LOCALIZATION AND PARTIAL IMMUNOLOGICAL

CHARACTERIZATION

OF *Fasciola hepatica* THIOREDOXIN

A Dissertation

by

RICHARD DWAYNE MCKOWN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2004

Major Subject: Veterinary Microbiology

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ABSTRACT

Localization and Partial Immunological Characterization

of *Fasciola hepatica* Thioredoxin. (December 2004)

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This study reports the localization and partial characterization of thioredoxin from the parasitic trematode *Fasciola hepatica*. Snails (*Pseudosuccinia columella*) were raised in culture and infected with *F. hepatica* so that Western blotting and immunohistochemical techniques could be utilized to determine the presence of thioredoxin in different stages of the parasite's development. The results of these experiments showed that thioredoxin was present in the tegument, gut epithelium, excretory canal epithelium and sperm, of the adult parasite as well as in the tegument and gut of the redia and cercaria intermediate stages. *In situ* hybridization was used to determine the localization and possible differential mRNA expression of two different *F. hepatica* thioredoxin isotypes (Fh2020.A and Fh2020.SL) in the adult parasite. The *in situ* hybridization results showed that both isotypes are expressed in the tegument and gut epithelium. Fh2020.A stains with a greater intensity possibly demonstrating a difference in the amount of expression between the two isotypes.

Recombinant *F. hepatica* thioredoxin expressed in bacteria using the pMAL™ Protein Fusion and Expression System was used to test its affects on the production of super oxide anion by murine peritoneal macrophages, bovine monocyte-derived

macrophages and bovine whole blood neutrophils, and nitric oxide production by mouse peritoneal macrophages and bovine monocyte-derived macrophages. The results of the cellular assays were not definitive due to the fact that the maltose binding protein (MBP) moiety of the recombinant thioredoxin, when tested alone, increased production of nitric oxide by bovine monocyte-derived macrophages. Consequently, since the MBP could not be effectively separated from the thioredoxin portion of the recombinant, allowing the thioredoxin affects to be tested independently, no true conclusions regarding its affects on the host immune cells tested could be drawn.

This is the first report of the localization of thioredoxin in both the adult *F. hepatica* as well as in specific intermediate stages of the parasite. These studies demonstrate the possible affects that a protein tag can have on experimental results and demonstrate how such data may be interpreted when a non-cleaved recombinant protein is used in cellular or other assays when compared to native or cleaved recombinant proteins.

DEDICATION

This dissertation and all the work done to get to this point are due entirely to my family, especially my wife Carol and daughter Genny. Without their support and encouragement over the years this never would have been completed.

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I would like to thank the individuals at Texas A & M University who have provided support, guidance and friendship during my tenure there, particularly the administrative and support staff of the Departments of Veterinary Pathobiology and Medical Biochemistry and Genetics.

Additionally, I would like to thank the administration and staff of the U.S. Meat Animal Research Center, and the Great Plains Veterinary Education Center, both in Clay Center, Nebraska for the generous use of their facilities.

Much of the work described within this dissertation would have been much more difficult to achieve without the assistance of the technical, support, and secretarial staffs at both of these facilities – for their unselfish help and good humor, I am deeply indebted.

Finally, I owe immeasurable thanks to Dr. Judy Ball for all of the effort and stress she dedicated to helping a graduate student she basically didn't know, but fought for anyway. May you be truly blessed!

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

The earliest evidence of the common liver fluke *Fasciola hepatica*, was found

between 1996 and 1999 at a large-scale, multi-period archeological site. The excavation

was undertaken at Karsdorf in the Saale-Unstrut Valley in Central Germany. Human

habitation of the site dated from the middle Neolithic to the Roman Iron Age,

approximately 3,500 BC to 200 AD. Within the excavation area, a cemetery dating from

the late Neolithic to the middle of the third millennium BC was discovered. Along with

horses and cattle, more than 70 human skeletal remains were found. Soil samples from

the pelvic area of a single human skeleton and that of a bovine burial were found to

contain the recognizable eggs of *F. hepatica*. Supportive evidence of the existence of

the full parasite life cycle at the site was also found, within the excavation area small

snail shells belonging to the genus *Lymnea* were uncovered (Dittmar and Teegen, 2003).

The earliest mention of *Fasciola* sp. in the historical literature was by Jean de

Brie in France, 1379, where he described the consequences of "sheep liver rot" in his

treatise on sheep management and wool production (Reinhard, 1957). However, as seen

from the evidence presented above, this is a very ancient parasite and coexistence with

man and his animals predates its first appearance in the literature. Since de Brie,

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This dissertation follows the style and format of Veterinary Parasitology.

F. hepatica has been reported from every continent of the globe with the exception of Antarctica. In addition to humans, cattle, and sheep, it has also been found in goats and at least 46 other species of both domestic and wild mammals, infected either naturally or experimentally (Boray, 1969). Taxonomically, it falls into Class: Trematoda, Order: Digenea, Family: Fascioloidae. Other members within the family include *Fasciola gigantica, Fasciolopsis buski*, and *Fascioloides magna*, all of which are of either human or veterinary importance. Both *F. hepatica* and *F. magna* (Figure 1.1) are found within the continental United States, and *F. gigantica* is present in Hawaii.

Figure 1.1: (A) Adult *Fasciola hepatica* and (B) adult *Fascioloides magna*.

History

As an adult parasite found in the livers of humans and sheep, *F. hepatica* had been known to farmers and scientists for hundreds of years with the first written descriptions appearing in Europe around 1523 (Reinhard, 1957). In 1668, Francesco Redi published the first drawing of an adult fluke that had been removed from the liver of a ram. Then in 1698, Govard Bidloo, an anatomy professor at The Hague, wrote and illustrated a 34-page book devoted entirely to the sheep liver fluke. In it he described finding the adult flukes in the livers of sheep, calves, and men, as well as observing the eggs within the adult fluke and attributing the means by which an animal becomes infected to the swallowing of the eggs or the flukes themselves. Throughout Europe, there were several outbreaks of sheep liver rot, the most severe being those in Holland in 1562 and 1674, and one in Germany in 1663. At the time, most people realized that while having something to do with the presence of the flukes in the affected animals' livers, the disease was attributed to divine justice or to the eating of "bad plants". The bad plant theory was related to the fact that the majority of cases of liver rot occurred in those animals that were pastured in low-lying or poorly drained areas. It was not until 1758 that the parasite was named *Fasciola hepatica* by Linnaeus. The Latin derivation of the name "fasciola" meaning fillet or small bandage, referring to its appearance, and "hepatica" meaning liver, referring to its location in the host (Borror, 1971).

During the winter of 1879-1880 a severe outbreak of liver rot occurred in Britain, resulting in the death of over three million sheep. It was after this that the Royal Agricultural Society of England offered a grant for the investigation into the natural history of the parasite that by this time was believed to be the direct cause of the disease.

So, in 1881 from work started one year before in the summer of 1880, and after more than 500 years since the first mention of the parasite, two individuals working in different laboratories, A. P. W. Thomas in England and Rudolf Leuckart in Germany, elucidated the complex life history of *F. hepatica*.

Description

When fully mature, the dorsoventrally flattened adult fluke reaches a size of approximately 30 mm long by 13 mm wide. The adults are somewhat leaf-shaped and have a narrow cephalic cone at the broad anterior end. Two anterior suckers are present, an oral sucker at the tip of the cephalic cone and a ventral sucker located at the level of the "shoulders". The ventral sucker functions as an organ of attachment, while the oral sucker is the opening to the pharynx and the digestive tract. The digestive tract consists of a pair of highly branched intestinal ceca that extends to the posterior end of the body. A single branched ovary lies to the right side of the midline and slightly posterior to the ventral sucker with the coils of the uterus situated between the ovarian branches. Two extensively branched testes, one anterior to the other, lie posterior to the ovary occupying a considerable portion of the remaining body. Numerous vitelline glands extend along the sides of the body from the area of the shoulders to the end of the body where they are confluent behind the testes (Olsen, 1974).

Life Cycle

As was shown by Thomas and Leuckart (Reinhard, 1957), *F. hepatica* exhibits a life cycle typical of all digenetic trematodes with two stages of multiplication; one that is sexual in the adult stage of the parasite and the other that is asexual in the larval or

intermediate stages. In short, the life cycle of the fluke consists of seven phases: 1) development to the adult in the definitive host; 2) passage of the eggs from the definitive host; 3) embryonation of the eggs in the environment; 4) hatching of the miricidia in water and its search for a snail intermediate host; 5) development of larval stages in the snail; 6) emergence of cercaria from the snail and successful encystment of the metacercaria; and 7) ingestion of the metacercaria by the definitive host.

Starting with the adult parasite in the biliary system of the liver of the definitive host, the adults lay on average between 8,000 and 25,000 eggs per day. While an individual fluke is hermaphroditic, cross-fertilization between two adult flukes is believed to be the most common form of sexual reproduction (Chen and Mott, 1990). The unembryonated eggs pass out of the liver via the common bile duct into the small intestine and are voided with the feces of the host. The anaerobic conditions found within the fecal mass prevent any development; therefore, only those eggs free in water will embryonate to hatching. Under the proper conditions, hatching can occur in as little as 9 to 10 days. Development is temperature dependant, slowing down but continuing at lower temperatures and ceasing at or below 10°C. When activated by light stimulation, the miracidium alters the permeability of the egg membrane and the resulting increase in internal pressure causes the operculum to rupture and open (Wilson, 1968). Upon hatching, the ciliated miracidia have a life expectancy of about 24 hours, during which time a suitable snail intermediate host must be found.

Some, but not all snails within the family Lymnaeidae will serve as suitable intermediate hosts. In North America, suitable hosts include members of the following

genera; *Fossaria*, *Lymnaea*, *Pseudosuccinea*, and *Stagnicola* (Kendall, 1950; Krull, 1934; McKown and Ridley, 1995). Once out of the egg, the free-living, non-feeding miracidium will begin its search for a snail. Chemotactic structures within the miracidium triggered by substances in the snail's mucus aid in locating and directing it towards the snail intermediate host. While the miracidium possesses energy reserves that enable it to live for upwards of 24 hours, the successful penetration and infection of the snail host is more likely to occur the sooner a snail is found. Once coming into contact with the snail, the miracidium will burrow in, lose its ciliated outer coat, and transform into a sac-like sporocyst. Each sporocyst produces numerous first generation or mother redia that will then migrate to the hepatopancreas of the snail where further asexual reproduction occurs. This multiplication results in the formation of numerous second generation or daughter redia. From these second-generation redia, multiple cercaria are typically produced within 5 to 7 weeks post-infection. Once released from the snail, the motile cercaria will swim free into the water and then attach to a substrate, usually plants or the undersurface of the water film, and secrete a double-walled protective cyst around themselves. These cysts become fully infective to a definitive host within 24 hours. The process of asexual reproduction within the snail allows a single miracidium to produce from 10 to 700 metacercaria. Estimates of the longevity of metacercaria in the environment vary with some studies reporting viability of upwards of 122 days in running water to as little as 95 days in stagnant water. Others have stated that under ideal conditions, metacercaria may remain viable for as long as one year on pasture and even live for a few months on dry hay (Haseeb et al., 2002).

Once ingested by any of a number of definitive hosts, the metacercaria now containing a juvenile fluke will go through a two-stage excystment. First, while under the reducing conditions of the rumen and at a temperature of approximately 39°C, the juvenile fluke becomes activated. After passing into the duodenum, the presence of bile enzymes and salts trigger the emergence of the immature fluke by activating enzymes present in the metacercaria. These cause a hole to open in the cyst wall allowing the escape of the parasite. Once free of the cyst, the newly excysted juvenile (NEJ) fluke will penetrate the gut wall and enter the celomic cavity of the host, usually within 24 hours. Once in the peritoneal cavity, the NEJ will migrate towards and penetrate the liver of the host. From here it will spend the remainder of its life within the liver of its host. After penetration of the liver capsule, the juvenile fluke will begin a period of migration, feeding, and growth within the liver parenchyma. After a time it will then penetrate a bile duct and enter the biliary system where it will complete its development to the adult stage. The prepatent period i.e., that time from infection until eggs are detectable in the feces of the host, will vary from one species of definitive host to another. Eggs can be found in mice in as little as 6 weeks, in sheep as long as 8-12 weeks, and in cattle, typically from 12-15 weeks. Once mature, adult flukes may live in the bile ducts of the host for varying periods of time, from 9-12 months in cattle, as long as 11 years in sheep, and 9-13 years in man (Chen and Mott, 1990).

Tegument

Throughout the course of its development over its various stages and habitats, one of the most studied structures of *F. hepatica* is the tegument (Threadgold, 1963a; Wilson, 1969; Southgate, 1970; Davies, 1978; Dunn et al., 1992), in particular that of the juvenile

and adult stages (Threadgold, 1963b; Threadgold, 1967; Bennett and Threadgold, 1973). The outer layer of the tegument is a continuous living cytoplasmic syncytium, consisting of two regions that are an anucleate surface syncytium and a deeper nucleated zone. Both of these are connected to each other by cytoplasmic bridges or tubules that come from the tegumental cytons, which are nucleated cells located beneath the underlying muscle layer (Threadgold, 1967; Bennett and Threadgold, 1973; Threadgold, 1976; Hanna, 1980). It is speculated that protein expression in the tegument regulated and plays an important part in the fluke's defense against the host's immune system (Hanna, 1979; Bennet et al., 1980).

Three types of nucleated cells are found within the nucleated layer of the tegument, which are active during various stages of the fluke's development within the mammalian host. These three cell types have been named type 0 (T0), type 1 (T1) and type 2 (T2) cells on the basis of the time frame in which they are functional in the parasite. The T0 cells are the first to become active and then only within the metacercaria and the newly excysted juvenile stage. These cells are known to produce granules that are then released at the apical surface of the juvenile fluke allowing for the continual turnover of the outer tegument in response to host antibody attachment (Hanna, 1980).

When the fluke starts to undergo its liver migration the T0 cells begin to differentiate towards becoming T1 cells and start producing T1 granules. These granules differ in size from those of the T0 cells (0.20 µm vs 0.12 µm) but are also membrane-bound, spherical and very electron dense. The T2 cells are of a separate cell type and occur only among groups of T1 cells, usually in a ratio of approximately 1:2-1:3. These

two cell types can be easily differentiated using several criteria including the large dense nucleolus within the irregularly shaped nucleus of the T2 cells. But most evident is the presence of great numbers of small biconcave secretion bodies found throughout the cytoplasm of the T2 cell (Threadgold, 1967). T2 cells only produce these granules after the fluke has reached the bile duct.

From the various studies dealing with the structure and function of *Fasciola* tegument, it has been suggested that it has an organization distinctly different from that of other flukes of veterinary importance. Also, it must be considered as both a secretory epithelium as well as a protective covering, with one function being no less important that the other (Threadgold, 1967, Bennett and Threadgold, 1973).

Veterinary Importance

The economic importance of *F. hepatica* infection worldwide in veterinary medicine is well established. It has been estimated that both direct and indirect losses in excess of $30,000,000 are incurred annually by cattle and sheep producers in the United States due to fascioliasis (Malone, 1986; Malone et al., 1982). In 1969, England reported the direct losses due to condemnation of between 600-700,000 bovine livers for a total loss of approximately £1 million ($1.7 million U.S.; Haseeb et al., 2002). Direct losses are defined as those due to the condemnation of infected livers at slaughter (Foreyt and Todd, 1976; Malone, 1986; Malone et al., 1982).

Indirect losses are those that include reductions in average daily gain and reduced feed conversion ratios in feedlot cattle (Hope-Cawdery et al., 1977), reduced milk production in dairy cattle (Randell and Bradley, 1980), and reduced herd performance in cow-calf operations (Dargie, 1986; Foreyt and Todd, 1982; Mage et al., 1989; Malone et

al., 1982). Indirect damages can amount to a greater monetary loss to the producer than liver condemnation. In one university study (Hope-Cawdery et al., 1977) using cattle of different age groups and dosed with varying numbers of metacercaria, 8-9 month old calves given 600 metacercaria (which averaged only 54 adult flukes present at slaughter) showed a 8% reduction in weight gain compared to uninfected controls when slaughtered at 54 weeks post-infection. An even greater reduction (28%) was seen in animals given 1,000 metacercaria at 14-15 months of age. It was also shown that even with antihelminthic treatment, the initial performance reductions remained until slaughter. While some compensatory gains may be made after treatment, the infected cattle never did reach the performance levels of the uninfected control group. In the same study, similar trends were seen at slaughter, with the group given 600 metacercaria showing a 2.3% or a 13-pound reduction in hot carcass weight and the 1,000-metacercaria group having a 10% or 55-pound reduction, when compared to the uninfected control animals.

As one would expect, similar findings are seen with naturally infected animals. Simpson et al. (1985) estimated that in mature cows and replacement heifers there was a 2-4% death loss, 6-12 % calf loss in pregnant animals and a 40-100 pound overall weight difference seen in *F. hepatica* infected animals due to fluke infection. In Louisiana, Loyacano et al. (2002) showed that when naturally infected Angus x Brangus heifers were treated, there were significant increases in body condition scores and total weight gains when compared to untreated animals. There was, however, no mention made of performance comparisons to non-infected animals.

