen, it is likely that the dog's immune response to the parasite is similar to that observed with other intracellular protozoal organisms such as *Leishmania spps*, *Toxoplasma gondii*, and *Trypanosoma cruzi*. Studies of the latter organisms have demonstrated that the most common mechanism employed by the host to eliminate these parasites is a cell-mediated immune response.[1,12,98]

A cell-mediated response is directed by CD4+ subset T cell helper 1 (Th1) lymphocytes, macrophages, and the secretory products of both lymphocytes and

macrophages.[19,50,58,89,99,132,139,144] Initially, Th1 lymphocytes become activated after recognizing antigen presented by antigen presenting cells such as macrophages that express major histocompatibility complex II (MHCII). Once activated, Th1 lymphocytes secrete interferon-gamma (IFN3), interleukin 2 (IL2), and tumor necrosis factor-alpha (TNFK), which in turn stimulate and activate macrophages to become more effective in killing the intracellular pathogen. In the process, macrophages produce and secrete cytokines such as interleukin 12 (IL12), interleukin 1 (IL1), interleukin 6 (IL6), TNFK, and transforming growth factor beta (TGF2) to name a few.[90,12,114,134] The release of IFN3 by Th1 lymphocytes inhibits the proliferation of Th2 lymphocytes thereby directing the immune response towards a cell-mediated response rather than antibody production.[133]

For years, the type of T helper cells that ultimately direct the immune response has been considered based on the type of pathogen involved. Intracellular parasites stimulate a Th1 response while extracellular parasites activate the Th2 response.[90,91] However, recently these "fundamental" reactions are being reexamined.[3] The speculation now is that the interaction between the host and the pathogen may in fact result from cytokines released by both Th1 and Th2 lymphocytes. Nevertheless, the interaction between T cell lymphocytes, macrophages, their secretory products, and other inflammatory cells or mediators is highly sophisticated and vulnerable to mechanisms employed by pathogens to evade destruction. Regulation and control normally by the immune response in addition to evasive mechanisms by the intracellular parasites can often interfere with the function of T cells and macrophages that may in turn affect the outcome of disease. Often, these interactions (positive and

negative) are inappropriate resulting in enhanced susceptibility of the host to the parasite or in harmful side effects for the host.

The immune response to *H. americanum* initially begins as an acute inflammatory response that is observed following the release of *H. americanum* merozoites from the "modified" host cell and the disintegration of the mucopolysaccharide material.[104] Cytokines known to mediate an acute inflammatory response are TNFα and IL1 both of which are released by activated macrophages.[19,47,114,132] Commonly, TNFα and IL1 induce fever, pain, loss of appetite, muscle wasting, and edema. As more merozoites are released, the inflammation and lesions become more chronic characterized by the formation of granulomas. The prime component of granulomas, macrophages, along with lymphocytes, both T and B cells, and lesser numbers of neutrophils, accumulate due to an overwhelming or persistent infection resulting from the inability of the inflammatory cells to rid the host of the invading organisms.[19] In addition, cytokines released during that time, mostly by macrophages, include TNFα, TGFβ , IL1, IL12, and growth factors such as platelet derived growth factor (PDGF), epidermal growth factor (EDF), and TGFβ, to name just a few.[1,19,25,47,53,60,71] The capacity to produce many cytokines during inflammation is the forte of macrophages and the sophisticated coordination between macrophages, their cytokines, and the functioning immune system is highly important to the host during an infection with an intracellular parasite.[14,53,60,76,78,89] Therefore, it appears logical that as the inflammatory response becomes more persistent and prolonged, an over abundance of cytokines may occur and potentially be the cause or stimulation for the production of clinical signs and possibly periosteal bone proliferation. Consequently, it is the type

and level of these cytokines that form a major component of this research. Although there is an overwhelming number of cytokines and other inflammatory mediators, the focus in this study is centered on two cytokines, TNFα and TGFβ and the possible role each may play in the disease process of American canine hepatozoonosis (ACH). The reasons for choosing these two cytokines are that both are common components and regulators of an inflammatory reaction including a cell-mediated response, both are secreted by macrophages, and both have been shown to potentiate bone proliferation.

Tumor Necrosis Factor Alpha (TNF-α)

TNFα is a proinflammatory cytokine with a broad spectrum of biological activities. Produced chiefly by macrophages, TNFα binds to receptors on virtually all cells throughout the body. Classically during early inflammation, the combined effects of IL-1 and TNF-α on endothelial cells and leukocytes trigger the induction of the systemic acute-phase response. This response includes the initiation of fever, an increase in the production of neutrophils, an increase in leukocyte adherence, and an increase in prostaglandins.[10,19,85,110] In most instances, the acute phase response generated results in the development of an adaptive immunity and removal of the inciting agent. However, in some instances the acute phase response or the adaptive immune response is inappropriate leading to either an under or an over production of inflammatory mediators which can lead to harmful side effects for the host. For example, marked elevations of IL-1, IL-6, and TNF-α can contribute to tissue wasting and organ failure.[53] In diseases such as malaria, researchers have found that at some critical point, the cytokine cascade leads to a rapid auto-amplification of TNFα

overproduction causing cerebral vascular lesions that are pathognomonic for the condition cerebral malaria.[60] In contrast, chronic, low-levels of TNFα can cause biochemical changes in glucose resulting in insulin resistance which can contribute to the development of type II diabetes mellitus.[10] TNFα has also been implicated in the formation of granulomas, the hallmark of chronic inflammation.[19,47]

Already alluded to in the Introduction is the fact that TNFα may play a role in ACH by inducing clinical signs such as fever and muscle wasting. However, TNFα may also have various effects on skeletal tissue.[47,145] Effects on skeletal tissue can be seen in people with arthritis and chronic rheumatic disease, where it increases bone resorption, stimulates enzyme release from synovial cells, and stimulates fibroblast growth.[60,129] *In vitro* studies have shown that TNFK is a potent stimulus for bone resorption.[48,45,60,122,146] Osteoclasts are both directly activated by TNFα and indirectly activated by a message generated by osteoblast.[45,48,60,120,146] In theory, the mechanism of indirect stimulation of osteoclasts by osteoblasts appear to involve the production of prostaglandin-E_2 (PGE_2) by osteoblasts.[146] In another report, stimulation of osteoclasts could be the result of either PGE_2 stimulation released by osteoblasts, or could involve another intermediate cell line such as lymphocytes that release tumor necrosis factor beta (TNF2) in response to monocyte-derived prostaglandins.[48] Therefore, TNFα not only influences osteoclasts to activate bone resorption but it may also indirectly influence osteoblasts through a synergistic relationship whereby the production and secretion of prostaglandins by osteoblast inhibit the osteoclast allowing for the production of bone rather than the resorption of bone to occur.[48]

One hypothesis proposed for the production of periosteal bone proliferation in canine hepatozoonosis is an elevation or increase in some humoral substance that activates osteoblasts. The support for this hypothesis is two-fold. First, periosteal bone proliferation occurs as a chronic lesion in the disease, first observed approximately 32 days post experimental exposure to *H. americanum.*[106] Granulomas are another chronic lesion occurring in ACH as a result of the prolonged, persistent infection. If granulomas, composed of activated macrophages secreting numerous cytokines, and periosteal bone proliferation both occur late in the infection, then it stands to reason that the persistence of infection and possibly the overproduction of cytokines from activated macrophages may in fact cause an elevation in certain cytokines in the blood stream. Similarly, if there is a rise in TNFα and possibly other monokines then there may be a connection with bone proliferation since clearly TNFα has been shown to have an effect on bone components. Secondly, multiple bones including humerus, femur, ulna, radius, scapula, and vertebral column, have lesions consistent with periosteal bone proliferation in ACH.[22,106,136] This fact suggests that a systemic rather than local effect stimulates the production of bone. For these reasons, a humoral substance rather than a localized phenomenon is the proposed mechanism responsible for periosteal bone proliferation in ACH.

Transforming Growth Factor Beta (TGF-β)

Considered one of the most pleiotropic and complex cytokines, TGF-β has numerous biological activities. TGF-β has been found to play a role in embryonic development, tissue repair and regeneration, the formation of bone and cartilage, as well

as in the initiation, evolution, and resolution of inflammation.[1,13,15,18,20,40,77,102,109,112] An overproduction of TGF-β due to derangement in regulatory mechanisms has been linked to immune defects associated with malignancy, autoimmune disorders, susceptibility to opportunistic infections, and tissue fibrosis as well as lesions common to chronic inflammatory reactions such as granulomas.[1,13,60,88,109] The major sources of TGF-β are platelets, bone cells, placental tissue, dendritic cells, and leukocytes, especially activated monocytes and macrophages.[53,67,102]

Pertinent to our research is the possible role TGF-β plays in the inflammatory process and in periosteal bone proliferation of ACH. The term "multifunctional" has been applied to TGF-β because it has multiple effects on the inflammatory process, similar to TNFα. When secreted by activated macrophages, TGFβ can enhance macrophages to produce other cytokines such as TNF-α and IL-1, can stimulate macrophages to undergo phagocytic and lysosomal activities, and can promote the development of fibrosis during conditions of chronic inflammation.[60,70,128,138] TGFβ influences osteoblasts and osteoclasts by stimulating their growth and their differentiation.[60,109] TGF-β not only plays a pivotal role in controlling the extent of bone formation and resorption in normal remodeling, but may also control bone formation in the disease state.[131] TGF-β stimulates osteoblast chemotaxis as well as osteoblast proliferation that in turn increases osteoblast precursors.[92] Numerous studies have shown that in rodents, subcutaneous injections of TGF-β triggers bone growth at the injection site. TGF-β has been found to inhibit the formation and function of osteoclasts and may possibly encourage apoptosis of osteoclasts.[92] In addition, TGF-β can both up-regulate and down-regulate the production of TNF-α.[109,119] Although the

interplay between local environmental conditions, other cytokines, and growth factors are important in bone growth and remodeling, the key role of TGF-β in the activation of bone cells and the formation of bone is quite significant.

As far as parasitic infections are concerned, TGF-β appears to support the survival of intracellular organisms such as *Leishmania spp., T. cruzi,* and *Mycobacterium avium* in vivo.[1,67] *In vitro* experiments have demonstrated that while typically induced by the parasite or secreted by activated macrophages, the amount of TGF-β can be correlated with the virulence and proliferation of the parasites within the macrophages.[12] In other words, it is believed that TGF-β suppresses the killing activity of macrophages. TGFβ counteracts the effector function of IFN3,. Additionally and most importantly, TGFβ counteracts the development of a protective cell-mediated immunity by inhibiting T cell proliferation, by antagonizing the secretion of IL2 by T cells and by inhibiting the proliferation of natural killer cells, thereby enhancing intracellular proliferation of the pathogen.[12,109] An example of this action is apparent during an infection with *M. avium.* An increased production and secretion of TGF-β by macrophages infected with *M. avium* is thought to result from TGF-β suppression of the antibacterial activities of the macrophages thus promoting survival of the intracellular bacteria.[67] Although still under investigation, the interaction between TGF-β production and secretion and the survival of intracellular parasites remains crucial in the study of intracellular parasites, not to mention crucial in our understanding of Th1-Th2 responses. Similarly, in the study of canine hepatozoonosis, the role of TGF-β is theorized to be important not only during the inflammatory response to the parasite, but also in the induction of periosteal bone proliferation.

Periosteal Bone Proliferation

The process of normal bone growth and remodeling involves a balance between bone formation by osteoblasts and bone resorption by osteoclasts. When this balance is interrupted, the outcome can lead to either osteosclerosis and hyperostosis or to osteoporosis and osteolysis.[75,117]

In humans, hypertrophic osteopathy (HO) or hypertrophic osteoarthropathy is a syndrome resulting in diffuse periosteal bone proliferation and associated joint involvement. In humans, this extensive bone proliferation has been found to occur secondary to either inflammation or intrathoracic neoplasms.[62] Similarly in dogs, HO has been associated with intrathoracic lesions such as neoplasms, granulomatous inflammation, and chronic pulmonary abscessation.[106] The bone lesion that accompanies ACH is similar to HO. ACH is characterized by random to diffuse deposits of new bone laid down on the periosteal surface of mature long bones similar to lesions found in HO.[29,106] Although numerous theories exist as to what initiates or activates periosteal bone proliferation in humans and dogs with HO and in dogs with ACH, the exact cause remains unknown.

Currently five theories exist on the pathogenesis of HO in dogs that include neurogenic, vascular/hypoxia, hormonal and humoral causes.[106,130,131] The fifth theory predicts a combination of these. The neurogenic theory postulates that neural impulses triggered by the intrathoracic lesion produces a neural reflex arc between the vagus nerve and other sympathetic nerves initiating an increase in blood flow to the extremities and subsequently the periosteum.[106] The basis for this theory lies in the resolution of bone lesions after vagotomy. The vascular/hypoxia theory proposes that

localized hypoxia occurs secondary to a shunt occurring in the lungs because of an intrathoracic mass.[106] This shunt theoretically causes blood to bypass the lungs resulting in the extremities receiving a large increase in poorly-oxygenated blood. This theory has been negated based on the fact blood gas values measured in dogs with HO are normal. The hormonal theory that appears in the human medical literature, proposes that an abnormal increase in estrogen or another unknown growth hormone, most likely produced by the thoracic neoplasm, is somehow connected with bone proliferation. However to date, no correlation between estrogen and bone proliferation has been confirmed. Lastly, the humoral theory postulates that a substance released at the site of disturbance circulates in the blood reaching the periosteum and initiating a periosteal response.[106] Although each of these theories attempts to explain a cause for HO, none consistently fits exactly with the clinical and pathological characteristics of HO or ACH. We hypothesize that cytokines are the likely key to the interaction and balance between osteoblast/osteoclast and periosteal bone proliferation.

In dogs infected with *H. americanum*, the humoral theory appears to be the most likely cause for generalized periosteal bone proliferation. According to the literature, many inflammatory mediators are known to trigger bone activity.[48,92] Such mediators as IFNγ, TNFα, IL-1, and TGF-β are well documented for their role in bone formation and resorption.[45] The complex role these and other mediators play in maintaining normal bone development implies that during an inflammatory process, these same mediators may have different and unpredictable effects on bone.

Bone-specific variant of alkaline phosphatase (BALP) is a bone isoenzyme of alkaline phosphatase. Alkaline phosphatase (ALP) is a membrane bound protein

synthesized by a variety of cells. BALP is the fourth ALP isoenzyme identified in humans.[4,116] Dogs have a bone isoenzyme that is similar to BALP found in humans.[4,46,116] Research has shown that an increase in the serum activity of BALP can indicate changes in bone over time.[116] Allen et. al stated that serum analysis of BALP and other serum markers of bone turnover may provide a tool for diagnosis and prediction of changes in bone and possibly musculoskeletal disorders of dogs.[4] Speculation is that fluctuations of serum levels of BALP may correlate with changes occurring in bone during inflammation or other disease processes.[4,30] Therefore, measurements of serum BALP could be a valuable tool for detecting dynamic changes in bone during the inflammatory reaction of dogs with hepatozoonosis.

B. Research Problem

Considerable information describing the clinical and pathologic features of canine hepatozoonosis and the phylogenetic characteristics of *H. americanum* have been well documented since ACH was first reported in North America and *H. americanum* was classified as a new species.[7,21,31,80,81,104,106,137] Yet, many questions concerning the parasite and the host:parasite relationship remain unanswered. The identity of cells that harbor developing asexual stages of *H. americanum* has yet to be determined. By light microscopy, the earliest evidence of infection is represented by an unidentified, large, modified cell found predominantly in skeletal muscle.[105] Some workers have mistaken this modified cell to be the parasite.[21] Light microscopic observations supported by immunohistochemistry clearly establish that this modified cell contains developing stages of *H. americanum*.[107] As the parasite undergoes

merogony, the integrity of elements that contain the meront and maturing organism breaks down releasing numerous merozoites. Once released, these merozoites incite an acute inflammatory reaction. Subsequently, unidentified cell(s) containing parasites are observed within the acute inflammation, then within granulomas, and finally within peripheral blood leukocytes. Though the identity of these cells have been speculated upon, the cell origin remains unclear. Knowledge of the host cell is still essential in understanding the pathogenesis of hepatozoonosis. Therefore, one of the objectives of this research is to define the characteristics of the cells, using immunohistochemistry and transmission electron microscopy.

Lesions produced by *H. americanum* in canids have been described using light microscopy. However, clinical signs, characterized by recurring periods of intense neutrophilia, weakness, pain and periosteal bone proliferation, have yet to be correlated with pathologic lesions. Similar to many other parasites, the inflammatory response in dogs with hepatozoonosis is clearly induced by parasite development.[49,66,86,113,115,121] The onset of inflammation is believed to occur following the release of merozoites from the meront body. Thus, another objective of this research is to perform a temporal investigation of dogs experimentally infected with *H. americanum* in order to determine if a relationship exists between the development and progression of clinical features and the development of asexual stages of *H. americanum*.

If the release of merozoites causes a localized inflammatory response that in turn stimulates inflammatory cells to secrete substances, such as cytokines, then it is likely that the levels of pertinent cytokines may peak at that time.[26,27,34,41,54,64,65,69] Curfs et al, assert that the ability or inability of cells and tissue to generate certain cytokines or

cytokine patterns in response to infection often determines the clinical course of an infection.[25] Hence, cytokines generated during the inflammatory response to *H. americanum* should contribute to the production of clinical signs, and possibly, to the formation of periosteal bone proliferation. Specific cytokines such as TNFα and TGFβ exert their activity during periods of inflammation. We hypothesize that a rise and fall of such levels of these cytokines may correlate with different stages of the parasite's development in the dog. Similarly, a rise in serum BALP can be used to detect increased metabolic activity in bone that would indicate periosteal bone proliferation. The fact that an acute inflammatory response occurs at the time of merozoite release from the meront body suggests that the levels of TNFα and TGFβ should rise and subsequently fluctuate during the parasite's development. To confirm this, bioassays for TNFα, TGFβ, and serum BALP will be performed to measure serum fluctuations as they correlate with development of *H. americanum*. The importance of detecting the time of merozoite release could be useful in tracking the parasite's development in the dog, in detecting bone activity such as periosteal bone proliferation, and in developing effective treatment and therapeutic strategies.

C. Experimental Hypotheses and Objectives

To explore and further expand our knowledge of the host:parasite relationship between *H. americanum* and the canine host, the following hypotheses and objectives have been formulated:

1. The host cell that harbors asexual stages of *H. americanum* is a macrophage. Immunohistochemistry (IHC) and transmission electron microscopy (TEM) will be used to investigate this issue.
2. Morphologic characteristics of asexual stages of *H. americanum* in dogs with hepatozoonosis can be further described using TEM and IHC.
3. The combination of clinical features (fever, weakness, pain, etc.), blood work, and the results of periodic muscle biopsies, can be correlated with the development of asexual stages of *H. americanum*.
4. The asexual stage at which *H. americanum* merozoites are released causes a local inflammatory reaction with subsequent systemic illness and an associated increase in serum levels of TNFα and TGFβ.
5. Periosteal bone proliferation occurs secondary to inflammatory mediators, such as TNFα and TGFβ, released during the inflammatory response to developing asexual stages of *H. americanum*.
6. The onset of periosteal bone proliferation can be determined using bone radiographs, bone scintigraphy and by measuring serum fluctuations in cytokines such as TGFβ and serum levels of BALP.

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CHAPTER II

AMERICAN CANINE HEPATOZOONOSIS: CORRELATION OF THE ASEXUAL CYCLE WITH THE CLINICAL AND PATHOLOGIC FEATURES OF THE DISEASE IN EXPERIMENTALLY INFECTED DOGS

Introduction

Knowledge of canine hepatozoonosis dates back to 1905 in India where the parasite, *Hepatozoon canis*, was described by Bentley and James.[4,13] In their descriptions, both Bentley and James reported that infected dogs showed no obvious signs of illness other than slight anemia and a mild febrile disturbance. Since then, canine hepatozoonosis has been reported in numerous countries in Africa, Southern Europe, the Far and Middle East, Asia, South America, and the Philippines.[3,9,12,18,21,23] Although parasitemia is often high, the Old World disease is usually nonclinical unless the dog has a concurrent immunosuppressive disease such as toxoplasmosis, ehrlichiosis, or babesiosis.[10,11,27] In 1978, Craig et al, recognized and described canine hepatozoonosis in Texas. The disease was considerably more severe than the disease in the Old World.[6] Dogs presented with intermittent fever, stiffness, and inappetence. The dogs developed marked leukocytosis, mild anemia, and periosteal bone proliferation. Between 1978 until 1997, additional cases with similar clinical signs were reported in Louisiana, Alabama, Georgia and Oklahoma, prompting a more critical examination of the causative agent.[10,14,24,31] In 1997, based on clinical and pathologic

features of the disease, the fine structure of the gametocytes, and the location of merogony, the causative agent of American canine hepatozoonosis (ACH) was identified as a new species, *Hepatozoon americanum*.[32] Definitive separation of *H. americanum* and *H. canis* was further supported by evidence of genetic, molecular, and definitive host differences.[2,17,32]

Amblyomma maculatum has been established to be a highly suitable host for *H. americanum*.[15] Dogs have been experimentally infected by ingesting infected ticks or oocysts isolated from ticks. Experimental infections documented that following the ingestion of oocysts, parasitic stages may be observed in various tissues, particularly striated muscle, 3 to 4 weeks later.[15,25] The earliest recognized lesion is a large "modified" canine cell (host cell) with an intracytoplasmic zoite. This "modified" cell becomes "encysted" by multilamellar mucopolysaccharide material and is referred to as the "onion skin" cyst.[15,22,25] The zoite within the host cell undergoes merogony with the production of merozoites that when freed from the encysted host cell, cause a localized neutrophilic inflammatory reaction that eventually results in the formation of a granuloma. Many mononuclear cells within the granuloma contain intracytoplasmic parasites. Many of these parasite-containing cells are found within vascular channels of the granuloma. These vascular channels presumably provide a mechanism by which the parasite reaches the peripheral circulation. This assumption is supported by the fact that subsequently in peripheral blood smears, a small number of mononuclear cells are found to contain parasites.

The clinical manifestations of ACH are believed to occur when the release of merozoites incites localized inflammation. In this study, clinical data gathered from a

temporal investigation of dogs experimentally infected with *H. americanum* will be correlated with the asexual development of *H. americanum* as determined by histopathologic examination of sequential muscle biopsies. This study will enhance previous observations about the disease and the pathologic lesions produced as well as clearly correlate the onset and progression of disease with parasite development. Additionally, features that may possibly influence the immune reaction and the activation of periosteal bone proliferation in dogs with ACH will also be discussed.

