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Barry Clayton Anderson
Portland State University

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DISSERTATION APPROVAL

The abstract and dissertation of Barry Clayton Anderson for the Doctor of Philosophy in Environmental Sciences and Resources: Biology were presented March 23, 2001, and accepted by the dissertation committee and the doctoral program.

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ABSTRACT

An abstract of the dissertation of Barry Clayton Anderson for the Doctor of Philosophy in Environmental Sciences and Resources: Biology presented 23 March 2001.

Title: The Response of Mice to Infection by the Parasitic Nematode *Trichinella*:

A comparison of *Trichinella spiralis* and *Trichinella pseudospiralis*

The intracellular parasite *Trichinella* is a genus in phylum Nematoda that contains six named species including *Trichinella spiralis* and *Trichinella pseudospiralis*. These parasites infect a large variety of wild and domestic animals, human beings and a few species of birds. The parasitic strategies and the pathological effects on the host between *Trichinella spiralis* and *Trichinella pseudospiralis* are quite different.

The purpose of this study is to gain an understanding of the physiological, immunological and pathological differences between these two species of *Trichinella* using infections in the mouse as a model. In the course of this research I have attempted to answer the following questions: A) Is cortisol a factor in the differences of the host response to *Trichinella spiralis* or *Trichinella pseudospiralis*? B) Are there differences in leukocyte response in the peripheral blood of *Trichinella* infected mice? C) Are there differences in the up-regulation or down-regulation of cell surface molecules on leukocytes in the spleens of *Trichinella* infected mice? D) Is there a difference in the degree of muscle damage (as measured by creatine phosphokinase) when infections by

the two species of *Trichinella* are compared? E) Are there differences in angiogenesis and collagen deposition in *Trichinella* infected mice and are these differences related to cortisol? F) Is nitric oxide a component in parasite killing and are there differences in nitric oxide production in host mice when the two species of *Trichinella* are compared?

My research has shown that there are significant differences in the parasitic strategies and pathological consequences in mice infected with one or the other of the two species of *Trichinella*. The two species appear to generate different immune and inflammatory responses from the host. *Trichinella pseudospiralis* is much less damaging to the host, generates a very different peripheral blood response, stimulates the production of substantially greater levels of serum cortisol, generates a significantly different profile in cytokine production, presents a very different cell surface antigen profile and does not produce a collagen nurse cell or generate an angiogenic response when compared to *T. spiralis*. In addition, I have shown a role for nitric oxide in parasite killing and a role for serum cortisol in larval survival. I have also shown that cortisol has no role in either collagen deposition or the angiogenic response in Balb/c mice under the experimental conditions detailed here.

THE RESPONSE OF MICE TO INFECTION BY THE
PARASITIC NEMATODE *TRICHINELLA*:
A COMPARISON OF *TRICHINELLA SPIRALIS* AND
TRICHINELLA PSEUDOSPIRALIS

by

BARRY CLAYTON ANDERSON

A dissertation submitted in partial fulfillment of the
requirements for the degree of

DOCTOR OF PHILOSOPHY
in
ENVIRONMENTAL SCIENCES AND RESOURCES: BIOLOGY

Portland State University
2002

DEDICATION

This work is dedicated to the glory of God whose saving Grace has allowed me to overcome the spiritual and physical obstacles that I found along the path less taken.

ACKNOWLEDGMENTS

MY GRATEFUL APPRECIATION TO:

DR. DAVID CLARK

AND THE MEMBERS OF THE COMMITTEE:

DR. MARY TAYLOR

DR. LESTER NEWMAN

DR. RICHARD FORBES

DR. JAMES R. PRATT

DR. PAVEL SMEJTEK

DR. GARY BRODOWICZ

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RANDY SMITH

AND ESPECIALLY TO MY WIFE CLAIRE AND MY MOTHER VIRGINIA (FOR
THEIR INFINITE PATIENCE AND SUPPORT THROUGHOUT).

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CHAPTER ONE

GENERAL INTRODUCTION

THE TAXONOMY AND BIOLOGY

OF *TRICHINELLA*

The intracellular parasite *Trichinella* is the single known genus in the superfamily Trichocephaloidea, of the order Stichosomida, class Adenophorea, and phylum Nematoda. In this genus, there are six named species: *Trichinella spiralis* (Owen, 1835), *Trichinella pseudospiralis* (Garkavi, 1972), *Trichinella britovi* (Britov and Boev, 1972), *Trichinella nativa* (Britov and Boev, 1972), *Trichinella nelsoni* (Britov and Boev, 1972) and *Trichinella murrelli* (Pozio and La Rosa, 2000). There are only minor morphological differences between adults of the various species of *Trichinella* (Skvortsova and Veretennikova, 1997). Their identification, therefore, is based not on morphology but on differences in their physiology, environment and genetics. For example, *T. pseudospiralis* infects birds and lacks an encapsulated stage during the muscular phase of the life cycle.

Trichinella species are obligate parasites of many species of warm-blooded vertebrates. They have been found in most areas of the world and in most habitats occupied by mammals or birds. *Trichinella* has been found to naturally infect various animals including fox, wolf, horse, polar bear, civet cat, whale, seal, hyena, dog, pig and rodent in addition to human beings. Uniquely, *T. pseudospiralis* has been found to

parasitize various species of meat-eating birds including the hawk, eagle and owl and is frequently found in scavengers such as the crow and vulture and has been reported to parasitize several species of mammal. Experimentally, *T. pseudospiralis* will infect chickens (Despommier, 1975).

The life cycle of the various *Trichinella* species consists of an intestinal phase and a muscle phase. As described by Despommier (1975), the cycle begins when the infectious (L1) larval stage housed in a nurse cell within a skeletal muscle fiber is ingested. The nurse cell is an important component of the host-parasite relationship with stimuli for development probably arising from both the parasite and the host. The mature nurse cell consists predominantly of collagen types IV and VI. (Polvere, et al 1997). One species, *T. pseudospiralis*, does not develop the collagen nurse cell or cyst. All species of *Trichinella* including *T. pseudospiralis* live within the host myofiber. *Trichinella* may live for months in short-lived animals and years in longer-lived animals.

When a host muscle fiber infected with mature L1 larvae is consumed by another animal, the myofiber is disrupted and the larval stage is released from the cyst by pepsin digestion in the new host's stomach. The L1 infectious stage is then carried to the small intestine. In the intestine, the larvae penetrate rows of columnar epithelial cells mostly in the jejunum and ileum. The mechanism of penetration is not fully understood; but the larvae of *T. spiralis* are known to secrete proteinases from the stycosome (Moczon and Wranicz, 1999). The cell membranes of adjacent epithelial cells fuse to form syncytia (Despommier, et al 1978). In the intestinal epithelial syncytia, the larvae consume nutrients and grow rapidly, requiring four molts to mature

to the adult stage. The maturing larvae reach the adult stage in approximately 30 hours (Wright, 1979). Mating occurs upon reaching sexual maturity and spermatozoa have been found in the females approximately 37-40 hours post-infection or 7-10 hours after maturity of the female is reached (Despommier, 1975). *Trichinella* females are one of the few nematodes that are larviparous. Depending on the host species and beginning at about 6 days post-infection, each female extrudes several hundred to a few thousand larvae over the course of the intestinal phase that may last another 10 days. Larval extrusion increases for five days then slowly decreases. The adult female worms are generally expelled by day 20 post-infection and the males of all species are expelled by 15-20 days post-infection.

The newborn larvae enter the lamina propria of the villus and most frequently penetrate the lymphatic vessels although they occasionally enter the capillaries. The larvae depart the lymph vessels and enter the general circulation through the thoracic duct. With the aid of its oral stylet, the newborn larvae exit the capillary or lymphatic vessel and penetrate a single muscle fiber of the host for the final development of the L1 larvae. Frequently, muscle fibers are parasitized by more than one larva.

Although the newborn L1 larvae may be transported in blood or lymph through several tissues, including the brain, lungs and smooth and cardiac muscle, it will only establish its intracellular niche in striated muscle. The specificity of *Trichinella* for skeletal muscle may be due to several factors. Hughes and Harley (1977) found that *Trichinella* larvae are electrotactic and migrate to striated muscle based on the electrical characteristics of the muscle. Experimentally, the larvae are attracted to a

120mV stimulus and repelled by a 90mV stimulus. Stewart and Carniga (1980). Despommier, et al (1978) suggested that those muscles most heavily vascularized were most frequently penetrated by the worm for its subsequent development. I have found that, in the mouse and rat, the most heavily parasitized skeletal muscles are the masseter, diaphragm, tongue and intercostal muscles.

After the newborn larvae penetrate the striated muscle fiber, they undergo a period of extremely active growth for approximately 12 days. It has been estimated that the larvae increase their body weight by approximately 40% per day during the first 6 to 8 days following muscle penetration (Despommier, 1975). Among the internal structures to develop during this time are the intestine and the stychosome. Within the stychosome are the stychocytes containing α and β granules as seen with the electron microscope. These granules are known to contain immunogenic proteins that are secreted in the worm's saliva (Jasmer and Neary 1994; Ko, et al 1994; Gerencer, et al 1992).

Changes in the host muscle cell resulting from larval penetration include hypertrophy of muscle cell nuclei (Despommier, et al 1991) and degenerative changes in the host mitochondria (Wandurska-Nowak and Boczon, 1993). Membrane whorls appear within the sarcoplasm and one finds the apparent initiation of DNA synthesis within host muscle nuclei ($2n \rightarrow 4n$) as measured by ^3H -thymidine incorporation (Jasmer, 1993). Other changes include the development of a nurse cell around the L1 stage. *Trichinella pseudospiralis* is the only species of *Trichinella* that does not develop a nurse cell. Vassilatis, et al, 1992 noted the translocation of at least one worm

excretory/secretory protein to the host nucleus. Transcript levels of the transcriptional regulatory factors MyoD1 and myogenin are altered indicating inactivation of certain host muscle genes that are involved in muscle cell differentiation (Jasmer, 1990).

Angiogenesis begins at approximately day 10 post-penetration resulting in neovascularization around the nurse cell (Baruch, et al 1991). The angiogenic factor vascular endothelial growth factor (VEGF) mRNA and the VEGF peptide itself have both been found within the myocyte following penetration by the larvae (Capo, et al 1998). By day 21, larval growth has essentially stopped for all species of *Trichinella* and the worm (now designated the L1 infectious stage) may remain within the host cell for months or years depending on several factors including the species of host and the host life span. Continuation of the *Trichinella* life cycle requires consumption of the L1 infectious stage by the appropriate host. The mechanisms responsible for many of the phenomena associated with the host-parasite relationship remain unclear. Very different sets of cellular phenomena are associated with *T. pseudospiralis* infections including the absence of both the nurse cell and angiogenic response.

THE HOST-PARASITE
RELATIONSHIP IN
TRICHINELLA INFECTIONS

Most investigators agree that the earliest immune reactions to the presence and growth of the infective larvae are acute inflammatory responses in the intestine. These responses include the typical inflammatory reactions such as infiltration by polymorphonuclear cells (PMNs), macrophages and a significantly higher number of mast cells and eosinophils (mastocytosis and eosinophilia respectively) (Wakelin, et al 1982). With continuing inflammation, increased numbers of CD4⁺ and CD8⁺ T lymphocytes are found in the blood (Ivanoska, et al 1990). It is suspected that, as granulocytes accumulate in the intestine, vasoactive amines such as histamine and 5-hydroxytryptamine (5-HT) are produced and secreted into the tissues. These substances may have a role in expulsion of the adult *Trichinella* after larvae are deposited and in the subsequent Th2 lymphocyte infiltration (Hermanek, et al 1994).

The responses of B-lymphocytes during the intestinal phase include secretion of antibodies primarily of the subclasses IgG2a in *T. spiralis* infections or IgG1 in *T. pseudospiralis* infections. (Ruitenbergh, et al 1980). Complement may also play a role and it has been suggested that complement is activated primarily via the alternative pathway during *Trichinella* infections (Hong, et al 1992).

Newborn larvae are antigenically distinct from the adults with the exception of a single shared antigen of approximately 57kDa (Wakelin, et al 1994; Phillip, et al 1980). There is still some controversy as to the immunological distinctions between

Trichinella species, but most investigators agree that no consistent antigenic differences are seen in the adult worms of different species. (Hegazy and Boulos, 1993; Ko, et al 1994; Wakelin, et al 1994;).

In many instances of parasitemia, the infection and subsequent tissue injury result in the activation of the inflammatory response. The acute inflammatory response is both localized and systemic. Frequently, serum clotting factors are produced that cause activation of the coagulation, kinin-forming and fibrinolytic pathways (Kuby, 1994). The release of various molecules including cytokines, chemokines and/or extracellular matrix (ECM) components at the inflammation site often result in the margination of immune cells to the vascular endothelium. This is followed by the rapid extravasation of these leukocytes into the tissues at the site of injury. The infiltrating cells may include polymorphonuclear (PMN) cells, macrophages, basophils, eosinophils and lymphocytes. The recruitment of leukocytes and other cells such as fibroblasts to an area of inflammation is mediated by a number of molecules (both membrane-bound and soluble). These molecules can be both detected and quantified using immunochemistry as will be detailed in the following chapters.

In addition, the acute phase response to an infection commonly includes fever, leukocytosis, increased secretion of adrenocorticotrophic hormone (ACTH) and cortisol. There is also increased production of serum amyloid A (SAA) and c-reactive protein (CRP) by hepatocytes in addition to nitric oxide (NO) production by a number of cell types including endothelial cells, lymphocytes and macrophages (Lincoln, 1997). The roles of cytokines, chemokines, ACTH, cortisol, SAA, NO, prostaglandins and CRP

are uncertain in *Trichinella* infections. It is generally agreed, however, that there are complex and important interactions between the immune and endocrine systems and there may be a particularly vital role for the hypothalamic-pituitary-adrenal (HPA) axis in infected mammals.

This acute inflammatory reaction is the result, in part, of the activation of tissue macrophages and the release of TNF, IL-1 (α and β), IL-6, IL-8, interferon- γ (IFN- γ) and other cytokines. These cytokines and chemokines are believed to be responsible for many of the systemic and localized effects observed during acute inflammation.

Regulation of ACTH is controlled by the trophic factor corticotropin-releasing factor (CRF) and by negative feedback from glucocorticoids. Corticotropin-releasing factor released by the hypothalamus is initiated by stress, disease and hypoglycemia. In addition to the negative feedback effect of glucocorticoids like cortisol, there exists a short negative feedback loop between the hypothalamus and the pituitary where increasing concentrations of ACTH suppress CRF secretion (Kannon, 1986). The hypothalamus, in turn, is also influenced by a number of inflammatory cytokines including interleukin-1 (IL-1) and tumor necrosis factor (TNF), and by the presence of exogenous endotoxins such as lipopolysaccharide (LPS) (Eskay, et al 1990). It is thought that the secretion of CRF, ACTH and cortisol may act to moderate the immune and/or inflammatory response and so help return the host organism to homeostasis (Griffin and Ojeda, 1992).

RESEARCH FOCUS

There are physiological and biochemical differences in mice infected with *Trichinella spiralis* when compared to *T. pseudospiralis* (Despommier, et al 1975). For example, there are significant differences in the host macrophage response to *T. spiralis* compared to *T. pseudospiralis* in formalin-fixed, paraffin-embedded tissues using monoclonal antibodies against murine myeloperoxidase. Experimental results have demonstrated that there is a very large cellular tissue response to the encysted *T. spiralis* and a substantially smaller response to *T. pseudospiralis* (Pruhiere, 1994). To explain these phenomena, some workers have speculated that *T. pseudospiralis* may mediate (or in some manner diminish) the host response (Gerencer, et al 1992; Boulos, et al 1993). The mechanisms for this immunosuppression remain unknown.

It has been also been shown that there is a significant increase in prostaglandin E2 (PGE2) in *T. spiralis* infected mice and a significant decrease in PGE2 in *T. pseudospiralis* infected mice when compared to uninfected controls (Mehdizadehkashi, 1995). Eicosinoids like PGE2 and the leukotrienes are lipid-derived molecules that are potent mediators of the inflammatory response in mammals. Increased eicosinoid levels may be associated with increases in serum cortisol and ACTH in mice (Thorburn, 1991; Thorburn, et al 1991) and in humans (Jorgensen and Sany, 1994; Panayi, 1993a; Panayi, 1993b).

T. pseudospiralis does not have an intracellular collagen nurse cell and does not stimulate angiogenesis in mice (Despommier, et al 1978). Furthermore, *T. pseudospiralis* does not alter the structure of the host myofiber in the same manner as

does *T. spiralis* and the other encapsulating species of *Trichinella* such as *T. nativa*, *T. britovi*, *T. mullerii* and *T. nelsoni* (see above). The mechanisms underlying these differences are not entirely known.

Several investigators have observed that, in some hosts, the animal responds to chronic inflammation or to a persistent antigenic stimulation by a granulomatous reaction (Penrith and VanVollenhoven 1994; Tang and Eaton, 1995; Geboes, 1994; Stadecker and Flores-Villanueva, 1994; Ng and Loo, 1993). For example, there is a T cell mediated granulomatous inflammation to the presence of the antigens produced and secreted by the eggs of the parasite *Schistosoma mansoni* in the liver (Boros, 1994). I have found no evidence to support a granulomatous reaction in *Trichinella*-infected animals that might lead to nurse cell formation. One group of workers however, reported that *T. pseudospiralis*-infected mice treated with the immunostimulant, Levamisole, showed what appeared to be the beginning of a membranous structure around the muscle stage of the larvae (Hegazy and Boulos, 1993).

The Levamisole data suggest that the nurse cell may be a host-derived structure and that the formation of the nurse cell may be suppressed in *T. pseudospiralis*-infected mice. Elevated cortisol levels such as those found in *T. pseudospiralis*-infected animals can suppress collagen synthesis through the down-regulation of procollagen I transcription. (Guller, et al 1995; Delaney, et al 1995). It is also possible that *T. pseudospiralis* does not secrete a collagen cyst (if the cyst is worm-derived) or does

not stimulate nurse cell collagen formation within the infected myofiber (if the cyst is host-derived).

The reported physiological and/or pathological differences between the two species of *Trichinella* may be based, at least in part, on differences in the host inflammatory and immune reaction to the presence of the parasite. These differences may include variations in the host levels of cytokines, chemokines or dissimilarities in the secreted products of the HPA axis. These contrasting observations may be the result of physiological differences between the two species of worm, or the differences may indicate an active moderation of the host response by the worm. Because cortisol suppresses the immune and inflammatory responses in mammals (Delaney, et al 1995; Haapasari, et al 1995), it is possible that cortisol may have a role in suppressing the host immune and inflammatory reactions to the presence of *Trichinella* in mice.