Reproductive performance in cattle is also affected by fluke infections. Rees et al. (1975) reported the 0.5% occurrence of prenatal infection in 1-3 week old Australian

calves. These were necropsy findings and no correlation was made between the clinical significance of finding the flukes and the calves' death. Rees speculated a possible decreased resistance to infection in advancing age and the effects this could have on the epizootiology of the disease as related to increased pasture contamination in intensively managed situations. Effects on fertility have been reported by López-Díaz et al. (1998), where studies demonstrated a delay of 39 days in the onset of first estrus when 4 month old heifers were given 600 metacercaria as compared to uninfected controls. In France, Mage et al. (1989) showed that when infected dairy cows were treated for *Fasciola*, there was a subsequent 23% increase in first insemination conception rates during the next breeding cycle when compared to non-treated control animals.

In sheep, wool growth and quality have been shown to be depressed in *Fasciola* infections with as few as 30 adult flukes (Clarkson, 1989; Dargie, 1986). Hawkins and Morris (1978) demonstrated that the number of flukes present can have a significant impact on wool quantity. They determined that an infection of 45 flukes decreased wool quantity by 14%, 117 flukes, 19%, and 230 flukes, 33%. Studies indicate that the nutritional status of an animal, in particular the intake of iron and protein, plays an important role in how severely it will be affected by fascioliasis (Berry and Dargie, 1978).

Medical Importance

The first record of human fascioliasis is that of Pallas in 1760 in a female patient found infected upon autopsy in Berlin (Grove, 1990). Whether or not fascioliasis was the cause of death or if these findings were simply incidental is unknown. At present, estimates suggest that between 2.4 to 17 million people in 61 countries are infected with

F. hepatica worldwide, with 180 million at risk (Haseeb et al., 2002; Mas-Coma et al., 1999; Rim et al., 1994). Geographically, when placed in order of the number of cases reported, the human distribution of fascioliasis is highest in South America (primarily Bolivia, Peru and Chile), followed by Europe, Africa, and Asia with the fewest cases being seen in Oceania (that area of the Pacific containing the islands of New Zealand, Australia, the Philippines, etc.). However, in many instances the distribution of human cases does not correlate well with those areas of the world where fascioliasis is a major veterinary concern (Chen and Mott, 1990; Estaban et al., 1998). With human *Fasciola* infection, the primary areas of high prevalence include the Andean countries of South America, northern Africa, Iran, and Western Europe (Mas-Coma et al., 1999).

Due to the complexities of the life cycle as stated above, as well as factors having to do with the snail intermediate hosts, human fascioliasis has an uneven geographic focal distribution even within those regions where it may be considered endemic. This can hold true even at the superegional level as is seen in Peru where prevalence ranges from a comparatively low level of 8.7% in the area of Cajamarca to 34.2% in the Mantaro Valley (Mas-Coma et al., 1999; Yanez, 2001). Several epidemiological characteristics are considered to contribute to this patchy distribution, two of which are the occupation of those individuals infected (primarily sheep and cattle herders/producers), and habitation (rural; Chen and Mott, 1990). As an example, Bjorland et al. (1995), reported an outbreak of fascioliasis among the Aymara Indians within the Altiplano region of Bolivia. In this instance it was shown that in 52% of the cases, the only factor associated with illness was the ingestion of aquatic plants while working animals in the fields. A tendency also exists towards familial clustering, in that frequently, multiple members of a

single household may become infected due to the sharing of contaminated food items (Bechtel et al., 1992; Chen and Mott, 1990; Rodriguez Hernandez et al., 1998).

While the liver and its associated biliary ducts are the most commonly affected organ systems, other ectopic locations have been reported with some frequency in infected individuals. Such unusual locations have included various subcutaneous sites (el-Shazly et al., 1993; Prociv et al., 1992), lung and pleural cavity (el-Shazly et al., 2002), intraocular (Cho et al., 1994), the wall of the cecum (Park, 1984), the epididymis, the abdominal wall, heart, pancreas, spleen, blood vessels, skeletal muscle, and brain (Chen and Mott, 1990). A particular syndrome known as halzoun or parasitic pharyngitis has been attributed to *Fasciola* in which immature flukes attach to the pharyngeal mucosa after the ingestion of raw liver, primarily from sheep or goats, frequently causing irritation and edema of the throat (Kerim, 1956). Recently, this condition has been attributed to the pentastomid *Linguatula serrata*, so some controversy as to the true etiology of this unique disease still exists (Drabick, 1987; Saleha, 1991; Schacher et al., 1969).

Protein Biochemistry

While fascioliasis and its causative agent *F. hepatica*, have been studied extensively, comparatively little research has been performed from a biochemical standpoint, in particular, protein biochemistry. The earlier works predominantly dealt with isolation and characterization of the specific protein of interest (Lammas et al., 1985; Rege et al., 1989), with few attempts at actually localizing the protein anatomically (Hanna and Trudgett, 1983; Hanna et al., 1988; Zurita et al., 1989). In recent years however, the localization of proteins using various methods such as

immunohistochemistry (Zurita et al., 1989), immunofluorescence (Marin et al., 1992; Stitt et al., 1992a), and immunogold labeling (Hanna et al., 1988; Marks et al., 1995; Smith et al., 1993), have become more routine in the study of *Fasciola* as well as numerous other helminth species (Bogers et al., 1995; Havercroft et al., 1991; Tuan et al., 1991).

Protein biochemistry studies to date consist primarily of protein isolation and localization within the adult parasites (Marks et al., 1995; Rege et al., 1989; Stitt et al., 1992b; Waite and Rice-Ficht, 1989; Waite and Rice-Ficht, 1992; Rice-Ficht et al., 1992). Some work has been carried out on newly excysted juvenile flukes or those immatures still found in the liver parenchyma (Carmona et al., 1994; Lammas et al., 1985; Stitt et al., 1992b). Very little work has been done regarding the isolation and characterization of larval stage proteins or with the localization or occurrence of adult proteins in larval stages, such as sporocysts or redia. The paucity of such studies is most likely due to the complexity of the parasite life cycle. Most of the work completed thus far has been performed on stages of the life cycle obtained relatively easily and not requiring the raising of the snail intermediate host.

One protein isolated from adult *F. hepatica* is thioredoxin (Richardson, 1994). First isolated from the bacteria *Escherichia coli* (Laurent et al., 1964), thioredoxins have since been shown to consist of a group of small redox proteins, with molecular masses of approximately 12kD. Since their original isolation, thioredoxins have been found in diverse groups of both prokaryotic and eukaryotic organisms from plants, viruses and bacteria, up to and including higher mammals. The thioredoxins appear to represent one of the most ancient functional small proteins known. Comparisons of amino acid

sequences have shown between a 50-70% homology among thioredoxins from related species. However, homologies of only 25-30% exist among those of distantly related species such as the bacterium *E. coli*, spinach, and humans (Follmann and Haberlein, 1995). While functional and species diversity exists between the various thioredoxins, only about 20 amino acids of the roughly 105-110 that comprise the protein are highly conserved, those being primarily found in and around the active site. The active site sequence of Cys-Gly-Pro-Cys is conserved among all species, whether plant or animal (Arner and Holmgren, 2000; Holmgren and Bjornstedt, 1995; Powis and Montfort, 2001).

Thioredoxins have been identified as multifunctional proteins that act together with nicotinamide adenine dinucleotide phosphate reduced form (NADPH) and thioredoxin reductase, thus composing the "thioredoxin system". A general protein disulfide-reducing system that are comprised of major cellular protein disulfide reductases, acting on the disulfide bonds in specific target proteins such as enzymes (ribonucleotide reductase, thioredoxin peroxidase and methionine sulfoxide reductase), hormones, and storage proteins (Arner and Holmgren, 2000; Follmann and Haberlein, 1995). Due to its negative redox potential of around -240 mV, reduced thioredoxin should be capable of reducing disulfide bonds in various biomolecules. In fact, it has been demonstrated that its actions are limited to an estimated 20-30 specific targets, encompassing processes that are primarily regulatory or catalytic in nature (Follmann and Haberlein, 1995).

Structural studies such as X-ray crystallography and two-dimensional proton nuclear magnetic resonance have shown the various thioredoxins to be compact globular proteins, each composed of a five-stranded beta sheet forming a hydrophobic core

surrounded by four alpha helices and the external surface. The conserved or active site amino acids link the second beta strand to the second alpha helix and form the first turn of the second helix. This tertiary structure protrudes from the molecule and forms what is known as the "thioredoxin fold" (Powis and Montfort, 2001).

Thioredoxin isoforms are present in most organisms with the mitochondria of those organisms having a separate but equally functional thioredoxin system. A variety of thioredoxin activities have been found be extracellular, such as inducing the chemotaxis of neutrophilic granulocytes, monocytes, and T-cells (Nordberg and Arner, 2001). Intracellularly, it can function as an antioxidant and a reductant cofactor, with its intracellular expression dependant on the cell cycle, suggesting its possible involvement in the redox regulation of the cell cycle itself (Nakamura et al., 1997). It has also been found to have functional activity within the nucleus as well as the mitochondria (Powis and Montfort, 2001). All eukaryotic cells contain numerous thioredoxins that are encoded on the nuclear genomes of these cells. When bacterial, mammalian and plant extracts are viewed, cytoplasmic thioredoxins are by far the most abundant forms overall (Follmann and Haberlein, 1995).

Thioredoxin has recently been shown to have protective activity against some host cellular immune functions. It exhibits protective action against the effects of tumor necrosis factor (TNF; Matsuda et al., 1991), hydrogen peroxide and activated neutrophils (Nakamura et al., 1997), as well as acts as an inhibitor of nuclear factor kappa B (NF-kB; Flohe et al., 1997). Another study (Fernando et al., 1992) demonstrated that thioredoxin is a component in the regeneration of proteins that have been inactivated by oxidative stress in endothelial cells.

It is well known that reactive oxygen species are an effective host defense mechanism against both intracellular and extracellular parasites (Callahan et al., 1988). Phagocytic cells such as eosinophils and neutrophils have been shown to kill parasites by undergoing a respiratory burst and releasing reactive oxygen species such as hydrogen peroxide (H_2O_2) and superoxide anions (O_2^-). With the discovery of thioredoxin in several parasite genera such as *Fasciola* (Richardson, 1994) and *Schistosoma* (Alger et al., 2002; Finken-Eigen and Kunz, 1997), and the growing evidence that thioredoxin acts as a redox-regulating molecule in the maintenance of cellular redox status (Nakamura et al., 1997), it is reasonable to speculate that thioredoxin may play a role in the protection of the parasite against the host immune response.

While the thioredoxin systems of parasitic organisms have been studied and comprehensive reviews written (Rahlfs et al., 2002), the majority of this work has dealt with protozoal parasites such as *Plasmodium* sp. (Krnajski et al., 2001; Muller et al., 2001), *Trypanosoma* sp. (Reckenfelderbaumer et al., 2000), and *Giardia duodenalis* (Brown et al., 1996). On a lesser scale, work dealing with the thioredoxin system of helminths has centered primarily around nematodes such as *Onchocerca volvulus* (Lu et al., 1998) and *Brugia malayi* (Ghosh et al., 1998), with little research directed towards the platyhelminths i.e., cestodes and trematodes (Table 1.1).

Table 1.1. National Library of Medicine - PubMed search results as of 3 May 2004 for journal articles related to thioredoxin.

Search Query	Number of Articles Found
thioredoxin	3219
thioredoxin + protozoa	151
thioredoxin + helminth	42
thioredoxin + nematode	23
thioredoxin + cestode	6
thioredoxin + trematode	11[1]
thioredoxin + Fasciola	5[1]

[1]Six references describing thioredoxin in *Schistosoma* sp. (Alger et al., 2002; Alger and Williams, 2002; Stadecker et al., 2001; Yu et al., 2001; Kwatia et al., 2000; Finken-Eigen and Kunz; 1997), and 5 references describing thioredoxin in *F. hepatica* (Maggioli et al., 2004; Jeffries et al., 2001; Salazar-Calderon et al., 2001; Salazar-Calderon et al., 2000; Shoda et al., 1999).

The first study of thioredoxin in *Fasciola* was that of Richardson (1994) reporting the expression of thioredoxin in *F. hepatica*. In this study, a series of three tegument specific clones were isolated from an adult *F. hepatica* λgt11 library. Using immunohistochemical staining, these clones were shown to be localized in the tegument surface of the spines of the parasite. Nucleotide sequence analysis showed that each of the clones shared an approximately 500 base pair region of homology with previously reported thioredoxins from various species. A later study by Salazar-Calderón et al. (2001), also using a cloned *F. hepatica* recombinant protein, showed this thioredoxin to be biologically active using an insulin reduction assay. This suggests that the protein

could be involved in protection of the parasite from reactive oxygen species produced by the infected host. A more recent study dealing specifically with the effects of *Fasciola* thioredoxin on host immune cell function is that of Shoda et al. (1999). In this work, recombinant *F. hepatica* thioredoxin was found to be only weakly antigenic to bovine T-cells and was considered to be a poor candidate for inducing protective immunity even after repeated stimulation.

The following chapters detail the investigation of *F. hepatica* thioredoxin localization in adult parasites as well as intermediate stages, and the effects recombinant thioredoxin has on the bovine and murine host immune responses, specifically as it relates to superoxide and nitric oxide production. The specific objectives for this study are as follows:

Objective 1- Localization of the tissue distribution of thioredoxin in various life cycle stages of *F. hepatica*. To accomplish this, an immunohistochemical technique using polyclonal antiserum produced against *F. hepatica* thioredoxin was used on paraffin-embedded sections of the various stages of the parasite.

Objective 2- Identification of the site or sites of *F. hepatica* thioredoxin mRNA expression and determination of differential expression of two of the known isotypes. This was accomplished using an *in situ* hybridization method utilizing probes of approximately 20-22 bases designed to differentiate between two isotypes of *F. hepatica* thioredoxin as previously reported by Richardson (1994).

Objective 3- Identification of the effect(s) of *F. hepatica* thioredoxin on host immune cell function. The effects of recombinant *F. hepatica* thioredoxin on superoxide production by mouse peritoneal macrophages, bovine monocyte-derived macrophages

and bovine neutrophils were measured. In addition, the effects on nitric oxide production

by mouse peritoneal macrophages and bovine whole blood neutrophils were examined.

CHAPTER II

MATERIALS AND METHODS

Fasciola hepatica Adult and Intermediate Stage Collection

Adult Flukes and Eggs

Adult worms, present in the common bile duct were removed from bovine livers obtained at a commercial slaughter facility. The bile duct was incised and the flukes were removed intact and placed in 1X phosphate buffered saline (PBS; 0.9% NaCl, 0.83 mM KH_2PO_4, 2.95 mM $Na_2HPO_4 \cdot 7H_2$), pH 7.4 (Life Technologies, Inc., Rockville, MD). Those to be used in later immunohistochemistry studies were then placed into a fixative solution consisting of 0.4% glutaraldehyde and 4.0% formalin in distilled water. They were allowed to remain in this fixative for no longer than four hours, at which time they were removed, rinsed in distilled water, then placed in 70% ethyl alcohol for storage until processing and embedding.

Adults used for egg collection were placed into tap water and kept at 4°C for 12-24 hours during which time the flukes expelled the contents of both the caeca and uterus. At the end of this period the adults were removed and the eggs were concentrated by use of a "Fluke-Finder" apparatus (Visual Differences, Moscow, ID), a screen mesh filtration system used to detect *F. hepatica* eggs in bovine and ovine feces. Briefly, the apparatus itself consists of two fine mesh screens of different pore size, each mounted within a short section of plastic pipe. Once fitted together, the material to be examined is washed through the device with coarse debris being caught by the upper screen while eggs and finer debris collected on the second screen, with the finest material being washed away.

After several washes, the apparatus is then separated and the contents of the second screen (eggs and fine material) are back-flushed off and into a container. The eggs are then further concentrated by gravity sedimentation, eggs and debris are placed in a graduated cylinder, then filled with water and the eggs are allowed to sink. The eggs, being heavier than some of the fine debris, will sink to the bottom of the cylinder faster with the debris remaining suspended for a longer period of time thus allowing it to be decanted. Once concentrated, the eggs are kept at room temperature (23°C) for embryonation with hatching usually occurring between day 19 and 21 of the incubation. When miracidia are first seen, the eggs are placed at 4°C for approximately three days then returned to room temperature. The resulting increase in both temperature and light will cause an almost simultaneous hatch of all viable eggs.

Miracidia

With the hatching of the eggs and release of the miracidia, the collection technique was dependent upon the proposed use of the miracidia. Those used to further the life cycle by snail infection were collected individually via aspiration using a fine tip glass pipette; whereas, those needed for protein and immunohistochemical studies had to be collected in much greater numbers. This was accomplished by a technique described by Faust et al. (1975) used for the collection of *Schistosoma mansoni* miracidia with minor modifications as described below. After hatching, the miracidia were placed into a 250-milliliter side arm flask to which a small glass test tube had been attached via plastic tubing. The flask was then filled with water to a point where the water level was slightly below that of the top of the opening of the side arm. This allowed the attached test tube to be filled with water allowing access to the miracidia. The entire flask apparatus was

then covered with aluminum foil with the exception of the end of the test tube, which was left open. At this point a light source was placed at the end of the test tube and the phototropic tendencies of the miracidia attracted them to the light and into the tube. By using this method almost all miracidia could be collected into a relatively small volume of water. Miracidia used for protein extraction were killed by placing them at $-20°C$, after which time they were pelleted by centrifugation. Excess water was removed and the pelleted miracidia were then flash frozen with liquid nitrogen and stored at $-80°C$ until further processing. Those to be used for immunohistochemical staining were fixed as described for the adult parasites and stored in 70% ethyl alcohol.