Material and Methods

A 20-week study was designed using ten *H. americanum*-naive, 17-week-old, mixed-breed castrated/spayed dogs. The dogs were littermates that were born, raised, and cared for in the tick-free laboratory animal facilities at Oklahoma State University in accordance with conventional laboratory animal practices. Baseline data included physical examination (PE), complete blood cell count (CBC), chemistry panel (Chem), fecal flotation, muscle biopsy, bone scintigraphy and bone radiographs. Baseline serum and subsequent serum samples were collected and stored at -80°C pending enzyme linked immunoassay (ELISA) testing and bioassay analysis (being reported separately). Body temperature, pulse, respiration, and visual examination were recorded daily. Blood was drawn weekly for CBC, Chem panel, and serum.

The dogs were randomized into two groups each consisting of two control dogs and 3 infected dogs. The two groups (Table 2.1) were designed to provide sequential muscle biopsies for evaluation of parasitic development and to allow for alternating scheduling of procedures during the course of the experiment. Each group was

scheduled for periodic muscle biopsies, bone scintigraphy, and bone radiographs. The site of consecutive muscle biopsies was alternated to ensure collection of normal muscle specimens not altered by previous surgery. Parasitic lesions observed in muscle biopsies and in muscle and other tissues at necropsy were classified according to criteria developed by Panciera et al.[25] The procedure used for bone scintigraphy and radiographs have been described in detail by Drost el al.[8] Four hundred oocysts were obtained from experimentally infected *A. maculatum* ticks, suspended in 15 ml of normal saline, and then deposited into the oropharynx of dogs 3636, 3638, 3639, 3641, 3643, and 3644.[15] Dogs 3637, 3640, 3642, and 3645 served as non-infected controls and were subjected to the same daily routines and procedures as the infected dogs.

Although data collection for the experiment was initially scheduled for 20 weeks post exposure (PE), the onset and progression of clinical disease was more rapid than anticipated and four dogs (3641, 3638, 3639 & 3643) were euthanized prior to 10 weeks PE and the last remaining dogs (3636 and 3644) were euthanatized at 10 weeks PE. Only the infected dogs were subjected to a complete necropsy at the time of euthanasia or at the completion of the experiment. Due to the severity of the clinical signs, pain and fever management were initiated using buprenorphine hydrochloride at 0.01 mg/kg BID and morphine at 0.5 to 1.0 mg/kg SQ per 4 hours; later, aspirin was used at 162.50 mg (1/2 tablet) BID.

Prior to euthanasia, each dog was anesthetized and blood was collected by cardiac puncture, followed by a fatal dose of pentobarbital. A comprehensive necropsy was performed immediately after death. Multiple specimens of striated muscle (biceps femoris, triceps, temporalis, and axial), numerous other body tissues, and sections of

long bones were collected. Tissues were fixed in neutral buffered formalin, processed routinely for paraffin embedding then sectioned at 5 microns and stained with hematoxylin-eosin. Specimens of bone were decalcified, processed for paraffin embedding, sectioned at 5 microns, and stained with hematoxylin-eosin. The remaining skeleton (skull, vertebral column, and legs) was further prepared for gross examination by submergence in water containing detergent followed by autoclaving at 15 psi for 30-60 minutes. This procedure allowed for easy removal of tissue overlying the bone. The bones were dried and examined for periosteal bone proliferation.

Results

Clinical Observations

Control dogs had neither evidence of illness or muscle pathology during the course of the experiment. Each of the experimentally exposed dogs developed severe illness (Table 2.2). The onset of illness was evident by fever and diminished activity in each dog. Within 12 to 24 hours following diminished activity, transient knuckling of the fetlocks, and reluctance to move developed. In all dogs, fever (greater than 104°F) developed prior to the onset of visible illness and remained consistently high throughout the period of observation. The daily average body temperature ranged from 104 to 105.9^{0}F (Figure 2.1) [See Appendix 1 and 2 for raw data]. Dogs developed bilateral ocular discharge, generalized muscle stiffness, and weakness. They became recumbent, refused to eat and exhibited severe pain during handling between 1 to 14 days following the onset of illness. Extensive limb edema and generalized muscle atrophy developed within a week after the dogs became recumbent. Muscle atrophy was especially

prominent in the temporalis muscle of all dogs. Dogs 3644 and 3636 had difficulty moving and bending their necks at eight weeks. At no time was diarrhea or coughing observed.

Clinical Pathology

The white blood cell counts (WBC) prior to the onset of illness averaged 16,200 cells/µl. With the onset of illness at approximately 3 weeks post exposure, WBC increased and continued to rise to an average of 114,250 cells/µl at 10 weeks (Figure 2.2)[See Appendix 3 for raw data]. WBC as high as 177,000 cells/µl were recorded. Leukocytosis was due to marked neutrophilia with a severe left shift. A mild to moderate monocytosis and mild regenerative anemia was also present. Serum alkaline phosphatase rose at approximately 3 weeks post-exposure and remained high. Other alterations included decreased serum protein and albumin. Creatinine kinase was elevated in only one dog (3644). Serum levels of calcium, phosphorus, sodium, potassium, and magnesium remained essentially normal. Other chemical abnormalities were not noted.

Description of the Onset and Progression of Parasitic Development with Pathologic Lesions

Accumulated observations from histologic examinations of sequential muscle biopsies, weekly blood smears, and necropsy results were used to detect progression of the asexual cycle of *H. americanum* with the development of pathologic lesions. Sequential bone scintigraphy, radiography, and necropsy results were used to determine the onset and severity of periosteal bone proliferation.

Week 3 and 4 Post-Exposure

Dog (3641) showed clinical signs as early as 29 days. Dog 3641's first muscle biopsy at 3 weeks post-exposure revealed a few encysted lesions and a number of vessels with a hyperplastic endothelial lining. One mononuclear cell containing a parasite was found in dog 3641's peripheral blood smear during week 3 (Figure 2.3). Muscle biopsy of dog 3639 at 4 weeks post exposure revealed numerous parasites in encysted lesions and a mild, randomly scattered infiltrate of neutrophils between individual myofibers. No inflammation was observed around the encysted lesions. No parasitic stages or pathologic lesions were found in muscle biopsies from dogs 3636 and 3638, at 3 weeks PE, nor in dogs 3644, and 3643 at 4 weeks PE.

Week 5 Post-Exposure

Due to the rapid progression of illness, dog 3641 was euthanatized on day 35. At necropsy, the dog had limb edema, decreased fat storage, and mild muscle atrophy. Mucous membranes were pale and the subcutaneous tissue and major muscle groups (legs, back, head, and diaphragm) contained diffuse petechiation. Other lesions included mild peripheral lymphadenopathy. Lymph nodes draining the legs were enlarged and wet. Mild myocardial petechiation, a mild accentuated hepatic lobular pattern, and diffusely scattered petechiation of the lungs were also noted. Other organ systems appeared essentially normal.

The progression of parasite development was evaluated from tissues collected from dog 3641 at necropsy. The majority of the lesions observed included numerous parasites in encysted lesions, a few meronts, and granulomas (Figures 2.4-2.9).

Although generalized, severe pyogranulomatous myositis was observed, inflammation was not observed surrounding the early-encysted stages. Bone lesions are reported in the section on bone pathology.

Week 6 Post Exposure

Biopsy specimens of skeletal muscle were available from 2 dogs (3636 and 3638) and necropsy specimens were available from 1 dog (3639). Lesions consisting predominantly of parasites in encysted lesions; and granulomas were less frequently observed. Encysted lesions were found in adipose tissue adjoining muscle in Dog 3638. Neutrophilic myositis was observed in specimens from the three dogs.

Dog 3639 was euthanatized on day 42 PE due to pain and persistent lateral recumbency. At necropsy, gross lesions were similar to those observed in Dog 3641; the lesions were however more severe. There were large numbers of parasitic stages within sections of skeletal muscle, cardiac muscle, and other tissues, as well as a generalized pyogranulomatous myositis. Bone lesions found at necropsy are summarized in the section on bone pathology.

Week 7 Post-Exposure

Muscle biopsies of dogs 3643 and 3644 revealed areas of marked pyogranulomatous inflammation, extensive separation of the myofibers (edema), along with diffuse myofiber atrophy and mild regeneration. Though early encysted stages of the parasite were most numerous, meronts and granulomas were also present.

Week 8 Post-Exposure

Dogs 3638 and 3643 were euthanatized due to deterioration in health during week 8. Both dogs had generalized subcutaneous edema and muscle atrophy. Numerous parasitic lesions were found in striated and cardiac muscle, and other tissues such as the tongue, spleen, lymph nodes, and adipose tissue. A rather unusual lesion found both grossly and histologically was extensive synovial tissue proliferation of both the femoral-tibial and humeral-radial joints. Dog 3643 also had a 1 cm red ulcer at the pyloric antrum (presumably secondary to aspirin administration).

Week 9 Post-Exposure

Dog 3636, whose muscle biopsy was positive at 6 weeks post exposure, had a third biopsy during this week. The biopsy revealed a severe pyogranulomatous myositis. There was marked muscle atrophy and individual muscle fibers undergoing vacuolar fiber degeneration. Muscle sections contain numerous parasites in various stages of development in addition to a large increase in the number of granulomas.

Week 10 Post-Exposure

Dogs 3636 and 3644 were euthanatized during week 10. Gross lesions were similar to those in the other 4 infected dogs (3641, 3639, 3638, 3643), but the lesions were extremely severe and diffuse (Figure 2.10). Both dogs had inflammation and parasitic stages in multiple sections of muscle including the tongue and diaphragm. In addition, parasitic stages were found in the spleen, multiple lymph nodes, salivary

glands, and pancreas. Dog 3636 also had synovial tissue proliferation of both the femoral-tibial and humeral-radial joints similar to that observed in dogs 3638 and 3643.

Bone Pathology

Bone scintigraphy and radiographs performed during the experiment corresponded well with gross examination of the bones at necropsy. Diffuse periosteal bone proliferation in dog 3641 during week 5 was considered quite advanced with lesions observed on the humerus, scapula, femur, pelvis, and the thoracic and lumbar vertebrae (Figure 2.11). In addition, bone scintigraphy performed on dogs 3639 and 3643 during the same week (week 5) revealed evidence of periosteal bone proliferation on long bones (radius, humerus and femur). By week 6, dogs 3638 and 3636 had detectable bone proliferation. Bone proliferation was detected by radiographs and scintigraphy in dog 3639 and by radiographs only in dog 3636. Dog 3638 had bone proliferation on the cervical, thoracic, and lumbar vertebra and bilaterally on the humerus, femur, ulna, and radius. Dog 3636 had mild proliferation bilaterally on the ulna. Dog 3644 had detectable bone proliferation during week 7 that was determined by both bone scintigraphy and radiographs. Bone proliferation in dog 3644 was noted on the skull, entire vertebral column, scapula, humerus, femur, tibia, fibula, ulna and radius.

Summary of Parasite Development and Pathologic Lesions

The earliest parasitic stages and pathologic lesions were observed during week 3 PE. At the same time, those dogs found to have evidence of *H. americanum* infection

began to developed clinical signs of illness, an increase in body temperature and leukocytosis. At the time of euthanasia (5 weeks), dog 3641 had developed generalized pyogranulomatous myositis, periosteal bone proliferation, and numerous pathologic lesions including granulomas that were associated with parasite development. The remaining experimentally exposed dogs consistently developed clinical signs similar to dog 3641 around the same time parasite-associated lesions were found in their skeletal muscle.

The most common sites for bone proliferation were the long bones, scapula, and os coxae. Bone proliferation was found as early as 35 days post exposure. Another prominent feature was the extent of bone proliferation on the cervical vertebrae in five out of the six parasitized dogs. Histologic lesions observed in addition to bone proliferation and marked pyogranulomatous myositis included extensive muscle atrophy and cellulitis, mild hepatocellular vacuolation, synovial cell hyperplasia and synovial tissue proliferation of multiple joints. Although mild, vasculitis was observed in sections of striated muscle in 4/6 dogs. No dog developed pneumonia or amyloidosis.

Discussion

Several reports describing the clinical, radiographic, and pathologic observations of natural cases of ACH exist.[6,7,214,24,31,32] Two reports describe successful experimental transmission of ACH.[15,22] In those studies, the dogs were of different ages, different sexes, and different breeds. Some of the dogs used in both studies were either immunosuppressed, splenectomized, or were recovering from acute ehrlichiosis. Those studies are arguably flawed because one study used a single control animal while the

other had none. On the other hand, animals used in this study were healthy, neutered littermates. By using unisexed littermates, both as infected and as unexposed controls, variation in the result that might result from sex hormones, age, and breed related factors were minimized.

The results of this experiment correlated well with clinical and pathologic observations of other reports of ACH.[6,7,10,11,14,15,22,24,25,31,32] Success of transmission achieved by using oocysts from *A. maculatum* that had fed on a dog with ACH clearly confirms that *A. maculatum* is a highly suitable vector/host for *H. americanum*. Similarly, the time interval, development, and morphology of the parasite as detected in this experiment were identical to those reported by Panciera et al.[25] A difference in this study was the disease progressed more rapidly and morbidity was more intense. Other features such as diarrhea, renal dysfunction, and amyloidosis which have been previously reported were not observed in this study.[22] The infective dose may have been a factor that influenced the rapid progression of disease. The reason why no dog developed diarrhea or renal dysfunction may be related to the progression of disease; with these lesions occurring more commonly in dogs with chronic ACH. Similar to other reports, a decrease in total protein and albumin is most likely as result of anorexia and cachexia[7,31], both of which have been found to be related to fever and elevations in tumor necrosis factor alpha.[28,29]

The onset of clinical disease in naturally infected dogs is believed to occur when merozoites are freed from the meront and disperse into the surrounding host tissue. Prior to the release of merozoites, the developing parasite is within a host cell that is itself surrounded by a zone of mucopolysaccharide material. Similar to other reports, at

no time prior to the release of merozoites were more than a few inflammatory cells observed surrounding early cysts. Thus, it seems possible that the mucopolysaccharide and the fact that the parasite is intracellular shields the parasite from immune detection and inflammatory cells. Protozoan parasites such as *Leishmania spp.*, *T. gondii*, and *T. cruzi* are intracellular parasites that have developed various mechanisms by which they evade the immune system.[5] It seems likely that *H. americanum* may also have mechanisms to evade recognition by the dog's immune defenses, and one mechanism may be to stimulate the host cell to produce the mucopolysaccharide material that shields the parasitized cell and parasite from immune detection. Since pyogranulomatous and granulomatous inflammatory reactions commonly occur during a cell-mediated immune response[28,29], in all probability the dog's immune response to *H. americanum* is a cell-mediated one that occurs once the merozoites are released into the surrounding tissues.

Fever and leukocytosis are good indicators of inflammation.[28,29] In this study, timing of muscle biopsies enabled the detection of parasitic development and associated pathologic lesions to be correlated with the onset of illness as determined by fever, weakness, recumbency, and leukocytosis. Dog 3641, necropsied during week 4 PE, had multiple parasitic stages including meronts, merozoites, and granulomas; marked leukocytosis and fever were also observed prior to euthanasia. Advanced clinical signs and pathologic lesions such as diffuse, severe, pyogranulomatous myositis were found to be closely related with a large concentration of parasitic stages found in muscle biopsies (6 and 7 weeks post exposure) and at necropsy. At approximately the same time granulomas were observed in samples of skeletal muscle of dog 3641, leukocytes

containing parasites in the peripheral blood were found. This finding was significant because it demonstrated the asexual cycle had progressed while at the same time the dog had advanced inflammation and clinical illness. In earlier studies the presence of gamonts in the peripheral blood occurred on or around the same day that granulomas with cells containing parasites within vascular channels were found.[15] Gamonts in the peripheral blood are considered the final stage in the parasite's development within the vertebrate intermediate host.[6,15,25] Thus finding gamonts in the peripheral blood strengthens the conclusion that the onset of fever and pain are associated with inflammation and inflammatory mediators incited by the immune detection of parasites once freed from the meront; since the release of merozoites occurs prior to peripheral blood stages of the parasite.[25] An additional conclusion strengthened by this finding is that vessels within the granuloma are the means by which the parasite enters the circulation since parasites were not found in the peripheral blood prior to granuloma formation. Whether or not the parasites secrete mediators that initiate the proliferation of vessels remains to be determined.

In 2000, Panciera et al. described skeletal lesions of ACH in both naturally infected dogs and experimentally infected coyote pups and dogs.[26] The authors noted that the onset of bone proliferation was detectable as early as 5 weeks. In the present study, dogs 3641, 3639, and 3643 all had bone proliferation by 5 weeks post exposure. The bone lesions appeared more widespread than previous reports, occurred on multiple bones, and were histologically similar to those dogs with more advanced ACH. Although extensive myositis and cellulitis associated with parasitic stages were observed, neither lesions containing parasites nor inflammation were observed directly

adjacent to the periosteum. In all cases, the tissue adjacent to the periosteum was thickened due to edema and often it was mildly congested. The edema was presumably associated with prolonged recumbency.

Periosteal bone proliferation in ACH has been compared to and considered a potential model for hypertrophic osteopathy (HO) in human beings and dogs.[26] Theories as to the development of hypertrophic osteopathy include bone proliferation secondary to inflammatory or neoplastic lesions that may lead to hypoxia, decreased blood flow to the extremities or to vagal or intercostal nerve dysfunction. Another theory postulates that hormones such as estrogen and growth hormones cause changes that stimulate bone proliferation. Observations in this study rule out a possible connection between a thoracic lesions and the production of bone. Similarly, the possible connection between local inflammation or adjacent parasitic development also is considered the factors that activate the development of periosteal bone proliferation.[26] However, our findings do suggest the possibility that bone proliferation in ACH is caused by factors released into the systemic circulation. Humoral factors such as cytokines, secreted and produced by inflammatory cells during times of prolonged inflammation, and have been found to stimulate bone proliferation as well.[1,26] Thus the potential interplay between certain cytokines, other inflammatory mediators, the dog's immune system, and the development of *H. americanum* remains an area of interest worthy of investigation.

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Table 2.1 Muscle biopsy, bone scintigraphy and radiograph schedule. Prior to experimentation, all dogs had a baseline muscle biopsy and bone scintigraphy followed by bone radiographs.

	Dog #	Sex[1]	Status[2]	Weeks Post-Exposure							
				3	4	5	6	7	8	9	10
	3636	MC	I	Bx1[a]			Bx2,BS1			Bx3,BS2	Nec
	3638	MC	I	Bx1			Bx2,BS1		Nec		
Group 1	3641	FS	I	Bx1		BS1[b],Nec[c]					
	3642	FS	C	Bx1			Bx2,BS1			Bx3,BS2	
	3645	FS	C	Bx1			Bx2,BS1			Bx3	
	3639	MC	I		Bx1	BS1	Nec				
Group 2	3643	FS	I		Bx1	BS1		Bx2,BS2	Nec		
	3644	FS	I		Bx1			Bx2,BS1		BS2	Nec
	3637	MC	C		Bx1			Bx2,BS1		BS2	Bx3
	3640	MC	C		Bx1			Bx2,BS1			Bx3

[1] MC-male castrated, FS – female spayed [2] I-Infected, C-Control

[a] Muscle biopsy (Bx); [b] Bone Scintigraphy and radiography (BS); [c]Euthanasia and Necropsy (Nec)

Location of Muscle Biopsies:	**Weeks**	**Anatomic Location**
	Bx 1	Left Biceps femoris
	Bx 2	Right Triceps
	Bx 3	Right Biceps femoris

Table 2.2. Summary of clinical and pathological observations

Dog no.	Fever[1]	Weakness, stiffness, QAR[2]	Recumbent	Leukocytosis[3]	Parasitic lesions observed[4]	Bone proliferation[5]	Euthanatized
3641	29d	30d	30d	3wk	3wk	5wk	5wk
3639	31d	32d	33d	4wk	4wk	5wk	6wk
3643	31d	32d	33d	3wk	7wk	5wk	8wk
3638	34d	37d	42d	4wk	6wk	6wk	8wk
3644	35d	33d	40d	5wk	7wk	7wk	10wk
3636	37d	37d	54d	5wk	6wk	6wk	10wk

Time is in days (d) or weeks (wk) elapsed following feeding of oocysts.
[1]Temperature of 104^0F or greater
[2]The day the animal showed physical signs of illness. QAR – Quiet, alert, and responsive
[3]White Blood cell count greater than 14,000
[4]Parasitic lesions observed on muscle biopsy
[5]The first indication of bone proliferation by scintigraphy or radiographs

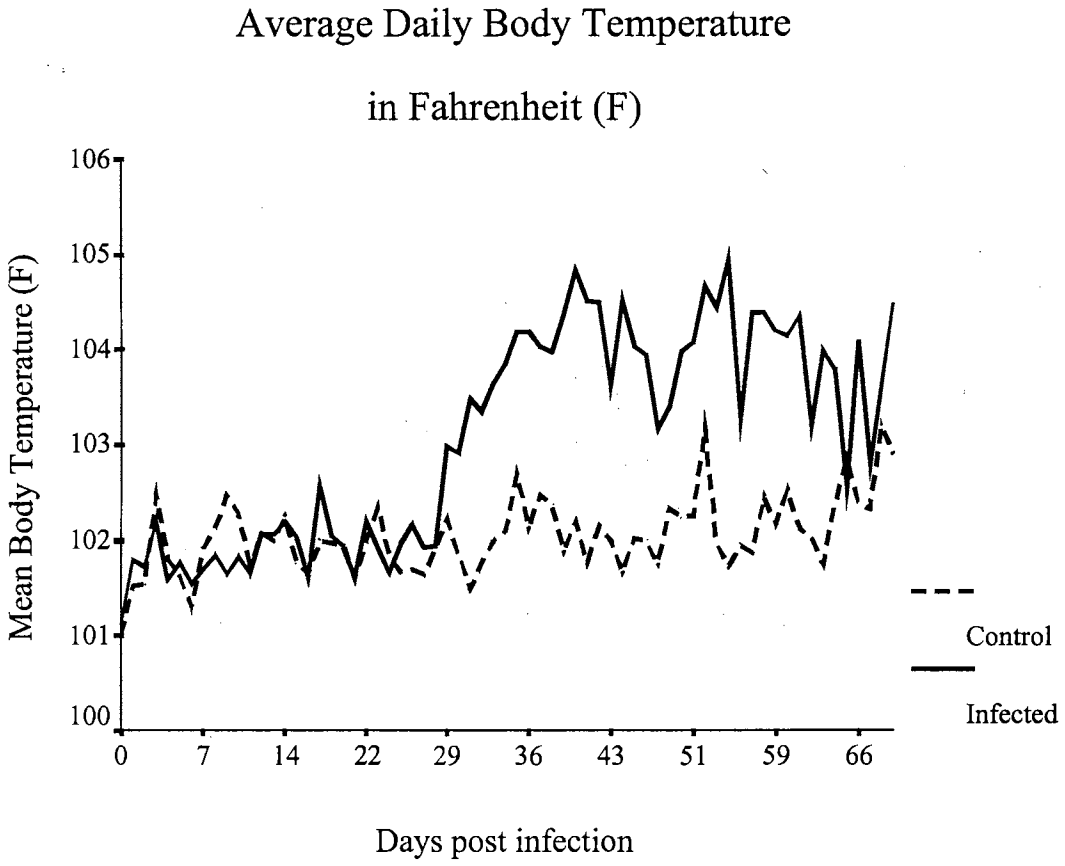


Figure 2.1. The graph illustrates the average daily temperature fluctuations of experimentally-exposed (infected) and control dogs. Note that around day 29 post exposure, the daily temperature of infected dogs began to rise and remained high throughout the remaining period of observation. The average daily temperature of infected dogs ranged from 104°F to 105°F.