My hypothesis is that the differences in the responses of mice to the two species of *Trichinella* are due to differences in the pathogenesis of the infection. I further hypothesize that *T. pseudospiralis* may be less damaging to the host than is *T. spiralis*. The unique pathology and extensive inflammatory and immune reactions in tissue infected with *T. spiralis*, but not seen in *T. pseudospiralis* infections, may be related directly to serum cortisol levels and/or to tissue damage and the subsequent wound-healing cascade.

The profound differences seen in the pathogenesis of the infection may be evolutionary divergence in the host-parasite relationship as it has developed over time. This postulated divergence suggests that *T. pseudospiralis* may be the most genetically

distinct of the various *Trichinella* species. These differences in the relationship between *T. spiralis* and *T. pseudospiralis* and the host are measurable phenomenon. A number of ways to establish how the host responds to *Trichinella* infection will be outlined below. Therefore, in order to ascertain the causes of these differences between *T. spiralis*, *T. pseudospiralis* and the host, I attempted to answer the following questions:

- Is cortisol a factor in the differences of the host response to *T. spiralis* or *T. pseudospiralis* in BALB/c mice?
- Are there differences in the leukocyte response in the peripheral blood of *Trichinella*-infected BALB/c mice when the two species of worm are compared?
- Are there differences in the up-regulation or down-regulation of cell surface molecules on splenic leukocytes in *Trichinella*-infected BALB/c mice?
- Is there a difference in the degree of damage to the host myofibers, as measured by serum creatine phosphokinase, when infections by the two species of *Trichinella* are compared?
- Are there differences in angiogenesis and collagen deposition in *Trichinella*-infected BALB/c mice and are these differences related to cortisol?
- Is nitric oxide a component in parasite killing in *Trichinella* infections and are there differences in the levels of nitric oxide in *T. spiralis*-infected animals when compared to animals infected by *T. pseudospiralis*?

In the following chapters, I present results on creatine phosphokinase (CPK) assays of the peripheral blood of *Trichinella*-infected mice and of uninfected controls. Creatine phosphokinase can be used to directly ascertain the degree of muscle damage

in mammalian myocytes. In addition, the peripheral blood level of the inflammatory and antimicrobial molecule nitric oxide (NO) was assayed in both *Trichinella*-infected mice and in uninfected controls.

To determine the role of cortisol in nurse cell formation and collagen synthesis, formalin-fixed, paraffin-embedded muscle tissue stained with Masson's Trichrome was examined using *Trichinella* infected mice, uninfected controls and in *Trichinella*-infected mice that had undergone adrenalectomy (ADX). Counts of surviving larvae were performed in infected animals and compared to infected mice that had undergone adrenalectomy before infection. I also performed larval counts in adrenalectomized mice that had replacement cortisol (dexamethasone) therapy.

A method suitable to demonstrate and quantitate the angiogenic response in infected mice was utilized in live whole-mount tissue employing a florescently-labeled lectin against a specific molecule found only on endothelial cells.

Finally, I present results on the host immune and inflammatory response to the presence of the parasite using flow cytometric analysis of cell surface clusters of determinates (CD) molecules. Results include the response of B cells, T cells, monocytes/macrophages and granulocytes within the spleen employing flow cytometry and using monoclonal antibodies to the appropriate CD antigens in uninfected control mice and in mice infected with one of the two species of *Trichinella*. I also present data on leukocyte responses in peripheral blood that were assayed manually using standard staining techniques.

CHAPTER TWO

NITRIC OXIDE AND CREATINE PHOSPHOKINASE IN MICE INFECTED WITH *TRICHINELLA SPIRALIS* OR *TRICHINELLA PSEUDOSPIRALIS*

The role of nitric oxide (NO) in the inflammatory response to infectious agents has been well documented (Klemm, et al 1995; Jacobs, et al 1995; Liew, et al 1997; Bogden, et al 1997; Mossalayi, et al 1999). Nitric oxide also has been demonstrated to have a role in the intestinal phase of *Trichinella* infections (Hogaboam, et al 1996).

Nitric oxide is a small, 30Da, multifunctional molecule with a half-life of approximately 30 seconds in biological systems. Nitric oxide (NO) is very toxic and is known to act as a mediator of cytotoxicity in host defense (Lincoln, 1997). As an uncharged gaseous molecule, it diffuses freely within cells and across cell membranes. Nitric oxide is produced by a number of different cell types including endothelial cells, neurons, certain lymphocytes and by activated macrophages (Lincoln, 1997).

Nitric oxide is synthesized by one of three isoforms of the enzyme nitric oxide synthase (NOS) (Ghosh, et al 1999; Pollack, et al 1991; Bredt, et al 1990). The Type I, Type II and Type III isoforms are distinguished from each other based on whether they are constitutively (cNOS) expressed or are induced (iNOS). Types I and III NOS are Ca^{2+} dependent and are expressed constitutively. Type II NOS is inducible and is Ca^{2+} independent. Cell types synthesizing Type II iNOS include macrophages, phagocytic mesangial cells, astrocytes, microglia, splenocytes, neutrophils and select subsets of T

lymphocytes (Barnes and Liew, 1995). Natural killer cells and T helper 1 (Th1) cells both synthesize Type II iNOS but Th2 cells do not synthesize NO (Lincoln, 1997). In addition, a large number of non-immune cells types including hepatocytes, fibroblasts and endothelial cells can synthesize Type II iNOS (Kroncke, et al 1995).

Control of Type II iNOS is regulated by a number of cytokines and other agents (Murphy, et al 1993; Wynn, 1997). Activating or enhancing agents include TNF, IFN- γ , IL-1, IL-2, certain heat shock proteins and low concentrations of NO (Kroncke, et al 1995). Contrarily, IL-4, IL-6, IL-8, high concentrations of NO or prostaglandin E2 (PGE2) may inhibit iNOS.

The cytotoxic effects of NO on target cells consist of damage to ion channels, transport proteins, mitochondrial membrane potential, Fe-sulfur and Zn-finger clusters in proteins, inhibition of mitochondrial enzymes and/or direct damage to DNA (Kroncke, et al 1995; Nussler and Billiar, 1993). Thus, NO may inactivate enzymes that are essential for energy metabolism and growth of invading parasites (James, 1995). Excess NO production by the iNOS can damage the host by killing lymphocytes, chondrocytes, pancreatic islets, vascular smooth muscle cells and hepatocytes (Nussler and Billiar, 1993). Although the toxic effect of NO on a variety of pathogens has been shown, no work has been reported on the possible role of NO in inflammation caused by the larval stage of the parasitic nematode *Trichinella*.

The role of NO in the inflammatory response to infectious agents has been well documented (Klemm, et al 1995; Jacobs, et al 1995; Liew, et al 1997; Bogden, et al 1997) In response to inflammatory stimuli, NO is produced in micro molar (μ M)

amounts by macrophages and neutrophils, Th1 lymphocytes and by non-immune cells including endothelial cells. Nitric oxide, whether constitutively produced or induced, reacts rapidly with other molecules in biological fluids as detailed below:

As can be seen, nitric oxide is present only transiently in serum and the final products of NO synthesis *in vivo* are nitrite and nitrate. Since the relative proportions of nitrite and nitrate are unpredictable, the best gauge of NO production is the sum of both nitrite and nitrate levels in serum (Ellis, et al 1998).

Stimulated macrophages appear to be a major effector cells in *Trichinella* infections and may be of particular importance in *T. spiralis* infected animals. Hirji, et al (1998) found that NO synthesis in rats could be initiated by stimulation of the macrophage CD8 marker. The CD8 marker of macrophages differs from the T cell CD8 in its ligand-binding region. This finding indicates a role for both stimulated macrophages and NO production in the killing of parasites *in vivo*. The findings of Hirji, et al was confirmed by Taylor-Robinson et al (1996) who found that NO played a significant role in the parasitemia of mice infected with *Plasmodium chabaudi*.

Thomas, et al (1997) and Maeda and Akaike (1998) found that INF- γ stimulated macrophages produced NO that severely damaged the microfilaria of *Brugia malayi in vitro*. Knockout or genetically altered mice deficient in iNOS (iNOS^{-/-})

displayed defective antimicrobial activity further demonstrating the importance of NO in parasite killing (Scharton-Kerston, et al 1997).

Certain parasites may be able to suppress NO synthesis (Ruhl and Collins, 1997). *T. pseudospiralis* has been reported to suppress the immune response in mammals (Alford, et al 1998). The results reported by Mehdizadehkashi (1995) showed a decreased level of prostaglandin E2 (PGE2) in *T. pseudospiralis*-infected mice and increased levels of PGE2 in *T. spiralis*-infected mice. High levels of PGE2 have been shown to be associated with low levels of NO and low levels of PGE2 have been associated with high levels of NO.

Creatine phosphokinase (CPK) is well accepted as an indicator molecule for ascertaining the level of muscle damage in mammals and in other species (Martin, et al 1997; Mizutani, et al 1996; Chen, et al 1997; Fukuuchi, et al 1996). In damaged muscle, the amount of CPK released may increase dramatically (Giltay, et al 1999; Pagel and Partridge, 1999). Creatine phosphokinase catalyzes the phosphorylation of adenosine-5'-diphosphate (ADP) to adenosine-5'-triphosphate (ATP) and free creatine (Qin, et al 1999; Felice and North, 1998). This reaction is monitored by following the formation of the end product, inorganic phosphate (Pi) (Ellis, et al 1998). Inorganic phosphate may then be quantitated. The level of inorganic phosphate released is equivalent to CPK activity and, therefore, can be correlated to the level of larval damage to the cell.

The aim of this research is twofold. First, to discover if there are differences in the amount of NO produced between *T. spiralis* and *T. pseudospiralis*. Secondly, to measure the levels of CPK activity in mice infected with *T. spiralis* or *T. pseudospiralis* determine which of the two species is more damaging to host striated muscle. To answer these questions, I measured the levels of CPK and NO in the peripheral blood of mice after infection with *T. spiralis* or *T. pseudospiralis* and in uninfected control mice.

Because lipopolysaccharide (LPS) is a known stimulant of the innate inflammatory response (Wolf, et al 1996; Drenth, et al 1996), it was used as my positive control.

MATERIALS AND METHODS

SERUM NITRIC OXIDE ASSAY

For each NO and CPK experiment, 15 BALB/cAnNTacfBR (BALB/c) inbred mice were used. The BALB/c mice were supplied by Taconic Laboratories, Inc.. All mice were female and approximately 3 months old. Five mice received *T. spiralis* larvae, five mice received *T. pseudospiralis* larvae and 5 mice were used as uninfected controls. The L1 infective stage used for infection were in the muscle of a maintenance infection in BALB/c mice. Larvae were counted manually in infected diaphragm tissue from the maintenance infections and fed to the recipient mice at 250 larvae per mouse. The post-infection blood samples were collected 14 days after infection. All mice were kept according to State of Oregon standards for the care of research animals. Food and water were supplied ad libitum. The mice were kept on a 12-hour day/night regimen and the bedding was pine shavings.

Blood samples (500 μ l/mouse) were collected from infected and control mice using the tail clip technique. The blood from the caudal vessels was drawn into non-heparinized collection tubes. Blood samples were allowed to clot for 30 minutes at room temperature, ringed, and centrifuged at 500xG for 10 minutes at room temperature to separate the serum from the cells.

Serum (150 μ l) was carefully withdrawn and transferred to a Centriprep (Amicon) 10,000mw cutoff concentrator for ultra filtration. Samples were centrifuged for 45 minutes at 4°C at 3000xG. The supernatant was transferred to a microfuge tube and

placed at 4°C. Each sample was assayed in triplicate using a colorimetric nitric oxide assay kit (Cayman Chemical). Following the directions of the manufacturer, a 40µl serum sample was diluted with 40µl of assay buffer, 10µl of enzyme cofactor and 10µl of nitrate reductase. Both standards and unknown samples were added to a 96 well micro titer plate and incubated for 3 hours at room temperature with shaking at 30 rpm on an Eberbach reciprocal shaker. At the end of 3 hours, Griess reagents numbers 1 and 2 were added to all samples and control wells. The color was allowed to develop for 10 minutes at room temperature. Plate contents were assayed at 640nm using an automated micro plate reader (Biotek). The triplicate readings from each sample were averaged. A standard curve was developed using nitrite/nitrate standards (Cayman Chemical) and was run with each set of samples. The standard curve was used to ascertain NO levels as µM nitric oxide/mouse.

CREATINE PHOSPHOKINASE ASSAY

Mice were kept and infected in the same manner and in the same numbers as the NO experiment detailed above. Blood samples (500µl/mouse) were collected from infected and control mice using the tail clip technique. The blood from the caudal vessels was drawn into heparinized collection tubes (Becton-Dickinson). Blood samples were centrifuged at 500xG for 10 minutes at room temperature. The serum was withdrawn into 15ml tubes and kept at 4°C. The CPK assay was performed using a CPK assay kit (Sigma Diagnostics) and following the manufacturer's directions. Briefly, serum samples were diluted 1:3 with Tris-HCl buffer (pH 7). One milliliter of creatine solution and 1ml of H₂O was added to each tube. Tubes were held in a 37°C

water bath for 5 minutes. To start the reaction, 100µl of ATP-glutathione solution was added to each tube. The tubes were held at 37°C for 30 minutes. The reaction was terminated by the addition of 1.6ml of cold (4°C) trichloroacetic acid (TCA). All tubes were centrifuged at 1500xG for 5 minutes and the supernatant transferred to a cuvet containing 4ml of H₂O, 1ml of acid molybdate and 250µl of Fiske and SubbaRow solution (1-amino-2-naphthol-4-sulfonic acid containing 0.8% sodium sulfite/sodium bisulfite). The tubes were held for 5 minutes at room temperature to allow the hydrolysis of phosphocreatine. A standard curve was developed using a known inorganic phosphate standard. All tubes were assayed for inorganic phosphate at 630nm in triplicate using a standard spectrophotometer and the results from each triplicate sample were averaged. All nitric oxide and creatine phosphokinase experiments were repeated four times in an identical manner for a total of 60 mice used for each assay.

Following the blood collections at day 14 post-infection and at an average of 31 days post-infection, all mice used in the CPK and NO experiments were sacrificed by cervical dislocation and the L1 infectious stage larvae present in whole mounts of the diaphragm were counted manually using a dissecting microscope at 30x.

For each LPS-mediated CPK or NO assay, four mice were given LPS at dosages of 6.25, 12.5, 25.0 or 50.0mg/kg/mouse diluted in 250µl of normal saline in a single injection intraperitoneally (ip). A fifth mouse was given 250µl of saline ip. Before any treatments, an initial blood sample was drawn from each mouse one week prior to LPS injection to serve as a baseline for NO and CPK. Blood was collected from the five

mice at 6 hours post-injection. The LPS-mediated CPK or NO assays were prepared as detailed above. The LPS experiment was repeated once for a total of 10 mice.

All results for the nitric oxide, creatine phosphokinase and LPS assays were statistically analyzed using the student's t-test.

RESULTS

NITRIC OXIDE

Levels of NO in the peripheral blood serum of infected animals were significantly different from controls ($P < 0.05$). As shown in Figure 2.1, it can be seen that the levels of serum NO in control mice were consistently below 1 μM NO/sample (average 0.9 μM NO/sample). In infected mice, those infected with *T. pseudospiralis* showed an average of 10.1 μM NO/sample while mice infected with *T. spiralis* had NO levels that averaged 6.3 μM NO/sample. The NO baseline results did not differ significantly from controls (data not shown). If the control animal level of NO is subtracted, *T. pseudospiralis* had approximately 2X the amount of NO/sample than did *T. spiralis*.

CREATINE PHOSPHOKINASE

As shown in Figure 2.2, there were significant ($P < 0.05$) differences in the in the amount of CPK present in the serum of *T. spiralis* infected mice when compared with serum from mice infected with *T. pseudospiralis*. The level of CPK in uninfected control mice was substantially ($P < 0.05$) lower than in infected animals. In *T. spiralis* infected mice, CPK levels averaged 20.1 μg Pi/sample. This contrasts with *T. pseudospiralis* infected mice that averaged 11.7 μg Pi/sample. Uninfected control mice and the LPS treated mice averaged 5.8 μg Pi/sample. The CPK baseline results did not differ significantly from the controls (data not shown).

LIPOPOLYSACCHARIDE

As shown in Figure 2.3, dosage levels of LPS at 6.25, 12.5, 25.0 or 50.0mg/kg/mouse resulted in the production of an average of 7.7, 12.9, 17.3 and 21.8 μ M/NO/blood sample. Mice receiving LPS showed an increase in blood NO in a non-linear but still dose-related manner. Thus, it appears that the level of nitric oxide is approximately proportional to the degree of inflammation as caused by LPS.

Mice injected with LPS showed levels of CPK that averaged 5.7 μ g Pi/sample that is not significantly different from the control animals (data not shown) suggesting that LPS does not stimulate CPK production under the experimental conditions detailed here.

Figure 2.1: Nitric Oxide. Nitric oxide data represents the average of four experiments.(n=60. *T.spiralis*: mean=6.26;SD=0.336. *T.pseudospiralis*: mean=10.3; SD=0.509. Control: mean=0.84, SD=0.089).

Figure 2.2: Creatine Phosphokinase in *Trichinella*-Infected Mice as determined by the measurement of inorganic phosphate. n=60. *T.spiralis*: mean=6.35, SD=0.129. *T.pseudospiralis*: mean=10.15, SD=0.129. Control: mean=0.85, SD=0.057.

Figure 2.3: Nitric Oxide after LPS Injection. LPS injected IP. Blood Drawn at 6 hours post-injection. n=8. 6.25mg: mean=7.96, SD=0.194. 12.5mg: mean=12.06, SD=0.658. 25.0mg: mean=17.44, SD=0.456. 50mg: mean=21.14, SD=0.965.

DISCUSSION

NITRIC OXIDE

The data shown in Figure 2.1 indicate that mice infected with *T. pseudospiralis* have a significantly ($P < 0.05$) larger NO response than do mice infected with *T. spiralis*. Levels of NO averaged more than 11 times higher in *T. pseudospiralis* than in controls and an average of 2 times higher than in *T. spiralis* infected mice. Because PGE2 is known to have a down-regulating effect on NO production (Hrabak, et al 1998), one might postulate that *T. spiralis* infected mice would demonstrate a lower serum NO level than would mice infected with *T. pseudospiralis* due to the higher levels of PGE2 in mice infected with *T. spiralis* (Mehdizadehkashi, 1995). We have demonstrated that this is the case. In addition, there are substantial differences in NO production between infected animals and uninfected controls.