Snail Culture

Snails (*Pseudosuccinea columella*) used in this study were obtained from a laboratory colony established by Dr. Robert K. Ridley of Kansas State University. These snails had been in continuous laboratory culture since first collected from Labette County, Kansas, in 1988. The snails were kept at room temperature in modified aquaria consisting of plastic storage boxes with lids. A modification (McKown and Ridley, 1995) of the technique described by Whitlock et al. (1976) was used in which a soil-bentonite-agar slope was poured at one end of the aquaria so that the snails could crawl out of the water. Preparation of the agar slope consisted of dissolving 2.5 g of bacteriologic agar in 400 ml of artificial spring water by gentle boiling. To this, 0.5 g gelatin was added. A mixture of 300 g soil and 5 g bentonite was then added to the boiling solution. This mixture was heated continuously and stirred for approximately 5 minutes. The warm soil agar mixture was then poured into the plastic containers, one end of which had been elevated so that the agar would form a gentle slope when cooled.

Water used in the snail culture aquaria was again prepared as described by Whitlock et al. (1976). Briefly, stock solutions of the following were made using double distilled deionized water 1) ferric chloride, 0.25 g/liter; 2) calcium chloride, 11.0 g/liter; 3) magnesium sulfate, 10.0 g/liter; 4) phosphate buffer - prepared by dissolving 34 g potassium acid phosphate in 500 ml double distilled deionized water, to which was added approximately 175 ml 1N sodium hydroxide until a pH of 7.2 is reached. Ammonium sulfate, 1.5 g, was then added to this solution and the volume brought to 1 liter with distilled water. Just prior to use, the following proportions of stock solutions were added to 1 liter of deionized water: 1) 0.5 ml; 2) 2.5 ml; 3) 2.5 ml; 4) 1.25 ml.

The snails were fed a commercial fish food (Tetramin, TetraWerke, Dr. rer.nat. Ulrich Baensch GmbH, D-4520 Melle 1, Germany) once daily. Enough food was placed in each aquarium so that all was eaten prior to the next feeding. It was found that any uneaten food would quickly decompose and foul the water, requiring more frequent cleaning of the aquaria.

Snail Infection

Viable miracidia were collected from egg cultures by pipette at the time of hatching. Laboratory raised snails having a shell length of less than 3 mm were used. Infection was accomplished by placing a single snail into a well of a 24-well microtiter plate containing enough artificial spring water to cover the snail. To this was added 5-10 active miracidia per well per snail. The snail was left in the well for 12-24 hours to allow sufficient time for miracidial penetration. At the end of the exposure period, the snail was removed and returned to a clean aquarium in which only other exposed snails were housed. Each well of the microtiter plate was then examined by use of a dissecting

microscope to determine if any miracidia remained. The progress of the infection was checked weekly beginning day 21 post-infection by randomly selecting one or two snails from the test group and crushing them to check for asexual stages of the parasite.

Sporocysts

The first intermediate stage collected, were sporocysts. Due to the close tissue association of this stage to that of the snail host, it was impossible to dissect the parasite away from the snail tissue. Consequently, the entire snail was fixed and processed for sectioning and microscopic examination. Prior to fixation each snail to be examined was relaxed and anesthetized by placing menthol crystals on the surface of the water. The snail was left until it no longer responded to external stimuli and was fully anesthetized which was indicated by a failure to withdrawal into the shell when touched. At this point the snail was placed into fixative consisting of 0.4% glutaraldehyde and 4.0% formalin in water. It was left for no longer than four hours after which the snail was briefly rinsed in distilled water then placed into 70% ethyl alcohol for storage until later processing and embedding. After each snail was embedded in paraffin, several 5 μm sections were cut with every fourth section being stained with hematoxylin and eosin by standard histological methods. Each of the stained sections was then microscopically examined for the presence of one or more sporocysts. If sporocysts were seen in a particular slide section the unstained sections preceding and following the stained section were saved for immunohistochemical staining.

Redia

As stated previously in the introduction, two redial stages are found in the snail, the first being what is termed the mother redia and the second the daughter redia. These

were collected separately from snails solely on the basis of the time of examination post infection. Mother redia were collected from day 10 through day 18 post-infection and daughter redia were collected any time after day 21 post-infection. One stage could be distinguished from the other by internal contents, i.e., whether they contained additional redia (mother) or cercaria (daughter). Each stage was collected by first anesthetizing the snail as described previously, then crushing the snail and teasing apart the tissues in order to separate the parasite stages from the snail tissue. Once separated from the snail tissues, the redia were aspirated via pipette and fixed as described previously in the combination glutaraldehyde/formalin solution for a short period then placed in 70% ethyl alcohol for storage. Additional redia were collected and placed into 1.5 ml microcentrifuge tubes, allowed to settle, then excess water was removed and the redia pellet was then flash frozen in liquid nitrogen and stored at –80°C for later protein extraction.

Cercaria

Cercaria were collected in one of two ways, either by crushing snails in the latter stages of infection, usually 35-40 days post-infection, or by collecting those cercaria shed naturally, which occurred around 45-70 days post-infection. Whatever the time of collection, the cercaria were treated differently dependent upon their subsequent use. Those used for protein analysis were frozen and stored as described for redia and those to be used for immunohistochemistry were fixed and processed as described for previous stages.

Metacercaria

When cercaria were seen in a specific aquarium, the snails were removed and placed into individual covered petri dishes where the cercaria were allowed to encyst on the sides of the dish. Once cercaria were no longer seen to be swimming in the petri dish, the snails were removed and the metacercaria were allowed to remain for a minimum of 24 hours in order to become fully encysted. At the end of the 24 hours period, the metacercaria were either scraped off the glass or were digested off using a 20% solution of sodium hypochlorite (5.25% household bleach) in distilled water, as described by Fried and Stromberg (1985). After the metacercaria were freed, they were rinsed with distilled water and either stored at 4°C, fixed, or frozen as described previously.

Mouse Infection

Each animal was inoculated *per os* with a suspension of 0.5 ml distilled water containing metacercaria. The suspension was administered via a small diameter (1 mm outside, 0.8 mm inside diameter) stomach tube attached to a 3 ml plastic syringe. Each mouse was anesthetized with methoxyflurane to lessen discomfort and to facilitate ease of tube placement. After intubation, the metacercaria were expelled from the syringe into the stomach. Each rodent was then returned to its individual cage and observed until recovery. Little or no discomfort appeared to be experienced by the mice and no complications occurred in relation to the procedure. All procedures were carried out as described and approved under Animal Use Protocol RF#93-685.

Juvenile Flukes

Juvenile flukes were collected from experimentally infected mice 14 to 40 days post-exposure. The process of collecting the young flukes, those still found within the

liver parenchyma prior to entry into the bile duct, was initiated by first removing the intact liver from a previously infected mouse host and placing it into PBS, pH 7.4. Once in the PBS the liver was either cut into 3-5 mm thick slices or carefully teased apart with blunt dissection and allowed to set for 30-60 minutes during which time any flukes present would extricate themselves from the surrounding liver tissue and remain free in the medium. Any flukes recovered were washed in fresh cold PBS then either flash-frozen and stored at −80°C for protein isolation or placed into fixative for use in immunohistochemistry.

Parasite Soluble Protein Preparation and Analysis

 <u>Preparation of Soluble Parasite Protein</u>

Protein was isolated from the following *F. hepatica* life cycle stages; adult worm, egg, miracidia, redia, cercaria and metacercaria. The initial step in the soluble protein isolation differed somewhat between the adult worms and all other stages. Adult worms were placed into a previously chilled mortar, covered with liquid nitrogen and ground into a fine powder using a pestle. Approximately 0.5 g of powdered tissue was transferred to a 30 ml capacity glass tissue grinder containing 15 ml of a protease inhibitor buffer on ice. The buffer consisted of a working solution of the Complete™ Protease Inhibitor Cocktail (Roche Diagnostics Corporation, Indianapolis, IN) prepared as per the manufacturer's instructions. Due to their small size, all other stages of the parasite were processed *en masse*. The intermediate parasite stage pellet was thawed in the presence of the protease inhibitor buffer and then placed directly into a 2 ml capacity glass tissue homogenizer. From this point on, the processing of the parasite protein was the same for all stages.

Once the powdered adult or pellet of one of the intermediate stages was placed in the glass tissue homogenizer on ice, it was homogenized for 5-10 strokes. The resulting suspension was transferred to a polycarbonate tube, centrifuged at 30,000 x g for 30 minutes at 4°C to pellet insoluble material. The supernatant was then aliquoted, flash-frozen in liquid nitrogen and stored at –80°C until used.

Determination of Protein Concentration

The protein concentration of each supernatant sample was determined by using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL). The microtiter plate protocol was followed as per the manufacturer's instructions. Each assay plate contained a standard curve prepared using dilutions of the 2.0 mg/ml bovine serum albumin (BSA) stock standard provided ranging from 0 µg/ml to 2,000 µg/ml. Absorbance of the wells was measured at 560 nm on an ELISA plate reader, and protein concentration was determined by regression analysis of the sample absorbance compared to the standard curve.

Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were separated according to molecular weight utilizing Ready Gel Precast denaturing 4-20% Tris-HCl gradient gels (50 µl comb, BIO-RAD, Hercules, CA). The samples were diluted 1:1 in 2X SDS sample buffer (6.25 ml 4X Tris-HCl/ SDS [6.05 g Tris base, 0.4 g SDS], 5.0 ml glycerol, 1 g SDS, 0.5 ml 2-ß- mercaptoethanol, 0.25 mg bromphenol blue) and boiled for 5 minutes prior to loading. The samples were loaded along with a lane containing a molecular weight standard (Kaleidoscope Prestained Standards, BIO-RAD, Hercules, CA) and the gel was electrophoresed in a Mini-PROTEAN II gel chamber (BIO-RAD, Hercules, CA) containing 1X SDS-PAGE

running buffer (3.02 g Tris base, 14.4 g glycine, 1 g SDS) at constant 180 volts using a

PowerPac 300 power supply (BIO-RAD, Hercules, CA) for approximately 45 minutes or

until the bromphenol blue tracking dye front reached the bottom of the gel.

Coomassie® Brilliant Blue Staining of Polyacrylamide Gel

Coomassie® Brilliant Blue (BIO-RAD, Rockford, IL) was used in order to resolve

the protein bands separated by polyacrylamide gel electrophoresis. The detection limit of

this procedure is 0.3 to 1 μg protein per band (Sasse and Gallagher, 1991), and is

dependent upon the nonspecific binding of the stain to the proteins. Briefly, after

electrophoresis, the polyacrylamide gel was removed from the gel apparatus and

transferred to a fixing solution consisting of 500 ml methanol, 100 ml glacial acetic acid

and 400 ml distilled water. The gel was then allowed to equilibrate with gentle agitation

for a minimum of two hours before being transferred to the staining solution. The

staining solution was identical to the fixation solution with the addition of 0.5 gm

Coomassie® Brilliant Blue R-250. The gels were stained for 2-4 hours, and were placed

into a destaining solution containing 5 ml methanol, 7 ml glacial acetic acid and 88 ml

distilled water until the desired staining intensity of the protein bands was achieved.

Polyacrylamide Gel Drying

To provide a permanent record of the gel results each gel was dried using the

DryEase™ Mini-Gel Drying System (NOVEX - Novel Experimental Technologies, San

Diego, CA) as per the manufacturer's instructions. Briefly, the gel was washed in

distilled water prior to equilibration in Gel-Dry Solution and was then sandwiched

between two sheets of cellophane, which were also equilibrated in Gel-Dry solution.

These layers were in turn clamped into a gel-drying frame, which was set upright to dry

at ambient temperature for 2-3 days. When the cellophane was dry to the touch, it was removed from the drying frame, the excess trimmed from around the gel, and allowed to dry completely for approximately 2 days. The gels were then stored at room temperature where they were stable indefinitely.

Electrophoretic Transfer of Protein from SDS-PAGE to Nitrocellulose

Once the proteins were separated using the SDS-PAGE procedure they were electrophoretically transferred to nitrocellulose paper utilizing a Mini Trans-Blot Electrophoretic Transfer Cell (BIO-RAD, Hercules, CA) as per the manufacturer's instructions. Briefly, after electrophoresis, the gel was removed from the SDS-PAGE apparatus and equilibrated in –20°C transfer buffer (3.03 g Tris base, 14.6 g glycine, 200 ml 100% methanol, QS to 1 liter with deionized water) for approximately 15 minutes. Nitrocellulose membrane (0.45 µm Trans-Blot® Transfer Membrane, BIO-RAD, Hercules, CA) and Whatman 3 mm CHR chromatography paper (Whatman International, Ltd., Maidstone, England) were cut to a size slightly larger than the size of the gel and were wetted in transfer buffer prior to the assembly of the transfer apparatus. The gel transfer "sandwich" was assembled in the following order: 1) pre-wetted fiber pad, 2) chromatography paper, 3) polyacrylamide gel, 4) nitrocellulose, 5) chromatography paper, and 6) fiber pad. The gel holder cassette was then closed, locked and placed into the electrode module within the buffer tank containing a Bio-Ice® cooling unit (BIO-RAD, Hercules, CA) and filled with cold transfer buffer. The cassette was placed with the nitrocellulose membrane on the anode (positive) side of the transfer apparatus allowing the negatively charged proteins to be transferred electrophoretically from the gel onto the nitrocellulose membrane. The transfer was completed by running at constant

voltage (100 V) for 60 minutes utilizing the PowerPac 300 power supply (BIO-RAD, Hercules, CA).

Protein Immunodetection

Western Blotting

In order to detect the thioredoxin protein of interest, Western Blotting was used whereby proteins are analyzed immunologically by means of labeled antibody. Antibody against *Fasciola* thioredoxin (Fh2020) was obtained from Dr. Charlene Richardson and Dr. Allison Rice-Ficht. Once the proteins were transferred onto a nitrocellulose membrane, the membrane was placed into a solution of 3% gelatin (3 g gelatin, 100 ml tris buffered saline (TBS; 10 mM Tris, 150 mM NaCl), pH 7.5, for 45 minutes. The membrane was washed 3 times for 10 minutes per wash using TBS containing a 0.1% concentration of Tween® 20 (Sigma-Aldrich, St. Louis, MO), then exposed to primary antibody (rabbit anti-Fh2020) at a 1:1000 dilution in 1% gelatin for 2 hours. Unbound antibody was removed by a series of three 10 minute TBS-Tween washes. The membrane was then exposed the alkaline phosphatase-labeled goat anti-rabbit secondary antibody (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) at a 1:5000 dilution in 1% gelatin for 1 hour. Unbound antibody was removed by a series of two 10-minute TBS-Tween washes, followed by a single 10 minute TBS wash. The protein bands were identified on the membrane by a color reaction utilizing the chromagens nitrotetrazolium blue chloride (NBT; Sigma-Aldrich, St. Louis, MO) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Sigma-Aldrich, St. Louis, MO). The NBT/BCIP solution was prepared using 33 µl NBT stock (50 mg/ml in 70% dimethyl formamide) plus 17 µl BCIP stock (50 mg/ml in 100% dimethyl formamide) per 5 ml alkaline phosphate substrate buffer

(100 mM Tris, pH 9.5, 100 mM sodium chloride, 5 mM magnesium chloride). The membrane was soaked with gentle agitation in this solution until the band or bands of interest were easily seen. The color reaction was terminated by rinsing the membrane several times in PBS. The membrane was patted dry with paper toweling and placed between several layers of toweling. A light-weight was added to the top and the membrane was allowed to dry flat for 24-48 hours.

Sample Handling for Immunohistochemistry

Fixation of Tissues

All parasite stages and mouse tissues were fixed using a combined solution of 0.4% glutaraldehyde and 4.0% formalin. Minute samples such as the intermediate stages of the parasite, (redia, cercaria, etc.) were first suspended in melted agar that was allowed to cool so that a large agar block was available for ease of further handling. This agar block and small newly infected snails were fixed whole. Adult flukes and mouse tissues were cut into pieces no more than 2 mm in thickness. Dependent upon the size/thickness of the specimen, each was fixed for approximately 1-2 hours, but no longer than 4 hours, even for the thickest sections of mouse liver. After the initial fixation period all samples were rinsed with distilled water then placed in 70% ethanol for storage.

Processing and Embedding of Tissues

All samples to be used for immunohistochemical study were processed and embedded in paraffin (Paraplast® Tissue Embedding Medium, Oxford Labware, St. Louis, MO) via standard histological procedures (Stevens and Wilson, 1996). Sectioning was done at 5-7 μm with each section then floated on warm water and then placed on a silanized slide.

Silanization of Glass Slides

For increased adherence of tissue sections to the glass slides used for both immunohistochemistry and *in situ* hybridization each slide was silane-coated prior to tissue placement on the slide. This procedure, commonly known as "subbing", was performed as follows. A large number of slides were coated at one time using staining racks with a 20-slide capacity. Solutions used in the procedure were placed in 300 ml staining dishes to facilitate rapid movement of a rack from one solution to the next. The slide were initially placed in 100% acetone for 2 minutes then drained on paper toweling. Next they were placed into a solution of 2% aminopropyltriethorsilane (silane; Sigma-Aldrich, St. Louis, MO) in acetone for 2 minutes, then drained. This was followed by three double-distilled deionized water washes of five dips each. Excess water was drained and the slides were dried in a 60-80°C oven overnight. The adhesiveness of the slide was checked by dipping it into deionized water; if properly coated the water quickly dispersed and the slide appeared dry, otherwise it remained wet in appearance.