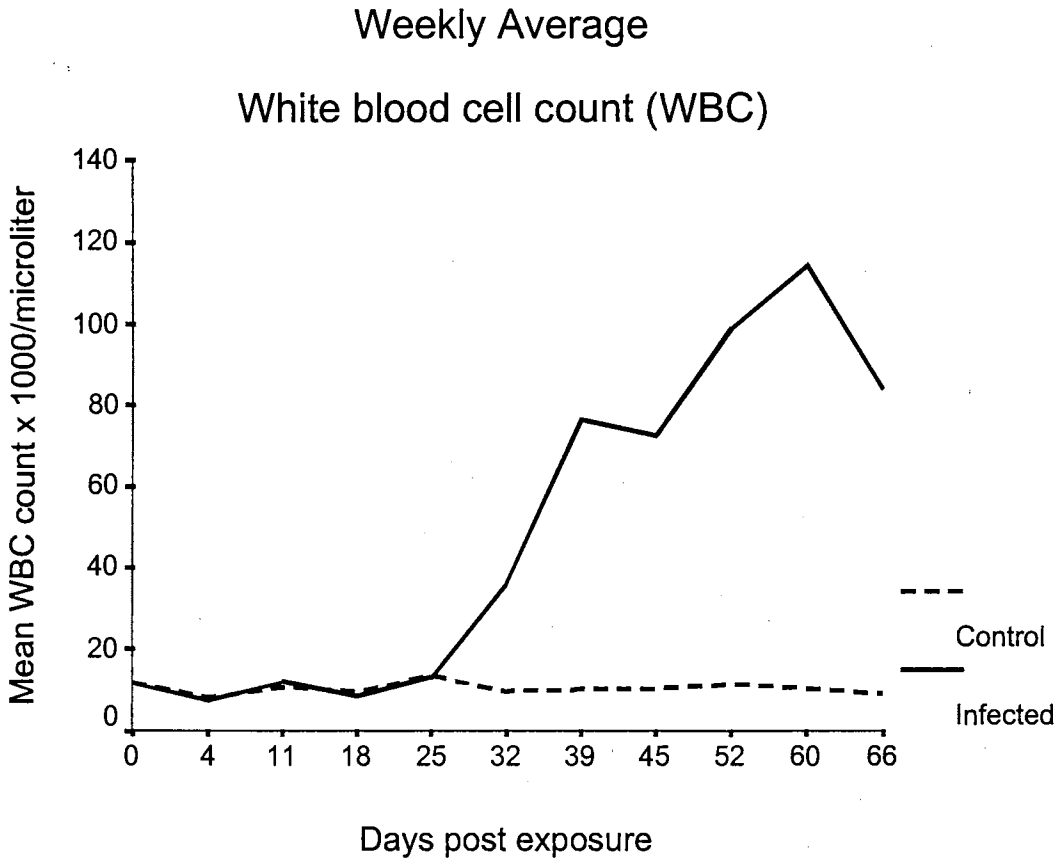


Figure 2.2. The graph depicts the average weekly WBC for experimentally-exposed (infected) and control dogs. Note at approximately Day 25, the average WBC increased dramatically in the infected dogs while the controls remains consistent throughout the study. At day 32, the average weekly WBC for the infected group was 36,000 cells/μl (n=6) while on day 60, the maximum average daily WBC was 114,300 cells/μl (n=2).

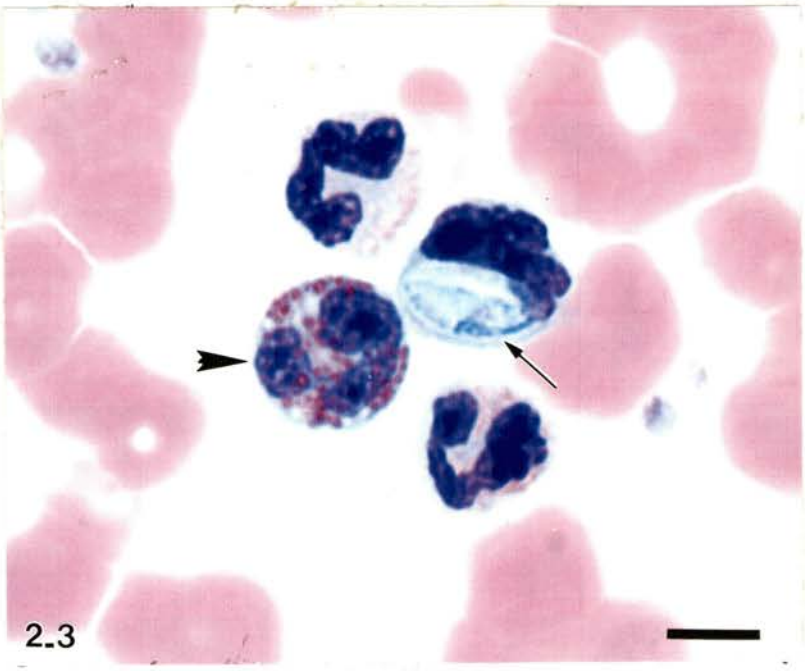
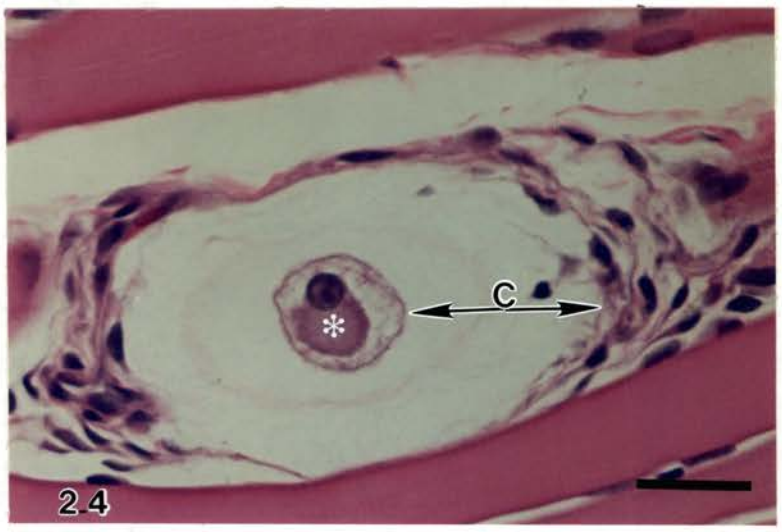


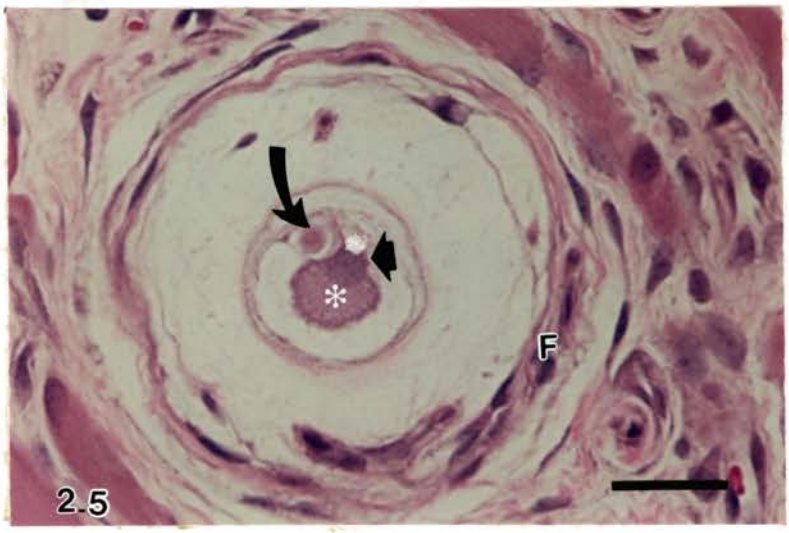
Figure 2.3. Peripheral blood smear of Dog 3641 at 3 weeks post-exposure. Note the largest cell, a macrophage, contains a visible crescent-shaped parasite (small arrow). Compare this cell with the eosinophil (arrowhead) that has prominent bright red cytoplasmic granules, and two neutrophils, one located at the top left and one at the bottom right. Neutrophils, smaller than macrophages, are characterized by a segmented nucleus and pale staining cytoplasmic granules. Bar = 10 μm.

Figure 2.4. An early mucopolysaccharide cyst (C) containing a centrally located host cell (asterisk). The host cell has a prominent nucleus. Note the lack of inflammation surrounding the cyst. Bar = 50 μm.

Figure 2.5. A slightly more advanced lesion demonstrating an "onion-skin" cyst containing a host cell (asterisk) with a large prominent nucleus (arrowhead). In the cytoplasm of this cell, a distinctly round body interpreted to be a parasite (curved) arrow) is observed. Fibroblasts (F) encircle the outer perimeter of the cyst. Again, note the lack of inflammation. Bar = 50 μm.

Figure 2.6. A developing meront (M) within a crescent-shaped host cell. The host cell nucleus (arrowhead) contains a large prominent nucleolus (arrow). Bar = 50 μm.


C
2.4


F
2.5

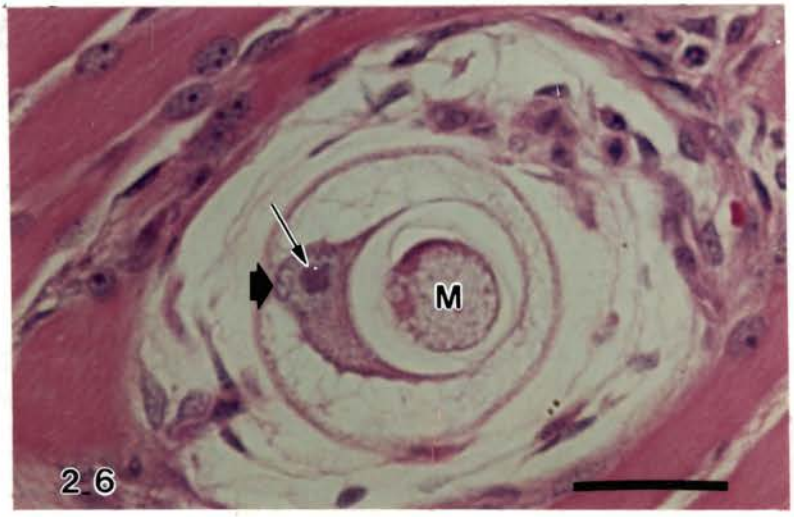
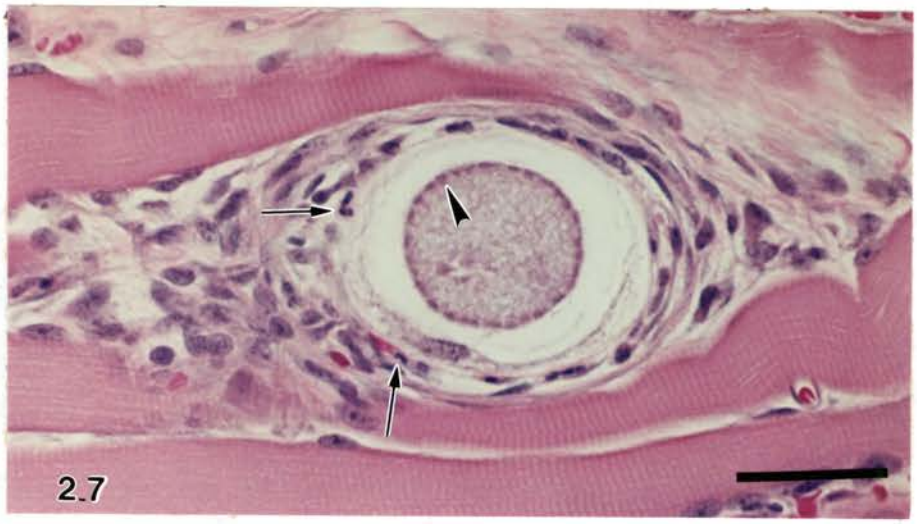

M
2.6

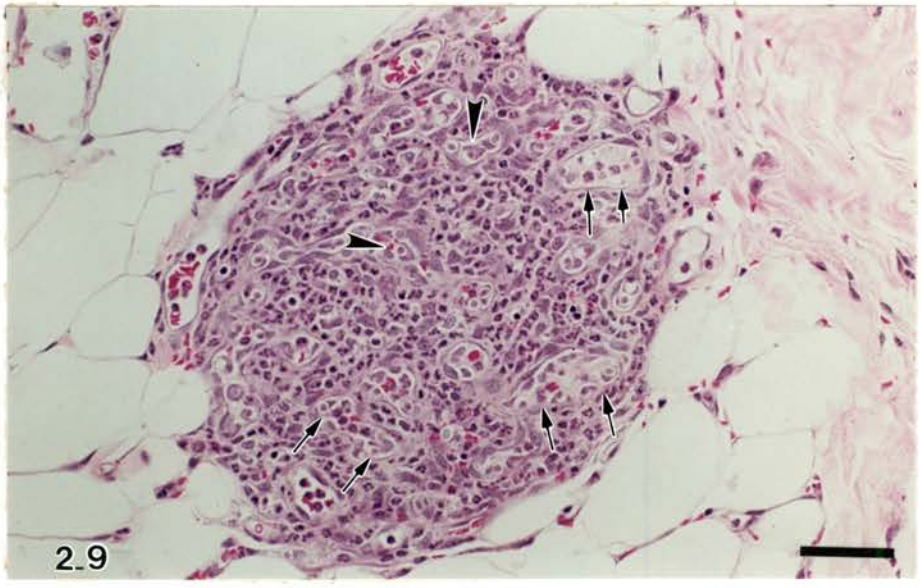
Figure 2.7. A further developing meront. Note the aggregation of nuclear material along the periphery of the meront body (arrowheads). Two neutrophils are found (small arrow) along the outer perimeter of the cyst wall. Bar = 50 μm.

Figure 2.8. The developing meront is beginning to infold. Note the lack of inflammation. Bar = 25 μm.

Figure 2.9. Persistent inflammation occurring secondary to the release of newly developed merozoites results in the formation of a granuloma. This particular lesion is a somewhat later stage composed of numerous vascular channels (arrows) with intraluminal host cells containing parasites (arrowheads). Bar = 50 μm.


2.7


2.8


2.9

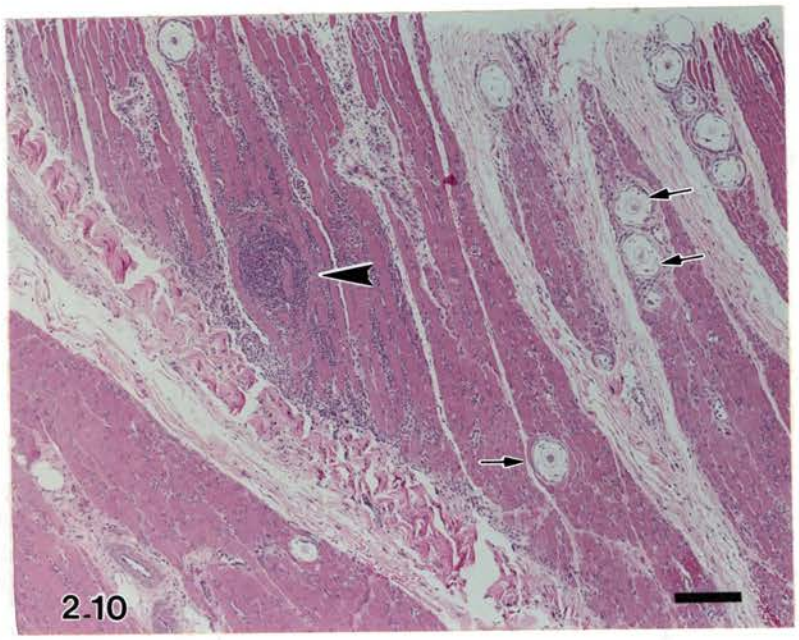


Figure 2.10. Low magnification of a section of skeletal muscle from Dog 3644 at 10 weeks post exposure. Multiple parasitic stages including early encysted stages (small arrows) and one granuloma (arrowhead) are observed. Accompanying the parasitic lesions is a severe pyogranulomatous myositis. Bar = 100 μm.

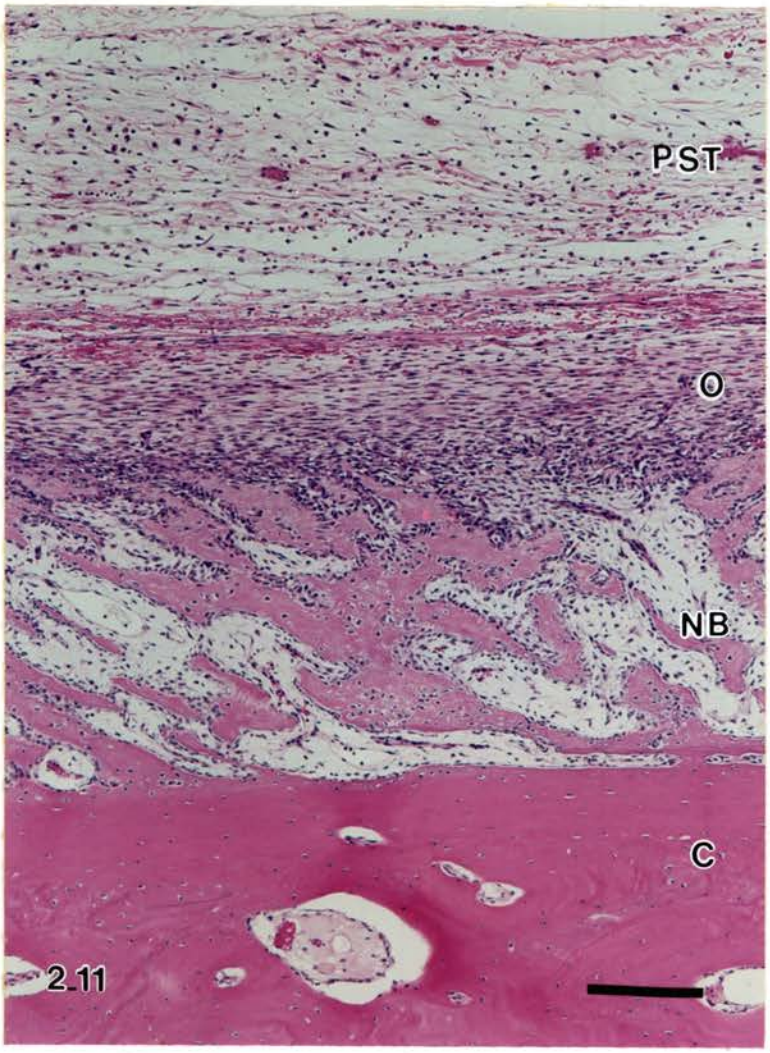


Figure 2.11 A section of humerus from Dog 3641 at 5 weeks post exposure. Above the highly cellular, active osteogenic zone (O) of the periosteum, the periskeletal tissue is edematous (PST). Below the periosteum, there is a thickened layer of perpendicular oriented new bone (NB) that overlies the original cortex (C). Bar = 50 μm.

CHAPTER III

AMERICAN CANINE HEPATOZOONOSIS: AN ULTRASTRUCTURAL AND IMMUNOHISTOCHEMICAL STUDY

Introduction

American canine hepatozoonosis (ACH) was first recognized in Texas in 1978.[5] The geographic distribution of the infection has expanded since 1978 to include cases in Louisiana, Alabama, Georgia, and Oklahoma.[10,15,22,28] Initially, the causative agent for ACH was believed to be a particularly virulent strain of *Hepatozoon canis*; however, based on morphologic, pathologic, and molecular characteristics, the causative agent of ACH is now recognized to be a distinct species, *Hepatozoon americanum*.[2,19,29]

ACH is a severe disease with marked myositis, vasculitis, and periosteal bone proliferation.[28] Clinically recognized by extreme neutrophilia, muscle wasting and generalized pain and weakness, ACH is progressive and often leads to death. Diagnosis of ACH is readily confirmed by the observation of parasitic stages in skeletal muscle biopsies. Infection occurs when dogs ingest oocysts of *H. americanum*. Following ingestion, the oocysts rupture in the intestinal tract and release numerous sporozoites that become widely distributed in various canine tissues particularly striated muscle. The route by which sporozoites are disseminated has yet to be defined. The earliest

recognized lesion of ACH is a large "modified" host cell harboring a trophozoite. The interval between oral exposure and the appearance of this early lesion is approximately 3 weeks in experimentally-infected canids.[17,23,Chapter 1] The so called "onion-skin" cyst is the most frequently observed histopathologic lesion. This lesion acquired its name "onion-skin" because of the multilamellar mucopolysaccharide deposit that surrounds a centrally located host cell that itself contains a developing trophozoite. The trophozoite within the host cell merogony. Eventually, the surrounding mucopolysaccharide material disperses and the merozoites are freed from the host cell. This release of the merozoites incites an acute localized inflammatory reaction that persists and eventually progresses to a granuloma. Many granulomas contain wide vascular channels; host cells harboring parasites are frequently observed in the intersitial space of the granuloma and in the wall and lumen of these vascular channels. Parasites (gamonts and possibly merozoites) reach the peripheral circulation via these vascular channels. The definitive host, *A. maculatum*, acquires the gamonts during ingestion of a blood meal. Gamogony and sporogonic development occurs within the gut cells of the tick.[18]

Although the clinical and light microscopic features of ACH have been well described, only two reports describe ultrastructural and immunostaining characteristics of the causative organism and lesions produced during an infection.[6,29] These reports do not clearly identify the cells that harbor asexual stages of *H. americanum*. The main objective of this study is to delineate morphologic and immunologic characteristics of these parasite-containing host cells that establish the identity of the host cells. Transmission electron microscopy (TEM) and immunohistochemistry (IHC) were used to identify the host cell and to establish a more detailed morphologic description of

three stages of *H. americanum:* the trophozoite stage within the "onion skin" cyst, the stage within the granulomas, and the peripheral blood stage.

Material and Methods

Samples of striated muscle and blood used in this study originated from muscle biopsies or necropsy tissue from three naturally infected and eight experimentally infected dogs (see Chapter 2: Materials and Methods). Experimental infection was achieved by feeding 400 *H. americanum* oocysts to *Hepatozoon*-naïve mixed-breed dogs. The experimentally-infected dogs were monitored daily for up to 10 weeks with periodic muscle biopsies followed by complete necropsy at the time of euthanasia or termination of the experiment. (See Chapter 1)

Sample Preparation for Transmission Electron Microscopy (TEM)

Specimens of skeletal muscle were collected at the time of muscle biopsy or within 10 minutes after euthanasia. Each specimen was diced into 1 mm cubes, instantly immersed into cold 2.5% glutaraldehyde fixative in 0.2M sodium cacodylate buffer, then stored until further processing. Capillary tubes were filled with EDTA blood drawn from infected dogs before euthanasia. The capillary tubes were centrifuged and the buffy coats were collected and immediately immersed in cold 2.5% glutaraldehyde fixative in 0.2M sodium cacodylate buffer and stored until further processing.