Nitric oxide can be used as an indicator of the degree of inflammation and is also an indicator of NO mediated cytotoxicity (Liew, et al 1997; Bogden, et al 1997; Hoagaboam, et al 1996). Our data supports these results and indicates a role for NO as a host defense mechanism against *Trichinella*. Our data also agrees with reports of other workers that NO is important in host defense against other helminthic parasites including cestodes (Kanazawa, et al 1993), trematodes (Oswald, et al 1994), and nematodes (Rajan, et al 1996; Marzio, et al 1990).

Macrophages are prominent responder cells in the muscle phase of *Trichinella* infections. Macrophages secrete NO as an important mediator of parasite killing *in vivo*. When other investigators used chloroquine to stop the macrophage production of

NO by iNOS, parasite killing was severely compromised in animals infected with the protozoan parasite *Trypanosoma cruzi* (Hrabak, et al 1998). Hayashi, et al (1996) used aminoguanidine, a structural analog of L-arginine, to block NO synthesis in mice infected with *Toxoplasma*. Blocking NO production in *Toxoplasma*-infected mice resulted in substantially increased parasite loads and lowered survival rates in infected animals. Our experiments with aminoguanidine in *Trichinella* infected mice (data not shown) resulted in increased parasitemia in both the intestinal and muscle phases. This finding further emphasizes the important role for NO mediated parasite killing in animals infected with *Trichinella*. Our findings with respect to the role of NO in parasite killing are supported by the work of Liew, et al (1999) in *Leishmania* infections; and by Burgner, et al (1999) and MacMicking, et al (1997) who reported NO protection against various viruses, bacteria, fungi, protozoa, helminths and tumor cells.

Trichinella spiralis infected animals have demonstrated levels of PGE2 that are considerably higher than those found in *T. pseudospiralis*. Hayashi, et al (1996) found that high NO production coupled with high PGE2 production acted to inhibit lymphocyte proliferation. We did not find this in *Trichinella* infections in BALB/c mice under the experimental conditions detailed here although, in *T. spiralis* infected mice, peripheral blood lymphocytes are present in significantly fewer numbers than those present in *T. pseudospiralis* infected mice (data not shown). This may be due to high PGE2 and low NO levels in *T. spiralis* infected mice when compared with *T. pseudospiralis* infected mice. In addition, Hayashi, et al (1996) found that inhibition of

both NO and PGE2 with indomethacin restored the lymphocyte proliferative response and cytokine production, thus preventing cell death. They suggested that these contrasting effects of NO might contribute to the chronic state of some parasitic infections. McCann, et al (1997) found that NO directly influenced the production of hypothalamic-induced or derived peptides including the prostaglandins and the leukotrienes.

McCann, et al (1997) reported that NO stimulates the release of corticotrophin-releasing factor (CRF) and adrenocorticotrophic hormone (ACTH) which, in turn, stimulates the release of cortisol. McCann further suggests that NO activates cyclo-and lipoxygenases thereby liberating PGE2 and leukotrienes respectively. Cortisol, in turn, down-regulates PGE2 production (Patel, et al 1999; Otsuki and Oku, 1995). This HPA axis feedback mechanism helps to explain our findings of high NO levels in *T. pseudospiralis* coupled with high cortisol levels and low PGE2 levels. It further helps to explain the lower NO and cortisol levels in *T. spiralis* coupled with high PGE2 levels.

The stimulated macrophage appears to be a major effector cell in *Trichinella* infections and may be of particular importance in *T. spiralis* infected animals. Hirji, et al (1998) found that NO synthesis in rats could be initiated by stimulation of the macrophage CD8 marker. The CD8 marker of macrophages differs from the T cell CD8 in its ligand-binding region. This finding indicates a role for both stimulated macrophages and NO production in the killing of parasites *in vivo*. This is further confirmed by the findings of Taylor-Robinson et al (1996) who found that macrophage-

derived NO plays a significant role in the parasitemia of mice infected with *Plasmodium chabaudi*. In addition, Thomas, et al (1997) and Maeda and Akaike (1998) found that INF- γ stimulated macrophages produced NO (specifically peroxynitrite) that was severely damaging to the microfilaria of *Brugia malayi* in vitro. Knockout mice deficient in iNOS (iNOS^{-/-}) displayed defective antimicrobial activity further demonstrating the importance of NO in parasite killing (Scharton-Kerston, et al 1997).

Despite the importance of macrophage derived NO in parasite infections, my results showed *T. spiralis* infected mice exhibit lower than predicted NO levels in serum. There are several possible explanations for the NO levels measured in *T. spiralis* infected mice. The most reasonable explanation was fostered by Milana, et al (1995) who demonstrated that higher than normal levels of PGE2 suppress the iNOS *in vivo*. This research also showed that low levels of PGE2 stimulate the iNOS. In addition, Kunz, et al (1996) showed that high levels of glucocorticoids like cortisol suppress iNOS through PGE2. Conversely, high levels of IL-1 generally correlate to an increase in NO production *in vivo* (Kroncke, et al 1995). This latter role for IL-1 does not appear to be a factor in *Trichinella*-infected BALB/c mice. The low levels of PGE2 and the high levels of IL-1 β in *T. pseudospiralis* infected mice and the high levels of PGE2 in *T. spiralis* infected mice (Mehdizadehkashi, 1995) may account for these observations. The seemingly paradoxical and conflicting roles of NO, PGE2 and IL-1 in inflammation and their relationship to the HPA axis has been documented by other researchers (Zidek and Masek, 1998). Despite this, NO appears to play a significant role in eliminating both species of *Trichinella* from BALB/c mice under the

experimental conditions detailed here. Nitric oxide is, therefore, an important effector mechanism in the non-specific immune response against *Trichinella* in mice.

CREATINE PHOSPHOKINASE

The level of muscle damage as measured by CPK was significantly higher in *T. spiralis* infected mice when compared to mice infected with *T. pseudospiralis* ($P < 0.05$) as illustrated in Figure 2.2. I had hypothesized that *T. spiralis*, because of the induction of changes to cellular architecture (Despommier, 1975) as described in the introduction, would cause more damage to muscle fibers than would *T. pseudospiralis*. My findings have confirmed that this is the case. There was an approximate increase of 170% in serum CPK (20.1 μ g versus 11.7 μ g) in *T. spiralis* infected mice when compared to *T. pseudospiralis* infected mice and an increase of approximately 345% (20.1 μ g versus 5.8 μ g) over controls. These data are supported by the findings of Gregor, et al (1999) and Yoshimura, et al (1999) in experiments with rats with artificially induced muscle damage. Measurement of CPK isoenzymes (CK-MB) has been shown to be an accurate diagnostic tool for ascertaining the level of ischemic damage in cardiac muscle (De Winter, et al 1998) and in amyotrophic lateral sclerosis (ALS) damaged skeletal muscle in human beings (Felice and North, 1998).

Muscle damage, as it occurs in all *Trichinella* infections, may stimulate an inflammatory response that is both related and proportional to the severity of the damage (Nathan, et al 1997; Panteghini, et al 1999). *Trichinella spiralis* infected animals have more inflammatory cells around the L1 larvae than is seen in *T. pseudospiralis* infected animals. The presence of these cells could be related to the severity of the damage and may contribute to cytokine variations. The inflammatory cells in *T. spiralis* infected mice may secrete products that cause muscle damage, inhibit

iNOS or modify the HPA axis. In addition, during the macrophage response to the presence of the parasite or parasite antigens, healthy cells may be damaged further exacerbating the inflammation and changing the CPK profile. Other workers have also noted a complex relationship between systemic inflammatory syndrome, muscle injury, cytokine release and the HPA axis in mammals (Martin, et al 1997; Bolton, 1996). Stewart, et al (1999) also noted a complex interplay between Th1 and Th2 subsets and cytokine release during *Trichinella spiralis* infections.

Trichinella spiralis and, to a lesser extent, *T. pseudospiralis* contribute to the ischemic (hypoxic) condition of *Trichinella*-infected myocytes. And, as stated above, the activity of CPK has been shown to be a reliable indicator of muscle damage (including ischemic damage) *in vivo* in human beings (Kawaguchi, et al 1997; Panteghini, et al 1999; Kemp, et al 1998; Cuono, et al 1998) and in rodents (Knipper, et al 1996). Assays for CPK have also been used as a model system to measure ischemic injury in the skeletal muscle of BALB/c mice (Carter, et al 1998). Mizutani, et al (1996) found a correlation between virally induced endocarditis in BALB/c mice and increased CPK activity. Other researchers have found a strong correlation between *Nematodirus* and *Strongyloides* helminthic infections in mammals and CPK levels (Haroun, et al 1996). *T. pseudospiralis* has been shown to cause increases in CPK in human beings (Jongwutiwes, et al 1998). Cortisol, however, has no apparent effect on CPK production *in vivo* as suggested by Goddard, et al (1997) allowing us to rule out cortisol as a mediator molecule in CPK production in *Trichinella* infected mice. It is

known, however, that CPK production can be linked to hypoxia and the uncoupling of the mitochondrial cycles (Kemp, et al 1998; Cuono, et al 1998; O’Gorman, et al 1996).

As was stated in the introduction, *T. spiralis* induces changes in the architecture of the infected myocyte including functional changes in the host mitochondria. This is not the case with *T. pseudospiralis*-infected animals. Therefore, I conclude that the presence of CPK in *T. spiralis*-infected BALB/c mice is directly proportional to the damage to the myocyte (including hypoxic damage) and that the myocyte damage caused by *T. pseudospiralis* is significantly less. Thus, this damage and the subsequent wound-healing cascade are the primary etiological events in the pathological and physiological sequelae seen in *T. spiralis*-infected mice but not in *T. pseudospiralis* infections.

LIPOPOLYSACCHARIDE

As seen in Figure 2.3, LPS induced NO production after an exposure time of 6 hours. This indicates that NO levels provide a reliable method to ascertain the degree of inflammation *in vivo*. Our data further shows that CPK is not influenced by LPS under these experimental conditions.

The measuring of NO_3^- and NO_2^- , the stable metabolites of NO synthesis in serum has been found to be a dependable indicator of the severity of the inflammatory response to parasites such as *Eimeria tenella in vivo* (Allen, 1997). Others have also shown the importance of cytokines like $\text{INF-}\gamma$, transforming growth factor- β (TGF- β) and tumor necrosis factor (TNF) in up-regulating NO synthesis during parasitic infections (Diefenbach, et al 1998; Vieira, et al 1996).

As hypothesized, LPS had a profound effect on NO production under the experimental conditions detailed here. As an exogenous priming agent, LPS stimulates an inflammatory response *in vivo*. This response includes the activation of the alternative complement cascade. The complement cascade in *Trichinella*-infected mice is activated primarily via the alternative pathway. Lipopolysaccharide reacts with LPS binding proteins produced by animals and human beings and this complex reacts with CD14 on monocytes and macrophages resulting in the release of IL-1, IL-6, TNF, NO and platelet activating factor (PAF). These cytokines and chemokines stimulate the production of prostaglandins and leukotrienes (Atlas, 1997). As discussed previously, NO production is stimulated by IL-1, TNF and PAF.

Under the experimental circumstances discussed here, LPS had no appreciable effect on CPK production in mice. This finding suggests that the mechanism(s) that stimulate CPK production may not be directly associated with IL-1, TNF or PAF and differs from the mechanism(s) that generate NO *in vivo*. Lipopolysaccharide is, therefore, an efficient positive control for measuring NO but not CPK production *in vivo* in BALB/c mice.

CHAPTER THREE

ANGIOGENESIS, COLLAGEN DEPOSITION, LARVAL SURVIVAL, AND PERIPHERAL BLOOD COUNTS IN MICE INFECTED WITH *TRICHINELLA* AND IN *TRICHINELLA*- INFECTED ADRENALECTOMIZED MICE

INTRODUCTION

ANGIOGENESIS

The intracellular parasite *Trichinella spiralis*, having entered the striated muscle niche, causes a number of profound changes to the striated muscle fiber. *Trichinella pseudospiralis* does not elicit these major changes but does cause minor changes. Despommier (1975) reported that *T. spiralis* elicits an angiogenic response beginning on day 12 post-muscle penetration and this response continues until a rete of new capillaries is formed around the nurse cell. *Trichinella pseudospiralis* does not cause the formation of a nurse cell with a capillary rete.

Angiogenesis leading toward neovascularization is considered an important factor for maintaining normal tissues and is especially important in tissues where rapid cell division and growth are occurring (Banda, et al 1982). For example, angiogenesis is significant for tumor growth and in wound healing. It is known that several growth factors are involved in stimulating the formation of new capillaries. These factors

include fibroblast growth factor (FGF), angiogenin, transforming growth factors α and β (TGF- α , and TGF- β), tumor necrosis factor (TNF) and vascular endothelial growth factor (VEGF) (Leibovich et al, 1987). In *Trichinella spiralis*-infected mice, VEGF mRNA is present in the sarcoplasm as early as day 7 post-penetration and is still detectable after 8 months (Capo, et al 1998). The capillary rete is in close proximity to the collagen nurse cell allowing exchange of nutrients and metabolic waste products. The collagen of the nurse cell may act as scaffolding for the dividing and migrating endothelial cells that will form the capillary rete.

One of the stimulators of angiogenesis is the macrophage secreted TNF. The macrophage production of TNF can be stimulated by pathogens including viruses, bacteria, fungi, protozoa, and helminths. Macrophages and granulocytes (including neutrophils) are attracted to wounds (Folkman and Klagsburn, 1987) and may respond to the type of cellular injury seen in *Trichinella* infections as can be observed by identifying these cells in and around the nurse cell complex.

Responding macrophages and granulocytes may induce VEGF production through the release of inflammatory cytokines. In addition, hypoxia within the infected myofiber may induce the production of VEGF. Since *T. pseudospiralis* does not seem to induce angiogenesis and, because serum cortisol levels are up to four times higher in *T. pseudospiralis* infected animals, I investigated the possibility that cortisol has a role in suppressing the wound healing response in mice infected with *Trichinella*. To accomplish this I utilized a florescently-labeled lectin obtained from *Griffonia simplicifolia*-1 (GS-1) to analyze the extent of angiogenesis and neovascularization in

Trichinella infected mice. This thiamine-labeled lectin is a marker for microvasculature and capillaries in skeletal muscle (Hansen-Smith, et al 1992a, Hansen-Smith, et al 1992b; Hansen-Smith, et al 1998). Specifically, GS-1 has an affinity for N-acetylgalactosamine groups.

COLLAGEN SYNTHESIS

Collagen synthesis is an essential part of the wound healing mechanism in vertebrates. Collagen synthesis is also part of the survival strategy of *Trichinella spiralis* and other encapsulating species of *Trichinella* and forms the nurse cell that surrounds the larvae. Collagen synthesis and deposition is inhibited by glucocorticoids (Delaney, et al 1995a; Guller, et al 1995) while collagenase activity is up-regulated (Delaney, et al 1995b). The unencapsulated *T. pseudospiralis*, using an unidentified mechanism, stimulates the host to increase the production of the glucocorticoid cortisol more than four fold over the level found in uninfected control mice. Because of the effect of cortisol on collagen synthesis, I examined muscle tissue in unadrenalectomized mice infected with *Trichinella* and in *Trichinella*-infected adrenalectomized mice using Masson's Trichrome. This staining method is used for (among other functions) the detection of all types of collagen.

LARVAL SURVIVAL

Postulating that the glucocorticoid cortisol may have a role in larval survival for *Trichinella* and particularly the unencysted *T. pseudospiralis*, I compared the numbers of surviving larvae in mice with normal adrenal function with mice that had been adrenalectomized (ADX) and thus have no endogenous cortisol. I also examined the effects of replacing cortisol in infected ADX mice with the synthetic drug dexamethasone. Dexamethasone produces a cortisol-like effect in mammals.

MATERIAL AND METHODS

PERIPHERAL BLOOD COUNTS

Three month old female BALB/c mice were used for all experiments. For all experiments involving adrenalectomy, 18 unadrenalectomized control mice were infected orally with 250 LI stage *Trichinella* from previously infected mice. Nine mice received *T. spiralis* and nine received *T. pseudospiralis*. A second group of 18 mice underwent adrenalectomy. These adrenalectomized mice were allowed to recover for 30 days before infection. Following recovery, the second group of mice was infected in an identical manner as described above (i.e.: nine mice with *T. spiralis* and nine mice with *T. pseudospiralis*). A third group of 18 mice also underwent adrenalectomy and following a 30 day recovery period, were infected as above. Beginning 7 days post-infection, 9 mice in this third group of ADX mice received 16µg dexamethasone (dissolved in 250µl of 10% v/v ethanol with 90% v/v normal saline)/mouse/day. The other 9 mice received 250µl of 10% v/v ethanol with 90% v/v normal saline without dexamethasone intraperitoneally (IP) daily for the course of the infection. Because dexamethasone is approximately 30 times stronger than cortisol (Casini-Raggi, et al 1995; Guthrie, 1991; Lane, et al 1990), I adjusted the standard dose of dexamethasone from the normal 480µg.kg/day/mouse to 16/µg/kg/day/mouse.

All mice were bled by the tail clip method at 10, 15, 20, 30, 45, 60, 75, 90, and 105 days post-infection and blood was collected in non-heparinized microtubes (Becton-Dickinson). One drop of blood from each mouse at each time point was used

to prepare a blood smear. The remaining blood was allowed to clot for 30 minutes at room temperature. The tubes were ringed, and centrifuged at 500xG for 10 minutes at room temperature to separate the serum from the cells. Serum was carefully withdrawn and transferred to a 30,000mw cutoff Centriprep concentrator (Amicon) for ultrafiltration. The supernatant was transferred to a microfuge tube and placed at 4°C. Fifty microliters of each serum sample was sent on ice to IDEXX Corp. for cortisol assay.

The blood smears prepared at each of these nine time points were stained with modified Wright's stain (DifQuik) for standard leukocyte counts and morphological analysis following the protocols of Cochran, (1999, personal communication) and Hoppman, (1999, personal communication) to determine the numbers of responding peripheral blood leukocytes. Mice were killed following the blood collection at each of these time points and the diaphragm of each mouse was removed for subsequent analysis (see below).

Before either the angiogenesis or collagen assays were performed, *Trichinella* larvae present in each diaphragm of each mouse and at each time point, were counted manually. Following the larval count, the diaphragms were cut in half and washed in sterile normal saline or in sterile Hank's balanced salts solution (HBSS). One half the diaphragm was used for the angiogenesis assay and the other ½ was used for the collagen assay (see below).