Immunohistochemistry

Protein Localization

Immunohistochemical staining and localization of the thioredoxin protein within various parasite life cycle stages was accomplished via the use of the Histomark[TM] Streptavidin-HRP System (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) using rabbit derived anti-thioredoxin primary antibody and biotinylated goat-derived anti-rabbit secondary antibody as provided in the Histomark[TM] kit. The staining procedure started by placing both a negative control slide (no primary antibody) through two xylene deparaffinizing steps of 5 minutes each. Each slide was then rehydrated through a

decreasing series of four one-minute ethanol steps, decreasing in concentration from 100% (twice), to 95% (once), to 80% (once), to TBS. The slides were then tipped and excess TBS allowed to drain and 3% hydrogen peroxide flooded over the slide and section for five minutes in an endogenous peroxidase-blocking step. This was followed by another TBS rinse after which the rabbit anti-thioredoxin primary antibody was applied at a 1:500 dilution and was allowed to incubate either for one hour at room temperature or overnight at 4°C.

At the end of the primary antibody incubation, the slides were rinsed with TBS and biotinylated goat anti-rabbit secondary antibody was applied to each section for a 30-minute incubation. The slides were rinsed with TBS and streptavidin peroxidase was applied to each section for a period of 30 minutes. The slides were again rinsed with TBS and the color detection reaction started by the addition of the chromogen diaminobenzidine (DAB; Diaminobenzidine Reagent Set, Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) as per the manufacturer's instructions. The proper color intensity was determined by viewing each section microscopically until the desired difference between intense positive staining and low background could be visually determined. At this point, each slide was rinsed with tap water and then counterstained with Harris's hematoxylin to provide a light blue background and nuclear staining against which to better visualize any positive brown staining. No positive staining should be seen on the negative control slide.

***In situ* Hybridization**

Preparation of Reagents and Glassware

All glassware used for *in situ* hybridization was rendered RNase free by baking at 180°C for four hours then stored covered under dust-free conditions. All prepared solutions were made using RNase-free water and stored in baked glassware.

Parasite Preparation and Fixation

Arrangements were made with a local abattoir, and a fresh *F. hepatica*-infected liver was obtained and the flukes collected and fixed within two hours of removal from the infected cow in order to minimize nucleic acid degradation. Fixation time was kept to a minimum to avoid over-fixation and all subsequent processing was done under the strictest RNase-free conditions with sections being cut within one week from the time of collection. All adult flukes, once removed from the bile duct, were rinsed in sterile PBS, pH 7.4 then placed into a fixative mixture of 0.4% glutaraldehyde / 4.0% formalin. After a period of fixation not exceeding four hours, the tissues were rinsed in distilled water and placed in 70% ethanol until processing.

Processing and Embedding

Tissues were processed and embedded according to standard histological techniques (Stevens and Wilson, 1996). Sections were cut to a thickness of approximately 5 μm, and attached to RNase-free silanized glass slides and stored under dust-free conditions at room temperature.

Design of Oligonucleotide Probes

DNA-antisense probes *S. mansoni* actin and *F. hepatica* tubulin were designed using the hybridization probe analysis option of Vector NTI, Suite5.5 sequence analysis

software (InforMax, Inc., North Bethesda, MD) using nucleic acid sequence information obtained from Genbank (http://www.ncbi.nlm.nih.gov; Accession numbers M80334, and AJ297256, respectively). Briefly, the nucleic acid sequence was entered into the Vector program along with the desired parameters of probe length ($\geq$50, $\leq$70 bases), melting temperature (Tm; $\geq$40°C, $\leq$65°C), %GC content ($\geq$35, $\leq$60), hairpin loop stem (>3), palindromes (<8) and nucleotide repeats (<4).

Nucleic acid sequences for Fh2020.A and Fh2020.SL were obtained from Richardson (1994). Because these isotypes are highly homologous, isotype-specific probes were designed by comparing the sequence differences at the 5' ends (the differences between the two isotypes were seen within the first 36 bases), and selecting probes that were complementary to a region of 20-25 bases from the 5' end. The selected probes were then analyzed using the hybridization probe analysis option of Vector NTI, Suite5.5 sequence analysis software (InforMax, Inc., North Bethesda, MD) to determine if they fit the parameters used to select the *S. mansoni* actin and *F. hepatica* tubulin probes.

All probes were checked for specificity for their respective proteins by performing BLAST sequence analysis (http://www.ncbi.nlm.nih.gov/BLAST).

Digoxigenin Tailing of Probes

Unlabeled oligonucleotide probes (Fh2020.A, Fh2020.SL*, S. mansoni* actin, and *F. hepatica* tubulin) were purchased from Integrated DNA Technologies, Coralville, IA. Digoxigenin labeling was carried out as per manufacturer's instructions using the DIG Oligonucleotide Tailing Kit, 2nd Generation (Roche Diagnostics GmbH, Mannheim, Germany).   Briefly, the required reagents were added to 100 pmol of unlabeled probe (*F.*

hepatica tubulin, *S. mansoni* actin, Fh2020.A or Fh2020.SL) in distilled water, incubated at 37°C for 15 minutes and the reaction stopped using 0.2 M EDTA. This procedure resulted in the binding of 10-100 DIG labels to each probe.

Dot Blot

In order to test that the DIG label was attached to the probes, a dot blot procedure was performed individually for each probe after the labeling reaction. The unlabeled controls were tested as well. Briefly, after each labeling reaction 1 µl (100 ng) of probe was spotted onto a nylon membrane (Zeta-Probe Membrane, Bio-Rad, Hercules CA), air-dried and UV irradiated to bind the probe to the membrane. The membrane was then washed three times for 10 minutes each wash in TBS buffer containing 0.1% Tween® 20 (Bio-Rad Laboratories, Hercules, CA). The membrane with its associated DIG-labeled probe dots was then placed in AP-conjugated sheep anti-DIG antiserum (1:500 dilution) in TBS buffer containing 1% gelatin at room temperature for 2 hours. The membrane was further washed with TBS buffer containing 0.1% Tween® two times for 10 minutes each and finally, once for 10 minutes. Label was detected using the chromagen NBT/BCIP. The NBT/BCIP visualization solution was prepared using 33 µl NBT stock (50 mg/ml in 70% dimethyl formamide) plus 17 µl BCIP stock (50 mg/ml in 100% dimethyl formamide) per 5 ml alkaline phosphate substrate buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM MgCl).

Calculation of Hybridization Temperature

The T_M for each of the probes was calculated using the following formula:

$$T_M = 81.5 + 16.6(\log M) + 0.41(\%G+C) - 0.61(\% \text{ formamide}) - 500/n$$

$M = Na^+$ concentration in moles/liter
n = shortest chain in the hybrid

For DNA:RNA hybridization such as that used in this study, 10-15°C was added to the result. The optimal hybridization temperature was calculated to be 25°C below the T_M of the hybridized probe.

In situ Hybridization Protocol

This protocol was based on the manufacturer's instructions for the Rembrandt® *In situ* Hybridization and Detection Kit (PanPath, Amsterdam, The Netherlands). Briefly, two sections were mounted per slide so that both a test and negative control could be processed simultaneously. Each slide was deparaffinized in two changes of xylene then placed in 100% ethanol. A proteolytic digestion step using hydrochloric acid and pepsin as provided in the kit was carried out on each section at 37°C for 30 minutes. Each slide was then dehydrated through a series of graded ethanols (70%, 80%, 95%, 100%, 100%), for one minute each, then air-dried. Each section was then covered with hybridization solution containing the appropriate probe at a concentration of 150-200 ng per section and incubated at 37°C for two hours. The hybridizations were carried out utilizing a PTC-200™ Thermal Cycler with block/heat pump assembly and model SG96P glass slide adapter (MJ Research, Inc., Watertown, MA). At the end of that time the coverslip was removed by soaking in TBS and the slide was further washed with three additional TBS rinses. At this time, 2-3 drops of conjugate (AP-conjugated anti-DIG as provided in the kit) was added to each section and incubated at 37°C for 30 minutes. The slide was then rinsed with TBS and a detection step using NBT/BCIP substrate was carried out at 37°C for 15 minutes after which the slides were washed with distilled water, dehydrated and coverslipped. Probes tested with this protocol included, *F. hepatica* tubulin (the

positive control), *S. mansoni* actin (an additional positive control), and thioredoxin isotype Fh2020.A and Fh2020.SL probes.   Negative control slides consisted of sections that had mRNA hybridization solution (DAKO Corp., Carpinteria, CA) without probe. Additionally, a negative control probe included in the Rembrandt[®] kit consisting of 100 bases of a DIG-labeled pSP vector sequence was utilized to detect non-specific binding. When this probe was applied to the fluke tissue under the same hybridization conditions as used for the test probes (*F. hepatica* tubulin, Fh2020.A, Fh2020.SL), no positive staining was demonstrated.

Immune Cell Collection and Isolation

<u>Murine Peritoneal Macrophages</u>

All mice used were 6-8 week old C57BL/10ScN, stock originally obtained from Dr. S.K. Chapes, Kansas State University. Peritoneal macrophages were obtained by intraperitoneal injection of thioglycollate as described by Meltzer (1981). Briefly, a solution of 3% thioglycollate medium (Sigma-Aldrich, St. Louis, MO) was prepared in distilled water then autoclaved to sterilize. Four to five days prior to the date macrophages were used, approximately 1-1.5 ml of media was injected into the peritoneal cavity of a donor mouse to initiate an inflammatory response. At the end of this period the mouse was euthanized via a gas anesthetic overdose. The abdomen was wetted with 70% ethyl alcohol and massaged gently between the fingers which increased the cell yield by freeing the inflammatory cells within the cavity (Smith et al., 1992). The skin was wetted again with 70% ethyl alcohol and a small incision made with scissors and the skin retracted to each side exposing the abdominal musculature. At no time during the procedure was the abdomen itself opened. Once the skin was reflected, the muscles were

picked up in a tent-like manner using sterile forceps. An 18 gauge needle attached a 12 ml syringe filled with cold PBS was inserted at the midline, while at the same time a small amount of PBS was injected to "push" away the intestines in order to avoid contamination of the abdominal cavity with gut contents. Each mouse was injected with 8-12 mls of PBS that was then withdrawn into the same syringe. Without removing the needle from the abdomen, the syringe was detached and the contents of the syringe placed into a sterile 50 ml plastic conical centrifuge tube. The syringe was again filled with cold PBS, reattached to the needle and PBS again injected into the abdominal cavity. This process was repeated 2-3 times resulting in a total yield of 30-36 mls of cellular fluid. If performed properly, contamination of the recovered cells with intestinal contents or blood does not occur. All procedures and manipulations of mice for the procurement of peritoneal macrophages were carried out under the approval of the United States Department of Agriculture Experimental Outline, Experiment # 5438-32000-013-08 and Texas A&M University Animal Use Protocol #9-173.

After the cells were harvested, the lavage fluid was centrifuged at 400 x g at 4°C for 7 minutes. The supernatant was discarded and a red blood cell lysis step was performed if necessary by adding 1 ml of Red Cell Lysing Reagent (Sigma, St. Louis, MO) to the cell pellet, resuspending the pellet then incubating at 37°C for 5 minutes. Following incubation, 40 mls of sterile PBS were added and the suspension was again centrifuged as described above to pellet the cells. The lysis step was repeated once more if red blood cell contamination was visible. The supernatant was discarded and the cells again washed in cold sterile PBS. After the final wash and centrifugation, the supernatant was discarded and the cells resuspended in 5 mls cold sterile PBS. At this time 200 µls

of cell suspension were removed and placed into a 500 µl microfuge tube for cell counting and differential staining. The remainder of the cells was placed on ice until the count was completed.

In order to plate a consistent number of cells into each test well, the number of cells obtained from each lavage procedure were counted using a hemacytometer. Briefly, 5 µl of cell suspension was added to 95 µl of 0.4% Trypan Blue Stain (Life Technologies, Rockville, MD) and gently mixed. Approximately 15 µl of the mixture was added to each side of a hemacytometer (Fisher Scientific, Pittsburgh, PA) and all cells in the four corner squares were counted, and cell concentrations calculated. Ideally, 1×10^5 cells per well were used in all experimental samples and all samples were run in triplicate.

Bovine Monocyte-Derived Macrophages

Bovine monocyte-derived macrophages were obtained from whole blood via a density gradient separation as described by Chitko-McKown et al. (2004). Briefly, 60 mls of blood were obtained via jugular venipuncture into a syringe containing 1 ml of 0.1 M EDTA as an anticoagulant. 15 mls of 1X PBS were added to each 50 ml polypropylene conical tube, then 15 mls whole blood were added and mixed with gentle agitation. The diluted whole blood was then underlayed with 14 mls of Ficoll-Paque™ Plus (Amersham Biosciences AB, Uppsala, Sweden) via gravity a 10 ml plastic pipette. The pipette was slowly removed so as to not disrupt the bilayer, the tube was capped then centrifuged at 913 x g for 45 minutes at room temperature. Following centrifugation, the mixture resolved into four layers. The uppermost layer contained serum and buffer (1X PBS), the next a cloudy layer of peripheral blood mononuclear cells (PBMCs), the next Ficoll-Paque™ Plus and the lowest contained red blood cells and neutrophils. The

plasma/PBS layer was pipetted off and discarded, and the PBMC layer was retained.

Contaminating red blood cells were removed using Red Cell Lysis solution, and the

PBMC pellet washed in cold PBS as described above. The resulting pellet was

suspended in 10 mls RPMI 1640 (Life Technologies, Rockville, MD) tissue culture

medium containing Penicillin G/Streptomycin Sulfate (Life Technologies, Rockville,

MD) and L-Glutamine (Life Technologies, Rockville, MD). A 200 µl aliquot was

removed for counting and differential staining. After the cell concentration was

determined, the cells were plated into a 24-well microtiter plate at a concentration of

$1x10^6$ cells per ml per well. The plate was then placed in a 37°C, 5% CO_2 incubator and

left undisturbed for one hour to allow the monocytes to adhere to the plate. The contents

of each well were pipetted up and down and the supernatant discarded with only adherent

cells, i.e. monocytes, remaining. One ml of fresh media containing 5% fetal bovine

serum (FBS) was then added to each well and the plate returned to the incubator and

allowed to remain until the following day when the cellular assay was carried out.

Bovine Whole Blood Neutrophils

Bovine neutrophils were obtained from the red blood cell and neutrophil layer

obtained after whole blood density gradient separation as described above. Once the

plasma and monocyte layer were removed, the RBC/neutrophil layer was suspended in

20 mls of cold 0.2 % NaCl for 30 seconds (Clark and Nauseef, 1998), which lysed the

majority of red blood cells and left the neutrophils intact. At the end of 30 seconds, 20

mls of cold 1.6% NaCl were added to restore the solution to physiological osmolarity.

The solution was centrifuged at 250 x g for 6 minutes at 5°C. Following centrifugation,

the supernatant was discarded and the RBC lysis procedure and centrifugation were

repeated 2 additional times, or until the neutrophil pellet appeared free of contaminating red blood cells. The pellet was suspended in 1 ml of Red Cell Lysing Reagent (Sigma, St. Louis, MO) and incubated for 5 minutes at 37°C to remove any remaining red cells. The neutrophils were then resuspended to a volume of 50 mls with cold PBS and centrifuged at 250 x g for 6 minutes at 5°C. The supernatant was discarded and the cold PBS wash was repeated two additional times. The remaining cell pellet was then suspended in 10 ml of cold PBS and placed on ice with an aliquot removed for counting and differential staining (Chitko-McKown et al., 1991).

Cytospin Preparation and Differential Staining

Differential staining of all cell isolates was used for confirmation of cell type recovered. This procedure was accomplished using of a Cytospin® 3 cell preparation system (Shandon Lipshaw, Pittsburgh, PA). Approximately 3×10^5 cells were resuspended in 500 µl medium and loaded into a Cytofunnel® (Disposable Sample Chamber, Shandon Lipshaw, Pittsburgh, PA). The cell suspension was centrifuged at approximately 48 x g for 4 minutes at room temperature to permit adherence of the cells onto Cytoslide® microscope slides (Shandon Lipshaw, Pittsburgh, PA). The slides were dried at room temperature and stained with a modified Wright's stain (Leukostat Stain Kit, Fischer Scientific, St. Louis, MO) and microscopically examined at 1000X to determine the types and percentages of cells present in the cell suspension.

Cellular Assays

Endotoxin Assay

The Limulus Ameboctye Lysate assay was used to detect the presence of LPS contamination in MBP that had been run through the Detoxi-Gel™ column as well as the

untreated MBP as a control. This assay was carried out as per manufacturer's instructions for the E-Toxate® Kit for the detection and semi-quantitation of endotoxin (Sigma-Aldrich, St. Louis, MO.).