Fixed muscle and buffy coats were postfixed in 2% osmium, dehydrated through a graded series of ethanol or acetone, and then embedded in polybed resin.[13] Embedded

tissues were trimmed, sectioned (approximately 1 micron thick), and stained with Mallory's Richardson's blue.[13] The tissue sections were examined by light microscopy. Ultrathin (silver-gold reflective) sections of samples containing specific tissue and/or parasitic stages were cut using an ultramicrotome and a diamond knife. Sections were collected on a 300-mesh copper grid then stained with uranyl acetate and lead citrate followed by examination using a JEOL 100CX II STEM transmission electron microscope operated at 80kV.[13] Electron micrographs were prepared, critically examined, and interpreted. Photomicrographs of the parasite, parasitic stages, and adjacent tissue were documented and described. References of ultrastructural characteristics of normal and abnormal inflammatory cells and tissues (macrophages, neutrophils, muscle, connective tissue, and blood vessels) were used to describe and support these findings.[4,9]

Sample Preparation For Immunohistochemistry

Specimens of skeletal muscle, approximately 0.25 cm thick were collected from the experimentally-infected dogs and fixed in 10% neutral buffered formalin at room temperature for 24 to 48 hours. Fixed tissues were then processed for routine paraffin sectioning, hematoxylin and eosin (H&E) staining followed by light microscopic examination.

Immunohistochemical staining was done using a DAKO Autoimmunostainer Model LV (Dako Corporation, Carpenteria, CA). Antibodies used in this study include MAC 387 (DAKO M0760, myeloid:histiocytic and granulocytic marker), CD3 and CD79a (DAKO A0452, DAKO M7051, markers for T and B lymphocytes

respectively), α-1-antitrypsin (DAKO A0012, macrophage marker), desmin and smooth muscle actin (DAKO M0760 and DAKO M0851, muscle markers), vimentin (DAKO M0725, marker for cells of mesenchymal origin), Von Willebrand factor VIII (DAKO A0082, vascular marker), and S-100 (DAKO Z0628, neuroendocrine marker). In addition, rabbit-origin antibody to *H. americanum* oocysts was used to delineate the parasite within host cells.[24] The procedure for each antibody including ACH immunostaining were performed using the recommended specification for each antibody and the accepted labeled streptavidin-biotin (LAB-SA) technique.[8] Dilutions for each antibody and their incubation times were previously determined in the histotechnic lab at the Oklahoma Animal Diagnostic Laboratory in Stillwater, Oklahoma. Appropriate controls, both negative and positive, were utilized to ensure antibody specificity. Following immunostaining, each slide was counterstained with Mayer's hematoxylin and then examined, interpreted, and photographed by light microscopy.

Sample Preparation For Double-label Immunofluorescence and Confocal Microscopy

Serial sections of skeletal muscle with lesions were deparaffinized, and rehydrated in phosphate-buffered saline containing 0.2% fishskin gelatin (PNS-FSG). Sections were heated in antigen-unmasking solution for antigen retrieval. Tissue sections were treated with 10% normal goat serum (diluted with PBS-FSG) for 20 minutes to avoid nonspecific binding and treated with DAKO-biotin blocking system solutions. Slides were washed in PBS-FSG and treated with polyclonal anti-*Hepatozoon* antibody at 1:100 dilution for 1 hour at room temperature (RT).

Biotinylated anti-rabbit antibody (1:200 dilution) raised in goat was applied to the slide for 30 minutes, followed by streptavidin conjugated with alexa 488 (green) or 568 (red) (1:1000) (Molecular Probes Inc., Eugene, Oregon) for 30 minutes. Slides were washed and treated with the second primary antibody (MHA, Vimentin, Lysozyme), and a serial section was incubated with appropriate negative control antibody for 1 hour. Secondary antibody (horse anti-mouse) conjugated with alexa 568 or 488 (Molecular Probes) was applied and slides were treated for 30 minutes. Confocal microscopy was performed using a Leica TCS SP laser-scanning microscope equipped with three lasers (Leica Microsystems, Exton, PA). The fluorescence of individual fluorochromes was captured simultaneously after optimization to reduce bleed through between channels (Photomultiplier tubes), using the Leica software. Individual confocal slices represent 0.5μm and optical slides were collected at 512 x 512 pixel resolution. The images were transferred to a Macintosh G3 (Apple Computer, Cupertino, CA) and NIH-image v1.62. (ftp://codon.nih.gov/pub/nih-image/) and Photoshop v4.0 (Adobe, CA) were used to assign correct colors to the channels collected. Co-localization of antigens is indicated by the addition of colors and is explained in the figure legend.

Results

Transmission Electron Microscopy

"Onion skin cysts". The "onion-skin cyst" was consistently located between muscle fibers (Figure 3.1). Ultrastructurally, the multilamellar material of the cyst was composed of an amorphous, electron-lucent ground substance and randomly scattered fine, electron-dense particles. This material possesses characteristics of

mucopolysaccharide ground substance. Capillaries, fibroblasts and collagen fibers were scattered within the lamellar material. Fibroblasts and collagen commonly bordered the outer edge of the cyst (Figure 3.2). Ultrastructurally normal striated muscle fibers were often compressed adjacent to the cyst. Very few to no inflammatory cells were found surrounding the "onion-skin" cysts. The ideal section of an "onion skin cyst" contained a centrally located canine cell with a trophozoite or a developing meront (Figure 3.3). In many instances, however, the plane of section did not intersect the host cell or the parasite (so called "empty cysts").[25]

The plasma membrane of the host cell was irregular with multiple undulations (Figure 3.4). Beneath and often fused with the plasma membrane were electron-lucent mucus granules or less electron-dense reticulated granules. Vesicles excreting product into the adjacent ground substance were commonly observed (Figure 3.5). The host cell cytoplasm was filled with electron dense aggregates of monoparticulate glycogen. By light microscopy, the host cell commonly had a prominent nucleolus and a finely granular to vacuolated cytoplasm. The meront was ultrastructurally characterized by a prominent pellicle, a central nucleus, multiple sized vesicles, small osmiophilic structures (micronemes), mitochondria, lipid vacuoles, and dilated rough endoplasmic reticulum (Figure 3.6). In no instance was the parasite found to be within a defined membrane such as a parasitophorous vacuole among the few parasites observed ultrastructurally and the many hundred observed microscopically..

Granuloma. In the granulomas, host cells containing parasitic stages had a plasma membrane that was irregular with numerous surface short (filopodia) and elongated (lamellipodia) undulations (Figure 3.7). Within the cytoplasm of the cell

were numerous elongated mitochondria that often formed a ring around the developing parasite. Other organelles commonly observed in these cells included numerous stacks of Golgi complexes that were often dilated, lysosomes that frequently contained electron dense material, and randomly scattered dilated rough endoplasmic reticulum. The host cell nucleus was oval to indented, with a finely granular to lacy chromatin arranged along the periphery of the nucleus.

Other components of granulomas included neutrophils and a few randomly scattered lymphocytes. Fibroblasts, collagen, and swollen capillaries were observed surrounding the inflammatory cells. Capillaries were characterized by endothelial cells with a voluminous cytoplasm containing numerous mitochondria, Golgi complexes and dilated rough endoplasmic reticulum (Figure 3.8).

Two different stages of the parasite were found within the cytoplasm of the host cells in the granuloma. One of these stages had features typical of an apicomplexan merozoite, a prominent apical complex, numerous micronemes, and microtubules, and a large nucleus often with a prominent nucleolus (Figure 3.9). Variable sized vesicles were randomly scattered throughout the cytoplasm of the parasite (Figure 3.10). The parasite's pellicle consisted of three layers; the outer and inner layers were electron dense and between these layers was an electron lucent region. Occasionally, an irregular pale zone surrounds these parasites. This pale zone could represent a parasitophorous vacuole; however, a distinct membrane was not observed surrounding any of the parasites in the granuloma.

The second morphologically distinct parasitic stage had features consistent with a developing gamont. Of the approximately 25 or more parasites observed in this form,

all were characterized as being round with a prominent pellicle, and a large round nucleus with irregularly dispersed granular chromatin (Figures 3.11 and 3.12). Other features included numerous vesicles; some crescent-shaped, loose ribosomes, and distended rough endoplasmic reticulum. An apical complex was not observed; however, this may have been due to the plane of section.

Peripheral blood. Cells found harboring parasitic stages in peripheral blood had characteristics similar to the host cells described in muscle (Figure 3.13). The cell had an oval nucleus that was bilobed or trilobed. The chromatin was lace-like to finely granular and was often found condensed along the periphery of the nucleus. Nucleoli were often observed to be electron-dense aggregates. Cytoplasmic organelles consisted of numerous mitochondria, often with short stubby cristae; abundant vacuoles that often contained phagocytized material; abundant, well-developed, and often swollen Golgi complexes; dilated rough endoplasmic reticulum; and loosely aggregated ribosomes.

Parasites within the cytoplasm of host cells were of two morphologically distinct forms in the peripheral blood (Figures 3.13, 3.14, 3.15). Each stage had a single nucleus. One form (Figure 3.14) was folded with both the posterior and anterior ends coming to a prominent point. A thick pellicle composed of an outer limiting membrane and an inner limiting membrane was observed. The inner membrane enclosed the parasite's internal organelles. The nucleus was oval with multiple condensed nucleoli often arranged in a ring at the nuclear periphery. The cytoplasm of the parasite was heavily granulated, filled with tubular mitochondria that were often clustered together, and rough endoplasmic reticulum. In addition, there were round electron dense bodies, lipid inclusions, small clear vacuoles, and numerous dilated Golgi complexes. The

other morphologically distinct form was smaller than the first form and had an elongated "tail-like" appendage (Figure 3.15). Features of the appendage included central axial microtubules and a bilayer consisting of an electron dense outer membrane and an intermediate pale zone (Figure 3.16). Intense and extensive examination of multiple parasites of this type failed to detect a distinct membrane that enclosed the parasite; however, there was always a clear space between the parasite and the host cell cytoplasm.

Immunohistochemistry and Confocal Microscopy

Host cells consistently stained strongly for α-1-antitrypsin. The host cells in granulomas stained consistently with MAC 387; however, host cells harboring earlier stages of the parasite did not stain with this antibody. Immunostained specimens examined by confocal microscopy revealed host cells harboring early parasitic stages were positive for vimentin and lysozyme and host cells within granulomas were positive for MAC 387 (Figures 3.17-3.19). All host cells failed to react with CD79a, CD3, and S-100. Additionally, the host cells containing parasites were also negative for desmin, smooth muscle actin, and Von Willebrand Factor VIII immunostaining.

All stages of parasite development were well delineated in immunostained preparations using conventional light microscopy and confocal microscopy. With light microscopy and immunohistochemistry using anti-*H. americanum* antibody, cells containing the parasite were observed within and outside of vascular channels. These vascular channels were delineated by positive staining with Von Willebrand Factor VII antibody (Figure 3.20). Randomly scattered T lymphocytes and vimentin positive

tissue fibroblasts were also found scattered in the granuloma. Early cysts and meronts were also frequently observed surrounded by vimentin positive tissue (fibroblast) and desmin positive tissue (striated muscle).

Discussion

Conflicting and ambiguous reports based on light and electron microscopic examinations exist in the literature about the identity of the host cells that harbor developing asexual stages of *H. americanum* in dogs with ACH.[5,6,15,28,29] In two previous reports wherein ultrastructural characteristics of the parasite and the host cell were described, the authors interpreted the host cell to be either macrophages or neutrophils. At least 2 and possibly more individual canine cells are necessary to support asexual development of *H. americanum*. These cells include: first, the cell harboring merogonic phases of development, and secondly, the cell that harbors stages after merozoites are freed and enter cells of the granuloma. The latter parasite-containing cells enter the circulation within the granuloma. It is not clear whether the parasite enters yet another host cell once in the peripheral blood.

Ultrastructurally host cells, during merogony, within granulomas or in peripheral blood have morphologic features of activated macrophages. They include undulations of host cell surface, increase in number and size of mitochondria and lysosomes, and increase and dilation of Golgi complexes and rough endoplasmic reticulum. Results of our immunohistochemical studies, positive staining for alpha-1-antitrypsin, lysozyme, vimentin, and random staining with MAC 387 confirms the host cell is of monocytic origin. Negative staining for lymphocytic, neuroendocrine

markers, smooth muscle actin and desmin further confirms the host cell is not of muscle, granulocytic, lymphocytic, or neuroendocrine origin. Further, there is evidence that host cells are not neutrophils in the fact that cells harboring *H. americanum* gamonts in peripheral blood smears with a modified CAE stain "...demonstrated the absence of red granular staining in the cytoplasm of infected neutrophils."[20] Failure to stain with CAE indicates that the cell is not a neutrophil.[8,11,19,21,30] Similarly, cytochemical analysis of leukocytes parasitized by *H. canis* by earlier workers demonstrated that the host cell was α-naphthyl acetate esterase (ANAE) positive and CAE negative.[16] Macrophages stain positive for ANAE and negative for CAE.[30]

The macrophage should be a more suitable host cell for *H. americanum* than a neutrophil because of the ability of macrophages to move from one tissue to another.[11] Free macrophages in the peritoneal cavity were found to migrate via the draining lymphatics into other organs such as the spleen.[27] Additionally, monocytes transformed into macrophages are often observed in capillary blood smears in parasitic infections such as ehrlichiosis, histoplasmosis, and leishmaniasis.[7] Neutrophils do not recirculate after leaving the peripheral circulation and the half-life of neutrophils is shorter than the half-life of macrophages.[7,11] The fact that macrophages can recirculate and live longer than macrophages may be important in explaining the possible mechanism by which the parasite reaches its destination after sporozoites are liberated from the oocysts following ingestion by the dog.

In this study, we found evidence that macrophages are producing and secreting proteoglycans. Proteoglycans, another name for "mucopolysaccharides", are commonly secreted by activated macrophages. In addition, activated macrophages are known for

synthesizing phospholipids, fatty acids, and substances such as proteoglycan-macrophage colony-stimulating factor and chondroitin sulphate E.[12,14] These and other secretions from macrophages, such as cytokines, most certainly play an important role in the cell-mediated immune response that develops concurrently with the parasite's asexual development. We speculate that early in the intracellular parasite's development, *H. americanum*, like other protozoans and tissue-invading helminths transforms their host cells as means to evade detection by the host's immune system. *H. americanum* stimulates their host cell to become activated and secrete materials like proteoglycans.[3,12] In the case of certain helminths, the parasite itself secretes substances such as high concentrations of mucopolysaccharide.[3] The host cell, now established to be a macrophage, is induced or activated by *H. americanum* to secrete the mucopolysaccharide ground substance.

Additional findings in this study include observations on components of the granuloma. Supporting our ultrastructural findings, there was strong positive staining of fibroblast (vimentin), blood vessels (Von Willebrand's factor), as well as randomly scattered positive immunostaining for T-cell lymphocytes within the granuloma. Fibroblasts and blood vessels were found commonly within granulomas that typically characterize chronic ACH infection. In addition to macrophages, T lymphocyte are the major components of a cell-mediated immune response; thus finding T-cell lymphocytes within the granuloma of dogs infected with ACH supports the hypothesis that dogs with ACH develop a cell-mediated immune response similar to other intracellular protozoal parasites such as *Leishmania spp., Toxoplasma gondii*, and *T. cruzi*.[3] Since cell-mediated immunity involves the initiation and maintenance by

cytokines secreted by macrophages and T cell lymphocytes, it seems likely that cytokines may play a role in chronic ACH infections.

An additional feature in the host:parasite:relationship was our failure to recognize a "parasitophorous" vacuole surrounding the intracellular parasite. According to Aikawa and Sterling, a parasitophorous vacuole is formed from the invagination of the host cell plasmalemma as the parasite enters the host cell.[1] The parasites of the family Plasmodiidae, Haemoproteidae, Leucocytozooidae, Eimeriidae, Lankesterellidae, and Toxoplasmidae are frequently observed within parasitophorous vacuoles. However, parasites of the genus Sarcocystis, Piroplasmia, Microsporea, and Haplosporea are not enclosed within a similar vacuole.[1,26] In the case of *H. americanum*, either the vacuole does not exist or the vacuole was overlooked. The probability of overlooking or failing to section through the vacuole is inconceivable given the multiple sections examined during this and other studies of ACH. Thus, the questions remain, could it be possible that the mode of entry for *H. americanum* does not require invagination of the host cell membrane or could the parasite possibly enter the host cell by a mechanism similar to those used by species of Piroplasmia, Microsporea, and Haplosporea and live directly within the cytoplasm of the host cell. The answer to this question remains to be determined but was beyond the scope of this study.

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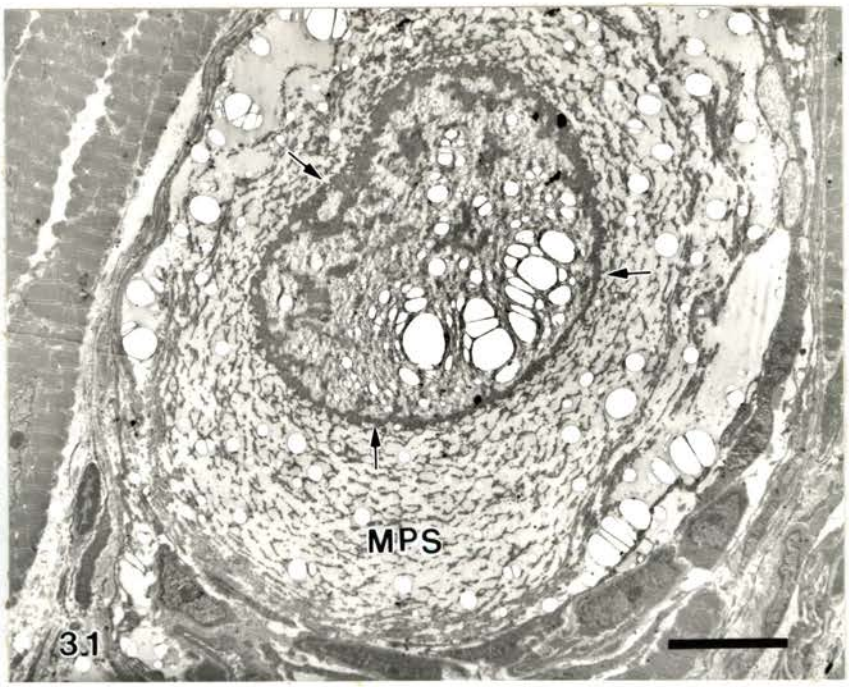


Figure 3.1. Electron micrograph illustrating an "onion-skin" cyst. The host cell (arrows) is surrounded by multilamellar material composed of an amorphous, electron-lucent ground substance and randomly scattered fine electron-dense particles both consistent with a mucopolysaccharide ground substance (MPS). The cyst is located between muscle fibers. Note the lack of inflammation surrounding edge of the cyst. Bar = 10 μm.

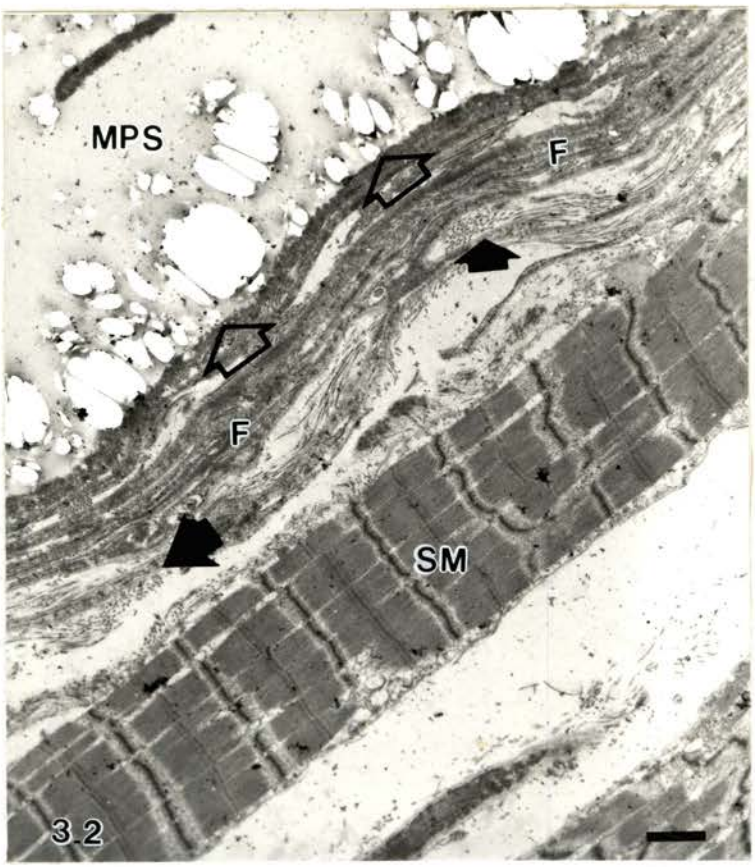


Figure 3.2. Electron micrograph illustrating the edge of an "onion-skin" cyst. Fibroblasts (F) and collagen (closed arrows) commonly border the outer edge of the cyst edge (open arrows). A small part of the mucopolysaccharide ground substance (MPS) is found in the upper left hand corner of the micrograph. Normal striated muscle fibers (SM) are observed adjacent to the compressed fibroblasts. Bar = 10 μm.