ANGIOGENESIS ASSAY

A rhodamine-labeled lectin from *Griffonia simplicifolia*-1 (GS-1) (Sigma Chemical Co.) was diluted in normal saline containing 0.001M N-acetylgalactosamine (N-agn) to a final lectin concentration of 25µg/ml. The rhodamine-labeled lectin/N-agn mixture was added directly to ½ the diaphragm in a sterile 15ml polypropylene tube and held for 60 minutes at 37°C. The treated tissues were then whole-mounted on freshly prepared poly-l-lysine coated slides. Photomicrographs were obtained using a Nikon camera attached to a Zeiss microscope. Both transmitted and epiflorescent light was used. Epiflorescent photomicrographs were taken at 1000x and subsequently analyzed for lectin binding. Capillary density was determined for each specimen at each of the nine time points. Capillary density as discernible with the lectin was quantified visually as 0 for no lectin binding to ++++ for maximum lectin binding.

COLLAGEN ASSAY

To determine collagen deposition in muscle, the remaining ½ diaphragm (see above) was placed for 6 hours in Bouin's fixative (25ml 10% formalin, 5ml glacial acetic acid and 75 ml saturated aqueous picric acid). After fixation, tissues were washed thoroughly with running tap water for 24-36 hours. Tissues were dehydrated in graded ethanol to xylene and embedded with paraffin. Tissue blocks were sectioned into 7µm sections with a microtome. Sections were placed on freshly prepared poly-l-lysine coated slides. The slides were heated to melt the paraffin and affix the tissue to the slide. The slides were then rehydrated from xylene through graded ethanol to water.

Sections were stained with freshly prepared Masson's Trichrome (Sigma Chemical Co.) following the manufacturer's directions. The stained slides were then examined for the presence of collagen. Transmitted light photomicrographs were taken of each specimen at a magnification of 1000x. The results presented are the average of two separate experiments for a total of 108 mice.

RESULTS

ANGIOGENESIS

As shown in Table 3.1, the beginning of endothelial cell growth and the formation of new capillaries appear at approximately day 15 post-infection in *T. spiralis*-infected mice and capillary density gradually increases reaching its maximum at approximately day 75 post-infection. The rhodamine-labeled lectin was also found binding to capillaries at the periphery of the muscle fiber. As hypothesized, *T. pseudospiralis*-infected mice showed no evidence of neovascularization. Mouse diaphragm examined microscopically at each of the nine time points indicated above showed no obvious differences in neovascularization patterns between mice with normal adrenal function and those that underwent adrenalectomy regardless of the species of *Trichinella* infecting the host. As can be seen in Figures 3.1 through 3.7, the progress of neovascularization is evident in *T. spiralis*-infected animals. The vascular rete is complete at approximately 75 days post-infection. No non-vascular binding to muscle fibers or to other cells in the observed field was seen.

LARVAL COUNTS

Total larval counts in diaphragm are summarized in Figure 3.8. It can be seen that *T. spiralis*-infected mice averaged 910 (range = 895-920) larvae in unadrenalectomized mice, 637 (range = 620-655) larvae in ADX mice and 1115 larvae (range = 1110-1140) in ADX mice with dexamethasone. *T. pseudospiralis*-infected mice averaged 805 larvae (range 800-813) in unadrenalectomized mice, 295 larvae

(range = 280-301) in ADX mice and 1038 larvae (range 1025-1050) in ADX mice receiving dexamethasone. Saline- treated infected ADX mice had larval counts of 875 for *T. spiralis* (range 870-880) and 810 for *T. pseudospiralis* (range = 805-820) which is not significantly different from the unadrenalectomized mice. The unexpected finding was the reduction in larval survival in ADX mice. This was notably more evident in *T. pseudospiralis*-infected animals. Conversely, a small number of the dexamethasone treated mice died of trichinellosis during the experiments and, at necropsy, showed lethal parasite loads of over 1500 larvae/diaphragm/mouse. In addition, examination of the small intestine at necropsy showed that the administration of dexamethasone also increased the numbers of surviving adults in both *T. spiralis* and *T. pseudospiralis*-infected animals. Adrenalectomized mice also had decreased intestinal worm burden when compared to unadrenalectomized mice (data not shown). Gross examination of both muscle and intestine in dexamethasone-treated mice showed a noticeable reduction in inflammation in infected mice. This reduction in inflammation was not apparent in non-dexamethasone-infected animals.

Table 3.1. Analysis of Capillary Density in Mice Infected With Either *T. spiralis* or *T. pseudospiralis* And in Uninfected Controls.
ADX = adrenalectomized. N=108.

	Day animal sacrificed							
	0	10	15	20	30	45	60	75
<i>T. spiralis</i>	0	0	+	+	++	++	+++	+++++
<i>T. pseudo</i>	0	0	0	0	0	0	0	0
<i>T. spiralis</i> ADX	0	0	+	+	++	++	+++	+++++
<i>T. pseudo</i> ADX	0	0	0	0	0	0	0	0
Uninfected Controls	0	0	0	0	0	Not Done	Not Done	0

Figure 3.1. The *T. spiralis* Cyst at Day 15 Post-Infection. Capillaries are present along the myofiber but angiogenesis has not begun. Small arrow depicts the collagen nurse cell. Large arrow depicts the capillaries. Magnification is 1000X. Capillary density is 0.

Figure 3.2. Neovascularization of the *T. spiralis* Nurse Cell at Day 20 Post-Infection. Small arrow depicts the nurse cell. Large arrow depicts the beginning of the capillary rete. Magnification is 1000X. Capillary density is +.

Figure 3.3. Neovascularization of the *T. spiralis* Nurse Cell at Day 25 Post-Infection. Small arrow depicts the nurse cell. Large arrow depicts the capillary rete. Magnification is 1000X. Capillary density is + 1/2.

Figure 3.4. Neovascularization of the *T. spiralis* Nurse Cell at Day 30 Post-Infection. Small arrow depicts the nurse cell. Large arrow depicts the capillary rete. Magnification is 1000X. Capillary density is ++.

Figure 3.5. Neovascularization of the *T. spiralis* Nurse Cell at Day 45 Post-Infection. Small arrow depicts the nurse cell. Large arrow depicts the capillary rete. Magnification is 1000X. Capillary density is ++.

Figure 3.6. Neovascularization of the *T. spiralis* Nurse Cell at Day 60 Post-Infection. Small arrow depicts the nurse cell. Large arrow depicts the capillary rete. Magnification is 1000X. Capillary density is + + +.

Figure 3.7. Neovascularization of the *T. spiralis* Nurse Cell at day 75 Post-Infection. Small arrow depicts the nurse cell. Large arrow depicts the capillary rete. Magnification is 1000X. Capillary density is +++. The capillary rete is complete.

Figure 3.8: Direct Larval Counts. n=60. See results for range.

PERIPHERAL BLOOD COUNTS

As can be seen in Table 3.2, there are considerable differences in the peripheral peak blood cell counts between the infected animals and the uninfected controls. The greatest leukocyte response in peripheral blood is seen in mice infected with *T. pseudospiralis* with a peak total white count increasing more than five-fold from 1,700 cells/ μ l to more than 10,000cells/ μ l at day 20 post infection. With the exception of eosinophils and monocytes, *T. pseudospiralis* elicits a substantially greater immune cell response in peripheral blood than does *T. spiralis*. Only eosinophils and monocytes are significantly elevated in *T. spiralis* when compared to *T. pseudospiralis*.

Table 3.2: Peak blood cell levels and serum cortisol at day 20 post-*Trichinella* infection. Cells were stained with modified Wright's Stain and counted manually. Results are based on the averages of four separate experiments (n=108). Three slides were made for each sample. Cortisol measurements were performed by IDEXX Corp.

PROFILE	<i>T. SPIRALIS</i>	<i>T. PSEUDOSPIRALIS</i>	CONTROL
TOTAL WBC	7900/ μ l	10000/ μ l	1700/ μ l
NEUTROPHILS	3901/ μ l	5060/ μ l	323/ μ l
LYMPHOCYTES	3456/ μ l	4390/ μ l	1343/ μ l
MONOCYTES	384/ μ l	323/ μ l	17/ μ l
EOSINOPHILS	349/ μ l	220/ μ l	17/ μ l
BASOPHILS	0/ μ l	0/ μ l	0/ μ l
CORTISOL	<0.2 μ g/dl	0.7 μ g/dl	<0.1 μ g/dl

COLLAGEN DEPOSITION

Collagen deposition around the nurse cell in *Trichinella*-infected mice, *Trichinella*-infected ADX mice and in *Trichinella*-infected ADX mice given replacement steroid treatment is shown in Figures 3.9 through 3.12. Masson's trichrome was used to detect collagen around the larvae in the intracellular niche. It can be seen that adrenalectomy does not change collagen deposition in *Trichinella spiralis* or *T. pseudospiralis* infected mice. Although not shown, dexamethasone made no difference in collagen deposition in ADX mice infected with either *T. spiralis* or *T. pseudospiralis*. No nurse cell formation was observed in *T. pseudospiralis* infected mice under any of the experimental conditions detailed here.

Figure 3.9. Masson's Trichrome stain of adrenalectomized BALB/c mouse diaphragm infected with *T. pseudospiralis* at day 35 post-infection. Large arrow depicts worm. Magnification is 1000X.

Figure 3.10. Masson's Trichrome stain of normal BALB/c mouse diaphragm infected with *T. pseudospiralis* at day 35 post-infection. Large arrow depicts worm. Magnification is 1000X.

Figure 3.11. Masson's Trichrome stain of normal BALB/c mouse diaphragm infected with *T. spiralis* at day 35 post-infection. Large arrow depicts worm. Small arrow depicts collagen deposition. Magnification is 1000X.

Figure 3.12. Masson's Trichrome stain of adrenalectomized BALB/c mouse diaphragm infected with *T. spiralis* at day 35 post-infection. Large arrow depicts worm. Small arrow depicts collagen deposition. Magnification is 1000X.

DISCUSSION

The collagen and angiogenic response in the skeletal muscle of BALB/c mice infected with either *Trichinella spiralis* or *T. pseudospiralis* was monitored over a period of 105 days using Masson's Trichrome or the lectin derived from *Griffonia simplicifolia*-1 (GS-1) respectively. No evidence of collagen deposition or angiogenesis was found in muscle cells infected with *T. pseudospiralis* infected ADX mice or in ADX mice treated with dexamethasone in this study. No differences were noted in collagen deposition or angiogenesis in *T. spiralis*-infected mice or *T. spiralis*-infected ADX mice. This is in contrast to the report by Hegazy and Boulos (1993) of the visualization of a membrane forming around *T. pseudospiralis* following Levamisole treatment (see Chapter I). Therefore, the absence of cortisol had no apparent effect on the formation of the nurse cell or neovascularization in *Trichinella*-infected mice.

The use of Masson's trichrome, which stains all types of collagen, demonstrated that collagen deposition in *Trichinella* infections was not dependent on or connected with the presence or absence of cortisol in the host's blood. *Trichinella spiralis*-infected ADX animals that received dexamethasone showed no changes in nurse cell formation or in angiogenic response.

The nurse cell found in *T. spiralis* infected animals appears to be species specific to *T. spiralis* and other encapsulating *Trichinella*. The enlarged nuclei of muscle cells infected with *T. spiralis* have been shown to be transcriptionally active for

Type IV and VI collagen (Polvere, et al 1997) suggesting that it is the host that produces the collagen that helps formulate the nurse cell. The nurse cell and enclosed parasite then form a complex that acts to maintain the larvae. And, although worm secreted antigens have been found in the nucleoplasm of infected muscle cells, it is uncertain whether these peptides have an active role in nurse cell formation (Vassilatis, et al 1992, Vasselitis, et al 1996). It has been shown, however, that the excretory/secretory products of both *T. spiralis* and *T. pseudospiralis* induce type IV collagen synthesis in murine 3T3 fibroblasts *in vitro* (Haehling, et al 1995). Despite these findings *in vitro*, my observations indicate that it is the host that deposits the collagen that becomes the nurse cell complex and that this phenomenon never occurred in *T. pseudospiralis* infected mice *in vivo* under the experimental conditions detailed here. It seems unlikely, however, that the host alone secretes a collagen-nurse cell complex that has such fortuitous benefits for survival of the enclosed worm.

The sequence of cellular events leading to collagen deposition and angiogenesis around the encapsulating species of *Trichinella* remain uncertain. However, because angiogenesis is only seen in the encapsulating species of *Trichinella*, this is strongly suggestive of a complex relationship between the collagen architecture, the ECM, the wound healing cascade and angiogenesis in *Trichinella*-infected BALB/c mice.

Since cortisol is a normal part of the HPA axis in mammals, cortisol would be expected to play a role in suppressing the inflammatory response and in restoring homeostasis in animals infected with parasites. *Trichinella pseudospiralis* does not form this nurse cell as was previously described. Other investigators that have reported

that collagen synthesis *in vivo* is suppressed by various immunosuppressive agents including cortisol and dexamethasone. (Delaney et al 1995a; Guller, et al 1995; Delaney, et al 1995b; Haapasaari, et al 1995). However, despite the role of cortisol in suppressing collagen synthesis as discussed below, cortisol does not appear to be a factor in nurse cell formation. In addition, dexamethasone did not suppress nurse cell formation in *T. spiralis*-infected mice under the conditions detailed here (data not shown).

As the level of IL-1 β and other cytokines and chemokines rise during an inflammatory reaction, the hypothalamus stimulates the production of cortisol by way of CRF and ACTH and cortisol then acts to suppress both the innate and adaptive immune responses. Suppression of the innate immune response includes the slowing or cessation of collagen synthesis by various cell types including fibroblasts (Davis, et al 1997; Crofford, et al 1997; Swolin-Eide and Ohlsson, 1998). However, my research shows that IL-1-induced cortisol synthesis appears to play no obvious role in the production or suppression of the collagen nurse cell in *Trichinella* infections and is therefore, not a factor in the absence of the nurse cell in *T. pseudospiralis* infected BALB/c mice.

There appears to be a role for IL-1 β in collagen synthesis and wound repair. Lertchirakarn, et al (1998) found that IL-1 β induced collagen synthesis in fibroblasts but this synthesis is tissue specific. This effect is probably due to IL-1 β induced PGE2 synthesis in certain cell types but not in others (Lertchirakarn, et al 1998). Mehdizadehkashi (1995) found that PGE2 levels were significantly higher in *T. spiralis*

infected mice than in *T. pseudospiralis* infected mice. Frisbie and Nixon (1997) found that corticosteroids like cortisol inhibit PGE2 synthesis and that IL-1 β induced collagen degradation may be inhibited by corticosteroids. IL-1 β is also known to decrease the formation of granulomatous tissue in the wound healing process when administered *in vivo*. (Rapala, et al 1997).

Because of the presence of high levels of PGE2 in *T. spiralis* infected mice, I maintain that PGE2 may be one of the major controlling mechanisms in collagen synthesis in *Trichinella* infected mice under the experimental condition detailed here. It is reasonable to assert, therefore, that cortisol suppresses the synthesis of PGE2 in *T. pseudospiralis* infected BALB/c mice. If this is the case, it may be the absence of PGE2 that prevents nurse cell formation in *T. pseudospiralis*-infected mice and the presence of high levels of PGE2 in *T. spiralis*-infected mice that contributes to nurse cell formation.

The data from mice with adrenalectomy with or without dexamethasone treatment showed no detectable differences between treated and untreated mice. Angiogenesis and collagen synthesis may be linked to each other and to the severity of the of the muscle damage and to the subsequent presence of angiogenic factors like TNF, VEGF, extracellular matrix (ECM) components and/or infected muscle cell hypoxia. Another possibility is that there may be some, as yet unidentified, angiogenic mechanism present in *T. spiralis* but not in *T. pseudospiralis*. It is known that the VEGF peptide and mRNA for VEGF is present in the sarcoplasm of *T. spiralis* infected myocytes as early as day 7 post-penetration (Capo, et al 1998). These workers have

speculated that it is muscle hypoxia that leads to the up-regulation of VEGF in infected cells. This is an interesting speculation but does not answer the question as to why the rapidly growing *T. pseudospiralis* does not appear to elicit an equivalent hypoxic condition and, therefore, stimulate the production of angiogenic peptides like VEGF with subsequent neovascularization of the muscle cell. An assay for VEGF mRNA or the VEGF peptide in *T. pseudospiralis*-infected myocytes may help to answer this question.

Also of interest is the fact that the degree of damage to the mitochondrial cycles as described previously (see Chapter 1) is more severe in *T. spiralis*-infected mice. The uncoupling of the mitochondrial cycles may contribute to the activation of the VEGF gene(s) in the myofibers of *T. spiralis* but not in *T. pseudospiralis*-infected-mice where mitochondrial and other damage is considered to be less severe. This assumption, if true, would suggest that it is damage and not worm growth that places oxygen demands on the myocyte that are answered by the up-regulation of angiogenic factors like VEGF. It must also be recognized that the angiogenic peptide TNF that is heavily secreted by activated macrophages may also have a role in stimulating angiogenesis. Of particular interest are the very large numbers of tissues macrophages present around the *T. spiralis* nurse cell but not around *T. pseudospiralis*.

Saito, et al (1998) and Kusano, et al (1998) found that monoclonal antibodies against matrix metalloproteases (MMP) completely inhibited IL-1 β and plasminogen induced collagen degradation. Matrix metalloproteases like collagenase and stromalysin are activated following the type of injury to basement membranes as might be found in

Trichinella infections (Acott, 1997, personal communication). Thus, there may be a relationship between collagen degradation via MMP and IL-1 β levels in host serum. Collagen degradation is an essential early step in wound repair mechanisms. *In vitro*, collagen has been shown to increase in response to IL-1 β levels in rat cardiac microvasculature endothelial cells (Okada, et al 1998). Rosenquist, et al (1996) found that endogenous PGE2 production had a stimulating effect on collagen deposition during inflammatory responses. Mice infected with *T. spiralis* but not *T. pseudospiralis* demonstrate high PGE2 levels. Conversely, Graham, et al (1997) found that corticosteroids suppress both type I collagen synthesis and collagenase production in human intestinal smooth muscle cells *in vitro* and may thus promote healing in inflamed tissues. *Trichinella pseudospiralis* stimulates very high levels of cortisol in the host when compared to *T. spiralis*-infected mice.

The presence of certain types of ECM components or collagen fragments may influence the type of T helper response (Th1 versus Th2) *in vivo*. The presence of ECM fragments might be expected due to an injury or other inflammatory event. The more severe the damage, the greater the number of ECM fragments that might be expected at the injury site. Interferon- γ and collagen tend to generate a primary Th1 response whereas IL-4 and IL-10 tend to generate a primary Th2 response (Lawrence, et al 1998; Mauri, et al 1996). Thus, the induction of one type of T helper cell appears to suppress induction of the other type and both types may be dependent on the presence of certain stimulatory molecules like those from the ECM.