<u>Insulin Reduction Assay</u>

The biological activity of the recombinant *F. hepatica* thioredoxin was determined by the dithiothreitol/insulin reduction assay described by (Holmgren, 1979) and further used and described by Salazar-Calderón et al. (2001) and Alger et al. (2002). This was a turbidity assay in which active thioredoxin served as a catalyst for the cleavage of insulin into its α and ß-chains by dithiothreitol (DTT), with the ß-chains forming an insoluble precipitate that was then detected spectrophotometrically by its absorbance at 630-650 nm.

Initially, a 10 mg/ml insulin stock solution was prepared by resuspending 50 mg of insulin powder (Sigma, St. Louis, MO) in 4 ml of 0.05 M Tris-HCl, pH 8.0 and adjusting the pH to 2.0-3.0 by the addition of 1.0 M hydrochloric acid. The pH was immediately brought up to 8.0 with 1M NaOH, and the volume adjusted to 5 ml with distilled water. The resulting solution was separated into 500 µl aliquots and frozen at −20°C. An insulin working solution (IWS) was prepared from the stock solution by adding 400 µl of 10 mg/ml stock solution, 400 µl phosphate buffer (PBS), and 16 µl of 500 mM EDTA to 3.2 ml of distilled water. The assay was carried out in triplicate in a 96-well microtiter plate with the following treatments: 1) Blank (IWS + water); 2) Control (IWS + water + DTT; demonstrates spontaneous insulin cleavage); 3) MBP Test (IWS + water + DTT + MBP at 0.23 µM / well); 4) LPS Test (IWS + water + DTT + LPS at 1 µM / well) and 5) TRX•MBP Test (IWS + water + DTT + TRX•MBP at 4 µM/well).

Each well had a final volume of 200 µl consisting of 150 µl IWS and 2 µl 33 mM DTT in all wells and additional reagents in the following proportions dependant upon the test reagent: TRX•MBP wells - 50 µl with no additional water; MBP alone and LPS wells - 1 µl MBP or LPS + 47 µl water. The test reactions were carried out on ice with the microtiter plate agitated slightly to insure mixing of all reagents. The plate was then placed within a microtiter plate reader and the absorbance was measured at 630 nm at 1 minute intervals for 15-60 minutes depending on activity/concentration of the test samples.

<u>Superoxide Assay</u>

The super oxide anion (O_2^-) assay was performed as described by Pick and Mizel (1981) to determine the production of superoxide anion by intact neutrophils and macrophages as a function of cytochrome C reduction. Briefly, 150 µl of cell suspension at a concentration of 1 x 10^6 cells per well were tested in triplicate in flat bottom, 96-well microtiter plates. Additional reagents were added in the following order and volumes: 20 µl superoxide dismutase (SOD, 3000 U/ml in colorless HANK's buffer, Life Technologies, Rockville, MD, appropriate test wells), 20 µl cytochrome C (Fc, 1 mg/ml in colorless HANK's buffer, all wells), 2 µl phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, St. Louis, MO,10 µg/ml in dimethyl sulfoxide, DMSO, Sigma-Aldrich, St. Louis, MO; appropriate test wells), and 10 µl recombinant *F. hepatica* thioredoxin (TRX, 10 µg/ml, kindly provided by Drs. W.C. Brown and L.K.M. Shoda, Washington State University, Pullman, WA; appropriate test wells), and brought to a final volume of 200 µl. The plate was incubated at 37°C for 15 minutes, removed and centrifuged at 450 x g for five minutes. The cell-free supernatant was then carefully removed and placed

into a new 96-well microtiter plate and kept on ice. The optical density of each well was then determined by reading the plate at 550 nm on a microtiter plate spectrophotometer. The μM concentration of O_2^- was determined by dividing the optical density (OD) reading by 9.5.

Nitric Oxide Assay

The procedure for this assay was the same for both macrophage types -- murine peritoneal macrophages and bovine monocyte-derived macrophages. The only difference between these assays was the chemical stimulus used, either lipopolysaccharide (LPS) for the bovine cells or peptidoglycan (Sigma - Aldrich, St. Louis, MO) for the murine cells. Purified LPS from *E. coli* O157:H7 was kindly provided by Dr. James Keen (United States Department of Agriculture - Meat Animal Research Center, Clay Center, Nebraska) and was prepared as previously described (Laegreid et al., 1998). Activity of the LPS was determined by using the E-Toxate Limulus Amoebocyte kit (Sigma - Aldrich, St. Louis, MO). Both cell type assays were carried out using 24-well microtiter plates with each column representing a single animal and each row representing a single treatment group. The four treatments consisted of 1) cells only, 2) cells plus stimulus, 3) cells plus thioredoxin and 4) cells plus stimulus plus thioredoxin. All treatments were run in triplicate.

Briefly, serum-free RPMI 1640 (Life Technologies, Rockville, MD) containing Penicillin G/Streptomycin Sulfate (Life Technologies, Rockville, MD) and L-Glutamine (Life Technologies, Rockville, MD) was added to all 24 wells of the microtiter plate, cells were then added at a concentration of 1×10^6 cells per ml per well. The cells were allowed to adhere for one hour at 37°C, 5% CO_2, the medium and non-adherent cells were

removed and medium was replaced with 1 ml fresh RPMI 1640 containing 5% FBS and antibiotics. At this time, either recombinant thioredoxin (10 μg; Shoda et al., 1999), peptidoglycan (20 μg), or both were added to the appropriate test wells. The cells were incubated for 30-36 hours after which the supernatant was collected and frozen at –80°C until nitric oxide assay.

The assay for nitric oxide was carried out as described by Stuehr et al. (1989) utilizing the Griess Reagent. Griess Reagent was made up of two separate solutions, Solution A: a 1% (w/v) solution of sulfanilamide (Sigma-Aldrich, St. Louis, MO) in a 2.5% solution of phosphoric acid (Sigma-Aldrich, St. Louis, MO) and Solution B: a 0.1% (w/v) solution of naphthylethylenediamine dihydrochloride (Sigma-Aldrich, St. Louis, MO) in 2.5% phosphoric acid. Each was kept separately at 4°C until used.

A sodium nitrite standard was established at the time of the assay using solutions of sodium nitrite (Sigma-Aldrich, St. Louis, MO) in concentrations of 0, 0.5, 1, 5, 10 and 25 μM. Triplicate 100 μl samples of each cell culture supernatant were plated into wells of 96-well, flat-bottom microtiter plates with wells containing 100 μl of medium acting as controls along with the appropriate standards. To all culture supernatants, medium controls and standards, 50 μl each of Solution A and Solution B were added and the plate incubated for 10 minutes at room temperature. The plate was then read at 550 nm on a microtiter plate reader to determine the optical density of each well, after which the nitrite concentration of all supernatant wells was determined by linear regression analysis onto the standard curve.

CHAPTER III

IMMUNOLOCALIZATION OF THIOREDOXIN (Fh2020.A) IN

ADULT AND IMMATURE STATES OF THE LIVER FLUKE,

Fasciola hepatica

Introduction

Thioredoxin, along with NADPH and thioredoxin reductase make up the thioredoxin system. Thioredoxins are small 12 kDa proteins first isolated by Laurent et al. (1964) from the bacteria *Escherichia coli*. Since its original isolation, thioredoxin has been found in diverse groups of both prokaryotic and eukaryotic organisms up to and including higher mammals. Within these species, thioredoxin performs or participates in a wide variety of biochemical processes (Arner and Holmgren, 2000; Nakamura et al., 1997), among them protein disulfide reduction, ribonucleotide reduction, light regulation of chloroplast enzymes, and sulfur metabolism. While functional and species diversity exists between the various thioredoxins, the active site sequence of Cys-Gly-Pro-Cys is highly conserved among all species (Follmann and Haberlein, 1995; Nakamura et al., 1997; Powis and Montfort, 2001).

With the discovery of thioredoxin in several parasitic genera such as *Fasciola* (Richardson, 1994; Salazar-Calderon et al., 2001), *Schistosoma* (Alger et al., 2002; Finken-Eigen and Kunz, 1997), *Echinococcus* (Chalar et al., 1999), *Plasmodium* (Rahlfs et al., 2002), and *Brugia* (Kunchithapautham et al., 2003) and with the growing evidence that thioredoxin acts as a redox-regulating molecule in the maintenance of cellular redox status (Nakamura et al., 1997), it is reasonable to speculate that thioredoxin may play a role in

the protection of certain parasites against the host immune response (Alger et al., 2002).

To this end, (Objective 1) experiments were designed to extend the knowledge gained by

earlier studies of *Fasciola hepatica* by Richardson (1994) and to localize, anatomically,

where thioredoxin was present in the parasite. Since previous work dealt with only adult

parasites, it was also deemed important to determine whether or not this protein was

present in earlier immature stages, those that could be considered free-living, eggs and

miracidia, as well as those stages associated with the snail intermediate host, i.e.,

sporocysts, redia and cercaria.

Materials and Methods

Parasites

      Adult flukes were obtained from bovine livers collected at a slaughter facility and

processed as described previously for either protein analysis or immunohistochemical

staining (Richardson, 1994). Immature stages of the parasite were collected from

laboratory-raised and -infected snails (*Pseudosuccinia columella*).

Snail Culture and Infection

      The culture system was based upon that described by Whitlock, et al. (1976) with

slight modifications as described by McKown and Ridley (1995). Snails were infected

with miracidia hatched from eggs collected from the gall bladders of the same cattle from

which the adult flukes were collected. Intermediate stages were collected from snails at

the appropriate day of infection to produce the desired parasite life cycle stage. They were

then fixed in a solution of 0.4% glutaraldehyde/4% formalin for no longer than four hours,

at which time they were transferred to 70% ethyl alcohol for storage.

Mouse Infection

Mice were infected as described for cotton rats (*Sigmodon hispidus*) by McKown et al. (2000). However, in this case, Metofane (methoxyflurane; Pitman Moore, Washington Crossing, NJ) was used as the anesthetic of choice (Texas A&M University Animal Use Protocol RF #93-685).

Tissue Processing for Immunohistochemistry

Processing and paraffin embedding were carried out using standard histological techniques. All sections were cut at a thickness of approximately 5 μm. All parasite stages were collected and processed *en masse* and separate from host tissue with minor exceptions. Due to the close association of this parasite stage to the tissue of the host, sporocysts were processed within the snail without being separated from the snail tissue. Each snail was fixed intact and the shell was then either removed or decalcified prior to further processing. In addition, some early stage juvenile flukes were processed *in situ* within the liver tissue of the mouse host while others were removed and processed individually.

Polyclonal Antibody Production

Polyclonal antiserum against a recombinant protein, *F. hepatica* thioredoxin (Fh2020.A) was kindly provided by Dr. Charlene Richardson and Dr. Allison Rice-Ficht of Texas A&M University. Antibody production was accomplished using standard techniques as described previously by Richardson (1994) utilizing New Zealand white rabbits and the injection of the Fh2020.A fusion protein produced in the pGEX vector.

Western Blotting

Parasite antigen, whether that of adult or immature stages, was prepared as described by Richardson (1994) then stored at –80°C until used. Protein electrophoresis and Western blot detection was performed using standard techniques (Sambrook et al., 1989). After transfer, the nitrocellulose membrane with the bound protein was blocked using 3% gelatin in TBS for one hour, then incubated in 1% gelatin with primary antibody at a concentration of 1:1000 for one hour at room temperature or overnight at 4°C. At the end of the primary antibody incubation the membrane was rinsed then incubated in secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit IgG; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) at a concentration of 1:500. Detection was with nitrotetrazolium blue chloride (NBT; Sigma-Aldrich, St. Louis, MO) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Sigma-Aldrich, St. Louis, MO) in alkaline phosphatase substrate buffer at room temperature.

Immunohistochemical Staining

Immunohistochemistry was performed using the Histomark^{TM} Streptavidin-HRP System (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) as per the manufacturer's instructions with a primary antibody dilution of 1:500 in TBS. A negative control slide of similar tissue was processed at the same time as the test slide only PBS not containing the primary antibody. After staining with DAB chromagen as per the manufacturer's instructions, all slides were counter-stained with Harris's hemotoxylin, dehydrated through graded ethanols to xylene, and coverslips were applied to each section using Permount$^{®}$ (Fisher Scientific, Pittsburgh, PA), a non-aqueous mounting media.

Results

<u>Western Blotting</u>

Soluble parasite protein extracts were collected from the following species and
stages: *Fascioloides magna* adult, *F. hepatica* adult, miracidia, redia, and cercaria.
Western blot results showed a 12 kDa band representing thioredoxin present in lanes
containing soluble parasite extracts of adult *F. magna* and adult *F. hepatica*, as well as in
those lanes containing homogenates of redia and cercaria. No bands were observed from
miracidial homogenates (Figure 3.1).
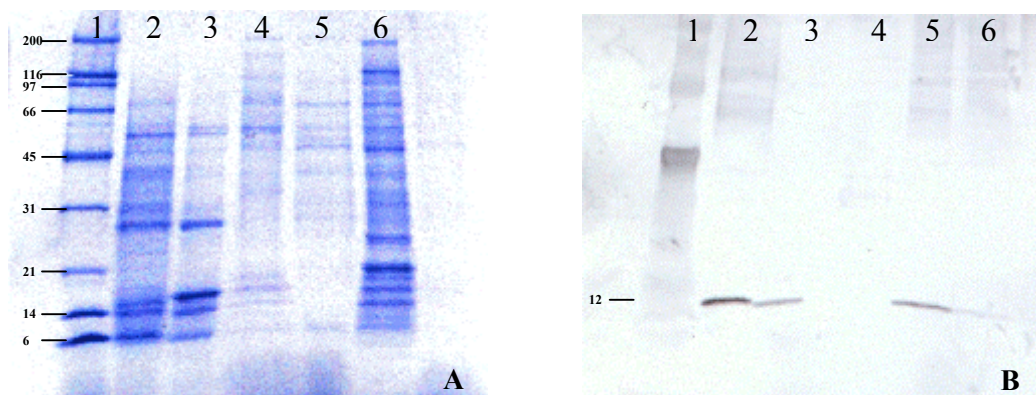
Figure 3.1: Coomassie Brilliant Blue (A) and Western blot (B) of *Fasciola hepatica*
whole parasite soluble protein. A) Proteins separated by SDS-PAGE on a 4-20%
gradient gel. B) Proteins transferred to nitrocellulose membrane. Primary rabbit anti-*F.
hepatica* thioredoxin (Fh2020) antibody and goat anti-rabbit alkaline phosphatase
conjugated secondary antibody. Lane 1 - molecular weight marker (kDa); lane 2 - adult
F. hepatica; lane 3 - adult *Fasciolodies magna*; lane 4 - *F. hepatica* miracidia; lane 5 - *F.
hepatica* redia; lane 6 - *F. hepatica* cercaria.

Immunohistochemistry

Immunohistochemical staining of 5 µm paraffin sections of various intermediate stages and adult parasites showed no staining of egg contents within the uterus but did show positive staining of sperm surrounding the eggs (Figure 3.2). There was no staining of sporocysts or surrounding snail tissue (Figure 3.3). Positive staining was seen in the tegument of redia (Figure 3.4) and the tegument and excretory vesicle of cercaria (Figure 3.5). Positive staining was seen in the tegument of a 28-day-old immature fluke but not in surrounding mouse liver tissue (Figure 3.6). Positive staining was also found in adult tegument and the outer layer of the associated spines (Figure 3.7). There was also positive staining found in association with the cecal epithelium as well as that of the excretory canal (Figures 3.8 and 3.9). Inconsistent staining of the vitelline glands was also seen, positive in some sections (Figure 3.8) but negative in others (Figure 3.9). This may have been due to slight differences in the fixation of the parasite or chromagen incubation times. Due to the inability to cut quality sections of metacercaria, no immunohistochemical staining was attempted on this stage of the parasite life cycle.