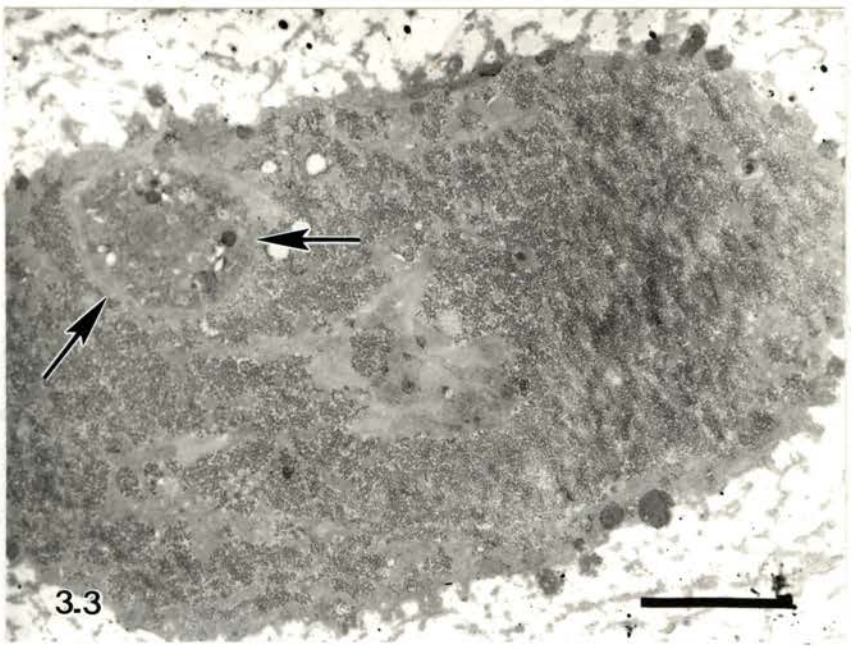
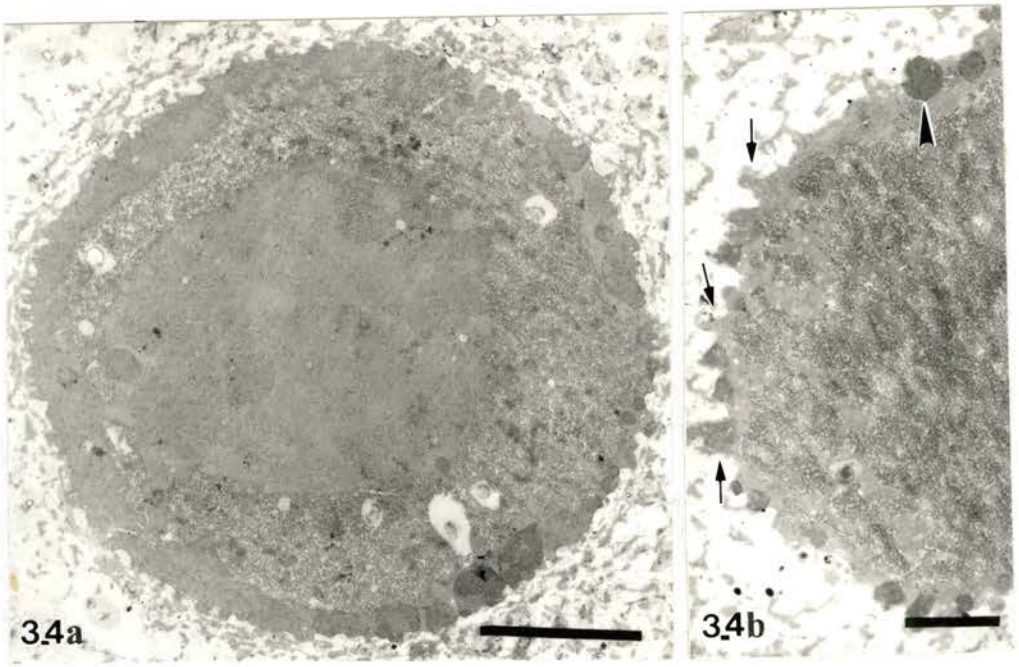


Figure 3.3. Electron micrograph of an oval shaped host cell containing a developing meront (arrows). The cell is within the center of an "onion-skin" cyst. Bar = 5 μm.



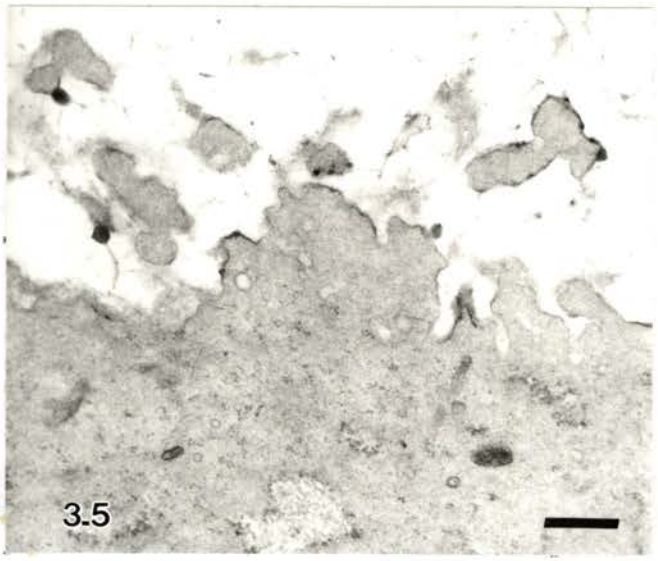


Figure 3.4. Electron micrograph of a centrally located host cell (a). The irregular, undulating plasma membrane (arrows) is illustrated in (b). Note the electron dense vesicle (arrowhead) on the membrane surface. Bars represent 5 μm (a) and 2 μm (b).

Figure 3.5 High magnification of the edge of a centrally located host cell. Note the excretion of material from the plasma membrane into the adjoining space. Bar = 0.5 μm.

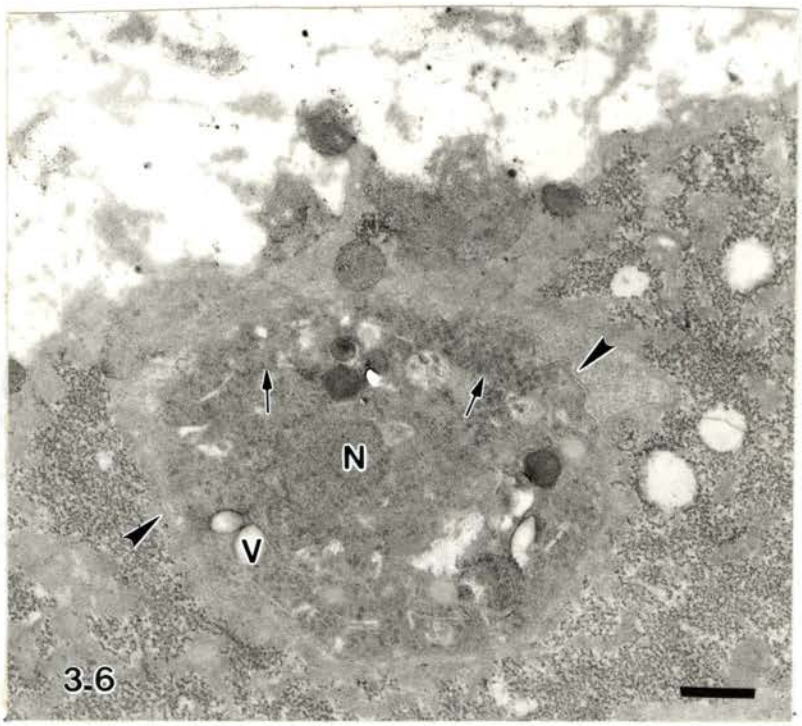


Figure 3.6. High magnification of a developing meront within a host cell. The meront is characterized by a prominent pellicle (arrowheads), a central nucleus (N), and multiple, variable-sized vesicles (V). Small osmiophilic structures (small arrows) are micronemes. Note the lack of a defined parasitophorous vacuole. Bar = 1 μm.

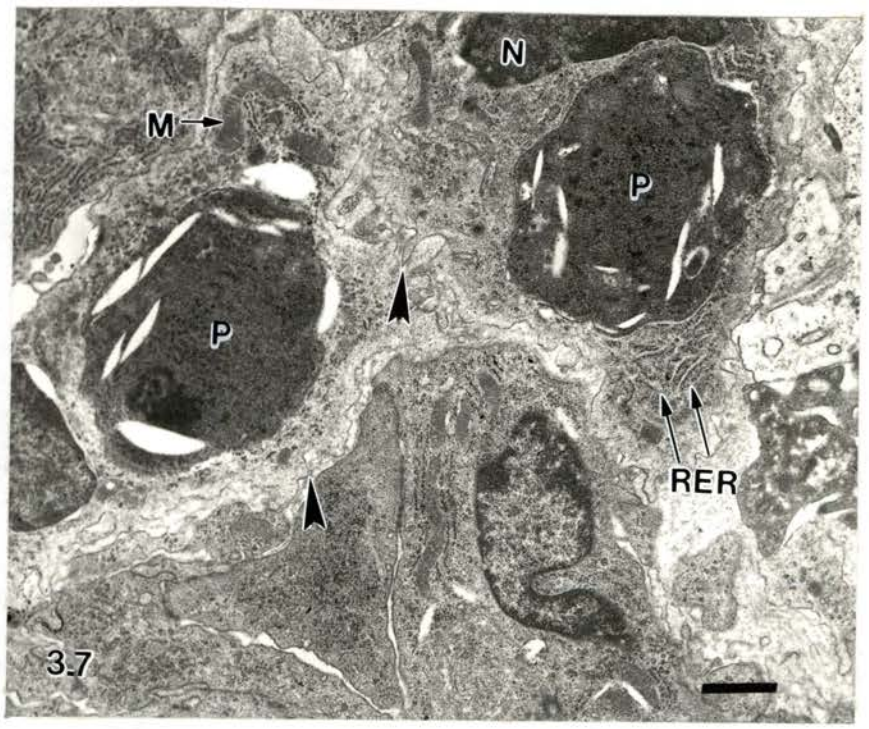


Figure 3.7. Electron micrograph of a granuloma. Two host cells each containing a developing parasite (P) are observed. A portion of the nucleus (N) of one of the host cells is found along the top right of the micrograph. The plasma membrane of both cells is irregular with numerous short and elongated undulations (arrowheads). In the cytoplasm of each cell, there are a number of mitochondria (M) and abundant rough endoplasmic reticulum (RER) that surround the parasite. Bar = 1 μm.

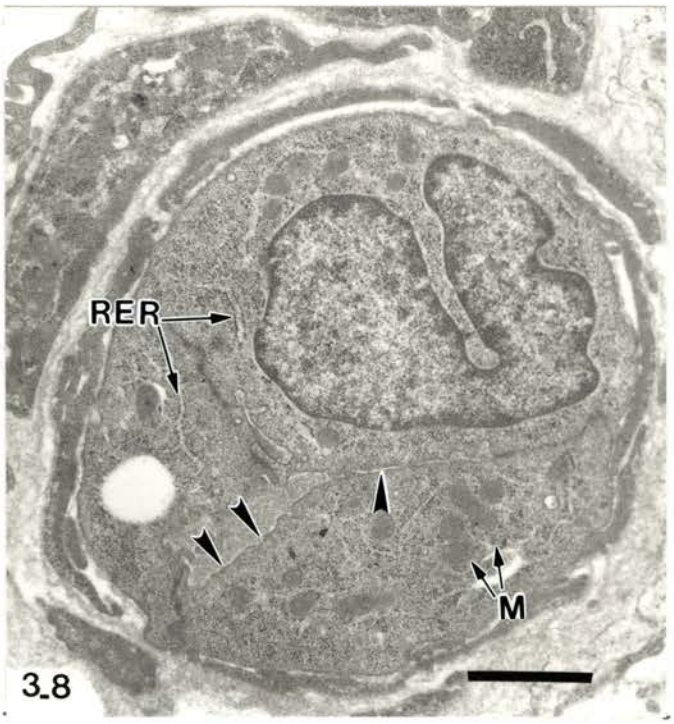


Figure 3.8. Electron micrograph of a large swollen capillary in a granuloma. The vessel is characterized by an expanded endothelial cell lining decreasing the size of the vascular lumen (arrowheads). A large number of mitochondria (M) and dilated rough endoplasmic reticulum (RER) are found within the cytoplasm. Bar = 1 μm.

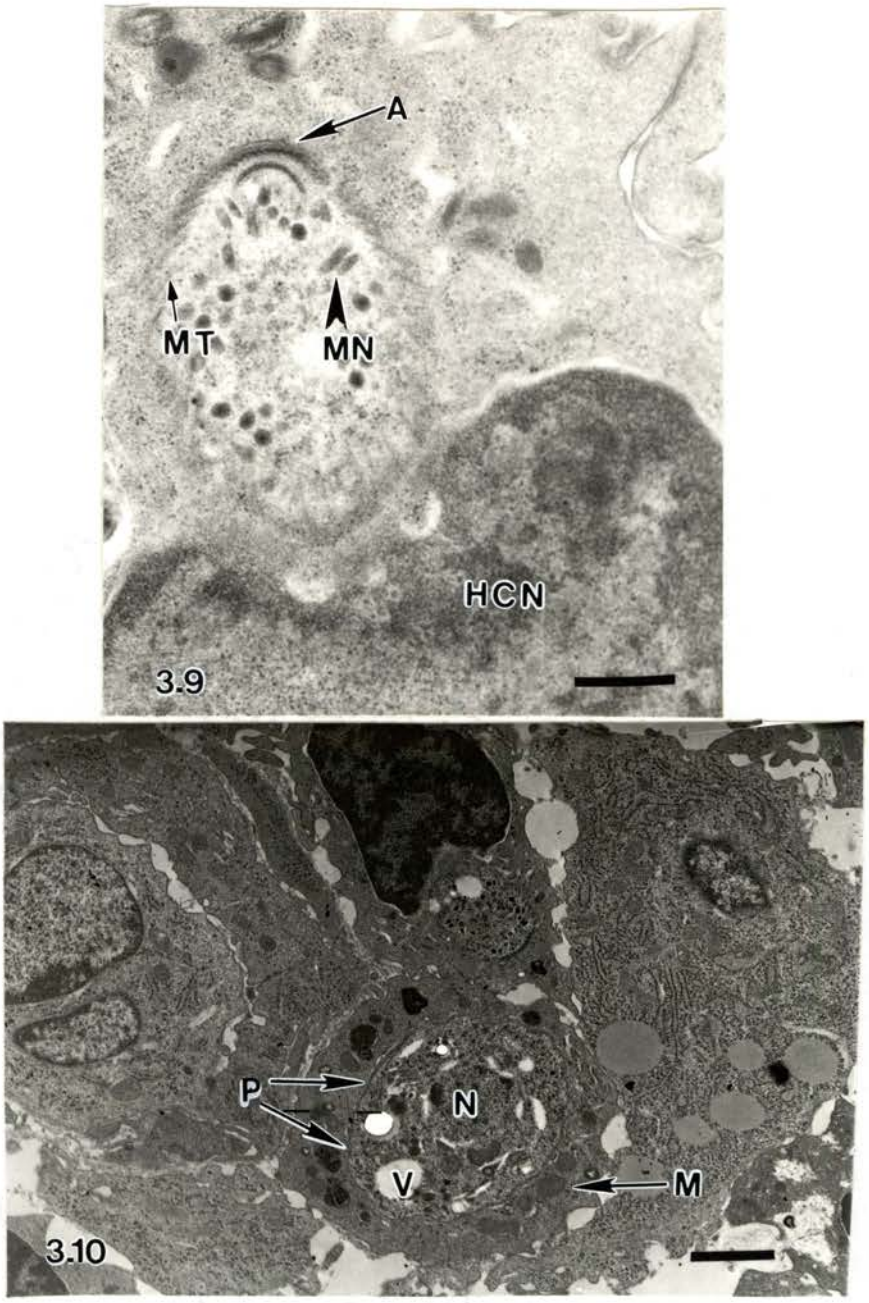


Figure 3.9 & 3.10. Cells within a granuloma. Both cells contain a parasite with features typical of an apicomplexan merozoite. Features such as a prominent apical complex (A), numerous micronemes (MN), and microtubules (MT), which are illustrated in the parasite in Figure 3.9. The host cell nucleus (HCN) is found at the bottom of the micrograph. The parasite in Figure 3.10 contains a number of variable sized vesicles (V), and a prominent pellicle (P). Bars represent 0.5 μm (Figure 3.9) and 1 μm (Figure 3.10).

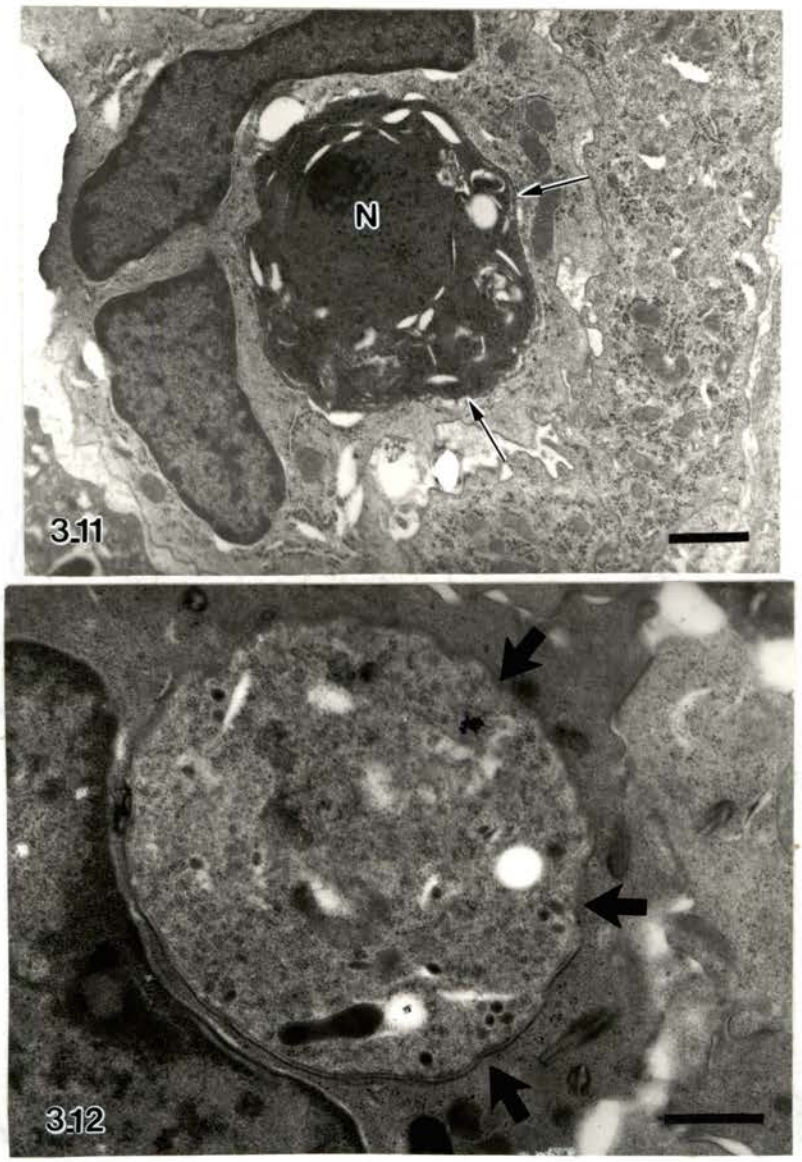
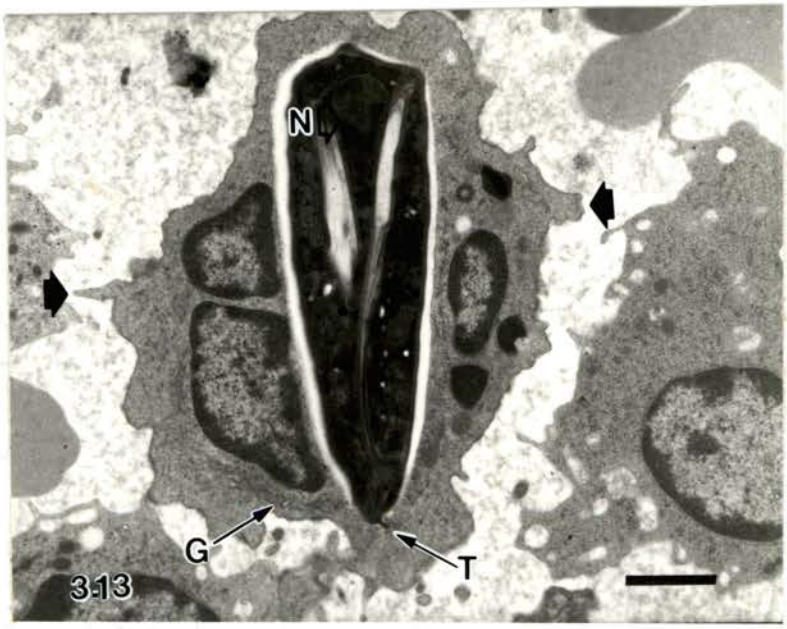


Figure 3.11 & 3.12. Electron micrograph of cells within a granuloma. Both cells contain parasites with features consistent with a developing gamont. These parasitic forms are rounded with a prominent pellicle (arrows), an often a large round nucleus (N). Bar represents and 1 μm (Figure 3.11) and 0.5 μm (Figure 3.12).

Figure 3.13. Electron micrograph of a cell (macrophage) in the peripheral circulation harboring a parasite. Note the irregular undulations of the plasma membrane of the host cell (short arrows) and the swollen golgi (G) within the cytoplasm. The parasite, folded onto itself, has a large nucleus (open arrow) that appears to be in the center of the parasite, and a short "tail-like appendage (T). Bar =1 μm.

Figure 3.14. Electron micrograph of a cell (macrophage) in the peripheral circulation harboring a visibly folded parasite. Note the posterior and anterior ends come to a prominent point (short arrows). Within the cytoplasm of the parasite, there is a cluster of mitochondria (M), dilated golgi (G), and a nucleus (N) located one end of the parasite. Bar = 1 μm.


N
G
T
3.13

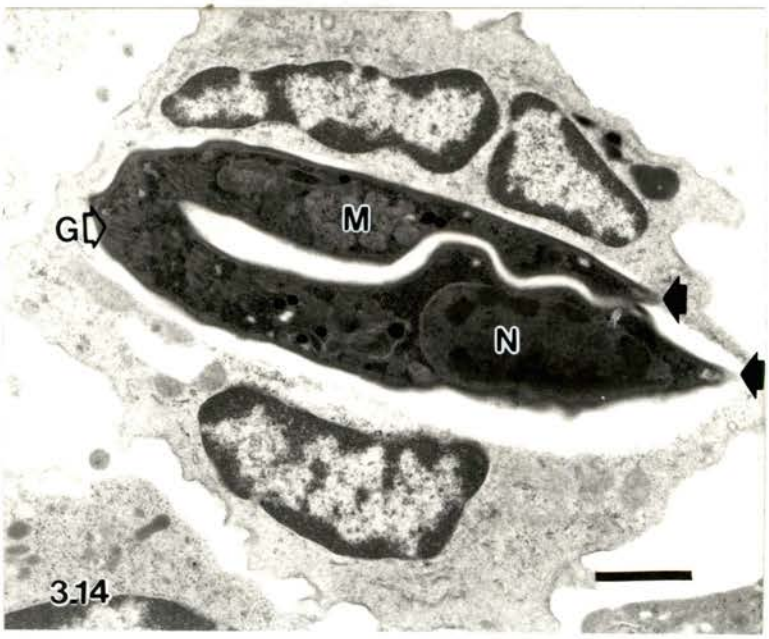
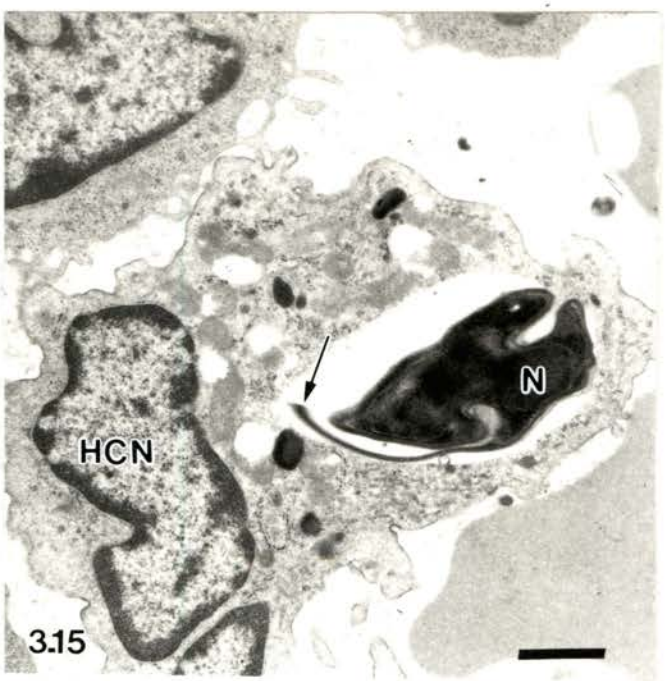

G
M
N
3.14

Figure 3.15 & 3.16. Electron micrograph of a cell (macrophage) in the peripheral circulation harboring an irregularly shaped parasite. The parasite clearly has an elongated "tail-like" appendage (arrow). The nucleus (N) of the parasite is located just above the attachment of the appendage. In figure 3.16, a higher magnification of the appendage illustrates a prominent bilayer membrane (arrows) consisting of an electron dense outer layer and an intermediate pale zone. Note the clear space between the parasite and the host cell cytoplasm and the lack of a distinct membrane enclosing the parasite (arrowheads). Bar represents 1 μm (Figure 3.15) and 0.25 μm (Figure 3.16).