Most researchers have found that, at least in the intestinal phase, the Th2 response dominates in mice infected with *T. spiralis*. I maintain that my data (based on NO production) indicate that, in the muscle phase, a Th1 response is most likely in *T. pseudospiralis*-infected-mice. If a Th1 response is also prominent in *T. spiralis*, NO production may be reduced or eliminated by the presence of PGE2. Whether the primary Th2 response remains an important factor in *Trichinella*-infected mice following the intestinal phase is unknown. An assay for the chemokine eotaxin that is produced only by Th2 cells would be instructive in determining T helper responses in *Trichinella* infections. Eotaxin is a chemoattractant and an activator of eosinophils, a granulocyte with an important role in the host response to parasitic infections like *Trichinella*.

Muscle damage that induces a wound-healing response in *T. spiralis* infected animals may account for some of the phenomena observed. The presence of collagen, fibronectin and such ECM macromolecules as proteoglycans and glycosaminoglycans and/or the degradation of hyaluronan may stimulate endothelial cell growth. These molecules are, in turn, chemoattractive and stimulate the production of angiogenic cytokines such as TGF- β and TNF by responding macrophages. It is plausible that the more severe damage seen in *T. spiralis* infections results in an angiogenic response not seen in *T. pseudospiralis* infected animals due, at least in part, to the presence of these angiogenic molecules. This is in addition to the effects of hypoxia and the production of VEGF by infected and damaged myocytes.

Kilcullen, et al (1998) reported that the presence and proliferation of macrophages at the site of wound repair is positively correlated to the proliferation of mesenchymal cells (especially fibroblasts). Proliferation of macrophages is also correlated with increases in reparative collagen, angiogenesis and the overall inflammatory responses to wounds *in vivo*. This report supports findings of a pronounced cellular infiltrate of macrophages in *T. spiralis* infected mice and a very sparse infiltrate of macrophages in *T. pseudospiralis* infected mice as measured immunohistochemically by myeloperoxidase assay (Prulhiere, 1994).

Other researchers have found that modulations of cytoskeletal and adhesion proteins during wound healing are critical events in the angiogenic cascade induced, in part, by collagen (Deroanne, et al 1996). *Trichinella spiralis* causes severe damage to skeletal muscle during invasion and modification of the myofiber following infection. It is reasonable to conjecture, therefore, that the generation of extracellular matrix fragments would be much more pronounced in *T. spiralis* than in *T. pseudospiralis* infected mice. The results of the research presented here support this assumption. Indeed, it has been noted that moderation of the ECM fragments *in vitro* results in the impairment of neovascularization (Kuzuya, et al 1998).

Other researchers have suggested a role for TGF- β 1 and type 1 collagen in wound healing in mice *in vivo* and that defective collagen deposition markedly reduces effective wound healing and subsequent neovascularization (Reed, et al 1998; DaCosta, et al 1998). Despite these findings, the precise sequence of events leading to angiogenesis *in vivo* remains unclear. Haas, et al (1998) maintain that IL-1 and PGE2

stimulate the endothelial cell production of matrix metalloproteases and that these events may be mediated by inflammatory cytokines and by the presence of macrophages at the site of the injury. This would tend to support the results reported here because of the very large number of responding macrophages at the site of myocyte injury in *T. spiralis* infected animals as mentioned above. Indeed, if matrix metalloproteases are necessary for angiogenesis and PGE2 and IL-1 are required for the production of these MMP, then this further substantiates my hypothesis that angiogenesis may be directly related to tissue injury, collagen deposition and the wound-healing mechanism in *T. spiralis* infected mice but not in *T. pseudospiralis* infected mice.

The evidence from Sephel, et al (1996) that endothelial cells are sequestered from stroma or provisional matrix by the basement membrane (BM) further supports the findings here. After breach of the membrane by the worm and the subsequent exposure to the matrix, endothelial cells migrate and proliferate. I suggest that it is the endothelial cell recognition of matrix architecture and specific matrix ligands that, in part, stimulates their expansion and proliferative behavior *in vivo*.

Researchers have found that the angiogenic response to injury in other mammals is non-specific (Malekan, et al 1998). Vascular endothelial growth factor (VEGF) is a key mediator of angiogenesis *in vivo* and is produced locally by injured cells including cells infected with *Trichinella* (Capo, et al 1998; Grad, et al 1998). In addition, there may be a complex mix of cytokines secreted following injury that have been dubbed the platelet-derived wound healing formula (PDWHF). This mix is known

to contain platelet-derived angiogenic factor (PDAF), platelet-derived growth factor (PDGF), TGF- β , platelet factor IV and a selection of cytokines like IL-1 β and TNF (Hiraizumi, et al 1996; Kurdy, et al 1996; Lee, et al 1998). The relative levels of these and other cytokines may vary during the course of the infection (Ramaswamy, et al 1996). Conclusions by these and other researchers (Ilan, et al 1998; Munno, et al 1998) lend credence to my hypothesis that it is the differences in degree of muscle damage and subsequent divergent inflammatory events during *Trichinella* infections that dictate the observed pathological outcomes. These events may, in part, determine the differences seen in the establishment of the specific intracellular niches for *T. spiralis* and *T. pseudospiralis*.

In conclusion, I have found no links between cortisol in angiogenesis or collagen deposition although it is possible that the high levels of serum cortisol may play some role in suppressing both angiogenesis and collagen synthesis. Taken together, my data suggest a complex set of events leading to the formation of the nurse cell in *T. spiralis* infected mice. The establishment of the intracellular niche, therefore, is substantially different from that seen in *T. pseudospiralis* infections. I suggest, based on my observations, that collagen deposition and angiogenesis may be linked to each other and to the wound healing response resulting from the pronounced muscle damage and hypoxia seen in *T. spiralis* infections but not in *T. pseudospiralis* infections. I base this suggestion on the data gathered from the infected adrenalectomized mice and in ADX mice that had dexamethasone replacement treatments in which no change in total collagen was noted and no differences in the angiogenic responses were observed

Examination of tissues for larval establishment revealed that larval numbers are greatly reduced in *Trichinella*-infected animals that had undergone adrenalectomy. However, this reduction in muscle larvae is significantly more pronounced in *T. pseudospiralis* infected mice than in *T. spiralis* infections. This result might be predicted because cortisol levels in *T. pseudospiralis* infected animals are almost four-fold higher than in *T. spiralis* although *T. spiralis* also has higher than control levels of cortisol as can be seen in Table 3.2. Because cortisol broadly suppresses the immune response in mammals as previously discussed, it is reasonable to hypothesize decreases in larval numbers in adrenalectomized animals. Other researchers have found that natural anti-inflammatory agents like cortisol down-regulate excessive inflammatory reactions against infectious agents and reduce the systemic inflammatory response syndrome (SIRS) *in vivo* (Iwagaki, et al 1997). Without cortisol to inhibit immune cell proliferation and inflammatory cytokine production, I observed an increase in parasite killing as measured by surviving larval numbers as seen in Figure 3.8. This reaction was reversed by the administration of dexamethasone. In fact, dexamethasone is a more potent inhibitor of the immune system than is cortisol even when the dose is adjusted to compensate for the 30-fold difference in pharmacological action between the two steroids (Casini-Raggi, et al 1995; Guthrie, 1991; Lane, et al 1990). This becomes especially apparent as larval numbers in dexamethasone treated animals are examined. Indeed, the infection was so severe in a number of mice that they died of trichinellosis. This was particularly true at about day 15 post-infection in *T. spiralis* infected mice as larvae became established in the intracellular niche with the subsequent muscle damage

that predictably accompanies these events. With this information in mind, it is reasonable to assert that low cortisol may be associated with a higher inflammatory reaction resulting in lower infectivity. Conversely, higher cortisol levels are associated with lower inflammatory reactions resulting in higher infectivity.

These basic assumptions are supported by the larval counts in mice infected with *T. spiralis* or *T. pseudospiralis* and in infected mice with surgically removed adrenal glands. I have observed that adrenalectomy also reduces the number of intestinal-phase adult worms and that this trend is reversed through the administration of dexamethasone. These phenomena were apparent for both species of worm although the reductions were less remarkable for *T. spiralis*. I suggest, therefore, that serum cortisol may play an important role in reducing the host immune and inflammatory responses in mice infected with *Trichinella* regardless of the species of worm. I further theorize that *T. pseudospiralis* is much more dependent on host serum cortisol than is *T. spiralis*. This may be due to the fact that *T. pseudospiralis* is unencysted and is therefore more exposed to the host cellular response than is *T. spiralis*. Conversely, *T. spiralis* may be more dependent on PGE₂ to moderate the immune response. Prostaglandin E₂ is believed to counteract ongoing inflammatory responses *in vivo* (Haveman et al 1999). However the mechanisms by which these two endogenous bioactive substances act are different. This is further evidence that the two species of *Trichinella* are eliciting quite different responses from the host

The results from an IL-1 β assays (data not shown) indicate that both species of worm stimulate the host to produce elevated amounts of this lymphokine. The levels of

IL-1 β are important in my research as another indicator of the degree of the inflammatory and adaptive immune responses in mice infected with one of the two species of worm. The IL-1 β , NO, and CPK data are all in agreement that the host is generating a significantly more massive response to *Trichinella spiralis* under the experimental conditions detailed here. The source of IL-1 β (although it probably primarily derived from activated macrophages) is of secondary importance. What is of importance is the quantity of IL-1 β secreted and the timing of the secretions during the course of the infection.

My observations have been confirmed by other researchers. Interleukin-1 β and TNF are produced at high levels throughout the course of the infection but drop off markedly during convalescence (Kavelaars, et al 1997; Ramaswasmy, et al 1996; Ishikawa, et al 1998; Lawrence, et al 1998; Faulkner, et al 1998). High levels of IL-1 β and other cytokines like INF- γ and TNF may stimulate the release of cortisol through the HPA axis. This, however, cannot be directly correlated with the substantially higher numbers of responding leukocytes in the peripheral blood of *T. pseudospiralis* infected animals. It must be noted that IL-1 β levels were higher in *T. spiralis* infected mice and, thus, IL-1 β levels do not directly correspond to high cortisol levels under the experimental conditions detailed here. The mechanisms responsible for generating a four-fold difference in cortisol levels in *T. pseudospiralis*-infected mice remain elusive.

It is interesting that my data shows that, despite less damage to the muscle and higher cortisol levels, *T. pseudospiralis* elicits a more massive immune cell response

from the host as measured in peripheral blood but not in tissues. In contrast, *T. spiralis* stimulates a substantially greater intracellular and tissue leukocyte response than does *T. pseudospiralis* suggesting again that the two species of worm elicit very different protective mechanisms from the host.

The higher cortisol levels in *T. pseudospiralis*-infected animals may be the result of an unidentified cytokine-generated host response. The response of peripheral blood macrophages is similar between *T. spiralis* and *T. pseudospiralis* and they are present in much higher numbers than in uninfected control mice. This macrophage response is true in blood but not in tissues as discussed previously. Macrophages are the primary source of IL-1 β but other cell types including neutrophils, epithelial cells and T cells may secrete this and other cytokines during an inflammatory reaction.

The neutrophil and lymphocyte counts in the peripheral blood are higher in *T. pseudospiralis* infected mice than in *T. spiralis* or controls. These observations tend to lend further support to my assertion that the host inflammatory and immune response differs significantly depending on the species of *Trichinella* that is infecting. And, although IL-1 β may play an important role in initiating an inflammatory reaction, it may be IFN- γ and TNF or some other cytokine that sustain the inflammatory response once it has started as has been suggested by Stadnyk and Kearsey (1996) and that, following an initial common innate response, the host expresses a dichotomous reaction that is dependent on a number of factors including differences in the wound healing cascade.

It has been shown that IFN- γ can activate the HPA axis without IL-1 β in humans (Ohno, et al 1998). IFN- γ is derived from macrophages and, to a lesser extent, other cells during inflammation. In mice infected with *Trichinella*, IL-4 and IL-6 also play a role in mediating the inflammatory response to the intestinal phase (Frydas, et al 1996). Among other functions, interleukin-4 generates a Th2 response whereas IL-6 activates the acute-phase response by hepatocytes. I suggest that cytokine production and T helper response changes as the host moves from the intestinal to the muscle phase of the infection and that this switch is dependent on the species of *Trichinella* infecting. Evidence of this switch may be presenting itself experimentally in NO and PGE2 production in infected host mice although it seems certain that the biochemical and cellular interplay is far more elaborate than that demonstrated here.

It has been well established that there is an immunosuppressive role for cortisol on the synthesis and actions of IL-1 β and other cytokines like IFN- γ during inflammatory events (Curti, et al 1996). It may be the high cortisol levels that have a role in lowering IL-1 β levels in *T. pseudospiralis*-infected mice when compared to *T. spiralis*-infected mice as I have demonstrated here. This immunosuppressive activity may extend to other cytokines as well including IL-6 and TNF. This suppression also applies to cells stimulated by LPS (P'aez, et al 1996). I found that, despite high cortisol levels, the peripheral blood inflammatory responses to *T. pseudospiralis* are pronounced and that IL-1 β levels are elevated and follow a pattern similar to that seen in *T. spiralis* infected animals but at a substantially decreased level.

The reasons for the hyperstimulation of the HPA axis in *T. pseudospiralis* infected animals remain unclear. As discussed above, I suspect that there may be other cytokines involved that stimulate the HPA axis in *T. pseudospiralis* infected mice. It seems likely that the two species of worm generate alternative T helper responses with the corresponding differences in cytokine production. This assertion is supported by Capo, et al (1998). These cytokines are probably derived from immune cells in the blood where significant differences are observed in peripheral blood cell counts between mice infected with *T. spiralis* or *T. pseudospiralis*.

In addition, it has been found that *T. spiralis* and other nematodes release an orthologue of macrophage inhibition factor (MIF or MIP-1 β) thereby inactivating a key subclass CC chemokine in the activation of T lymphocyte-dependent adaptive immunity (Pennock, et al 1998). The production of such molecules by *T. pseudospiralis* has not been demonstrated.

It is also noteworthy that I have observed that *T. spiralis* infected mice show a massive infiltrate of macrophages both inside and outside the nurse cell by day 21 of infection. It is possible that IL-1 β produced by macrophages inside the collagen cyst does not activate the HPA axis in a manner similar to that seen in *T. pseudospiralis*. It is also reasonable to posit that the very high peripheral blood leukocyte response stimulated by *T. pseudospiralis* generates a greater response by the HPA axis than does the encysted *T. spiralis*. And, finally, it is possible that, in *Trichinella* infected mice, cortisol may not prevent the proliferation of peripheral blood leukocytes but may act to

prevent activation of these leukocytes through mechanisms that have yet to be elucidated.

In summary, I believe that I am observing a different inflammatory and immune response that is dependent on the species of infecting worm. It is reasonable, therefore, to postulate other, as yet unidentified, factors mediating the host response. A Th1 response would generate a different set of cellular phenomena than would a Th2 response. The subsequent differences in cytokine production generating different cellular responses could help explain my observations. It should also be remembered that Th1 cells have iNOS and Th2 cells do not. Because NO is much more pronounced in *T. pseudospiralis* infected mice (see Chapter 2), this suggests a primary Th1 response in *T. pseudospiralis* infection in the muscle phase. This could account for variations in cytokine production if *T. spiralis* is generating a primary Th2 response. Conversely, although I am aware of no data that assigns a role for PGE2 (or any lipid-derived mediator) in the transition of naïve T cells to mature Th1 or Th2 lymphocytes, it is possible that the high levels of PGE2 may have a role in determining which type of T helper response dominates the muscle phase in *Trichinella* infections.

Cortisol does not appear to play a role in either angiogenesis or collagen deposition for either species of worm. Cortisol, however, appears to have a role in larval survival for *T. pseudospiralis*. Tissue and blood leukocyte responses in host mice infected with one of the two species' of *Trichinella* are very different in both numbers and in cell type. Once again, interleukin-1 β , cortisol and PGE2 appear to be key molecules in mediating the host immune reactions to the two species of worm.

The biochemical and cellular interactions between a eukaryotic parasite and its eukaryotic host are exceedingly complex. Therefore, because *T. spiralis* and *T. pseudospiralis* may generate a different immune profile (including possible differences in the Th1 and Th2 responses), the following chapter addresses the up-regulation and down-regulation of leukocyte surface molecules using flow cytometric analysis in an attempt to further identify the precise mechanisms involved in the host response to *Trichinella* infections in mice.

CHAPTER FOUR

FLOW CYTOMETRIC ANALYSIS OF CELL SURFACE ANTIGENS IN MICE INFECTED WITH *TRICHINELLA*

INTRODUCTION

FLOW CYTOMETRY

Chapter Four is directed toward the study of the leukocytes in the spleens of BALB/c mice infected with *Trichinella spiralis* or *Trichinella pseudospiralis* during the muscle phase of the parasite life cycle. Most of the *Trichinella* antigens from the intestinal phase would have been carried to the intestinal lymph nodes and Peyer's patches. Some antigens would have been transported to the liver and spleen. However, most of the cells involved in the adaptive response in the spleen are carried by the blood. During the collection period for spleen cells at designated time points, the immune cells collected are most likely to be reacting to the antigens of the migratory L1 larvae, muscle penetrating L1 larvae and to cells involved in the innate immune response.

In all animals that demonstrate inflammatory responses, the progress of the immune and inflammatory reactions to infectious agents like viruses, bacteria, protozoa and helminths can be ascertained by assaying for the up-regulation and down-regulation

of various leukocyte surface clusters of differentiation (CD) molecules. As a group of molecules, they serve a variety of biological functions for the cell on which they reside; including adhesion, recognition, binding of specific ligands, communication, differentiation and homing. As secondary lymphoid tissue, the spleen provides an easily accessible organ for the study of the up-regulation and down-regulation of CD markers during inflammatory events *in vivo* using an assay method known as flow cytometry.

Mature myeloid and lymphoid cells of the immune system respond to inflammatory stimuli in a number of different ways. These responses include the production of cytokines, chemokines, the clonal expansion of certain leukocyte subsets and the up- or down-regulation of various cell surface molecules. The up- or down-regulation of these surface molecules may vary depending on the type of invading organism. The inflammatory response of leukocytes in peripheral blood, lymph nodes or spleen can be monitored and quantitated using a number of different assay methods including immunochemistry, immunohistochemistry and flow cytometry.

The flow cytometer or fluorescence-activated cell sorter (FACS) analyzes cells based on size, granularity and/or the presence of fluorescent-tagged monoclonal antibodies to selected cell surface markers. Generally, a flow cytometer is able to analyze up to five different markers on the same cell. The cytometer then assigns each cell type to a specific quadrant on a dot plot based on the above parameters. These quadrants are subsequently analyzed by the cytometer's built in analytical software.