Figure 3.2: Photomicrograph of *Fasciola hepatica* uterus containing eggs (*). A) Negative control slide without rabbit anti-*F. hepatica* thioredoxin (Fh2020) primary antibody. B) Similar section with rabbit anti-*F. hepatica* thioredoxin (Fh2020) primary antibody. No positive staining of eggs or their contents are seen in either section. However, sperm (S) within the uterus and surrounding the eggs, exhibit positive staining. (400X)
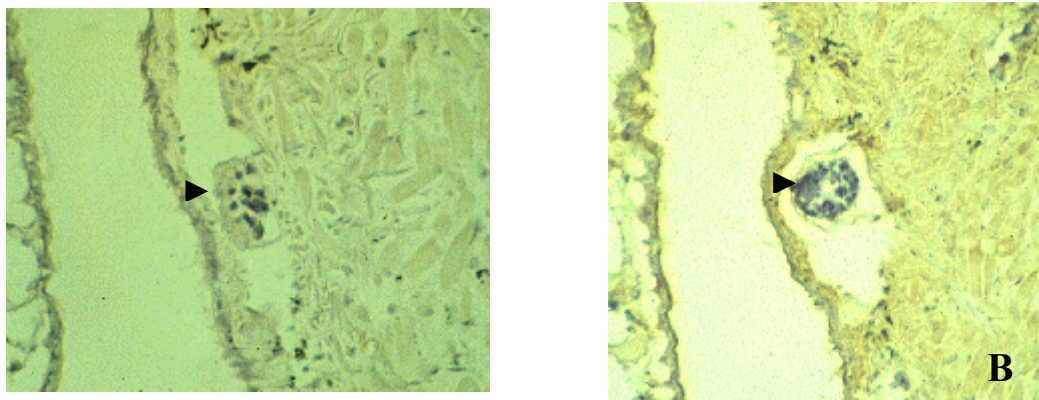
Figure 3.3: Photomicrograph of 3-day post-infection *Fasciola hepatica* sporocyst (►) within the tissues of a *Pseudosuccinea columella* snail intermediate host. A) Negative control slide without rabbit anti-*F. hepatica* thioredoxin (Fh2020.A) primary antibody. B) Similar section with rabbit anti-*F. hepatica* thioredoxin (Fh2020.A) primary antibody. No positive staining of parasite or snail tissue is present on either section. (400X)
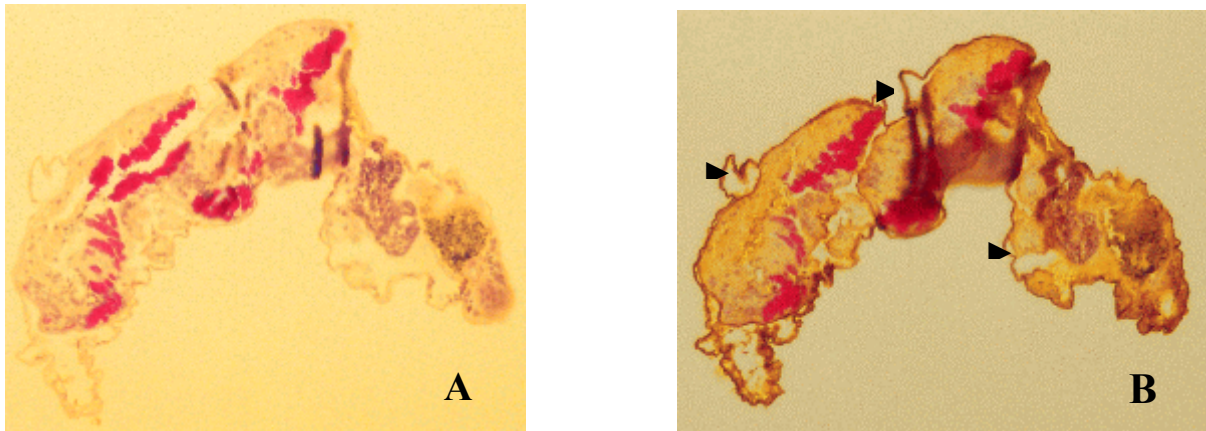
Figure 3.4: Photomicrographs of serial sections of a *Fasciola hepatica* 30-day post-infection redia dissected from the snail intermediate host, *Pseudosuccinea columella*. A) Negative control slide without rabbit anti-*F. hepatica* thioredoxin (Fh2020.A) primary antibody. B) Next 5 μm section with rabbit anti-*F. hepatica* thioredoxin (Fh2020.A) primary antibody. Section shows positive staining of the tegument surface (►). (200X)
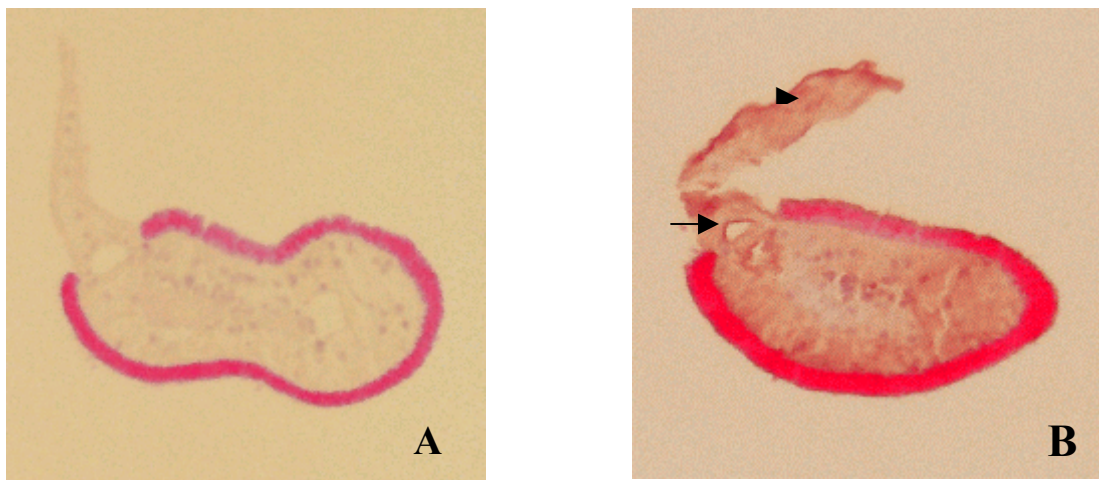
Figure 3.5: Photomicrograph of naturally shed *Fasciola hepatica* cercaria from experimentally infected *Pseudosuccinea columella*. A) Negative control slide without rabbit anti-*F. hepatica* thioredoxin (Fh2020.A) primary antibody. B) Similar section with rabbit anti-*F. hepatica* thioredoxin (Fh2020.A) primary antibody. Positive staining can be seen in the area of the outer tegument of the tail (►) as well as in the epithelial lining of the excretory vesicle (⟶). (200X)
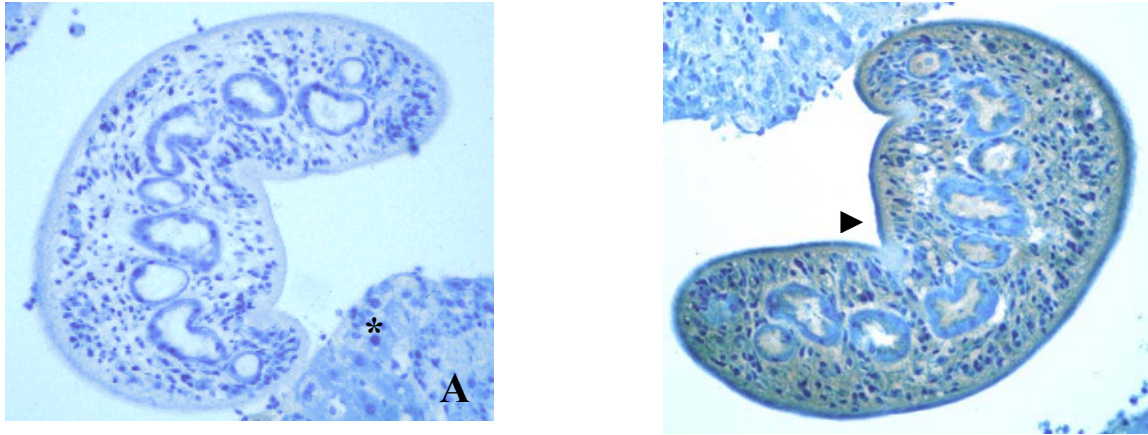
Figure 3.6: Photomicrographs of serial sections of 28-day post infection immature *Fasciola hepatica* within the liver of the mouse host (*). A) Negative control slide without rabbit anti-*F. hepatica* thioredoxin (Fh2020.A) primary antibody. B) Next 5 μm section with rabbit anti-*F. hepatica* thioredoxin (Fh2020.A) primary antibody. Positive staining can be seen particularly in the area of the outer tegument (►). No positive staining of mouse liver tissue can be seen. (200X)
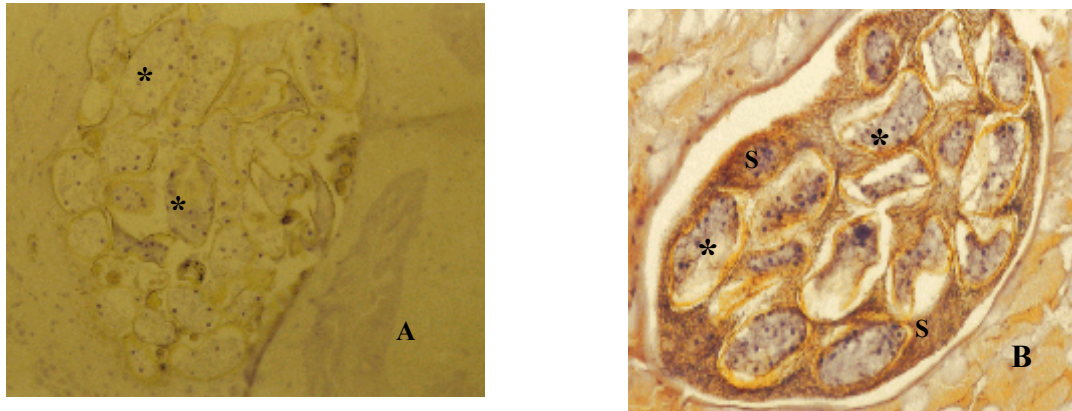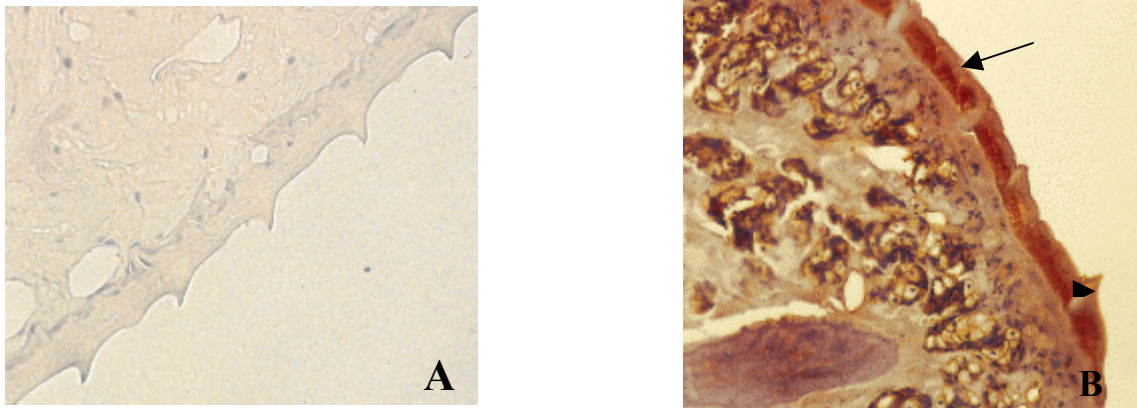

Figure 3.7: Photomicrograph of adult *Fasciola hepatica* tegument and spines. A) Negative control without rabbit anti-*F. hepatica* thioredoxin (Fh2020.A) primary antibody. B) Test section with rabbit anti-*F. hepatica* thioredoxin (Fh2020.A) primary antibody showing positive staining of the tegument (—►) and spine surface (►). Vitelline glands (*) appear positive in this section but not in subsequent slides (Figure 3.8) where sections of the glands are also present. (200X)
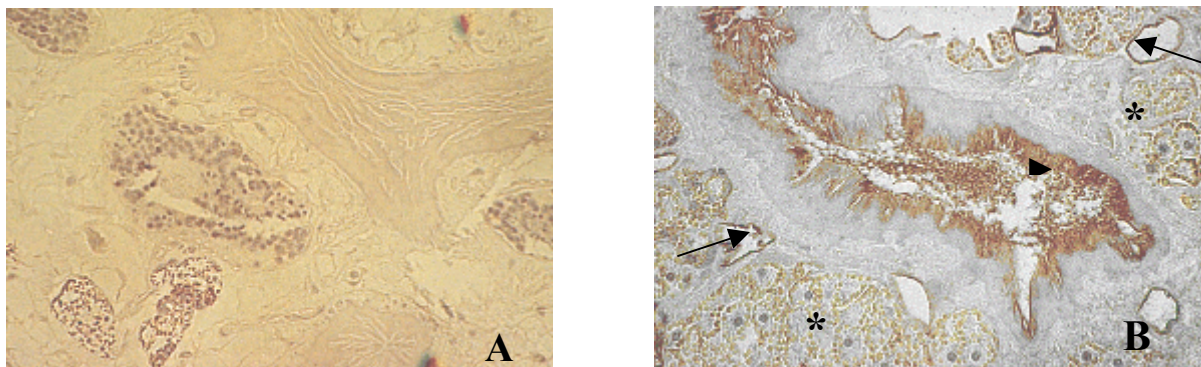
Figure 3.8: Photomicrograph of adult *Fasciola hepatica* intestinal cecum and surrounding tissues. A) Negative control slide without rabbit anti-*F. hepatica* thioredoxin (Fh2020.A) primary antibody. B) Similar test section with anti-*F. hepatica* thioredoxin (Fh2020.A) primary antibody showing positive staining of the cecal epithelium (▶) and lining of the excretory canal (⟶▶). No apparent staining of the vitelline glands (*) present on this section. (200X)
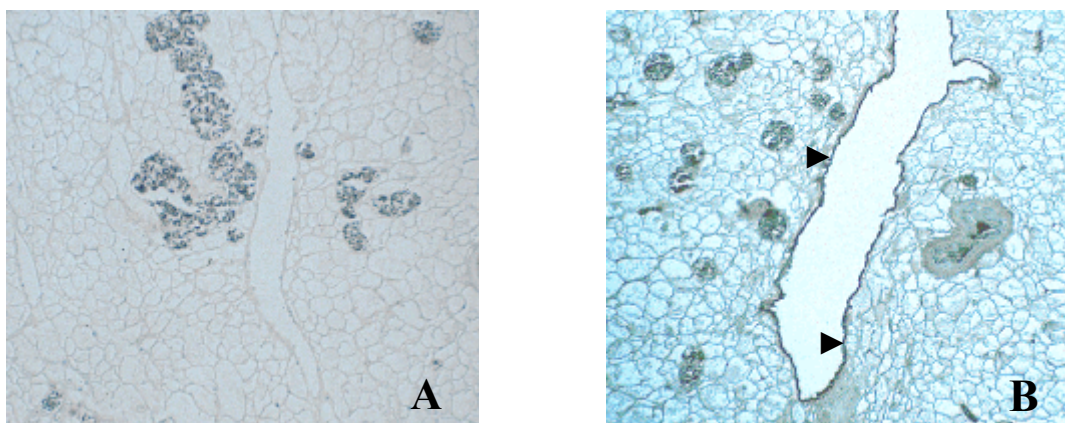
Figure 3.9: Photomicrograph of adult *Fasciola hepatica* excretory canal and surrounding tissues. A) Negative control slide without anti-*F. hepatica* thioredoxin (Fh2020.A) primary antibody. B) Similar section with anti-*F. hepatica* thioredoxin (Fh2020.A) primary antibody showing intense staining of the epithelial lining of the excretory canal (▶). (200X)

Discussion

It is well known that reactive oxygen species are an effective host defense mechanism against both intracellular and extracellular parasites (Callahan et al., 1988). Phagocytic cells such as eosinophils and neutrophils have been shown to kill parasites by undergoing a respiratory burst and releasing such reactive oxygen species as hydrogen peroxide and superoxide anion (Clark et al., 1986; Klebanoff, 1992; Miller and Britigan, 1997). A number of functions of thioredoxin have been elucidated to date, one being that of an antioxidant, as well as a modulator of apoptosis, cell growth and differentiation, and also a regulator of DNA-binding activity of several transcription factors. With the localization of thioredoxin within adult *Fasciola* and *Fascioloides* parasites, as well as within several of the other life cycle stages, this could add further support to the idea of it being involved in possible protective functions for the parasite.

During the portion of its life cycle during which *Fasciola* is parasitic within the mammalian host, two organ systems in particular are exposed to host defense mechanisms, these being the tegument and the cecal epithelium. As shown by this study, *Fasciola* thioredoxin is found in locations that would deem it appropriate for a protein that is involved in a protective role against possible host cellular defenses. Once ingested by the definitive host, the newly excysted juvenile fluke spends little time within the confines of the digestive tract. It quickly penetrates through the intestinal wall and enters the peritoneal cavity on its way to the liver. During this migration the newly emerged fluke is exposed to various host cellular defenses by way of peritoneal macrophages, eosinophils and neutrophils. Not only are these cells encountered during the tissue disruption of migration but also during feeding by the parasite as well. During

feeding, blood as well as tissue debris is actively ingested by the flukes (Cheng, 1986; Halton, 1997), exposing them internally to various host cellular components. Consequently, both the external surface of the fluke as well as the internal lining of its digestive tract are bathed in host cellular debris and metabolic products.

The demonstration of thioredoxin's association with the tegument of the redia may also indicate a protective function. Of the intermediate stages of *Fasciola*, the sporocyst and redia are exposed to snail host defense mechanisms to a greater extent than any other. Of these two stages, the redia showed immunohistochemical evidence of the presence of thioredoxin while the sporocyst stage did not. In fact, both stages are found within the tissues of the snail intermediate host and it would be expected that each would trigger and be equally susceptible to host defenses. However, while there are several differences between the two stages, one of possible importance to this discussion is that the sporocyst is non-feeding, whereas the redia is a feeding stage and can cause considerable tissue damage to the host. This extensive disruption of host tissue could promote a much greater host response to the presence of the redia than that elicited by the much smaller and less damaging sporocyst (Kendall, 1965). Thus, different host responses to each of these parasite stages could generate the production of thioredoxin or the activation of the thioredoxin system as a defense response in one stage but not the other.