N
HCN
3.15

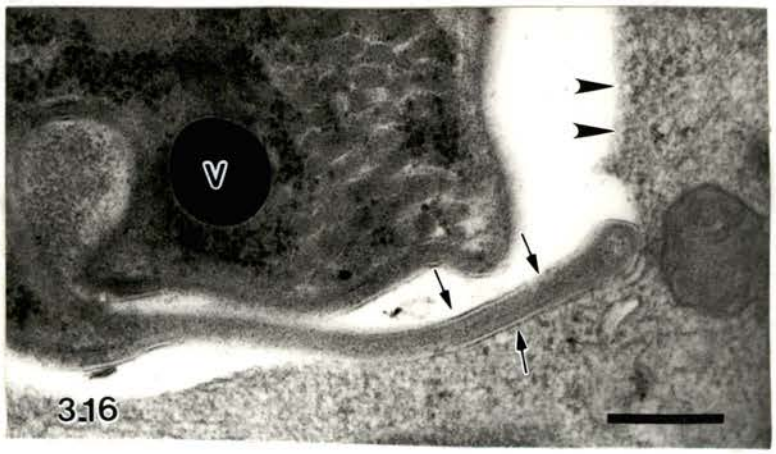
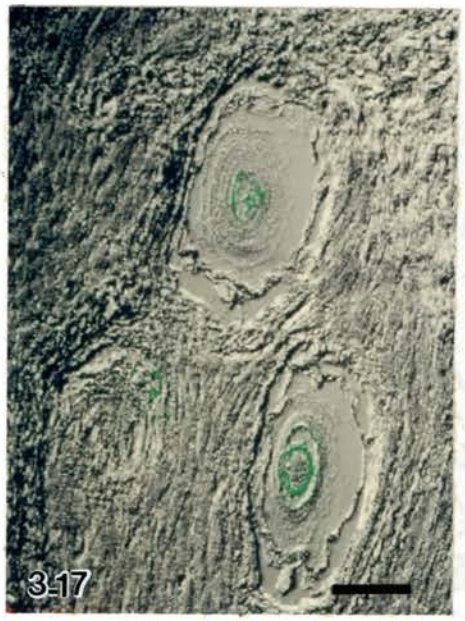

V
3.16

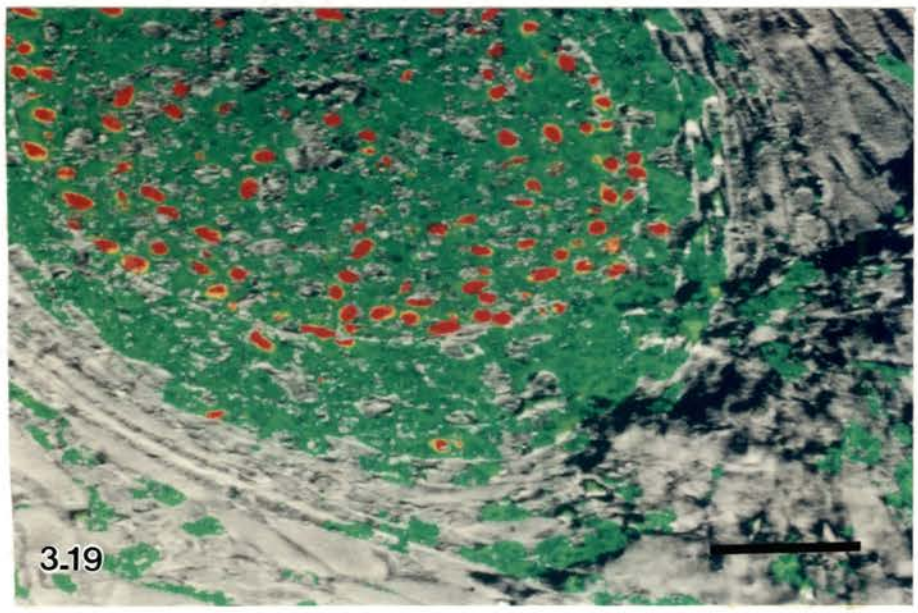
Figure 3.17. Two early encysted lesions with central host cell (cytoplasm green) positive for lysozyme immunostain. Bar = 25 μm.

Figure 3.18. Double immunostaining with vimentin and anti-*H. americanum* antibody followed by confocal microscopy of a host cell harboring a parasite. The cytoplasm of the host cell is positive (green) for vimentin. The parasite is positive for *H. americanum* (bright red). Bar = 10 μm

Figure 3.19. Double immunostaining with MAC 387 and anti-*H. americanum* antibody followed by confocal microscopy of a granuloma. Host cells are positive for MAC 387 (green) while the parasites stain bright red, positive for *H. americanum* antibody. Bar = 25 μm.


3_17


3_18


3_19

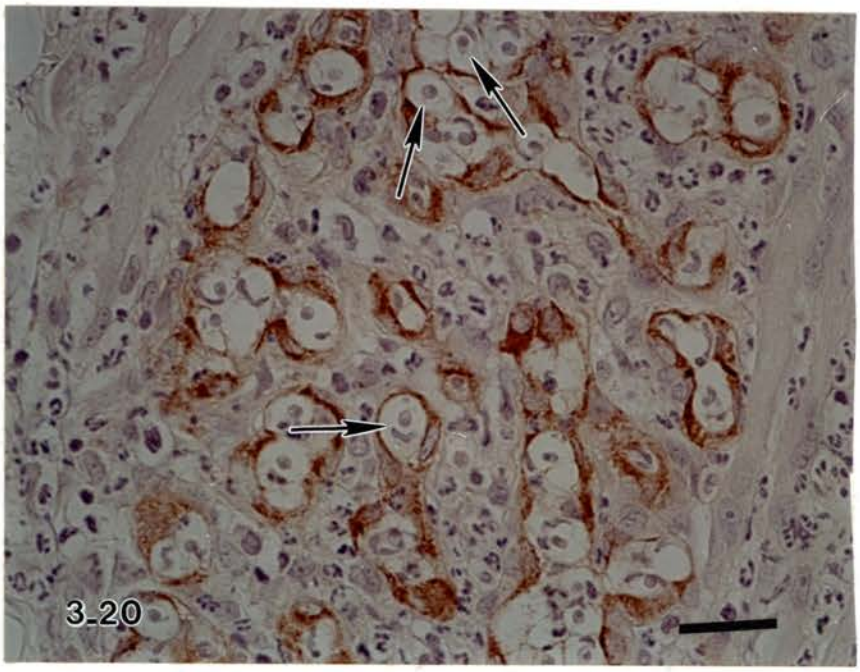


Figure 3.20. High magnification of a granuloma with blood vessels clearly delineated by Von Willebrand's Factor immunostaining. Note the host cells containing parasites within the vascular lumens (arrows). Bar = 50 μm

CHAPTER IV

AMERICAN CANINE HEPATOZOONOSIS: CORRELATION BETWEEN CHANGES IN SERUM LEVELS OF TRANSFORMING GROWTH FACTOR BETA (TGFβ), TUMOR NECROSIS FACTOR ALPHA (TNFα) AND THE PROGRESSION OF INFLAMMATION AND PERIOSTEAL BONE PROLIFERATION

Introduction

American canine hepatozoonosis (ACH) is an emerging tick-transmitted disease of dogs caused by *Hepatozoon americanum*.[5,20,24] The disease is chronic, progressive, and often fatal. Prominent features of the disease include fever, severe pyogranulomatous inflammation, and pain. The onset of inflammation follows the release of *H. americanum* merozoites from the host cell into adjoining tissue. The release of merozoites usually occurs 3 to 4 weeks after experimental exposure to *H. americanum*. Persistent and prolonged inflammation incited by the release of parasites results the formation of granulomas. An unusual feature of ACH is disseminated periosteal bone proliferation that occurs as early as 5 weeks following experimental exposure to the organism.[21] The bone lesion is similar to the well-characterized but pathogenetically obscure syndrome hypertrophic osteopathy (HO).[21]

Many theories have been proposed to explain the possible mechanisms of periosteal bone proliferation in both HO and in dogs with ACH; however, the causes for both remain unknown.[5,21] One cause of bone proliferation is speculated to involve

humoral factors, such as cytokines, that are released at the site of inflammation.[4] Hypothetically, these humoral factors enter the circulation, reach bone, and then activate the periosteum to form new bone.[7] During an inflammatory reaction, macrophages secrete and produce many cytokines.[7] Cytokines such as tumor necrosis factor (TNF) and transforming growth factor beta (TGF-β) released by macrophages have been well documented for their role in inflammation. Additionally, TNF-α enhances osteoclastic activity whereas TGF-β stimulates osteoblastic activity.[4,12,14,17,19] TGF-β also appears to play a pivotal role in the extent of bone formation and resorption during normal remodeling and during times of disease.[3,16,25] Further, macrophages are essential for coordinating both inflammatory and immune responses.[Chapter 2] Since macrophages secrete TNFα and TGFβ during an inflammatory reaction, it seems logical to predict that the levels of TNFα and TGFβ will rise as macrophages become activated and the inflammatory reaction becomes more persistent and prolonged. Given that these two cytokines are known to influence bone, prolonged secretion of these cytokines into the systemic circulation may play a role in activation of the periosteum. The inflammatory response and periosteal bone proliferation observed in a dog with ACH are clearly associated with parasitic development. Thus, our observations support the hypothesis that levels of TNFα and TGFβ become elevated in ACH and the prolonged elevation of these cytokines stimulates periosteal bone proliferation. To validate this hypothesis, bioassays that measure serum fluctuations of TNFα, TGFβ, and serum bone-specific variant of alkaline phosphatase (BALP) were performed. BALP, an isoenzyme of alkaline phosphatase (ALP), present in dog serum is considered an indicator of bone activity over time. Thus, serum fluctuations of BALP were calculated

with the intention of detecting bone growth during an experimental infection of ACH.[2,22]

The purpose of this investigation was to determine whether a relationship between fluctuations in serum levels of TNFα, TGFβ, and BALP with both the onset and progression of inflammation and periosteal bone proliferation in dogs with experimental ACH. Furthermore, this study was undertaken to provide preliminary data to support the theory that humoral factors, in particular cytokines TNFα and TGFβ, activate periosteal bone proliferation secondary to persistent releases secondary to prolonged inflammation in dog with ACH.

Materials and Methods

Ten *H. americanum*-naïve, mixed breed unisex, dogs were used in a broad-based 10-week temporal study of ACH (see Chapter 2: Material and Methods). The dogs were littermates that were born, raised, and cared for in tick-free isolation facilities at Oklahoma State University in accordance with conventional laboratory animal practices. Prior to experimentation, a physical examination (PE), complete blood cell count (CBC), chemistry panel (Chem), fecal flotation, muscle biopsy, bone scintigraphy and bone radiographs were performed on each dog. During experimentation, weekly blood samples were collected for CBC and Chem panel. Whole blood samples, collected before, sequentially during the 10-week study, and at necropsy, were collected from each dog. Specimens were centrifuged at 2000 rpm for 7 minutes and the serum was collected and stored at -80^0C pending cytokine bioassays and BALP analysis.

Six of the dogs were each fed 400-*H. americanum* oocysts and four were

designated as unexposed controls. Each dog was monitored daily by body temperature, pulse, respiration, and physical examination. Skeletal muscle samples from periodic muscle biopsies and from necropsy were collected for light and transmission electron microscopy, immunohistochemistry, and immunologic studies of parasitic development and tissue response. Periodic radiographic and scintigraphic evaluations were also done following the procedures of Drost et al.[8] The six experimentally infected dogs were subjected to a complete necropsy. Due to unexpected, rapid development of illness, 4/6 dogs were euthanized before the planned completion of the study. At necropsy, the entire skeleton was collected and prepared for gross examination by submergence in water containing detergent followed by autoclaving at 15 psi for 30-60 minutes. This procedure allowed easy removal of tissue overlying the bone. The bones were dried and systematically examined for periosteal bone proliferation, then compared to radiographic and scintigraphy findings.

Bioassay for TNF-α

TNF-α bioactivity was measured using a mouse fibroblast cell line (WEHI 164) that is exquisitely sensitive to TNF-α.[25] The cells were cultured to exponential growth, and 0.1 ml of a $4x10^5$ cells/ml suspension of cells was placed into each well of a 96-well flat bottom culture plate, and incubated overnight (37°C, 5% CO_2). The next day, serial two-fold dilutions of canine serum samples or recombinant human TNF-α (Genzyme), 0.05 ml each, was added to the wells in triplicate. In addition, 0.02 ml of Actinomycin D (0.05μg/ml-Sigma) was added to each well. Following overnight incubation, 0.025 ml of 5 mg/ml MTT,3-(4,5-dimethylthiazoyl)2,5-diphenyl tetrazolium

bromide (Sigma), was added to each well. After additional 30 minutes of incubation, all culture supernatant was removed, replaced with 0.1 ml of 0.1 N HCl isopropanol, and the plates were incubated for an additional 30 minutes. Following incubation, 0.1 ml of distilled water was added to each well and the optical density (O.D.) was read at 540 nm. A standard curve was generated from known concentrations of human recombinant TNFα. Canine serum samples were compared to the standard curve to quantitate the level of bioactive serum TNFα (using linear regression in the statistical program SAS). The positive control for each plate was human recombinant TNF-α. Negative controls included cells in culture media and Actinomycin D. All media for dilutions or propagation of the cells was Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

Bioassay for TGF-β

TGF-β assay was done using a modified procedure described by Lotz et al.[16] Briefly, CCL64 cells, obtained from American Type Culture Collection (Bethesda, MD), were maintained in 1% FBS-Minimum Essential Medium (MEM) media. For use in the assay, the cells were dislodged from the 75 cm^2 tissue culture flasks by treatment with Alsever's-trypsin-versene solution, washed twice, resuspended in 1% FBS MEM media and plated at $10x10^5$ cells/well in 96-well flat bottom plates. The cells were incubated for 12 hours and the medium was then removed. Serum (0.1 ml of a serial two-fold dilution of 1:4 diluted dog serum), acid activated dog serum (see next), or standard human recombinant TGFβ was added along with 0.1 ml of fresh medium to designated wells. A portion of undiluted dog serum was acid activated by taking 0.2 ml

of serum and placing it into a 1 ml vial along with 0.05 ml of 5N HCl and incubated for 2 hours; after which acid was neutralized by adding 0.07 ml of 0.7 moles of HEPES + 1.4 moles of NaOH. The pH was measured to confirm neutralization. The acid activated serum was then diluted 1:4. Following the addition of samples or human recombinant TGFβ, the plates were incubated at 37°C for 24 hour, following which each well was pulsed with 0.025 ml of [^{3}H]thymidine for 12 hours. The media was removed following incubation and 0.1 ml of fresh media and 0.1 ml of distilled water was added to each well. The plates were placed in the –80^{0}C freezer overnight. The next day, plates were allowed to thaw so that cells would rupture and release thymidine incorporated within the propagating cells. The ruptured cells and released thymidine were prepared for harvesting and reading. Harvesting of the cells was done using a Packard Filtermate Harvester and Unifilter96 plates. A Unifilter96 plate is a plate with 96 wells that each contain a small filter. Material within a regular 96-well plate is filtered through these small filters and cells and other compounds such as [^{3}H]thymidine are collected. In this study, the process of harvesting began with the ruptured cells along with the media containing the released thymidine being collected from the test plate and filtered thru the Unifilter96 plate. The wells in the test plate were rinsed with distilled water and the water was filtered again through the Unifilter 96 plate. Next, 0.1 ml of 1N NaOH (200μl/well) was added to each well of the test plate and incubated for 5 minutes. The wells were again filtered through the Unifilter96 plate and rinsed again with distilled water. Once harvesting was complete, 0.03 ml of scintillation media was added to each well of the Unifilter96 plate and each plate was read on a scintillation counter. The canine serum samples were compared to a standard curve generated from

the human recombinant TGFβ to quantitate the level of bioactive serum TGFβ and latent (acid activated) serum TGFβ present in each serum sample. A standard curve was generated using linear regression in Microsoft Excel. Once the concentration of TGFβ for each sample was calculated, the data were analyzed using SPSS Base 10.0 for Windows, Chicago, Illinois. Human recombinant TGFβ was used as the positive control and standard. Cells only in culture media were used as negative controls. All media used for dilutions and for cell propagation contained 1% FBS in MEM.

Serum Assay for BALP

Serum analysis of total ALP, BALP, corticosteroid-induced alkaline phosphatase (CAP) and liver alkaline phosphatase (LAP) was done by Dr. Walter E. Hoffmann at the University of Illinois following the procedures described by Sanecki.[22] In brief, total ALP was determined by hydrolysis of para-nitrophenyl phosphate in 1.0M diethanolamine buffer containing 0.5 mM $MgCl_2$ (pH 9.8) on the Hitachi 704 biochemical analyzer (Boehringer Mannhein Diagnostics, Indianapolis, Ind.) BALP was determined by combining 100μl of serum with 100 μl of 5 mg/ml wheat germ lectin (WGL) (Sigma Chemical Co., St Louis, MO) in a microcentrifuge tube that was incubated at 37^0C for 30 minutes. The tube was centrifuged for 5 minutes in a microcentrifuge (16,000 x g) and the supernatant fluid analyzed for non-binding AP activity, as described for determination of total AP, and multiplied by 2 to correct for dilution. Because a large but somewhat variable portion of CAP activity is also precipitated by WGL, both an aliquot of the WGL supernate and an aliquot from the original serum sample were simultaneously assayed for CAP activity using the

levamisole inhibition assay as previously described.[13] Final calculations were done as follows: LAP = 2(total AP in WGL supernate-CAP in WGL supernate); CAP = Levamisole assay; BALP = Total serum AP - (CAP + LAP). Due to number of serum samples and the cost involved to do them all, only 65/107 were analyzed. Serum samples chosen for analysis included baseline, weekly, and necropsy samples, In the case of control dogs, serum samples at the completion of the experiment were compared to samples taken at necropsy from the principal dogs.

Statistical Analysis

Appropriate nonparametric statistical testing was used to evaluate changes in serum levels of TNF-α and TGF-β. A significant difference was defined as $P = \leq 0.05$. Descriptive statistics of the data were calculated using the analytical software program SPSS Base 10.0 for Windows, Chicago, Illinois.

Results

Clinical Observations and Parasitic Development
(Table 4.1)

The onset of illness began with fever and diminished activity in 6/6 dogs. Daily average temperature ranged from 104 to 105.9^{0}F (Figure 4.1)[See Appendix 1 and 2 for raw data.] and persisted throughout the course of illness. Leukocytosis (WBC > 14,000/μl) was observed as early as 3 weeks post exposure. The average daily WBC ranged from 16,200 to as high as 177,600/μl (Figure 4.2)[See Appendix 3 for raw data.].

Early encysted stages, characterized by a trophozoite within a host cell, were observed in muscle biopsies as early as 3 weeks post exposure. At approximately 5 weeks, with the formation of granulomas, the presence of gamonts in circulating monocyte/macrophages indicated that the parasite had completed its asexual cycle or at least had undergone one asexual cycle. Randomly distributed moderate to severe pyogranulomatous myositis was observed. The intensity of myositis was greater in dogs that survived longer (3636 and 3644) (Figure 4.3) than dogs euthanatized earlier in the study due to profound illness.

Bone Pathology

Gross Findings. The onset and location of bone lesions are summarized in Table 4.2. Bone proliferation was detected as early as 5 weeks post exposure. The extent of bone proliferation was rated according to the amount of bone surface affected.[16] Radiographic and nuclear scintigraphic observations determined that on the average the surface of each bone having periosteal proliferation was less than 25% initially; however at necropsy, 50% to as high as 100% of a given bone surface was affected. The majority of the affected bone surfaces contained firm irregularly thickened plaques of bone that formed concentrically and to a degree, symmetrically around the diaphysis of the long bones. Firm thickened irregular plaques of bone formed also on the hipbones, scapula, and vertebral column. Bone proliferation of the pelvis and scapula were quite severe in 4/6 dogs. Dogs 3636 and 3644, euthanatized on day 72, developed the most severe bone lesions. Dog 3638, 3643, and 3644, developed bone proliferation

on the metatarsal bones, in addition Dog 3644 also developed bone lesions on the metacarpal bones.

Microscopic Findings. Periosteal bone proliferation was detected in dog 3641 at necropsy on day 35. Histologically, the bone lesions were characterized by irregular thickening of the periosteum (Figure 4.4). Loosely arranged periskeletal tissue was expanded by edema fluid and vascular changes that included vascular proliferation and endothelial cell hypertrophy. Mild to focally severe congestion and hemorrhage were also observed within the periskeletal tissue. The fibrous layer of the periosteum was also loosely arranged (edematous). Just beneath the fibrous layer was a markedly thickened dense population of small angular to stellate osteoprogenitor cells. Below the osteoprogenitor cell layer was an irregularly thickened layer of new bone formation characterized by numerous pale thin perpendicularly oriented trabeculae. More superficial in this new bone layer were haversian canals that were small and lined by plump osteocytes. Closer to the original cortex, the haversian canals became larger and were lined by a flattened layer of osteocytes. Microscopic examination of Dog 3644 revealed multiple "pseudocortices" characterized by a layer of new bone with a central core of marrow that overlaid the original cortex.