This type of analysis makes it possible, for example, to differentiate B cells from all other leukocytes in a sample by first tagging all leukocytes with a pan-leukocyte

phycoerythrin (PE)-labeled monoclonal antibody against CD45. A fluorescein-isothiocyanate (FITC) labeled anti-CD19 monoclonal antibody would then be added to distinguish all B-cells within this group of CD45-tagged leukocytes. This data allows a quantification of subsets of cells within a larger population of leukocytes. At the same time, it would be possible, for example, to distinguish between and quantify T helper versus T cytotoxic lymphocytes based on florescent tags to differentiate between the CD4 and CD8 markers respectively. Another useful function of flow cytometry is the analysis of activation of molecules on cell surfaces.

The flow cytometer, as used in this study, was used to delineate the up-regulation or down-regulation of selected cell surface antigens in mice infected with one of two species of *Trichinella*. This data provides a better understanding of the timing and progress of the inflammatory and immune reactions to *T. spiralis* infected mice as compared to *T. pseudospiralis* infections.

CELL SURFACE ANTIGENS

ASSAYED IN THIS STUDY

Thymocytes and mature T lymphocytes in mice express CD3 (Leo, et al 1987). The cytoplasmic domain of the CD3 molecule participates in signal transduction events and the activation of biochemical pathways following antigen recognition (Isakov, et al 1995; Nakano, et al 1996). The CD3 molecule consists of seven integral membrane proteins that are non-covalently associated with each other and with the T cell receptor (TCR) α/β or γ/δ heterodimers. During T cell activation, CD3 (as a coreceptor with the CD4 or CD8 TCR) is associated with binding the major histocompatibility complex (MHC) of antigen presenting cells (APC) and acts in signal transduction (Kruisbeek and Shevach, 1991). The molecule CD3 was used in this study to identify and quantify all mature T cells in the spleen of *Trichinella*-infected BALB/c mice.

The CD4 molecule is a 55kDa transmembrane glycoprotein of the immunoglobulin (Ig) superfamily expressed on thymocytes, macrophages and on a sub-population of $\alpha\beta$ -or γ/δ -positive MHC class II-restricted T cells (T helper cells). It is also present in low density on myeloid and intrathymic precursor cells and on pluripotent hematopoietic cells (Fredrickson and Bosch, 1989; Wineman, et al, 1992; Wu, et al 1991a; Wu et al 1991b; Godfrey, et al 1994). The CD4 molecule serves as a cell-cell adhesion molecule with invariant affinity for the class II MHC. It is presumed to bind the nonpolymorphic part of the class II MHC molecule in the immunoglobulin-like $\alpha 2$ or $\beta 2$ domains where there is an interaction of MHC restricted T cells with APC. The

CD4 molecule has a signal transducing function. The molecule is associated with T cell activation and is allied with a lymphocyte-specific protein tyrosine kinase p55^{lck}. In this application, CD4 will be used to identify and quantify a subset of T cells, specifically the T helper lymphocyte in *Trichinella*-infected BALB/c mice.

The CD8 α molecule is composed of either a 34kDa homodimer or associated with the 34kDa CD8 β in a multimeric or dimeric complex. CD8 is expressed on most thymocytes and on a sub-population of MHC class I restricted T cells (cytotoxic T lymphocytes [CTL]) and T suppressor cells (Abbas, et al 1991; Macdonald, et al 1990a; LeFrancois, 1991; Wang and Klein, 1994; Wu, et al 1995; Suss and Shortman, 1996). The CD8 molecule is an antigen co-receptor and reacts with the nonpolymorphic immunoglobulin-like $\alpha 3$ domains of class I MHC on APC. Like CD4, CD8 works with the protein tyrosine kinase p55^{lck} (Janeway, 1992), thus participating in T cell activation. The CD8 molecule may transduce signals itself or it may facilitate TCR/CD3-mediated signal transduction after binding class I MHC. Also, like CD4, it is uncertain if CD8-mediated signals are activating, inhibitory or if these signals are essential for the T cell response to antigen and MHC (Abbas, et al 1991). As used in the present study, CD8 will be used to determine the response of this subset of T lymphocytes to the presence of *Trichinella* in BALB/c mice.

The common leukocyte antigen is designated CD45 and is found on all cells of the hematopoietic system except erythrocytes and platelets (Ledbetter and Herzenberg, 1979; Van Ewijk, et al 1981). The CD45 molecule is a group of integral membrane proteins that range from 180 to 220kDa and, in mice, are all products of a single gene

complex on chromosome 1. Alternative splicing of mRNA results in the production of several polyfunctional isoforms that include CD45RO, CD45RA and CD45RB. The CD45 molecule is a member of the protein tyrosine phosphatase (PTP) family. The intracellular portion consists of two PTP catalytic domains while the extracellular portion is highly variable (Johnson, et al 1997). The CD45 molecule is involved in signal transduction and in modifying signals from other surface markers. It is, therefore, involved in T cell recognition and in the regulation of various activation pathways through a tyrosine kinase. As used in this study, CD45 will mark all leukocytes in a sample. Secondary florescent markers will identify subsets of leukocytes within this group in *Trichinella*-infected BALB/c mice.

The molecule CD11b/CD18 (Mac-1) is a significant surface antigen and a heterodimeric member of the integrin family of leukocyte surface molecules. Mac-1 functions as a complement receptor for iC3b, a fragment in the complement cascade, and as an adhesion molecule whose ligand is the intracellular adhesion molecule-1 (ICAM-1) (Springer, et al 1978; Springer, et al 1979; Lub, et al 1996). Mac-1 is expressed on granulocytes, macrophages, dendritic cells, and NK cells. Mac-1 is rapidly up-regulated on neutrophils following activation (Vremec, et al 1992; Leenen, et al 1994). As a complement receptor of iC3b, Mac-1 stimulates the phagocytosis of iC3b-opsonized particles. The molecule CD11b/CD18 will be used in this study to monitor the activation of cells of myeloid lineage (especially macrophages and neutrophils) in response to the presence of *Trichinella* in BALB/c mice.

The transmembrane glycoprotein CD19 is a 90kDa protein differentiation antigen specific for B cells (Krop, et al 1996a). The CD19 molecule is present throughout B cell differentiation but is shed when cells become terminally differentiated plasma cells (Krop, et al 1996b; Fearon, 1993). The CD19 antigen associates with surface B cell Ig, CR-1 and TAPA-1 to promote cellular activation (Krop, et al 1996b). The CD19 molecule may be involved in the clonal expansion of B cells, maturation of memory B cells and B cell activation via T cell dependent antigens (Rickert, et al 1995; Engle, et al 1995). In the present study, CD19 will be used to quantitate the clonal expansion of B cells during the specific humoral immune response to *Trichinella* in BALB/c mice.

The surface antigen CD44 is a sulfated acidic integral membrane protein of 80 to 200kD. The molecular weight depends both on the addition of chondroitin sulfate and on the degree of glycosylation (Leslie and Trowbridge, 1982; Schmits, et al 1997; Kahto, et al 1994). The CD44 molecule is expressed on the surface of T cells, thymocytes, B cells, granulocytes, macrophages and on a variety of non-immune cells such as neural cells, erythrocytes, epithelial cells, myocytes and fibroblasts (Leslie and Trowbridge, 1982). The antigen is heavily expressed on the surfaces of cells in certain mouse strains including BALB/c (Lynch and Ceredig, 1989). The amino terminal of CD44 is expressed on thymus-derived progenitor or bone marrow cells. It is also expressed in peripheral blood on activated B cells, CD4⁺ T cells and on memory cells (Spangrude, et al 1989; MacDonald, et al 1990b; Sprent and Tough, 1994; Budd, et al 1987). The CD44 antigen may also play a role in extravasation, acting as a homing receptor that binds to molecules in high endothelial venules (HEV). Binding to HEV is

a prerequisite for the extravasation of leukocytes from blood to lymphoid tissues such as the spleen and lymph nodes (Hyman, et al 1986). The ligands for CD44 include collagen, fibrinectin hyaluronate and HEV addressin. The molecule CD44 mediates adhesion of leukocytes and, as used in this study, will serve as an additional indicator of an activation response as measured by splenic leukocytes of BALB/c mice infected with *Trichinella*.

The adhesion molecule CD54 (ICAM-1) is a 95kDa Ig superfamily member expressed on lymphocytes, HEV, macrophages and dendritic cells (Scheynius, et al 1993). Up-regulation of ICAM-1 occurs in response to inflammatory mediators such as cytokines, chemokines and LPS. It is also the ligand for both leukocyte-functioning antigen (LFA-1)(CD11aCD18) and Mac-1 (CD11b/CD18) (Springer, 1990; Springer, 1994). The adhesion molecule CD54 participates in general inflammatory reactions and in antigen-specific immune reactions. ICAM-1 also participates in rolling (margination) interactions with the endothelium prior to extravasation. (Scheynius, et al 1996; Isobe, et al 1992; Xu, et al 1994). Like CD 44, CD54 will be used to monitor the inflammatory response to *Trichinella* by splenic leukocytes in BALB/c mice.

Surface antigen CD62L (L-selectin) is a 90kD receptor with lectin-like domains that bind sialylated oligosaccharide determinates on HEV in peripheral lymph nodes (Gallatin, et al 1983; Siegelman, et al 1990). In mice, L-selectin is expressed on most immunocompetent thymocytes, T cells, B cells, neutrophils, eosinophils and monocytes (Reichert, et al 1986a; Reichert, et al 1986b; Reichert, et al 1986c; Iwabuchi, et al 1991; Lewinsohn, et al 1987). This selectin appears to be required for neutrophil

immigration in response to inflammation and lymphocyte homing to peripheral lymph nodes (Pizcueta and Luscinskas, 1994; Ley, et al 1995). L-selectin is rapidly shed from cell surfaces upon activation. Thus, the levels of CD62L may be used to differentiate mature memory/effector cells from naïve cells (Jung, et al 1988; Kishimoto, et al 1989; Mobley and Dailey, 1992). As used in this study, the loss of CD62L from cells surfaces during *Trichinella* infection will act as an indicator of the severity of the immune reaction to the presence of the worm in BALB/c mice because there is a direct correlation between CD62L loss and the vigorousness of the inflammatory reaction.

The molecule CD69 (very early activation antigen) is a 28-34kDa homodimer that is disulfide-linked and composed of differentially-glycosylated subunits (Yokoyama, et al 1988; 1989). The expression of CD69 is induced very rapidly upon the activation of macrophages, T cells, B cells and NK cells in response to inflammatory mediators such as the cytokines IL-1, TNF and IFN- γ and to exogenous priming agents like LPS (Ziegler, et al 1994; Brandle, et al 1994). The surface molecule CD69 will be used to monitor the activation of lymphocytes in the presence of *Trichinella* in BALB/c mice.

MATERIALS AND METHODS

Eight female three-month old BALB/c mice were infected with 250 *Trichinella* larvae from previously infected mice. Four of the mice received *T. spiralis* and the other four received *T. pseudospiralis*. These eight mice are called the infected group. In addition to the infected group, a separate group of four mice were injected IP with either 100µg, 200µg or 300µg of LPS dissolved in sterile normal saline or with normal saline without LPS. The priming agent LPS was used to determine the CD response to a known activator of the inflammatory reaction in mammals. Four untreated and uninfected control mice were female BALB/c mice of the same age as the infected and LPS groups and were maintained in an identical manner as the infected and LPS groups according to Oregon law for the care of research animals. Food and water were supplied ad libitum.

Beginning day 7 post-infection, two mice from the infected group, one infected with *T. spiralis* and one infected with *T. pseudospiralis*, and one uninfected control mouse were sacrificed by cervical separation. The spleens were removed, placed in sterile saline and placed at 4°C. The spleen was washed twice in sterile normal saline, placed in Isoflow sheath fluid (Coulter) and manually minced. Minced spleen was filtered through a 750µm metal grid and the filtered cells were placed in new sterile polypropylene tubes and placed at 4°C. One milliliter of erythrocyte-lysing reagent (Tris-Cl buffered NH₄Cl [pH 7.2]) was added and the cells were held for 10 minutes at 4°C. The remaining unlysed leukocytes were fixed with 100µl of 10%

paraformaldehyde added to the lysing mixture. The fixed leukocytes were allowed to stand at 4°C for 10 minutes. One hundred microliters of each leukocyte suspension was removed to a fresh microfuge tube and 900µl of Isoflow sheath fluid was added to each tube. This exact procedure was repeated using the remaining mice at days 14, 21 and 28 post-infection.

Mice injected with LPS or saline were sacrificed by cervical dislocation at 6 hours post-injection and the spleens were harvested and treated in a manner identical to the infected and control mice. The leukocytes from the infected group, the uninfected control group and the LPS treated mice were then exposed to specific monoclonal antibodies as detailed below.

As a pan-T cell marker, phycoerythrin (PE)-labeled Armenian hamster antimouse CD3 monoclonal antibody (Sigma) diluted 1:100 in sterile PBS (pH 7.2) containing 0.075mg/ml sodium azide was added to selected tubes of spleen cells. Alternatively, as a pan-leukocyte marker, PE-labeled rat antimouse CD45 monoclonal antibody (Sigma) diluted as above was added to other selected tubes of spleen cells. All PE-labeled antibodies used in these experiments were added at a final concentration of 1µg of antibody to 500µl spleen cells.

Following addition of the pan-leukocyte or T cell markers, one of the following was added to select tubes of spleen cells: Fluorescein-isothiocyanate (FITC)-labeled rat antimouse CD4, FITC-labeled rat antimouse CD8, FITC-labeled rat antimouse CD19, FITC-labeled rat antimouse CD44, FITC-labeled Armenian hamster antimouse CD54,

FITC-labeled rat antimouse CD62L, FITC-labeled rat antimouse CD11b or FITC-labeled Armenian hamster antimouse CD69.

All FITC-labeled antibodies used in these experiments were supplied by Pharmingen. The antibodies were diluted in sterile PBS (pH 7.2) and added at a final concentration of 1 μ g of antibody to 500 μ l of spleen filtrate.

The antibody/spleen cell mixture was held at room temperature for 30 minutes. Cells were then centrifuged at 500xG for 10 minutes at room temperature. Cells were washed in sterile PBS (pH 7.2) and centrifuged at 500xG at room temperature for 10 minutes. The wash was removed from the cells with suction and the cells were resuspended in 1ml of Isoflow sheath fluid. A FACScan flow cytometer (Becton-Dickinson) or an Epics XL cell sorter (Coulter) was used to determine the relative proportions of various cells types and/or cell surface antigens using FACScan or Epics XL analytical software.

These experiments were repeated four times using identical protocols (n = 32). The results are the averages of pooled data sets. The LPS experiment was repeated once (n = 8).

RESULTS

Interpretation of the number of cells distributed in appropriate quadrants of the dot plot showed that cells bearing the CD19 antigen increased in mice infected with *Trichinella* when compared to controls (Figures 4.1 through 4.4). Of total lymphocytes counted, CD19⁺ cells averaged 23.5, 25.6, 28.9, and 25.7 percent of lymphocytes on days 7, 14, 21, and 28 days post-infection in *T. spiralis* infected mice (+/- 2%). In *T. pseudospiralis* infected mice CD19⁺ cells averaged 17.5, 21.3, 20.9, and 20.5 at the same time points (+/- 1.5%). This compares to 16.1, 15.7, 15.1, and 16.2 percent of cells in uninfected controls (+/- 2%). Thus, it appears that *T. spiralis* infected mice have a substantially larger CD19 B cell response in spleen than *T. pseudospiralis* infected mice and that *Trichinella* appears to stimulate a significant B cell response in mice when infected mice are compared to uninfected controls.

Cells characterized by CD11b surface molecules in control mice averaged 48.9, 51.2, 50.3, and 49.9 percent of total leukocytes on days 7, 14, 21, and 28 respectively (+/- 1%). In *T. spiralis* infected mice, the percentages rose to 65.7, 78.1, 86.3, and 54.9 (+/- 3%). This compares with averages in *T. pseudospiralis* of 58.8, 69.2, 84.5 and 54.4 (+/-4%) on days 7, 14, 21, and 28 post-infection respectively as can be seen in Figures 4.5 through 4.8). Infected mice showed significant increases in CD11b when compared to controls.

Cells marked with CD62L in *T. spiralis* infected mice averaged 9.7, 9.2, 7.8 and 7.9 (+/- 2.5%) percent of lymphocytes on days 7, 14, 21 and 28 post-infection. *T.*

pseudospiralis infected mice averaged 11.1, 9.9, 8.5 and 8.9 percent (+/- 2%) of lymphocytes at these time points. Control mice averaged 14.6, 16.2, 15.9 and 14.8 percent (+/- 1.5%) on days 7, 14, 21, and 28 respectively (Figures 4.9 through 4.12). Thus, CD62L showed significant decreases in infected mice when compared to controls.

Cells marked with CD69 rose from levels of 2.1, 2.3, 1.9 and 2.1 percent (+/- 0.3%) of total lymphocytes on days 7, 14, 21, and 28 respectively. This is compared to 13.6, 21.1, 19.3, and 10.4 (+/- 1.5%) in *T. spiralis* infected mice and 11.8, 18.7, 19.7 and 6.9 percent (+/-1.0%) in *T. pseudospiralis* infected mice on days 7, 14, 21 and 28 respectively (Figures 4.13 through 4.16). The molecule CD69 was significantly increased in mice infected with *Trichinella* when compared to uninfected controls.

Cells bearing the CD8 surface antigen that are primarily MHC class I-restricted T lymphocytes (cytotoxic T lymphocytes) demonstrated substantial increases in the spleen. In uninfected control mice, cells marked with CD8 averaged 3.01, 3.3, 3.1 and 3.3 percent (+/- 0.3%) of total lymphocytes on days 7, 14, 21 and 28 respectively. In *T. spiralis* infected mice, CD8 cells rose to an average of 5.1, 8.3, 9.4 and 7.2 percent (+/- 0.8%). *T. pseudospiralis*-infected mice averaged 6.4, 7.6, 13.7 and 13.2 percent (+/- 1.2%) of lymphocytes on days 7, 14, 21 and 28 post-infection respectively (Figures 4.17 through 4.20)). Cells marked with CD8 remained significantly elevated in *T. pseudospiralis* infected mice at day 28 post-infection even as CD8 began to drop in *T. spiralis* -infected animals.

The results of the injections of LPS at 100µg, 200µg or 300µg per mouse are presented in Figure 4.21. Briefly, the surface antigens CD69, CD11b and CD54 responded by increasing in a dose-related manner. Cells with the antigen CD69 increased from 1.56% to 3.98%. Cells with the antigen CD11b increased from 67.8% to 86.6%. Cells with the CD54 antigen increased from 8.3% to 22.5%. The antigen CD62L dropped from 10.4% to 6.7%.