While not localizing thioredoxin anatomically, in studying its production in *S. mansoni*, Alger et al. (2002) recorded positive bands via Western blotting in the following parasite stages: cercaria, 3-hour-old schistosomula, and in adult male and female flukes as well as egg secretory products and soluble egg proteins. Some of the

differences noted between that study and those of our current work with *Fasciola* can be explained by the differences in the life cycles of these two parasites. Starting with the adult fluke in the common bile duct of the definitive host, eggs are shed into the bile, then pass into the small intestine, are mixed with intestinal contents and expelled in the feces with little or no contact with the host immune system. Whereas, adult *S. mansoni* reside within the mesenteric vessels of the infected host and eggs are shed into the host vasculature and must pass through the vessel wall and the wall of the intestine before entering the intestinal lumen and then being voided with the feces. During this passage through the host tissue, a significant host response is elicited. To counter this response, the production of thioredoxin by the developing miracidia could enable the eggs to pass, without triggering such a tremendous host reaction so as to prevent the passage of the eggs to the outside. Even with this mechanism in place, numerous eggs are trapped, ectopically, particularly within the liver of the host with extensive granulation tissue formation surrounding the egg as the end result. This could simply be due to the period of time the egg spends in contact with host tissue. For if the eggs, once released, pass rather quickly through the host tissue and enter the intestinal lumen, they are exposed to comparatively little contact with host defense mechanisms and thus any thioredoxin antioxidant activity could be sufficient to allow the eggs to pass unhindered. However, if they are carried by the circulation to a location where their entry into the intestine is prevented, the duration of time spent exposed to host defenses is greatly increased and thioredoxin production could be effectively overwhelmed by the host with the resulting granuloma formation, characteristic of schistosomiasis.

As previously stated, production of thioredoxin has been identified in cercaria of both *Fasciola* and *Schistosoma*. Once again, differences in the life cycles of the two parasites could explain why this protein appears to be present in greater abundance in *Schistosoma* than in *Fasciola*. The major difference in the two is that cercaria of schistosomes initiate infection by actively penetrating the skin of the definitive host, while the cercaria of *Fasciola* are simply a motile stage used to get away from the snail intermediate and to locate a suitable substrate upon which to encyst. These cercaria never come into contact with the definitive host and its cellular defenses and only briefly with those of the snail intermediate host. Also, during their development, the cercaria are encased within the redia and are thus not exposed to the snail immune system. Being essentially protected by the redia, which does exhibit a strong thioredoxin presence in its tegument, there would be little necessity for the cercaria to generate a defense it didn't require at that particular time.

The other interesting finding of thioredoxin in *Fasciola* was its apparent occurrence in sperm within the uterus. While this finding has not been reported previously in a parasitic species, the expression of thioredoxin has recently been shown to occur in both murine and human spermatozoa (Miranda-Vizuete et al., 2001; Yu et al., 2002). Both studies suggest that this sperm-specific thioredoxin could be important in regulating spermatogenesis. One major difference in these findings and those in *F. hepatica* is that the mammalian sperm specific thioredoxin is just that, specific to spematozoa and no other tissues, whereas fluke thioredoxin is found in various tissues throughout multiple stage in the parasite life cycle.

While considerable evidence can be found as to the role thioredoxin and the thioredoxin system play in the antioxidant defenses of numerous species, its role in most helminth parasites remains to be elucidated. Experimental evidence of the existence of thioredoxin and the thioredoxin system in different life cycle stages of *Fasciola* as well as the anatomical locations of the protein in this parasite, lends credence to its possible role in the redox balance and in the antioxidant defenses of *F. hepatica*. While considerable work remains to be done, these few insights into the possible functions of thioredoxin in this parasite show promise for future study.

CHAPTER IV

LOCALIZATION OF *Fasciola hepatica* THIOREDOXIN ISOTYPES Fh2020.A AND Fh2020.SL IN ADULT *Fasciola hepatica* BY *IN SITU* HYBRIDIZATION

Introduction

Thioredoxins are low molecular weight (12 kDa) proteins that are maintained in their active, reduced form by the flavoenzyme thioredoxin reductase. Along with NADPH, these three compounds function as a general protein disulfide reducing system and make up what is commonly known as the "thioredoxin system" (Holmgren and Bjornstedt, 1995; Nakamura et al., 1997), which plays an important role in maintaining the redox environment of the cell. It would appear that most, if not all, organisms from bacteria to the higher mammals, have at least one complete thioredoxin system, and that with increasing organismic complexity there are also an increasing number of functional thioredoxin systems within the organism. In many organisms with multiple thioredoxins, a mitochondrial system frequently exists in parallel to a cytoplasmic one (Powis and Montfort, 2001; Rahlfs et al., 2002). The intracellular expression of thioredoxin is dependent upon the cell cycle suggesting its possible involvement in the redox regulation of the cycle itself (Nakamura et al., 1997).

In previous work on *Fasciola hepatica* thioredoxin (Richardson, 1994), two isotypes designated Fh2020.A (555 base pairs) and Fh2020.SL (491 base pairs) were found to have extensive homology, however, significant variability was present within their initial 30 base pair sequences (Richardson, 1994). Using this information and that

from other workers studying multiple thioredoxins within an organism, probes were designed for each of the two *Fasciola* isotypes. An *in situ* hybridization method was developed in an attempt to determine whether or not these two isotypes were expressed in different anatomical locations within the adult parasite (Objective 2).

Materials and Methods

Parasites

Adult *F. hepatica* were obtained at slaughter from the liver of a single naturally infected cow. The slaughter facility was located in Hastings, Nebraska, however the origins of the affected animal are unknown.

Fixation

All adult flukes, once removed from the bile duct, were rinsed in sterile PBS, pH 7.4 then placed into a fixative mixture of 0.4% glutaraldehyde/4.0% formalin. After a period of fixation not exceeding four hours, the tissues were rinsed in distilled water and placed in 70% ethanol until processing.

Processing and Embedding

Tissues were processed and embedded according to standard histological techniques. Sections were cut to a thickness of approximately 5 µm, and attached to RNase-free silanized glass slides and stored under dust-free conditions at room temperature.

Design of Oligonucleotide Probes

DNA-antisense probes for the control proteins *Schistosoma mansoni* actin and *F. hepatica* tubulin were designed using the hybridization probe analysis option of Vector NTI, Suite 5.5 sequence analysis software (InforMax, Inc., North Bethesda, MD) using

nucleic acid sequence information obtained from Genbank (http://www.ncbi.nlm.nih.gov; Accession numbers M80334, and AJ297256, respectively). Briefly, the nucleic acid sequence was entered into the Vector program along with the desired parameters of probe length ($\geq$50, $\leq$70 bases), melting temperature (Tm; $\geq$40°C, $\leq$65°C), %GC content ($\geq$35, $\leq$60), hairpin loop stem (>3), palindromes (<8) and nucleotide repeats (<4).

Nucleic acid sequences for Fh2020.A and Fh2020.SL were obtained from Richardson (1994). Because these isotypes are highly homologous, isotype-specific probes were designed by comparing the sequence differences at the 5' ends (the differences between the two isotypes were seen within the first 36 bases), and selecting probes that were complementary to a region of 20-25 bases from the 5' end. The selected probes were then analyzed using the hybridization probe analysis option of Vector NTI, Suite5.5 sequence analysis software (InforMax, Inc., North Bethesda, MD) to determine if they fit the parameters used to select the *S. mansoni* actin and *F. hepatica* tubulin probes. All probes were checked for specificity for their respective proteins by performing BLAST sequence analysis (http://www.ncbi.nlm.nih.gov/BLAST).

Digoxigenin Tailing

Unlabeled oligonucleotide probes (Fh2020.A, Fh2020.SL, *S. mansoni* actin, and *F. hepatica* tubulin) were purchased from Integrated DNA Technologies, Coralville, IA. Digoxigenin labeling was carried out as per manufacturer's instructions using the DIG Oligonucleotide Tailing Kit, 2nd Generation (Roche Diagnostics GmbH, Mannheim, Germany). Briefly, the required reagents were added to 100 pmol of unlabeled probe (*F. hepatica* tubulin, *S. mansoni* actin, Fh2020.A or Fh2020.SL) in distilled water, incubated

at 37°C for 15 minutes and the reaction stopped using 0.2 M EDTA. This procedure resulted in the binding of 10-100 DIG labels to each probe.

Dot Blot

In order to test that the DIG label was attached to the probes, a dot blot procedure was performed individually for each probe after the labeling reaction. The unlabeled controls were tested as well. Briefly, after each labeling reaction 1 µl (100 ng) of probe was spotted onto a nylon membrane (Zeta-Probe Membrane, Bio-Rad, Hercules CA), air-dried and UV irradiated to bind the probe to the membrane. The membrane was then washed three times for 10 minutes each wash in TBS buffer containing 0.1% Tween® 20 (Bio-Rad Laboratories, Hercules, CA). The membrane with its associated DIG-labeled probe dots was then placed in AP-conjugated sheep anti-DIG antiserum (1:500 dilution) in TBS buffer containing 1% gelatin at room temperature for 2 hours. The membrane was further washed with TBS buffer containing 0.1% Tween® two times for 10 minutes each and finally, once for 10 minutes. Label was detected using the chromagen NBT/BCIP. The NBT/BCIP visualization solution was prepared using 33 µl NBT stock (50 mg/ml in 70% dimethyl formamide) plus 17 µl BCIP stock (50 mg/ml in 100% dimethyl formamide) per 5 ml alkaline phosphate substrate buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM MgCl).

Calculation of Hybridization Temperature

The T_M for each of the probes was calculated using the following formula:

$$T_M = 81.5 + 16.6(\log M) + 0.41(\%G+C) - 0.61(\% \text{ formamide}) - 500/n$$

$M = Na^+$ concentration in moles/liter
n = shortest chain in the hybrid

For DNA:RNA hybridization such as was used in this study, 10-15°C was added to the result. The optimal hybridization temperature was calculated to be 25°C below the T_M of the hybridized probe.

<u>*In Situ* Hybridization Protocol</u>

All glassware used for *in situ* hybridization was rendered RNase-free by baking at 180°C for four hours then stored under dust-free conditions. All prepared solutions were made using RNase-free water and stored in baked glassware.

This protocol was based on the manufacturer's instructions for the Rembrandt® *In Situ* Hybridization and Detection Kit (PanPath, Amsterdam, The Netherlands). Briefly, two sections were mounted per slide so that both a test and negative control could be processed simultaneously. Each slide was deparaffinized in two changes of xylene then placed in 100% ethanol. A proteolytic digestion step using hydrochloric acid and pepsin as provided in the kit was carried out on each section at 37°C for 30 minutes. Each slide was then dehydrated through a series of graded ethanols (70%, 80%, 95%, 100%, 100%), for one minute each, then air-dried. Each section was then covered with hybridization solution containing the appropriate probe at a concentration of 150 – 200 ng per section and incubated at 37°C for two hours. The hybridizations were carried out utilizing a PTC-200™ Thermal Cycler with block/heat pump assembly and model SG96P glass slide adapter (MJ Research, Inc., Watertown, MA). At the end of that time the coverslip was removed by soaking in TBS and the slide was washed with three additional TBS rinses. At this time, 2-3 drops of conjugate (AP-conjugated anti-DIG as provided in the kit) was added to each section and incubated at 37°C for 30 minutes. The slide was then rinsed with TBS and a detection step using NBT/BCIP substrate was carried out at 37°C for 15

minutes after which the slides were washed with distilled water, dehydrated and cover slipped. Probes tested with this protocol included, *F. hepatica* tubulin (the positive control), *S. mansoni* actin (an additional positive control), and thioredoxin isotype Fh2020.A and Fh2020.SL probes. Negative control slides consisted of sections that had mRNA hybridization solution (DAKO Corp., Carpinteria, CA) without probe. Additionally, a negative control probe included in the Rembrandt® kit consisting of 100 bases of a DIG-labeled pSP vector sequence was utilized to detect non-specific binding.

Results

Probe Analysis

The Vector analysis showed that the *S. mansoni* Actin and Fh2020.A probes were of high quality with no indication that there would be any structural inhibition of hybridization. When the same analysis was performed on the Fh2020.SL probe, a hairpin was detected that might, under certain conditions, render it non-functional. In addition, the BLAST search of each of the two thioredoxin probes testing probe lengths of 32, 26, and 21 bases indicated that the 21 base probe had the highest specificity to *F. hepatica* thioredoxin. Calculation of melting temperatures determined that at the 21-base length, the melting temperature of the two thioredoxin isotypes would be identical. Table 4.1 lists the probe sizes, sequences, and hybridization temperatures for all of the probes utilized. Dot blot analysis showed that all of the probes were labeled with sufficient DIG tails to be visualized upon hybridization and color development.

Table 4.1: *In situ* probe sequences and hybridization temperatures.

Probe	Number of Bases	Sequence (5'-3')	Hybridization Temp. (°C)
S. mansoni Actin	23	atcttctccatatcatcccagtt	43-48
Fh2020.A	21	gagccgcatcgtggcaaaacg	41-46
Fh2020.SL	21	gggcagagaaccgttaaggtt	41-46
F. hepatica Tubulin	50	ttgtaaatcggagtctccatgataa gtaccagttgggtcaattccgtgct	39-44

In Situ Hybridization

Using DIG-tailed probes and the Rembrandt® *in situ* hybridization kit, successful positive staining was achieved in 5 μm sections of adult flukes with all probes tested, *F. hepatica* tubulin (Figure 4.1), *S. mansoni* actin (Figure 4.2), and thioredoxins Fh2020.A (Figure 4.3) and Fh2020.SL (Figure 4.4). Fh2020.A and Fh2020.SL signals were primarily located in the tegument of the worm as well as in the cecal and excretory canal epithelium. Interestingly, it was also located in the vitelline glands, a finding not apparent in the immunohistochemical trials. This could be due to the natural color (golden brown) of the glands and the difference in the colors of the two chromagens used in the different techniques, immunohistochemistry - DAB (brown) and *in situ* hybridization - NBT/BCIP (black). Without counter-staining, the negative control tissues were difficult to discern under brightfield conditions (Figure 4.5) and phase contrast was used to better visualize the sections (Figure 4.6). As stated previously, the negative control slides consisted of both the kit negative control probe and hybridization solution without the respective test probe. In all cases, the negative control sections showed no

positive staining indicating no non-specific background. This would also indicate that

the positive staining seen was specific for the probe sequence tested.
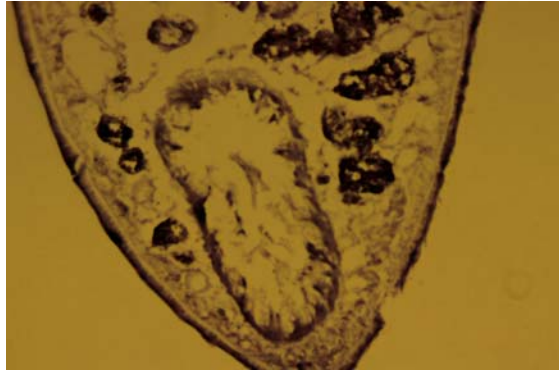
Figure 4.1: Section of adult *Fasciola hepatica* showing positive *in situ* hybridization staining of tissues by a 50-base digoxigenin-labeled *F. hepatica* tubulin probe (150 ng), 2 hour incubation at 37°C with 20 minute chromagen exposure. (200X)
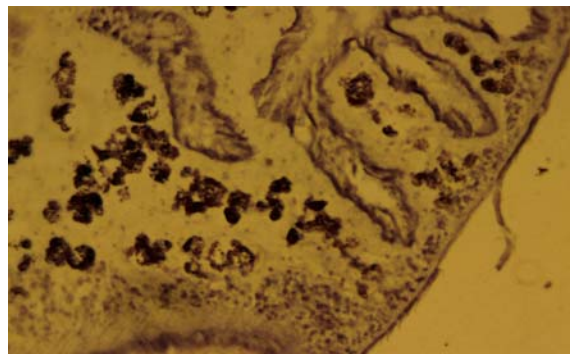
Figure 4.2: Section of adult *Fasciola hepatica* showing positive *in situ* hybridization staining of tissues by a 23-base digoxigenin-labeled *Schistosoma mansoni* actin probe (150 ng), 2 hour incubation at 37°C with 20 minute chromagen exposure. (200X)
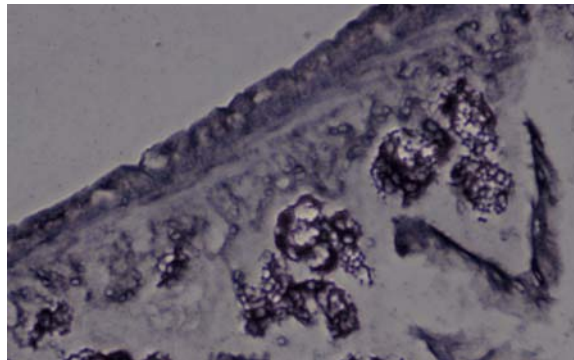
Figure 4.3: Section of adult *Fasciola hepatica* showing positive *in situ* hybridization staining of tissues by a 21-base digoxigenin-labeled Fh2020.A *F. hepatica* thioredoxin probe (150 ng), 2 hour incubation at 37°C with 20 minute chromagen exposure. (400X)
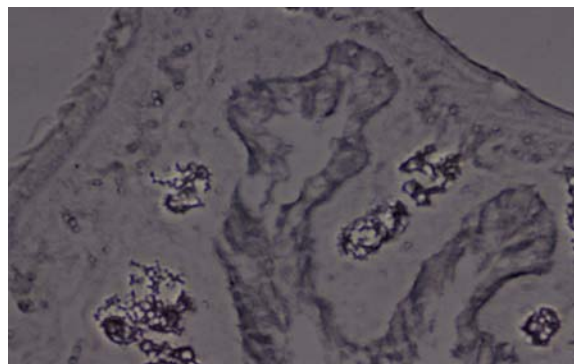
Figure 4.4: Section of adult *Fasciola hepatica* showing positive *in situ* hybridization staining of tissues by a 21-base digoxigenin-labeled Fh2020.SL *F. hepatica* thioredoxin probe (150 ng), 2 hour incubation at 37°C with 20 minute chromagen exposure. (200X)
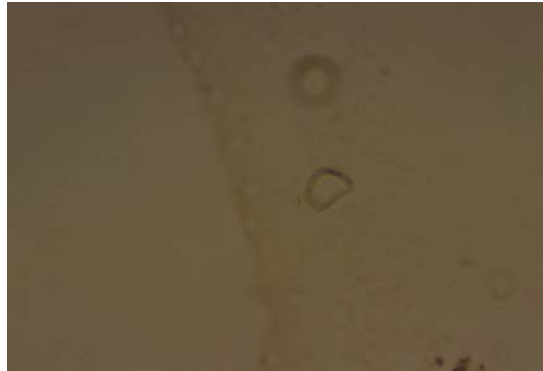
Figure 4.5: Section of adult *Fasciola hepatica* without digoxigenin-labeled probe (negative control) showing no non-specific *in situ* hybridization staining of tissues, 2 hour incubation at 37°C with 20 minute chromagen exposure (Brightfield). (200X)
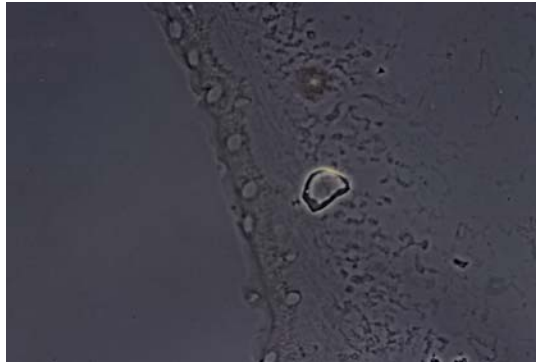
Figure 4.6: Section of adult *Fasciola hepatica* without digoxigenin-labeled probe (negative control) showing no non-specific *in situ* hybridization staining of tissues, 2 hour incubation at 37°C with 20 minute chromagen exposure (Phase Contrast - same field of view as seen in Figure 4.6). (200X)