TNFα Bioassay Results

A total of 107 serum samples were analyzed for TNFα. In all cases, a normal standard curve was generated; however, both controls and experimentally-infected dogs had TNFα levels that were consistently below detectable limits throughout the test interval.

TGFβ Bioassay Results[See Appendix 4 for Raw Data]

A total of 107 serum samples were analyzed. Statistically there was no difference in the level of acid activated serum (active TGFβ) between control and infected dogs (Figure 4.5). There was a significant difference (P=.025) between serum levels without acid activation (latent TGFβ) in infected dogs compared with control dogs (Figure 4.6). Graphically, infected dogs demonstrated 2 sharp spikes beginning at day 35 and then again on day 52. Beginning around day 18 began, control dogs began a slow decline in the level of serum TGFβ until approximately day 45 when the level of TGFβ again began to slightly increase, level off, and then decrease at the end of the experiment.

Serum Assay Results for ALP and BALP[See Appendix 5 for Raw Data]

Statistically, there was a significant difference (P<0.0005) between the levels of ALP in infected versus control dogs (Figure 4.7). Graphically, the experimentally-infected group demonstrated a sharp rise in serum ALP levels on day 32. Throughout the experiment, ALP remained higher in the experimentally infected group than the control group. There was no statistical difference in serum levels of BALP between control and infected dogs (Figure 4.8). Graphically, the control group demonstrated a slow decline from baseline at day 0 to an average of 30 U/L at day 71. The average level of BALP of dogs experimentally infected on the other hand was slightly lower than in controls. There was a sharp spike at approximately day 35 and a gradual increase at the end of the experiment.

Discussion

The purpose of this experiment was to investigate a hypothesized association between serum levels TNFα and TGFβ, and serum levels of BALP and ALP, with inflammation and bone lesions in dogs with experimentally-induced ACH. Observations from periodic muscle biopsies, weekly blood evaluations, and necropsy results were used to described the development of *H. americanum*, and the onset and progression of clinical and pathological lesions occurring secondary to parasitic development. Sequential bone scintigraphy, radiography, and gross examination of the skeleton of experimentally exposed dogs were used to detect and characterize the onset and severity of periosteal bone proliferation. Control dogs had no evidence of illness or bone proliferation. In contrast, all experimentally exposed dogs developed severe illness in addition to extensive periosteal bone proliferation.

The onset of inflammation, determined by a rise in temperature and WBC, began consistently and concurrently with the detection of early parasitic development in muscle biopsies at approximately three weeks post exposure. Early parasitic stages were found similarly at 3 weeks post exposure. During this time, an increase in TNFα was expected; however, serum TNFα was undetectable by bioassay techniques. Even though TNFα, an intrinsically pyrogenic cytokine, can be detected in dogs using bioassays[25], the production of most cytokines are temporary with a half life that is very short and concentrations that may be below detectable limits even during intense inflammation.[11,15] Also, cytokines are produced transiently and usually act locally in a combined autocrine and paracrine manner.[1] Thus, peak serum concentrations of TNFα may not be detected if blood wasn't collected at the correct time. Alternatively, the

highest levels of TNFα may not be present in serum but rather in muscle at the site of inflammation. Furthermore, TNFα may undergo rapid decay either in vivo or following blood collection.[11,15] Still another reason that fluctuations in serum TNFα was not detected could be due to an overwhelming infection and an increase in the regulation of TNFα. One study reported that large amounts of TGFβ released during chronic inflammation antagonizes the affects of TNFα.[15] Additional regulation of TNFα can be due to binding of serum TNFα to soluble receptors, internalization by target cells, or the production of auto-antibodies to TNFα which can depleted the level of circulating TNFα.[1,7,11] In addition, a number of others factors in serum such as other proteins can interfere with the results of bioassays such as the one used to detect TNFα in this study.[1,11]

The results of the bioassay for serum TGFβ were inconclusive. While serum levels of latent TGFβ were significantly different between control and experimentally exposed dogs, the active form (acid-activated) was not significantly different. High concentrations of the latent form of TGFβ can exert some activity; however, the active form of TGFβ is 200-fold more effective.[18] Fibronectin and alpha-2-macroglobulin in serum can bind to the free from of TGFβ. Most often, TGFβ bound to fibronectin is reported to be active; while that bound to α-2 macroglobulin is inactive. In one report, latent forms of TGFβ obtained from serum were shown to be in the form of TGFβ -a2-macroglobulin complex.[18] It is possible that α-2 macroglobulin acts as a scavenger to inactivate the release of TGFβ thereby resulting in less active TGFβ.[237] Other factors that may affect the amount of serum TGFβ, both latent and active, and thus the outcome

of the bioassay, include the confluency of cells, the growth stage of the cells, the affect of storage on serum, media, and other components used in the bioassay, as well as the influence of other growth factors and cytokines in the serum.[10] For example, TNFα and TNFβ are known to inhibit CCL64 proliferation and thus if present in serum can affect the outcome of a TGFβ assay. Other cell mediators such as epidermal growth factor and fibroblast growth factor have been shown to stimulate proliferation of CCl64 cells, thus resulting in an erroneously high level of TGFβ.

Bone lesions, detected as early as 5 weeks post exposure were characterized by generalized periosteal bone proliferation and were perceived to be more proliferative than bone lesions described by others.[6,21,23] Histologically, bone lesions were similar to those described by Panciera et al.[6] Although periskeletal edema, vascular changes, and mild to moderate hemorrhage were present, at no time was there histologic evidence of inflammation or parasitic stages intimately associated with bone proliferation.

The relationship between bone proliferation and serum levels of BALP, ALP, TNFα and TGFβ, was inconclusive. There was a significant difference in the level of serum ALP between controls and experimentally-infected dogs. Beginning around day 32, ALP levels increased rapidly and remained high in the experimentally exposed group as compared to the control group. This increase in ALP may represent an increase due to stress, or possibly represents a disease with increased osteoblastic activity.[9] Based on the results, there were no detectable levels of LAP or CAP, and statistically BALP was not significantly different between the control and experimentally exposed groups. BALP did appear to mildly increase around day 35 in the experimental group thus in fact may represent the reason for an increase in ALP.

In summary, the data collected in this study concerning the role humoral factors, such as TGFβ and TNFα, have on bone proliferation in dogs with experimental ACH were inconclusive. Even though there was a rapid and large increase in ALP, and a slightly variable increase in TGFβ following the onset of clinical disease in experimentally exposed dogs, the results are inconclusive. However, the fact that once increased, the level of ALP remained high and in the case of TGFβ, both latent and acid-activated serum levels remained slightly higher than control dogs is encouraging. These two observations suggest that with further research and better techniques, the relationship between humoral factors and their role in the activation of periosteal bone proliferation may eventually be proven.

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Table 4.1 Onset of clinical features

Dog no.	Fever[1]	Leukocytosis[2]	Parasitic lesions observed[3]	Bone proliferation[4]	Euthanatized
3641	29d	3wk	3wk	5wk	5wk
3639	31d	4wk	4wk	5wk	6wk
3643	31d	3wk	7wk	5wk	8wk
3638	34d	4wk	6wk	6wk	8wk
3644	35d	5wk	7wk	7wk	10wk
3636	37d	5wk	6wk	9wk	10wk

Time is in days (d) or weeks (wk) that have elapsed following feeding of oocysts.

[1]Temperature of 104^0F or greater

[2]White Blood cell count greater than 14,000

[3]Parasitic lesions observed on muscle biopsy

[4]The first indication of bone proliferation by scintigraphy and radiographs

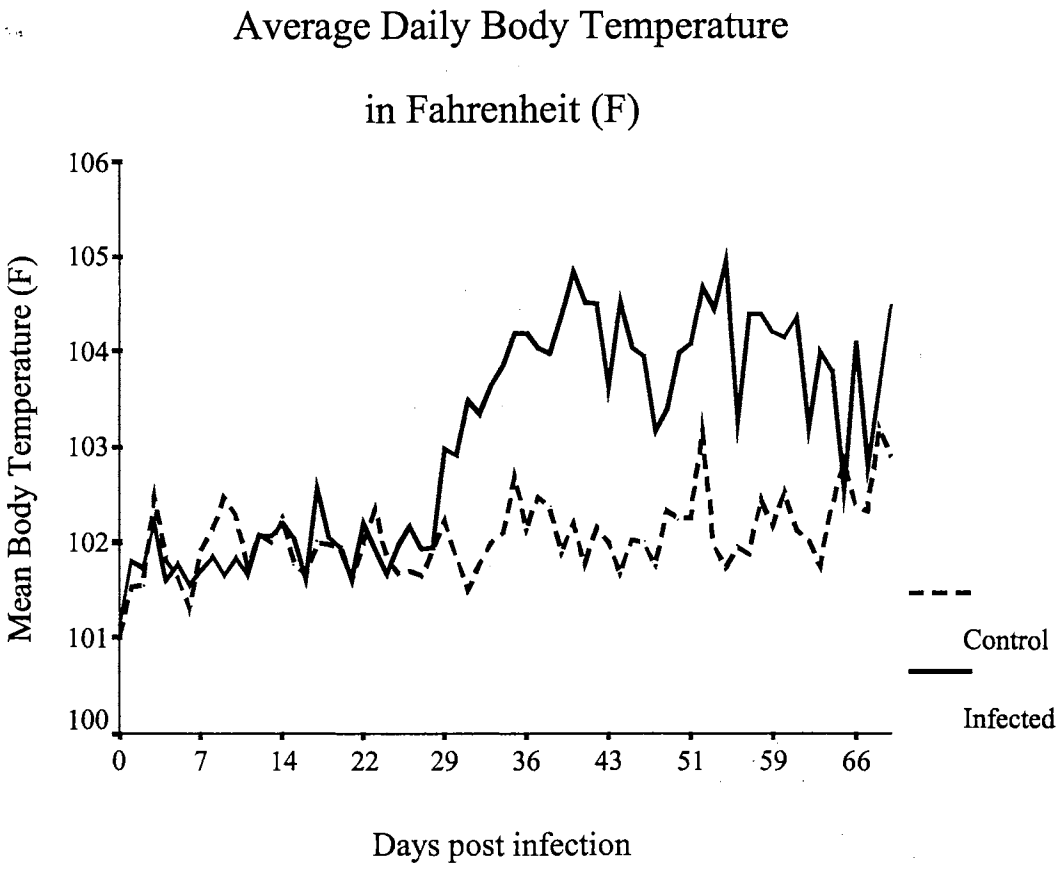

Figure 4.1. The graph illustrates the average daily temperature fluctuations of experimentally-exposed (infected) and control dogs. Note on approximately day 28, the temperature of infected dogs began to rise while control dogs remained somewhat consistent throughout the time period. Average daily temperature fluctuations of infected dogs ranged from 104°F to 105°F.

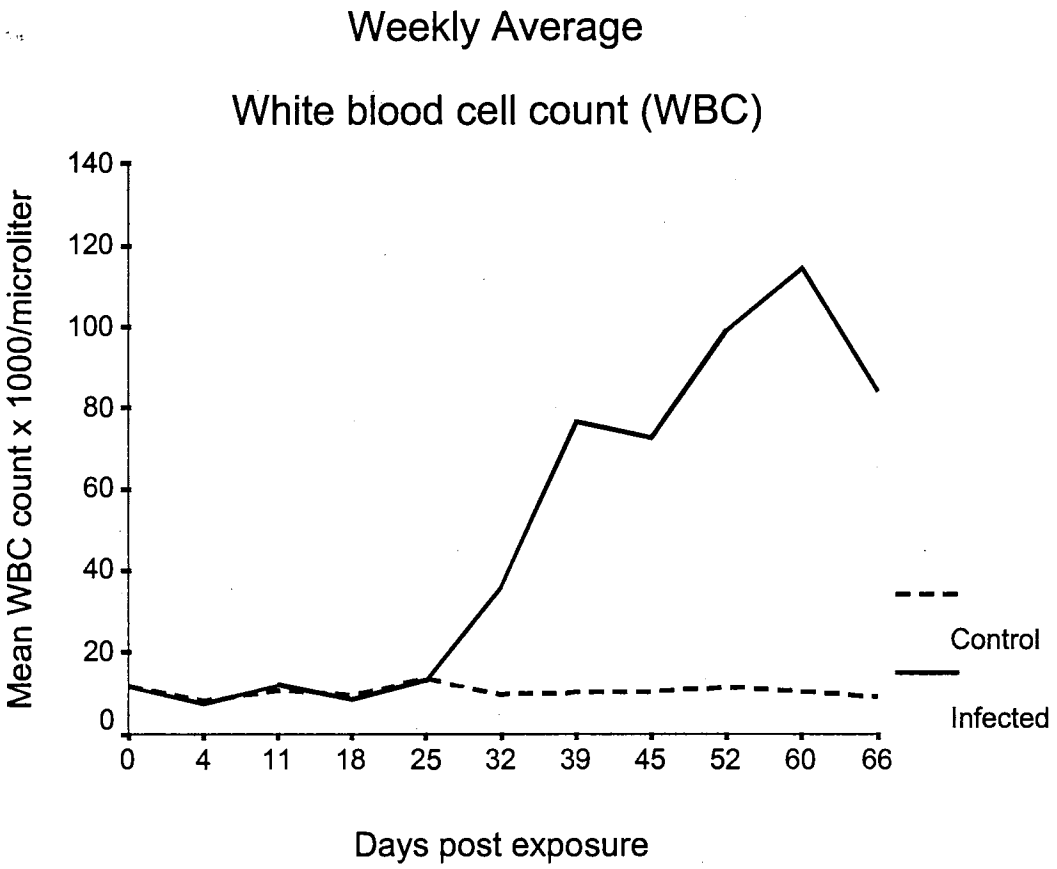

Figure 4.2. The graph depicts the average weekly WBC for experimentally-exposed (infected) and control dogs. Note at approximately Day 25, the average WBC increased dramatically in the infected dogs while the controls remains consistent throughout the study. At day 32, the average daily WBC for the infected group was 36,000 cells/μl (n=6) while on day 60, the maximum average daily WBC was 114,300 cells/μl (n=2).

Table 4.2 Onset, method of detection, and location of bone lesions

Dog #	Onset of Bone Lesions (Days Post-Exposure)	Location of Bone Lesions	Euthanatized (Days Post-Exposure)	Additional Necropsy Bone Lesions
3636	46	ulna, bilaterally	71	maxilla, mandible, skull, scapula, femur, radius, humerus, tibia, fibula, pelvis, vertebral column
3638	46	radius, ulna, femur, tibia, fibula	56	maxilla, mandible, scapula, humerus, metatarsal bones; pelvis, vertebra column
3639	36	radius, ulna, femur, tibia	42	small random areas on skull, mandible, scapula, humerus, fibula, pelvis, vertebral column
3641	35	thoracic and lumbar vertebrae, humerus, radius, ulna, femur, tibia	35	small random areas on skull, mandible, scapula, pelvis,
3643	36	humerus, radius, ulna, femur, tibia	56	mandible, skull, scapula, fibula, pelvis, metatarsal bones, vertebral column
3644	53	skull, vertebral column, scapula, humerus, radius, ulna femur, tibia	71	mandible, metacarpal and metatarsal bones, pelvis,

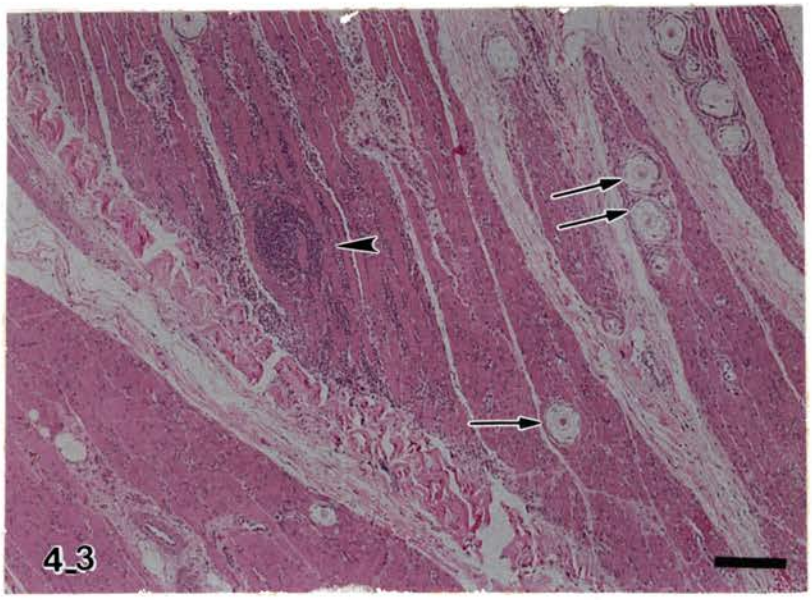

Figure 4.3. Low magnification of a section of skeletal muscle from Dog 3644 at 10 weeks post-exposure. Multiple parasitic stages including early encysted stages (small arrows) and one granuloma (arrowhead) are observed. Accompanying the parasitic lesions is a severe pyogranulomatous myositis. Bar = 100 μm.

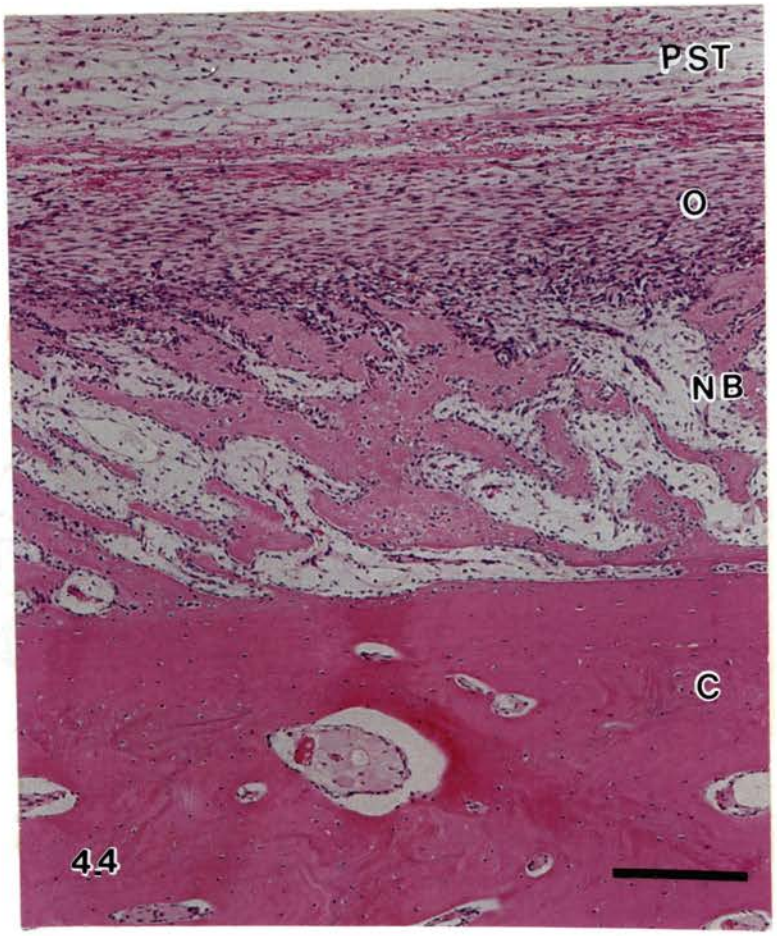

Figure 4.4. Microscopic section of the humerus from Dog 3641 at 5 weeks post-exposure. Above the highly cellular, active osteogenic zone (O) of the periosteum, the periskeletal tissue is edematous (PST). Below the periosteum, there is a thickened layer of perpendicular oriented new bone (NB) that overlies the original cortex (C). Bar = 50 μm.

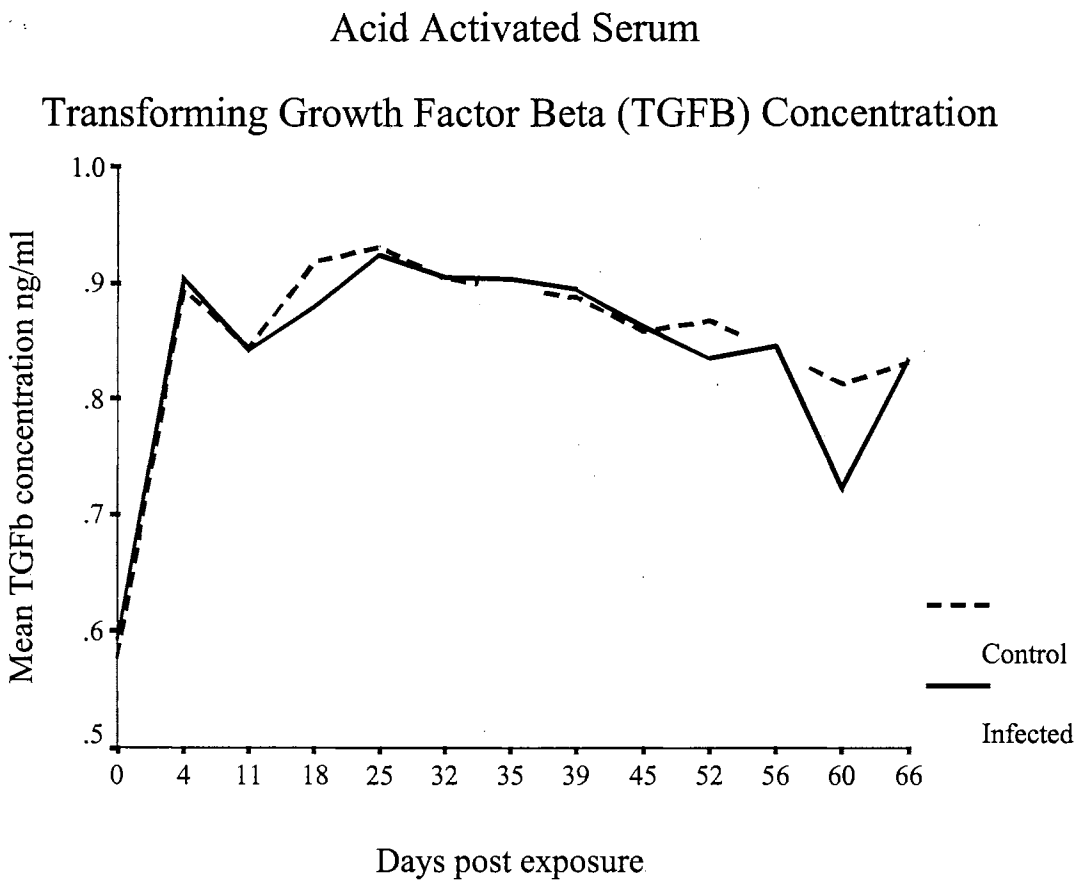

Figure 4.5. Graph illustrating the changes in the level of TGFβ concentrations in acid-activated serum. Individual data points represent the average concentration of each group: control and experimentally-exposed (infected).