The cellular markers CD44 and CD54 and CD4 were elevated in infected animals when compared to controls (data not shown). However, the differences in the up-regulation of these two molecules in the spleen of animals infected with either *T. spiralis* or *T. pseudospiralis* were insignificant when compared with each other.

Figure 4.1: CD19. Data is the average of four separate experiments. Only splenic B-cells are represented. n=32.

Figure 4.2a: CD45/19. Uninfected control. Total cell acquisition plot. Percent gated:
R1 = 100.00; R2 = 16.16 R3 = 1.75; R4 = 35.33.

Figure 4.2b: CD45/19. Uninfected control. Quadrant R2 acquisition plot.
Percent gated: UL = 16.01; UR = 16.21; LL = 17.68; LR = 50.09.

Figure 4.3a: CD45/19. *T. pseudospiralis*-infected mouse. Total cell acquisition plot. Percent gated: R1 = 100.00; R2 = 34.06; R3 = 2.54; R4 = 15.96.

Figure 4.3b: CD45/19. *T. pseudospiralis*-infected mouse. R2 quadrant acquisition plot. Percent gated: UL = 17.25; UR = 17.45; LL = 19.70; LR = 45.60.

Figure 4.4a: CD45/19. *T. spiralis*-infected mouse. Total cell acquisition plot. Percent gated: R1 = 100.00; R2 = 36.51; R3 = 2.03; R4 = 13.44.

Figure 4.4b: CD45/19. *T. spiralis*-infected mouse. R2 quadrant acquisition plot. Percent gated: UL = 21.75; UR = 23.50; LL = 8.85; LR = 45.90.

Figure 4.5: CD11b/MAC-1. Data represents the average of four experiments. n=32.

Figure 4.6a: CD45/11b. Uninfected control. Total cell acquisition plot. Percent gated: R1 = 100.00; R2 = 20.34; R3 = 5.05; R4 = 22.57.

Figure 4.6b: CD45/11b. Uninfected control. R3/4 quadrant acquisition plot. Percent gated: UL = 0.00; UR = 48.15; LL = 0.25; LR = 51.60.

Figure 4.7a: CD45/11b. *T. pseudospiralis*-infected mouse. Total cell acquisition plot. Percent gated: R1 = 100.00; R2 = 37.02; R3 = 1.55; R4 = 15.36.

Figure 4.7b: CD45/11b. *T. pseudospiralis*-infected mouse. R3/4 quadrant acquisition plot. Percent gated: UL = 0.00; UR = 54.60; LL = 0.60; LR = 44.80.

Figure 4.8a: CD45/11b. *T. spiralis*-infected mouse. Total cell acquisition plot. Percent gated: R1 = 100.00; R2 = 12.91; R3 = 7.15; R4 = 37.12.

Figure 4.8b: CD45/11b. *T. spiralis*-infected mouse. R3/4 quadrant acquisition plot. Percent gated: UL = 0.40; UR = 72.80; LL = 5.80; LR = 21.00.

Figure 4.9: CD 62L. Data is the average of four separate experiments. n=32

Figure 4.10a: CD45/62L. Uninfected control. Total cell acquisition plot. Percent gated: R1 = 100.00; R2 = 22.24; R3 = 45.52; R4 = 1.43.

Figure 4.10b: CD45/62L. Uninfected control. R2 quadrant acquisition plot.
Percent gated: UL = 17.17; UR = 20.12; LL = 8.96; LR = 53.75.

Figure 4.11a: CD45/62L. *T. pseudospiralis*-infected mouse. Total cell acquisition plot. Percent gated: R1 = 100.00; R2 = 21.44; R3 = 57.07; R4 = 2.03.

Figure 4.11b: CD45/62L. *T. pseudospiralis*-infected mouse. R2 quadrant acquisition plot. Percent gated: UL = 0.68; UR = 12.81; LL = 19.74; LR = 66.77.

Figure 4.12a: CD45/62L. *T. spiralis*-infected mouse Total cell acquisition plot. Percent gated: R1 = 100.00; R2 = 25.75; R3 = 54.08; R4 = 1.95.

Figure 4.12b: CD45/62L. *T. spiralis*-infected mouse. R2 quadrant acquisition plot. Percent gated: UL = 0.56; UR = 9.36; LL = 16.35; LR = 73.73.

Figure 4.13: CD69/Early Activation Antigen. Data is the average of four separate experiments. n=32

Figure 4.14a: CD45/69. Uninfected control. Total cell acquisition plot. Percent gated:
R1 = 100.00; R2 = 17.81; R3 = 5.06; R4 = 24.32.

Figure 4.14b: CD45/69. Uninfected control. R2 quadrant acquisition plot.
Percent gated: UL = 0.00; UR = 2.80; LL = 13.80; LR = 83.40.

Figure 4.15a: CD45/69. *T. pseudospiralis*-infected mouse. Total cell acquisition plot. Percent gated: R1 = 100.00; R2 = 31.11; R3 = 5.40; R4 = 31.02.

Figure 4.15b: CD45/69. *T. pseudospiralis*-infected mouse. R2 quadrant acquisition Plot. Percent gated: UL = 0.05; UR = 18.70; LL = 1.70; LR = 79.55.

Figure 4.16a: CD45/69. *T. spiralis*-infected mouse. Total cell acquisition plot. Percent gated: R1 = 100.00; R2 = 35.41; R3 = 6.73; R4 = 22.24.

Figure 4.16b: CD45/69. *T. spiralis*-infected mouse. R2 quadrant acquisition plot. Percent gated: UL = 0.10; UR = 19.25; LL = 1.10; LR = 79.55.

Figure 4.17: CD8 Positive cells. Data is the average of four experiments. Only splenic cytotoxic T-cells are represented. n=32.

Figure 4.18a: CD45/8. Uninfected control. Total cell acquisition plot. Percent gated:
R1 = 100.00; R2 = 28.30; R3 = 12.23; R4 = 34.24.

Figure 4.18b: CD45/8. Uninfected control. R2 quadrant acquisition plot. Percent gated: UL = 3.01; UR = 2.85; LL = 88.72; LR = 5.42.

Figure 4.19a: CD45/8. *T. pseudospiralis*-infected mouse. Total cell acquisition plot. Percent gated: R1 = 100.00; R2 = 31.79; R3 = 1.95; R4 = 10.92.

Figure 4.19b: CD45/8. *T. pseudospiralis*-infected mouse. R2 quadrant acquisition plot. UL = 0.40; UR = 13.70; LL = 30.05; LR = 55.85.

Figure 4.20a: CD45/8. *T. spiralis*-infected mouse. Total cell acquisition plot. Percent gated: R1 = 100.00; R2 = 28.47; R3 = 2.31; R4 = 14.70.

Figure 4.20b: CD45/8. *T. spiralis*-infected mouse. R2 quadrant acquisition plot. Percent gated: UL = 0.40; UR = 9.15; LL = 38.50; LR = 51.95.

Figure 4.21: CD Response to LPS. LPS injected IP. Blood harvested at 6 hours. CD11b, CD69, CD54, CD19 and CD62L show dosage dependent changes. n=8.

DISCUSSION

The Antigen CD11b

Cells with the surface markers CD11b/CD18 showed considerable increases in mice infected with *T. spiralis* or *T. pseudospiralis* when compared to uninfected controls and showed important differences when compared to each other. CD11b is the α_m chain of this β_2 family of integrins. The CD11b subunit α_m binds CD54, complement component iC3b and extracellular matrix proteins. The β chain is synonymous with CD18 that associates with CD 11a, b, c or d. The molecule CD11b is a leukocyte adhesion receptor and mediates the adhesion of neutrophils and monocytes to endothelium during extravasation. The molecule also participates in the phagocytosis of complement-opsonized molecules (Lee, et al 1999).

Elevation of CD11b, as a surface antigen on granulocytes, monocytes and macrophages is a well-known indicator of infection in human beings and may be used to prognosticate outcomes for patients with viral or bacterial disease (Weirich, et al 1998). Workers have found a correlation between elevated CD11b levels and progression of HIV infection (Benito, et al 1997). In cattle infected with the parasite *Theileria annulata*, cells showing increased expression of CD11b have been found in thymus, spleen and lymph nodes. This was especially evident in nodes draining the site of infection (Forsyth, et al 1997). It has also been noted, however, that up-regulation of CD11b may not be sufficient to sustain phagocytosis in infected animals (Dosgone, et

al 1997). Nevertheless, up-regulated CD11b may be involved in the activation of NK cells *in vivo* in cooperation with LFA-1, CD54 and other cytokines (Ito, et al 1996).

It is noteworthy that CD11b up-regulation in mice infected with *T. spiralis* averaged a 26% increase on day 7 post-infection compared with controls. This compares with mice infected with *T. pseudospiralis* averaging a 17% increase in CD11b at the same time point, when compared to controls. This suggests either an ongoing inflammatory response to the adult worms in the intestinal phase and/or the beginning of the systemic inflammatory reaction to the L1 larvae that are present in the skeletal muscle at this time. I believe that, at 7 days post-infection, it is both. At this point in time, however, there is no way to discriminate between the responses to the intestinal phase and the muscle phase. As stated in Chapter 3 however, I noted that the two species of worm appear to elicit different sets of immune phenomena in the host. I suggest that the immune response to the newborn larvae in muscle may also elicit different responses from the host depending on the species of worm infecting when compared to the host response to the intestinal phase. These differences might be expected to present themselves as quantifiable differences in the CD responses on various types of responding leukocyte subsets that are measurable by flow cytometric analysis.

At days 14 and 21 post-infection, an increase of 35% and 41% respectively was noted in CD11b in *T. spiralis* infected mice over uninfected controls. For mice infected with *T. pseudospiralis*, the percentages were considerably lower averaging 26% and 30% at these same time points as compared to uninfected controls. These results

suggest that adhesion molecules like CD11b may be up-regulated to allow extravasation of neutrophils and other responder cells to the site of inflammation as has been previously noted by Wang, et al 1998 and Benton, et al 1996). The major sites of inflammation at these time points might now be expected to be in striated muscle as the adult worms are expelled from the intestine and intestinal inflammation subsides. The expression of CD11b in splenic leukocytes returns to near normal levels by day 28 post-infection although it remains slightly elevated when compared to uninfected control animals.

Up-regulation of CD11b is also linked to ischemia-induced tissue injury and the production of certain cytokines like TNF and INF- γ . After cytokines return to pre-infection levels, CD11b is shed from cell surfaces and returns to levels approximating those seen before infection (Lin, et al 1996). This information supports my findings and suggests that, in *Trichinella* infections, the insertion of the larvae into the intracellular niche allows the animal to return to homeostasis. It is also recognized that leukocyte activation as measured by CD11b may remain slightly elevated for varying periods of time following infection/injury events in both human beings and in mice (Engervall and Lundahl, 1998; Paugam, et al 1997).

As stated above, in *Trichinella* infection the adults have generally been expelled by day 14 post-infection and my data suggests that the surviving L1 larvae are still eliciting a full inflammatory response at day 21 post-infection. This response appears to be diminished to near-normal levels by day 28. However, my investigation of responder cells in peripheral blood after day 28 indicates an ongoing generalized leukocytosis that

is evident for as long as day 75 post-infection (data not shown). This is particularly notable in *T. pseudospiralis* infected mice. This data supports the general observation that helminthic infections tend to be chronic and that low level inflammatory reactions continue into the convalescent period.

The Antigen CD62L

The selectin CD62L is found on a subpopulation of T and B lymphocytes, monocytes, neutrophils and other leukocytes and functions in rolling interactions and as a homing receptor for lymphocytes. The molecule also mediates leukocyte traffic using glycosylation-dependent cell adhesion molecule-1 (Gly-CAM-1) as its ligand along with CD34 in high endothelial venules (HEV) (Siegelman, et al 1990; Carlos and Harlan, 1994; Lawrence and Springer, 1991). The selectin CD62L is also involved in the activation of platelets (Kiuchi, et al 1998).

As one of the selectin family that includes E-selectin (CD62E) and P-selectin (CD62P), CD62L is involved in inflammatory responses to infection and/or injury events in mammals. Unlike the P- and E-selectins, CD62L is shed from cells following inflammatory activation and is not generally present on endothelial cells. During inflammatory events, CD62L is down-regulated on leukocytes but up-regulated on platelets. My protocol for the flow cytometric analysis of CD62L eliminated platelets and erythrocytes from consideration and included only those lymphocytes that expressed CD62L.

Cells with the CD62L molecule's expression on the cell surface was markedly lower in *T. spiralis* infected mice than in controls at all time points examined. And,

although *T. pseudospiralis* infected mice also showed a loss of CD62L from lymphocyte surfaces, it was retained in considerably higher amounts on these lymphocytes than on those of *T. spiralis* infected mice. The down-regulation of CD62L in *Trichinella* infected mice suggests an ongoing systemic inflammatory response that peaks at approximately day 21 and subsides by about day 28 post-infection. These observations are in agreement with the work of other investigators who have found that down-regulation of CD62L occurs concomitantly with the up-regulation of other cell surface molecules including CD44, CD54 and CD11b (Aziz, et al 1996; Condliffe, et al 1996). In addition, Fanelli, et al (1997) have suggested that there may be a direct correlation between the degree of shedding of CD62L from leukocyte membranes and the up-regulation of both CD62L and CD63 on platelets. These observations may be correlated with the severity of the inflammatory response to both injury and certain haemostatic disorders such as antiphospholipid syndrome.

The treatment of BALB/c mice with increasing doses of the priming agent lipopolysaccharide (LPS) resulted in the down-regulation of CD62L in a dose-dependent manner. This corresponded to an up-regulation of CD11b in a dose dependent manner as can be seen in Figure 4.21. Condliffe, et al (1996) also noted that the use of the priming agent LPS caused a detectable loss of CD62L from the surface of neutrophils. This same researcher noted a correlation between CD62L down-regulation and increases in the presence of inflammatory cytokines like TNF and platelet activating factor (PAF) following injury. However, no correlation was found between PAF and CD11b up-regulation and CD62L loss but a correlation was found

between TNF, CD11b up-regulation and CD62L loss. In other words, platelet activation during an inflammatory event is concomitant with, but not dependent on, neutrophil activation events that are signaled by the loss of CD62L from leukocyte surfaces. Condliffe, et al (1996) suggests, therefore, that neutrophil activation and recruitment to the sites of injury is dependent on a number of factors including the coordinated regulation of adhesion and activation molecules.

In parasitic infections, adhesion molecules such as CD62L have a role in antibody-dependent cell-mediated cytotoxicity (ADCC). Trottein, et al (1997) has found that the carbohydrate determinate sialyl Lewis(x) (CD15), CD11b (Mac-1) and Cd62L are required for activation of macrophage effector function against *Schistosoma* targets *in vivo*. The sialylated form of CD15 Lewis(x) (sLe^x) is the ligand for P-, E- and probably L- selectins (CD62P, CD62E and CD62L) *in vivo*.

My data shows that CD62L as measured by flow cytometry in lymphocyte populations follows a predictable pattern of down-regulation in mice infected with either *T. spiralis* or *T. pseudospiralis*. From the literature and my results, I suggest that the observed patterns of CD62L down-regulation in infected mice may be the result of the presence of collagen related peptides at the site of injury. Kehrel, et al (1998) found that CD62L and CD63 recognize the collagen sequence glycine-proline-hydroxyproline (gly-pro-hyp) leading to up-regulation on platelets and down-regulation on leukocytes. I suggest that the presence of extracellular matrix (ECM) proteins such as collagen fragments have a role in the down regulation of CD62L in *Trichinella* infections in mice and in the subsequent activation and adhesion of phagocytic cells to endothelium.

These ECM components may be present in higher amounts in *T. spiralis* infected animals as compared to *T. pseudospiralis* due to the increased muscle damage in *T. spiralis* infections as measured previously by creatine phosphokinase (CPK) assays (refer to Chapter 2). This further demonstrates that *T. spiralis* is significantly more damaging to the myofiber than is *T. pseudospiralis* and that this damage and subsequent inflammatory response results in measurably different cellular phenomena. These differences are of both type and strength. The possible role for cortisol and/or PGE2 in these events is unknown at this time.

I have also shown that down-regulation of CD62L is a measurable event by flow cytometric analysis. This down-regulation may correlate with the severity of the infection and with the corresponding severity of the muscle damage in *Trichinella* infected mice. Analysis of CD62L, therefore, is efficacious in comparing the pathological consequences of *T. spiralis* and *T. pseudospiralis* infections in mice.

The Antigen CD69

The surface antigen CD69 was much higher in *Trichinella* infected mice than in controls but the differences between the two species of *Trichinella* were not significantly different. This suggests that lymphocyte activation as measured by CD69 is approximately the same for both species of worm.

The surface antigen CD69 is expressed constitutively on platelets, neutrophils and eosinophils but can be induced transiently on T, B and NK cells following activation (Testi, et al 1994) and serves as the earliest inducible and detectable glycoprotein at the cell surface in these cell lines. The molecule CD69 is a type II membrane protein that is

characterized by having the -NH₂ terminal inside the cell and the -COOH terminal outside. The antigen contains a C-lectin domain that has been associated with glycoprotein endocytosis and is probably involved in binding surface residues on parasites such as *Trichinella* and certain bacteria (Lee, et al 1999).

Cross-linking of CD69 following the binding of its ligand initiates Ca⁺⁺ influx and subsequent activation of phospholipase A2. Therefore, it may be active in arachidonic acid hydrolysis and in IL-2 activated NK cell target lysis and platelet activation (Testi, et al 1994). It should be remembered here that arachidonate hydrolysis is a seminal biochemical event in the production and secretion of a number of inflammatory lipid mediators including the prostaglandins (through cyclooxygenase), the leukotrienes (through 5-lipoxygenase) and PAF (through an acetyl transferase). Activation of CD69 on T cells can be induced and measured both *in vitro* and *in vivo* within hours of stimulation by intracellular parasites like HIV using flow cytometry (Perfetto, et al 1997). With this information in mind, I suggest that the high PGE2 levels seen in *T. spiralis*-infected mice but not in *T. pseudospiralis*-infected animals are due to factors other than CD69. These purported other factors remain to be elucidated.

Because CD69 is expressed constitutively on neutrophils, eosinophils and platelets, all measurements focused on those quadrants of the flow cytometric dot plots that indicated lymphocytes. My data shows that *Trichinella* does induce an early activation of CD69 on T and/or B lymphocytes or NK cells *in vivo* as there are important differences between *Trichinella*-infected animals and uninfected controls. My data also

shows that CD69 is expressed at low levels even in non-infected control animals but at basal levels that never exceeded 2.5% of lymphocytes as determined by flow cytometry.

Trichinella infected animals showed a substantial (more than six-fold) rise in lymphocytes marked with CD69 when contrasted with uninfected controls although my data indicate no appreciable differences in the host response to the two species of worm as measured by CD69 up-regulation. Prince and Lape-Nixon (1997) found that CD69 is an excellent tool for measuring the mitogenic response of lymphocytes to stimulation. These same researchers and Nielson, et al (1998) suggested that the pathogenesis and clinical outcome for certain parasitic diseases like HIV may be predicted using a CD69 assay. The crucial role for CD69 in T cell activation was demonstrated by Krowka, et al in 1996. These researchers blocked the up-regulation of CD69 using the HIV-derived protein GP120 causing a significantly reduced T cell activation *in vitro* even when T cells were stimulated with the priming agent and mitogen phytohemagglutinin.

The predictability of the pathogenesis and sequelae of inflammatory events using CD69 is meaningful to my research on *Trichinella*. I support this statement with the data from the LPS injection experiments that showed a large dose-dependent upward shift in CD69. Based on these observations, T cell and/or B cell activation as measured by CD69 appears to be approximately equal in mice infected with *T. spiralis* or *T. pseudospiralis*. Other (non-lymphocyte) cell responses may also be predicted using CD69 as has been demonstrated by Neilson, et al (1998) who showed that activation of

peripheral blood mononuclear cells (PBMC) may be predicted accurately both *in vitro* and *in vivo* in individuals infected with HIV.

Lim, et al (1998) found that activation of both CD4⁺ and CD8⁺ T lymphocytes could be reliably predicted by assessing the percent of cells gaining the CD69 marker in human beings infected with HIV. They also demonstrated that CD69⁺ cells were 51% (+/- 3%) higher in HIV infected individuals than in uninfected controls. I have also shown that CD69 is significantly elevated in mice that are infected with either *T. spiralis* or *T. pseudospiralis*. However, CD69 is much more markedly elevated in my studies than was found in humans infected with HIV. This may be due to the high level of acute tissue injury seen in *Trichinella* infections as measured by CPK assay. Therefore, it is reasonable to suggest that CD69 up-regulation is positively correlated with the degree of inflammation resulting from a *Trichinella* infection/injury event *in vivo*.

Although CD69 is constitutively expressed on human monocytes, it may be inducible on murine bone-derived macrophages in the presence of IFN- γ plus LPS or in the presence of TNF (Marzio, et al 1997). In support of my findings, these same researchers also found that LPS alone was sufficient to induce expression of CD69 on murine leukocytes. In addition, they found that nitric oxide production is stimulated in the presence of INF- γ and by anti-CD69 monoclonal antibody suggesting a possible role for CD69 as a signaling receptor for an uncharacterized ligand. Lahn, et al (1998) found that CD69 activation may be an indicator of the type of T cell response. Other researchers found that TNF may be a key mediator in activating either an α/β or γ/δ

response in T lymphocytes in response to bacterial (Terres and Pajares, 1998), parasitic (*Candida*) or LPS induced inflammation (Angulo and Fulcher, 1998).

The Antigen CD19

The constitutive surface antigen CD19 is found on B lymphocytes and changes during the course of an infection. This change can be quantified using flow cytometry (Ginaldi, et al 1998; Cheek, et al 1997). Tomazic, et al (1997) have found substantial increases in the percentages of CD19 lymphocytes in human beings with neuroborreliosis or tick-borne encephalitis; thus, flow cytometry may be used to characterize this lymphocyte subset during infection. Conversely, blocking CD19 results in marked abnormalities in cytokine production (Mistchenko, et al 1998). However, there is not always a correlation between CD19 and infection. Sabatier, et al (1997) have found that CD19 levels vary depending on the type of infectious agent and, in some cases, may be even lower than in uninfected controls.

I have shown that there is an increase in most types of peripheral blood leukocytes (PBL) in *Trichinella* infected animals (see Chapter 3). There are also significant increases of CD19⁺ lymphocytes in the spleen of infected animals when compared to uninfected controls as demonstrated by flow cytometry. Although *T. spiralis* infected animals have fewer PBL than do those infected with *T. pseudospiralis* (refer to Table 3.2), splenic CD19⁺ cells are notably higher in *T. spiralis* infected mice. The compartmentalization of immune cells has been demonstrated by other researchers. Rosenmann, et al (1998) maintains that lymphocyte subsets may evolve independently in peripheral lymphoid organs from those in peripheral blood. I have shown that CD19⁺

lymphocytes are present in the spleens of BALB/c mice at percentages that approximate those found in healthy human beings. These CD19⁺ cells average approximately 12.4% of lymphocytes but rise dramatically during parasitic infections like those caused by the Hepatitis B virus (Choong, et al 1996). It is also noteworthy that CD19 remains on activated B lymphocytes during clonal expansion but is not expressed on terminally differentiated plasmacytes (Fehr, et al 1998). These same researchers suggest that CD19, which contains nine tyrosine residues as possible phosphorylation sites, acts as a signal-transducing element for complement receptor 2 (CR2) and that circulating memory B cells are fully dependent on CD19. This correlates with my data that shows an increase in cells bearing CD19 in the spleen in *Trichinella* infected mice and that the cells marked with CD19 remain higher than controls in the spleen during convalescence (data not shown). I have noted that this number gradually declines after the intracellular niche is established and the host returns to homeostasis.

Once again, it must be noted that *T. spiralis* is eliciting a larger B-cell response than is *T. pseudospiralis*. As stated in the introduction, it has been noted that the types of IgG secreted by plasma cells during *Trichinella* infections also varies. It is not possible at this juncture to determine how much of the B cell response at day 7 post-infection is due to the host response to the intestinal phase, however B cell clonal expansion continues to rise though day 21 post-infection before beginning to decline slowly. It should also be remembered that CD19 is not expressed on terminally differentiated plasmacytes which means that the actual number of cells present of B cell

lineage is actually much higher than is indicated by the flow cytometric percentages analyzed using CD19. Therefore, flow cytometric CD19 analysis in *Trichinella*-infected mice indicates increasing clonal expansion of B cells and, by extrapolation, continued expansion of antibody-secreting plasmacytes

Interleukin-4 (IL-4) plays a primary role in the dichotomous activation of Th1 or Th2 subsets *in vivo* and plays a role in activating CD19⁺ cells during an infection-stimulated inflammatory activation (Collins, et al 1998). In addition, IL-1 β is expressed intracytoplasmically in CD19⁺ cells *in vivo* in infected human beings (Gray, et al 1996). Other cytokines expressed by CD19⁺ cells during activation include IL-6, IL-10 and TNF.

The Antigen CD8

The expression of CD8 on cytotoxic T lymphocytes (CTL) is of particular importance in parasitic infections such as those caused by *Toxoplasma gondii* and *Trypanosoma brucei brucei* (Purner, et al 1996; Bakheit, et al 1996). As part of the cell-mediated immune response, CTL play a vital role in eliminating intracellular parasites. However, CD4⁺ cells act in a cooperative manner with CD8⁺ cells in animals infected with both intra- and extracellular parasites. *Trichinella* is both intra- and extracellular during its life cycle within the host and, therefore, may elicit a complex and overlapping inflammatory and adaptive immune reaction.

I have shown that there are significant increases in CD8⁺ cells in mice infected with either *T. spiralis* or *T. pseudospiralis* when compared to uninfected controls. There is a gradual increase in CD8⁺ cells beginning at approximately day 7 post-

infection and peaking at about day 21 post-infection. After day 21, there is a decline in CD8⁺ marked cells in the spleens of mice infected with *Trichinella*. This may be due, in part, to the resolution of the intestinal phase and/or the establishment of the L1 larvae in the intracellular niche. Cells marked with CD8 in uninfected control mice remain at 3% of lymphocytes. These results are supported by Scheller, et al (1997) who found that, in animals infected with the intracellular parasite *Plasmodium berghei*, CD8⁺ (and CD4⁺) cells increased up to six-fold and that CTL are essential for parasite clearance from infected animals. These same researchers have suggested that, at least for animals infected with *Plasmodium*, NO production is regulated by both CD4⁺ and CD8⁺ T lymphocytes. As can be seen in Figures 4.17-4.20b, *T. pseudospiralis* elicits a significantly higher CD8⁺ response in BALB/c host mice than does *T. spiralis*. This difference in the numbers of CTL present in the spleen could help explain (in part) the very substantial differences measured in the host production of NO in infected mice. Kumar and Tarleton (1998) have also discussed the primary importance of the cell-mediated immune response in parasitic infections but also confirmed that the humoral response is essential for parasite elimination.

Circulating and splenic CTL are seen to increase dramatically initially but decline slowly during the convalescent period in animals infected with *Loa loa* (Dennis, et al 1997) or *Trichostrongylus axei* (Pernthaner, et al 1996). This supports my findings that splenic CD8⁺ cells, although beginning to decline, remain at approximately two and four times the control levels in *T. spiralis* and *T. pseudospiralis* infected mice at day 28 post-infection respectively. The importance of CTL in parasitic infections was

demonstrated by Russo, et al (1996) who showed that the blocking of CD8 with monoclonal antibody caused a 200-fold increase in parasite burden in mice infected with *Trypanosoma cruzi*. Interestingly, these same workers found that the increase in parasite burden was organ specific, suggesting again that immune cell activation and surface marker up-regulation may be compartmentalized *in vivo*.

As mentioned above, that since *Trichinella* is present both intra- and extracellularly in infected animals, it is, therefore, reasonable to predict a complex mix of inflammatory and immune responses to this particular nematode. Large extracellular parasites like *Schistosoma mansoni* elicit a primary Th1 CD8⁺ response and it has been postulated that Th2 CD8⁺ cells function to regulate Th1 (Pearce and Pedras-Vasconcelos, 1997). In addition, adoptive transfer of CD8⁺ splenocytes from mice infected with *Toxoplasma* to naïve mice gave protection against a lethal challenge of *T. gondii*. The intracellular *T. gondii*, *Plasmodium* and *Leishmania* also elicit a primary CD4⁺/CD8⁺ Th2 response and a pulse with parasite antigen forces secretion of large quantities of INF- γ , a prime activator of macrophages *in vivo* (Kasper and Kahn, 1998). Despite this, my NO data indicates a primary Th1 response in *T. pseudospiralis* infected mice. This may be due to the fact that *T. pseudospiralis* is unencapsulated and, therefore, more susceptible to the MHC class 1-restricted CD8⁺ T cell.

My data suggests a primary role for a CTL-mediated response to *Trichinella* in mice. This is in addition to a pronounced humoral response. Again, *T. pseudospiralis* shows a CD8 response more than 50% higher at peak than does *T. spiralis*. I suggest again that this may be due to the exposure of the unencapsulated L1 worm and L1

worm antigens to phagocytes (especially macrophages) and the subsequent presentation of these antigens to T cells. In addition, the excretory/secretory antigens in the saliva of *T. pseudospiralis* may be more readily presented to MHC I-restricted CTL than those secreted by the encapsulated *T. spiralis*.

SUMMARY AND CONCLUSIONS

In conclusion, I will summarize my findings and attempt to answer the questions posed at the beginning of this research. Based on the data presented, it was shown that there are considerable differences in the infectious strategies when one examines *T. spiralis* and *T. pseudospiralis* infections in mice. These differences are of both kind and degree. The two species of this nematode generate a different immune, physiological and inflammatory response from the mouse host. Cortisol is obviously a component in the ability of *T. pseudospiralis* to establish an intracellular skeletal muscular niche. To a much lesser extent, cortisol may play a role in the survival of *T. spiralis*. Cortisol appears to have no bearing on the deposition of collagen or on the initiation of angiogenesis in either *T. spiralis* or *T. pseudospiralis* infected mice. It is also apparent that there may be two very different immune defensive mechanisms at work depending on which species of worm is infecting the host.

There are noteworthy differences in the type, location and numbers of responding immune cells to the two species of worm. *T. pseudospiralis* generates an extensive peripheral blood response that is generally larger than that seen in *T. spiralis*. *Trichinella spiralis*, while also eliciting a large peripheral blood response, generates a much more pronounced tissue infiltrate of immune cells than does *T. pseudospiralis*. Based on the quantitative assays of cells in peripheral blood, it is reasonable to assume that there is a more important role for eosinophils and macrophages in *T. spiralis* -

infected animals. Conversely, *T. pseudospiralis*-infected mice have a more pronounced neutrophilia and higher numbers of peripheral blood lymphocytes than do *T. spiralis*-infected mice. As demonstrated by flow cytometry, splenic lymphocytes are markedly elevated in *Trichinella* infections in mice. Cytotoxic T lymphocytes are more pronounced in the spleen of *T. pseudospiralis*-infected animals and B cells are more abundant in *T. spiralis*-infected mice. This suggests that the humoral response may be playing a larger role in *T. spiralis* infections and that the cellular response may be more important in the host defense against *T. pseudospiralis*.

The flow cytometric analysis, as demonstrated by the dot plots, shows that there are substantial differences in the up-regulation or down-regulation of certain cell surface antigens when the two species of infecting worm are compared to each other and to uninfected controls mice. Levels of CD19 and CD8 both rose significantly in *Trichinella*-infected animals. As stated previously, the humoral response as measured by CD19 is markedly higher in *T. spiralis*-infected animals whereas the cell-mediated response as measured by the CD8⁺ population is much more prominent in *T. pseudospiralis*-infected mice. The surface antigens CD11b and CD69 rose to notably higher levels in infected animals when compared to uninfected controls. On average *T. spiralis* generated higher number of CD11b and CD69 surface antigens than did *T. pseudospiralis*. Whether or not this difference is related to cortisol is unknown. The decrease in CD62L was also more conspicuous in *T. spiralis*-infected animals and was lower on day 7 post-infection suggesting that the inflammatory response to the ongoing intestinal phase is also more pronounced in *T. spiralis*-infected animals. The differences

between the two species of worm with regard to CD44 and CD54 up-regulation were insignificant and unremarkable although both antigens were present in much higher levels in infected animals than in uninfected controls. This finding may be explained in part by the fact that CD44 is expressed heavily on many cells types and is essential for the extravasation of these cells in the spleen. The molecule CD54 or ICAM-1 is also necessary for leukocyte binding and is up-regulated in inflammatory reactions. Because CD44 and CD54 were up-regulated in a very similar manner in mice infected with either species of *Trichinella*, my findings suggest that CD44 and CD54 have equally important functions in *Trichinella* infections regardless of the species of worm infecting.

From the analysis of creatine phosphokinase in infected animals, I have shown that *T. spiralis* inflicts substantially more damage to the host myocyte than does *T. pseudospiralis*. This difference in myofiber damage (including ischemia) and in the type and degree of the ensuing inflammatory reactions, cellular infiltration and wound healing mechanisms may have important consequences for the subsequent differences seen in the angiogenic and collagen deposition responses seen in *T. spiralis* and not in *T. pseudospiralis*-infected animals. Indeed, based on these observations, one might speculate that it is the damage to the extracellular matrix and the subsequent invasion by macrophages that stimulate the cascade of extracellular and sub-cellular events leading to both neovascularization and collagen synthesis in *T. spiralis*-infected animals.

The investigations of collagen deposition, nurse cell formation and angiogenesis have shown that there are major differences in the strategy of the two species of *Trichinella* in the intracellular niche. These differences appear to be specific to the species of nematode that is infecting. Cortisol does not appear to play any appreciable role in either the deposition of collagen to form the nurse cell or in the subsequent neovascularization of the nurse cell in *T. spiralis*-infected mice. In addition, cortisol has no discernible role in mice infected with *T. pseudospiralis* except in its effect on larval survival. My results indicate that *T. pseudospiralis* is dependent on cortisol for the survival of both adults and larvae in the infected host. The mechanisms leading to the very high levels of cortisol in *T. pseudospiralis*-infected mice remain elusive. Using a developing method for flow cytometric study of intracellular cytokine generation in *Trichinella* infections may shed light on the phenomena. However, I suspect that PGE2 may have a role in down-regulating cortisol and NO in animals infected with *T. spiralis*. Indeed, PGE2 inhibits NO production as previously discussed. In addition, because Th1 cells secrete NO and Th2 cells do not, PGE2 is probably acting to suppress Th1 cell-production of NO in *T. spiralis*-infected BALB/c mice.

Nitric oxide appears to be an important mediator of parasite killing *in vivo* in *Trichinella*-infected mice. Interference with NO production using aminoguanidine (data not shown) resulted in considerably larger or lethal parasite burden in both *T. spiralis* and *T. pseudospiralis*-infected mice. I have shown that NO production is not related to muscle damage. If there were a relationship between muscle damage as measured by CPK and NO production, NO production in *T. spiralis*-infected animals would have

been much higher than in *T. pseudospiralis*-infected mice. Again, I seem to be observing a different response by the host animal that is dependent on the species of infecting worm. It should be remembered that natural killer cells and Th1 cells both express type II iNOS but Th2 cells do not. I have shown that, during the muscle phase, both species of *Trichinella* induce NO production indicating the activation of a primary Th1 response. It also suggests that *T. pseudospiralis* induces a more vigorous Th1 cell-mediated response than does *T. spiralis*. However, only further investigation will identify these details of the host response.

In conclusion, I have shown that *T. pseudospiralis* generates a very different immune and inflammatory response in the host than does *T. spiralis*. These differences are of both kind and degree. I have demonstrated the efficacy of flow cytometry in the evaluation of the response of mice to specific infectious or inflammatory agents *in vivo*. I have shown that there are significant differences in the up-regulation and/or down-regulation of most of the cell surface antigens examined. I have shown that there are important variations in the response of leukocytes in the peripheral blood of infected animals. I have clearly demonstrated that *T. spiralis* is more damaging to the host than is *T. pseudospiralis* as measured by creatine phosphokinase. I have shown that, in *T. spiralis*-infected mice, cortisol has no evident role in the formation of the nurse cell or in the subsequent neovascularization of the intracellular niche. I have also shown, however, that cortisol does play a major role in larval survival in *T. pseudospiralis* - infected mice and, to a lesser extent, in *T. spiralis*-infected animals. I have clearly established that there is a vital role for nitric oxide in the killing of *Trichinella* larvae *in*

vivo. I have suggested that the presence of extracellular matrix components and the very pronounced recruitment of macrophages to the site of injury may be crucial in establishment of the intracellular niche in *T. spiralis*-infected mice. And, finally, although I have elucidated some of the mechanisms responsible for the pathogenesis and sequelae of trichinellosis in mice, it is obvious that a great deal more work remains to be done on *Trichinella* and its parasitic relationship with animals.

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