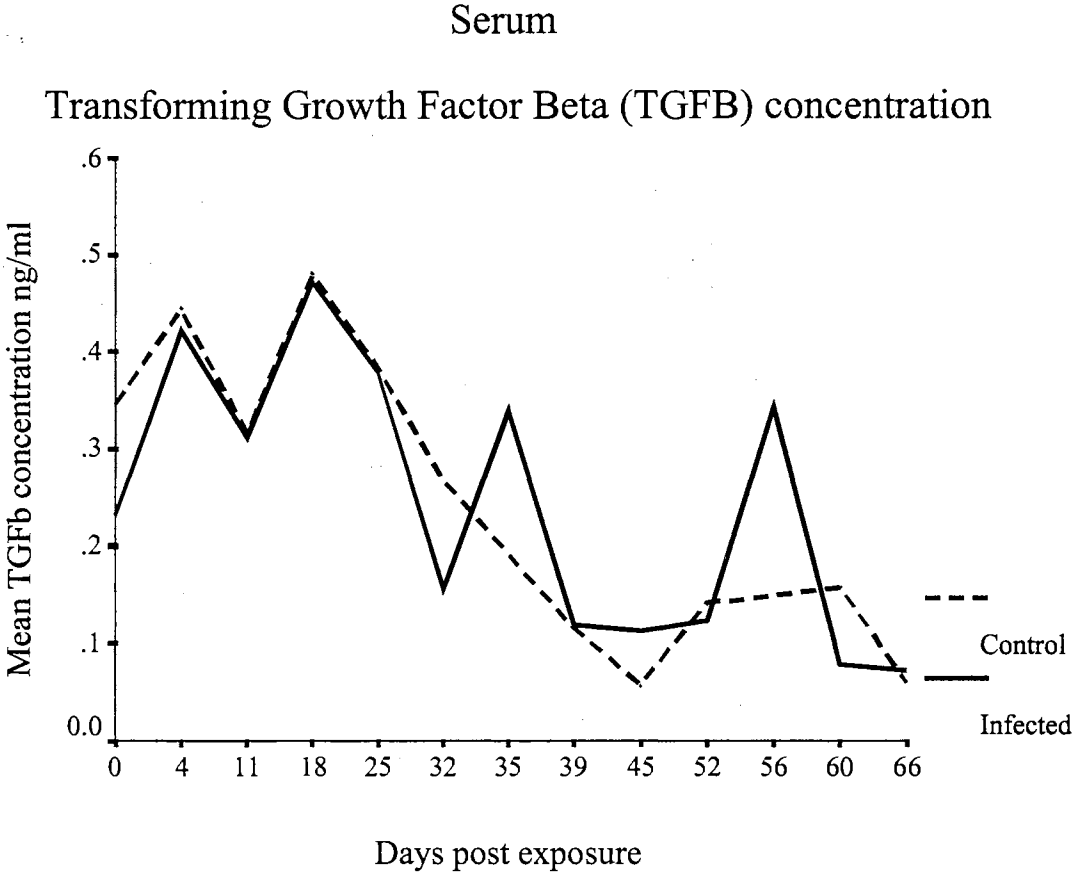

Figure 4.6. Graph illustrates fluctuations in serum TGFβ concentrations between control and experimentally-exposed (infected) dogs. Data points represent the average change in each group per days post exposure. Note that the infected group had multiple large spikes of TGFβ, while serum levels of TGFβ in control animals slowly decreased with less fluctuation during the period of observation.

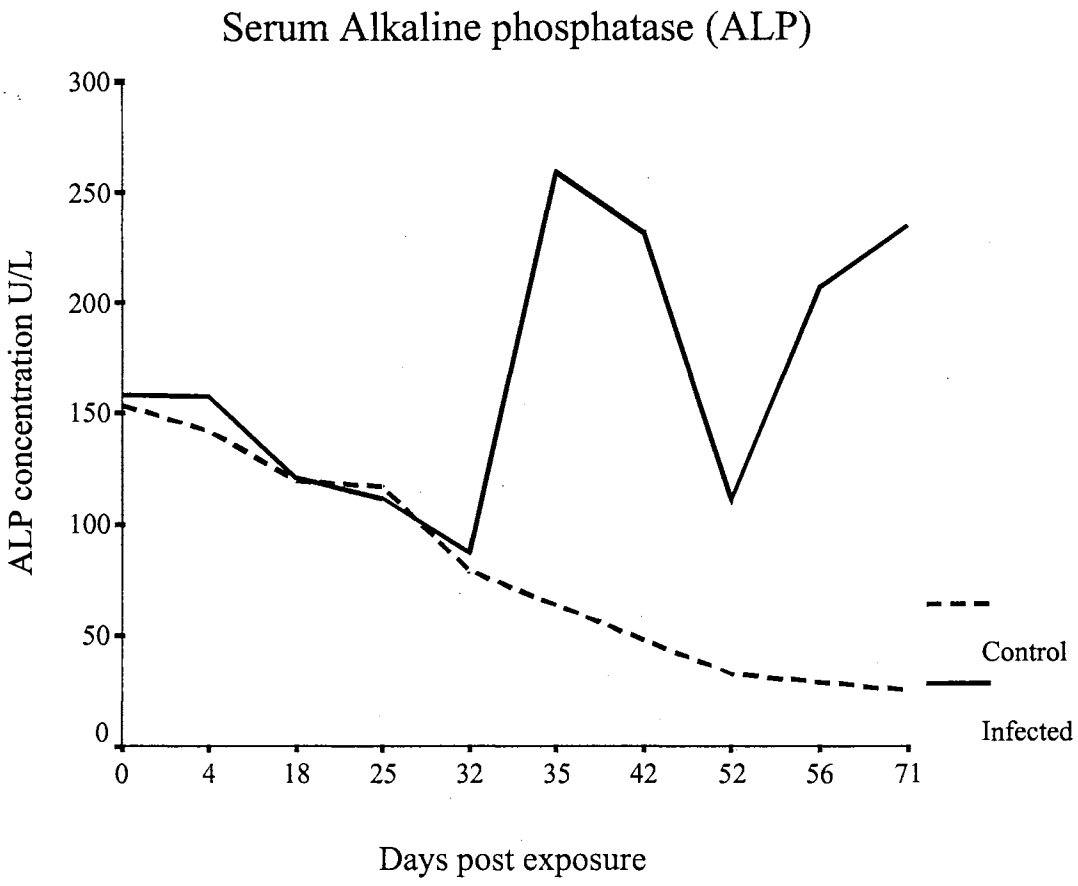

Figure 4.7. Graph illustrates the changes in serum concentrations of ALP in both control and experimentally-exposed (infected) dogs. Data points represent the weekly average ALP. Note that around day 32 post exposure, infected dogs had a rapid increase in the amount of ALP while ALP levels in control dogs slowly decrease during the period of observation.

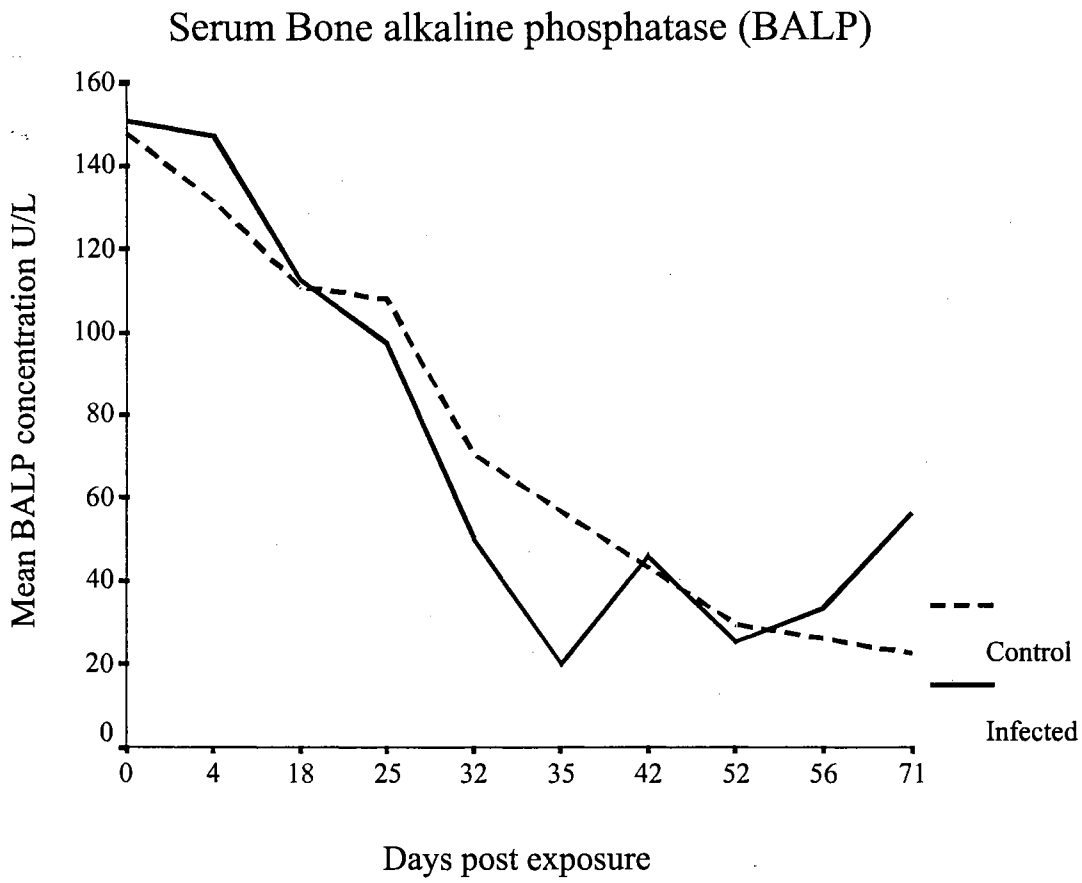

Figure 4.8. Graph illustrates the serum level of BALP during the 10-week study. Data represents the weekly average serum concentration of BALP between control and experimentally-infected (infected) dogs. Note a slow decrease in both groups; however, infected dogs did appear to fluctuation slightly with a slow rise occurring around day 35 days post exposure.

Appendix 1. Daily temperature for experimentally infected dogs. Note Day 0 is baseline temperature.

Days post exposure	DATE	3636	3638	3639	3641	3643	3644
0	2/10	100.6	100.7	101.0	101.0	101.6	101.8
1	2/16	101.6	101.7	101.2	102.4	102.4	101.5
2	2/17	101.7	102.3	101.3	102.0	101.3	101.8
3	2/18	102.2	102.9	101.9	102.2	102.2	101.9
4	2/19	101.8	101.9	101.3	101.7	101.4	101.5
5	2/20	101.3	102.3	100.8	102.2	102.2	101.8
6	2/21	101.8	101.4	101.3	101.8	101.4	101.6
7	2/22	101.6	101.8	101.4	102.1	101.8	101.5
8	2/23	101.9	101.9	101.5	102.3	102.0	101.5
9	2/24	101.4	101.8	101.3	102.2	102.0	101.4
10	2/25	102.1	102.2	101.3	102.2	101.9	101.3
11	2/26	101.7	101.9	101.7	102.0	101.1	101.5
12	2/27	102.1	102.9	101.7	102.4	101.9	101.4
13	2/28	102.3	101.9	101.6	102.7	101.8	102.1
14	3/1	101.7	102.2	102.0	102.5	102.8	102.0
15	3/2	102.5	102.4	101.8	102.2	101.7	101.6
16	3/3	101.2	101.9	101.8	101.7	101.5	101.6
17	3/4	102.4	102.5	101.8	102.8	102.1	101.3
18	3/5	102.2	102.0	101.8	102.5	101.7	102.1
19	3/7	102.0	101.4	102.2	102.2	101.9	101.9
20	3/8	101.5	101.5	101.5	102.1	101.7	101.4
21	3/9	102.9	103.1	101.2	102.7	102.0	101.3
22	3/10	102.3	101.9	101.6	102.4	101.9	101.5
23	3/11	101.8	101.8	101.6	102.3	101.0	101.5
24	3/12	101.1	102.0	102.1	104.2	101.1	101.3
25	3/13	102.2	102.3	101.7	103.5	101.7	101.6
26	3/14	101.7	102.0	101.5	102.8	102.0	101.6
27	3/15	101.4	101.6	101.9	102.9	102.0	101.9
28	3/16	102.1	102.5	103.4	104.5	103.1	102.3
29	3/17	101.5	102.4	103.7	104.5	103.7	101.7
30	3/18	101.8	103.3	104.6	104.8	104.6	101.8
31	3/19	101.6	102.9	105.0	104.3	104.7	101.6
32	3/20	101.9	103.8	104.9	104.1	105.3	101.9
33	3/21	103.4	104.3	104.2	103.6	104.4	103.2
34	3/22	103.8	104.2	104.6		104.4	104.0
35	3/23	103.7	104.7	104.2		104.1	104.3
36	3/24	104.7	104.4	102.8		103.8	104.5
37	3/25	104.6	105.3	102.7		102.8	104.5
38	3/26	104.4	104.9	104.2		103.9	104.5
39	3/27	104.6	105.8	104.7		104.4	104.7
40	3/28	105.4	104.7	104.4		103.9	104.2
41	3/29	105.4	105.2	104.3		103.7	103.9
42	3/30	102.2	104.5			104.0	103.8
43	3/31	104.5	105.0			104.7	103.9
44	4/1	103.6	104.7			104.2	103.7

Appendix 1. Daily temperature for experimentally infected dogs. Note Day 0 is baseline temperature.

Days post exposure	DATE	3636	3638	3639	3641	3643	3644
45	4/2	103.4	105.0			103.8	103.6
46	4/3		102.1			103.6	103.8
47	4/4	102.0	104.1			103.7	103.8
48	4/6	103.9	104.1			103.3	104.6
49	4/7	103.2	105.2			103.6	104.3
50	4/8	105.6	105.2			104.0	103.9
51	4/9	103.6	105.1			104.1	105.0
52	4/10	104.7	105.2				
53	4/11	105.9	104.3			101.6	101.3
54	4/13	104.4					104.4
55	4/14	105.0					103.8
56	4/15	105.0					103.4
57	4/16	105.0					103.3
58	4/17	104.7					104.0
59	4/18	104.5					101.9
60	4/19	105.1					102.9
61	4/20	104.1					103.5
62	4/21	103.5					101.6
63	4/22	104.3					103.9
64	4/23	103.5					102.1
65	4/27	105.1					104.5

Appendix 2. Daily temperature for control dogs. Note Day 0 is baseline temperature.

Days post exposure	DATE	3637	3640	3642	3645
0	2/10	101.5	100.6	100.8	101.1
1	2/16	101.8	100.7	101.7	101.9
2	2/17	100.8	101.5	101.8	102.1
3	2/18	103.2	101.9	102.3	102.5
4	2/19	101.6	101.9	101.9	101.8
5	2/20	102.2	100.7	102.1	101.5
6	2/21	102.1	100.8	101.4	100.9
7	2/22	102.4	101.8	101.8	101.7
8	2/23	102.6	101.7	102.0	102.2
9	2/24	102.1	102.7	102.3	102.8
10	2/25	102.4	101.8	102.3	102.6
11	2/26	102.3	101.8	101.6	101.3
12	2/27	101.8	101.8	102.5	102.2
13	2/28	101.6	102.0	102.4	102.0
14	3/1	102.6	102.3	102.3	101.8
15	3/2	102.0	101.5	101.5	102.1
16	3/3	101.9	101.4	101.9	101.4
17	3/4	101.9	102.3	102.0	101.8
18	3/5	102.6	102.1	101.7	101.5
19	3/7	102.6	101.2	102.1	101.9
20	3/8	102.4	101.1	101.7	101.2
21	3/9	102.9	101.5	102.0	101.6
22	3/10	102.5	102.0	102.5	102.4
23	3/11	102.2	101.2	102.1	101.8
24	3/12	101.9	101.6	101.6	101.6
25	3/13	101.4	101.6	102.5	101.3
26	3/14	101.6	101.1	102.2	101.7
27	3/15	102.0	101.5	102.5	101.7
28	3/16	102.6	102.2	102.5	101.6
29	3/17	102.7	101.6	101.7	101.4
30	3/18	101.6	101.5	101.0	101.9
31	3/19	101.8	101.8	101.7	101.8
32	3/20	102.5	102.2	102.0	101.3
33	3/21	101.9	101.9	102.8	101.8
34	3/22	102.7	102.7	103.1	102.3
35	3/23	102.3	101.8	102.7	101.8
36	3/24	103.1	102.4	102.7	101.7
37	3/25	102.7	102.0	102.9	101.9
38	3/26	102.1	102.0	101.6	101.9
39	3/27	102.5	101.6	103.0	101.7
40	3/28	101.3	101.8	102.6	101.4
41	3/29	102.2	101.9	102.5	102.0
42	3/30	102.3	101.9	102.8	101.8
43	3/31	101.8	101.5	101.8	101.6

Appendix 2. Daily temperature for control dogs. Note Day 0 is baseline temperature.

Days post exposure	DATE	3637	3640	3642	3645
44	4/1	101.8	101.1	102.8	102.4
45	4/2	102.0	102.1	102.2	101.7
46	4/3	102.6	101.4		101.3
47	4/4	102.5	102.0	102.5	
48	4/6	101.9	102.8	102.3	102.0
49	4/7	102.1	102.6	102.7	101.6
50	4/8	103.8	102.9	103.6	102.4
51	4/9	101.7	102.0	102.4	101.8
52	4/10		101.6	101.6	102.0
53	4/11	102.5	102.3	101.5	101.5
54	4/13	101.3	102.1	102.7	101.4
55	4/14	102.1	103.0	102.5	102.2
56	4/15	102.2	102.0	102.9	101.6
57	4/16	103.7	102.0	102.3	102.1
58	4/17	102.1	102.4	103.2	101.9
59	4/18	102.2	102.1	102.2	101.6
60	4/19	101.9	101.5	101.9	101.7
61	4/20	102.2	102.0	103.1	102.1
62	4/21	103.4	102.1	102.8	103.1
63	4/22	103.0	102.1	102.2	102.3
64	4/23	102.1	102.3	102.8	102.1
65	4/27	104.0	102.0	103.3	102.3

Appendix 3. Weekly white blood cell count (x1000 cells/μl) for each dog.

Dog #	2/10 Baseline	2/19	2/26	3/5	3/12	3/19	3/26	4/1	4/8	4/16	4/22
3636*	10.3	6.8	11.0	10.6	15.9	14.7	49.3	37.4	62.7	112.5	85.1
3637	16.0	8.8	12.8	11.7	13.1	10.0	12.0	11.2	12.8	11.0	9.9
3638*	13.8	6.5	13.1	8.5	13.0	20.3	55.3	65.8	177.6		
3639*	9.8	6.8	15.3	6.6	9.6	37.0	136.2				
3640	11.1	7.0	9.0	8.9	10.5	7.3	8.4	7.7	11.2	7.6	7.6
3641*	11.3	8.9	12.2	10.7	16.2	90.1					
3642	9.8	7.3	8.6	8.6	13.0	9.0	10.3	10.9	10.9	9.4	8.5
3643*	11.4	6.8	10.7	9.4	14.5	41.5	90.4	126.6	96.8		
3644*	12.8	7.9	9.4	5.5	9.2	12.2	50.9	60.5	59.5	116.0	82.6
3645	10.7	9.1	12.8	9.5	17.2	12.6	10.0	11.8	11.1	13.6	10.6

*Experimentally-infected dogs

Appendix 4. Transforming growth factor beta (TGFβ) data for experimentally-infected and control dogs.

Weekly and necropsy serum TGFβ levels.

Days post exposure	Infected Group	Control Group
Baseline (0)	0.2312 ± 0.20[a] (6)[b]	0.3466 ± 0.22[a] (4)[b]
4	0.4228 ± 0.10 (6)	0.4437 ± 0.10 (4)
11	0.3171 ± 0.17 (6)	0.3174 ± 0.11 (4)
18	0.4718 ± 0.15 (6)	0.4792 ± 0.12 (4)
25	0.3798 ± 0.10 (5)	0.3850 ± 0.11 (4)
32	0.1560 ± 0.12 (5)	0.2682 ± 0.11 (4)
35	0.3395 ± 0.11 (1)	-
39	0.0705 ± 0.09 (5)	0.1147 ± 0.11 (4)
42	0.3988 ± 0.20 (1)	-
45	0.1124 ± 0.17 (5)	0.0577 ± 0.08 (4)
52	0.1235 ± 0.10 (4)	0.1412 ± 0.16 (4)
56	0.3791 ± 0.12 (2)	-
60	0.0782 ± 0.13 (2)	0.1569 ± 0.13 (4)
66	0.0725 ± 0.08 (2)	0.0598 ± 0.07(4)

[a] Mean TGFβ concentration (ng/ml) ± SD
[b] Number of dogs (3 samples each). Days 35, 42, and 56 were necropsy serum samples.

Weekly and necropsy serum acid activated TGFβ levels.

Days post exposure	Infected Group	Control Group
Baseline (0)	0.5919 ± 0.42[a] (6)[b]	0.5762 ± 0.39[a] (4)[b]
4	0.9040 ± 0.03 (6)	0.8943 ± 0.03 (4)
11	0.8424 ± 0.03 (6)	0.8440 ± 0.03 (4)
18	0.8800 ± 0.11 (6)	0.9184 ± 0.11 (4)
25	0.9248 ± 0.03 (5)	0.9314 ± 0.03 (4)
32	0.9045 ± 0.09 (5)	0.9040 ± 0.06 (4)
35	0.9033 ± 0.05 (1)	-
39	0.8933 ± 0.07 (5)	0.8877 ± 0.04 (4)
42	0.9671 ± 0.02 (1)	-
45	0.8624 ± 0.07 (5)	0.8584 ± 0.04 (4)
52	0.8348 ± 0.09 (4)	0.8676 ± 0.03 (4)
56	0.8229 ± 0.03 (2)	-
60	0.7225 ± 0.04 (2)	0.8132 ± 0.02 (4)
66	0.8345 ± 0.02 (2)	0.8310 ± 0.03(4)

[a] Mean TGFβ concentration (ng/ml) ± SD
[b] Number of dogs (3 samples each). Days 35, 42, and 56 were necropsy serum samples.

Appendix 5. ALP and BALP serum levels

Days post-exposure	ALP		BALP	
	Infected	Control	Infected	Control
Baseline (0)	158.4±39.8[a] (5)[b]	153.5±36.1 (4)	150.0±35.6 (5)	147.75±37.4 (4)
4	157.3±28.1 (3)	142.0±29.4 (4)	147.3±30.1 (3)	131.5±27.5 (4)
18	120.7±22.3 (6)	119.5±23.2 (4)	112.7±21.6 (6)	111.0±19.8 (4)
52	111.5±63.0 (4)	32.5±41.32 (4)	25.3±16.7 (4)	29.5±38.9 (4)
66	235.0±21.2 (2)	25.0±48.6 (4)	56.5±9.2 (4)	22.5±45 (4)

[a] Mean ± SD.
[b] Number of dogs sampled (1 sample each).

VITA

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