Discussion

As a technique for the detection of DNA and RNA sequences in tissues, *in situ* hybridization has been used extensively since its first introduction by Gall and Pardue (1971) for the localization of specific DNA sequences on chromosomes. Its specific uses in the detection of parasite gene sequences are abundant (Rice-Ficht et al., 1992; Tuan et al., 1991; Velasquez et al., 1999; Zurita et al., 1989), as are those references of its use in the detection of thioredoxin (Lippoldt et al., 1995; Mansur et al., 1998; Rundlof et al., 2000). Consequently, the use of this technique seemed appropriate for the detection of thioredoxin isotypes in adult *F. hepatica*.

To test this hypothesis, a 50-base *F. hepatica* tubulin sequence was selected and a DIG tailing kit was used to label the probe with the greatest number of DIG labels available for detection. Also, a standardized *in situ* hybridization kit was used to eliminate the possibility of reagent RNase contamination and to use reagents at an established concentration in a protocol that was known to work under a variety of conditions and circumstances. This strategy was well founded and positive results were obtained in all cases with negative controls having no signal and positive controls exhibiting strong signal (Figures 4.1-4.6).

From these results, it would appear that the two different mRNA isotypes of *F. hepatica* thioredoxin are produced in the same anatomical locations within the adult fluke. The *in situ* findings complemented those of the immunohistochemical experiments in that it appeared that the thioredoxin protein production and RNA transcription are coincident. One difference seen with the *in situ* hybridization was the difference in the intensity of staining between the two isotypes. In this procedure a concentration of 150

ng of labeled probe per section was used for both the Fh2020.A and the Fh2020.SL, however, the staining intensity of the Fh2020.A (Figure 4.4) was much greater than that of the Fh2020.SL (Figure 4.5). This increased intensity could indicate a difference in the amount of expression between the two isotypes, with Fh2020.A being expressed in greater quantities. This may also indicate a difference in function between the two or simply a redundancy within the *Fasciola* thioredoxin system. Alternatively, it could indicate that the chromogen remained on one slide longer than the other, or was simply an artifact resulting from human error, but due to the repeatability of the results, this was most likely not the case.

Richardson (1994) reported identifying two different sized transcripts, one 530-bp and the other 1.5kb (apparently unprocessed transcript) for *F. hepatica* thioredoxin. Additional work by Richardson (1994), utilizing reverse-transcriptase polymerase chain reaction with thioredoxin isotype-specific primers, further demonstrated that the 530-bp transcript was composed of both trans-spliced and cis-spliced thioredoxin transcripts. By using a ribonuclease protection assay and subsequent visualization of the RNA products, it was determined by comparison of the relative intensities of the bands that the cis-spliced message (Fh2020.A) was more abundant than the trans-spliced message (Fh2020.SL). Thus, the *in situ* hybridization results and the comparative staining intensities of the Fh2020.A and Fh2020.SL probes correspond exactly with what was found in the earlier study.

At the light microscopic level, it would appear from the *in situ* results that both Fh2020.A and Fh2020.SL are expressed in the same or similar tissues. Although the message is produced in the same anatomical region, there is the possibility exists that

different cell types produced the isotypes. One way to confirm or refute this would be by using electron microscopy so that the transcripts could be detected at a specific cellular location.

Coupling the results of this work and knowledge of the various functions of thioredoxin in other organisms, a case can be made as to what possible functions this protein may have in parasitic trematodes. *F. hepatica* thioredoxin could be involved in a protective antioxidant function against oxidative stresses as has been shown in previous studies (Arner and Holmgren, 2000; Holmgren, 2000; Powis and Montfort, 2001). Anatomically, its location would correspond well with areas where the fluke would be exposed to the greatest stresses from both its environment and the host's defenses. These anatomical sites include the outer tegument and the gut lining, both of which are exposed to host defense factors both cellular and chemical, as well as the rigors faced by living in a sea of bile and its enzymatic components. Secondly, when one considers these anatomical sites (tegument and gut) as well as the presence of thioredoxin in the epithelium of the excretory canal and associated with the vitelline glands, these organs are undergoing rapid cellular division and metabolism. As seen in other organisms, one of the biological activities of thioredoxin includes that of cofactor, where it plays an important function in the growth of several cell types. In areas of intense cellular growth and proliferation, thioredoxin could provide a source of reducing equivalents for ribonucleotide reductase which catalyzes the conversion of nucleotides to deoxynucleotides, the first step in DNA synthesis (Powis and Montfort, 2001).

The results of this study indicate that first, the general anatomical location of both isotypes of thioredoxin studied are very similar and second, the intensity of staining of

the two isotypes is dissimilar, possible indicating a difference in the degree of expression between the two. The fact that the two isotypes are expressed differently is interesting in that it could simply be a means of redundancy in the system insuring that there is always a source for the protein even if for some reason one "pathway" is non-operational, or it could be that the two types perform different functions. Without further work at the cellular or even sub-cellular level, the locations of the two isotypes remain general at best, and without further molecular characterization the true function or functions of the two isotypes will remain uncertain as well.

CHAPTER V

EFFECTS OF RECOMBINANT *Fasciola hepatica* THIOREDOXIN ON NITRIC OXIDE AND SUPER OXIDE ANION PRODUCTION IN BOVINE AND MURINE IMMUNE CELLS

Introduction

It is well known that reactive oxygen species are effective host defense mechanisms against both intracellular and extracellular parasites (Callahan et al., 1988). Phagocytic cells such as eosinophils, neutrophils and macrophages have been shown to kill parasites by undergoing a respiratory burst and releasing such reactive oxygen species as hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and superoxide anions (O_2^-), as well as reactive nitrogen species such as nitric oxide (NO).

The redox activity of thioredoxin and the thioredoxin system have been shown in species within many classes of parasitic organisms (Krnajski et al., 2001; Lu et al., 1998; Rahlfs et al., 2002; Richardson, 1994). In addition to the three primary components of the thioredoxin system; thioredoxin, thioredoxin reductase and NADPH, there are other protein, enzyme and metabolite components that are dependent upon reduced thioredoxin for their functional ability, whether that is for host defense against infection or parasite defense against host response (McGonigle et al., 1997; Rahlfs et al., 2002). Within the digenea, a complete thioredoxin system has been reported from *Schistosoma mansoni*. Experimental results indicate that the system plays a significant role in the redox balance and antioxidant defenses of the parasite (Alger et al., 2002; Rahlfs et al., 2002). A functional thioredoxin system also appears to be present in *Fasciola hepatica*

(Richardson, 1994; Salazar-Calderon et al., 2001). Because no functional catalase system and only a trace of glutathione peroxidase activity having been described in *Fasciola*, this may place even greater importance on this parasite's thioredoxin system.

Previous studies have shown that *F. hepatica* is attacked by the host's immune response at the earliest stages of the infection. Smith et al. (1992) showed that antibody-coated, newly excysted juvenile (NEJ) flukes were attacked and damaged by various host immune cells, particularly eosinophils, neutrophils, macrophages and mast cells, while still passing through the wall of the small intestine and into the peritoneal cavity. When compared to *S. mansoni* schistosomula, NEJs were considerably more resistant to killing by reactive nitrogen intermediates (Piedrafita et al., 2000). Using 7-8 week old naive male white Wistar rats, NEJs experienced mortalities of only 7-15% even in the presence of what were considered high nitric oxide levels of 75-97 µM. By comparison, schistosomula experienced 78-87% killing at equal or lesser levels of nitric oxide.

The immune response elicited by infection with *F. hepatica* varies depending upon the species of host involved. Utilizing this response as a criteria, various natural and experimental hosts have been deemed either susceptible (mice and sheep) or resistant (rats and cattle) to infection by *F. hepatica* (Boray, 1969; Smith et al., 1992). Smith et al. (1992) showed that while murine (susceptible) peritoneal macrophages respond to challenge with *F. h*epatica by producing reactive oxygen intermediates, they do so with much lower concentrations than do laboratory rats (resistant). Rats, whose primary peritoneal response is mediated by eosinophils, will produce upwards of 30 times more reactive radical per animal than observed in mice.

The redox function of thioredoxin has been reported and reviewed extensively (Follmann and Haberlein, 1995; Nakamura et al., 1997; Nordberg and Arner, 2001; Powis and Montfort, 2001; Shao et al., 2002). The occurrence of thioredoxin and the thioredoxin system in various classes of parasitic organisms is also well documented (Alger et al., 2002; Henkle-Duhrsen and Kampkotter, 2001; Rahlfs et al., 2002) and its occurrence in *F. hepatica* has been described (Richardson, 1994; Salazar-Calderon et al., 2001). Abo-Shousha, et al. (1999), have shown that in humans infected with either *F. hepatica*, *S. mansoni*, or a combined infection, there were detectably higher levels of both superoxide anion and nitric oxide produced by monocytes from these individuals than from monocytes of non-infected controls. This would indicate that these reactive intermediates could play a role in the host defense against the parasite.

In the presence of such an onslaught from the host, the parasite must bring into play some type of defensive mechanisms. Jefferies, et al. (1997), demonstrated that when either sheep or human resting neutrophils were exposed to adult *F. hepatica* excretory-secretory (ES) products there was a significant increase in nitric oxide production, while at the same time there was no change in superoxide anion production in either cell type when exposed to ES products. El-Ghaysh et al. (1999), in similar *in vitro* studies using *Fasciola gigantica*, convincingly demonstrated that there was a significant inhibition of superoxide production by PMA-stimulated sheep neutrophils when exposed to both parasite somatic extracts and ES products.

In the current investigation, two reactive intermediates, superoxide anion (O_2^-) and nitric oxide (NO) were chosen for study (Objective 3) due to the fact that they are

produced by cells that are involved in the host immune response to parasite infection, i.e. neutrophils and macrophages, and both have been reported previously to be involved specifically with the host response to *F. hepatica* infection (el-Ghaysh et al., 1999; Haslett et al., 1989; Smith et al., 1992).

Materials and Methods

Endotoxin Assay

Since the recombinant thioredoxin was produced in a bacterial system the determination of the presence or absence of endotoxin associated with the recombinant was deemed necessary. Endotoxin contamination of Maltose Binding Protein (MBP) was measured using the E-Toxate® assay (Sigma-Aldrich, St. Louis, MO). The principle of this assay is that a lysate of circulating amebocytes from the horseshoe crab, when exposed to minute quantities of endotoxin, will increase in opacity as well as viscosity to the point of gelling dependent upon the concentration of endotoxin present.

The assay was run as per the manufacturer's instructions. Briefly, after reconstituting the Working Solution, a series of standard dilutions were prepared using endotoxin-free water (Sigma-Aldrich, St. Louis, MO) within a concentration range of 400 to 0.015 endotoxin units (EU) per milliliter. The unknown sample along with negative and positive control samples were then mixed and incubated in a 37°C water bath for one hour. At the end of the incubation the tubes were removed and inverted. A sample was considered positive for the presence of endotoxin if the solution formed a hard gel in the bottom of the tube. All other results, clear liquid, increased turbidity, soft gel, or increased viscosity were considered negative.

Insulin Reduction Assay

The biological activity of the recombinant *F. hepatica* thioredoxin was

determined by the dithiothreitol/insulin reduction assay described by (Holmgren, 1979)

and further used and described by Salazar-Calderón et al. (2001) and Alger et al. (2002).

This is a turbidity assay in which active thioredoxin serves as a catalyst for the cleavage

of insulin into its α and ß-chains by dithiothreitol (DTT), with the ß-chains forming an

insoluble precipitate that was then detected spectrophotometrically by its absorbance at

630-650 nm.

Initially, a 10 mg/ml insulin stock solution was prepared by resuspending 50 mg

of insulin powder (Sigma, St. Louis, MO) in 4 ml of 0.05 M Tris-HCl, pH 8.0 and

adjusting the pH to 2.0-3.0 by the addition of 1.0 M hydrochloric acid. The pH was

immediately brought up to 8.0 with 1M NaOH, and the volume adjusted to 5 ml with

distilled water. The resulting solution was separated into 500 µl aliquots and frozen at –

20°C. An insulin working solution (IWS) was prepared from the stock solution by

adding 400 µl of 10 mg/ml stock solution, 400 µl phosphate buffer (PBS), and 16 µl of

500 mM EDTA to 3.2 ml of distilled water. The assay was carried out in triplicate in a

96-well microtiter plate with the following treatments: 1) Blank (IWS + water); 2)

Control (IWS + water + DTT; demonstrates spontaneous insulin cleavage); 3) MBP Test

(IWS + water + DTT + MBP at 0.23 µM / well); 4) LPS Test (IWS + water + DTT +

LPS at 1 µM / well) and 5) TRX•MBP Test (IWS + water + DTT + TRX•MBP at 4

µM/well). Each well had a final volume of 200 µl consisting of 150 µl IWS and 2 µl 33

mM DTT in all wells and additional reagents in the following proportions dependant

upon the test reagent: TRX•MBP wells - 50 µl with no additional water; MBP alone and LPS wells - 1 µl MBP or LPS + 47 µl water. The test reactions were carried out on ice with the microtiter plate agitated slightly to insure mixing of all reagents. The plate was then placed within a microtiter plate reader and the absorbance was measured at 630 nm at 1 minute intervals for 15-60 minutes depending on activity/concentration of the test samples.

Maltose Binding Protein Control

Since the thioredoxin used in the cellular assays was a recombinant bound to a MBP moiety, a covalently linked MBP control was tested for stimulatory activity in one assay and cell type. MBP was obtained from the laboratory of Dr. W.C. Brown at Washington State University. To correctly determine the concentration of MBP to be used in the assay, all calculations comparing MBP with recombinant thioredoxin were based upon the molecular weight of each, free MBP: 42.7 kDa and recombinant thioredoxin: 54.7 kDa.

Murine Peritoneal Macrophages

Six to eight week old C57 Black/10ScN mice (n = 10) were used as outlined in USDA Protocol, Experiment # 5438-32000-013-08 and Texas A&M Animal Use Protocol #9-173. The original stock was kindly provided by Dr. S.K. Chapes, Kansas State University. Macrophages were obtained by the method described by Meltzer (1981), using 3% thioglycollate medium injected into the peritoneal cavity to stimulate macrophage recruitment, with activated cells collected 4-5 days post-injection via cold PBS lavage. The lavage fluid was centrifuged at 400 x g at 4°C for 7 minutes to

pellet the cells, which consisted of peritoneal macrophages and a small number of contaminating red blood cells. Red blood cells were removed using Red Cell Lysis Solution (Sigma-Aldrich, St. Louis, MO) as per the manufacturer's instructions. The cells were brought up to a 50 ml volume with cold PBS, pelleted by centrifugation, and resuspended in 10 ml cold PBS. An aliquot was taken and a Cytospin preparation was made (Figure 5.1) to determine the cell types present, and a total cell count was performed using a hemacytometer. The cells were then transferred into 24-well microtiter plates at a concentration of 1×10^6 cells per well.
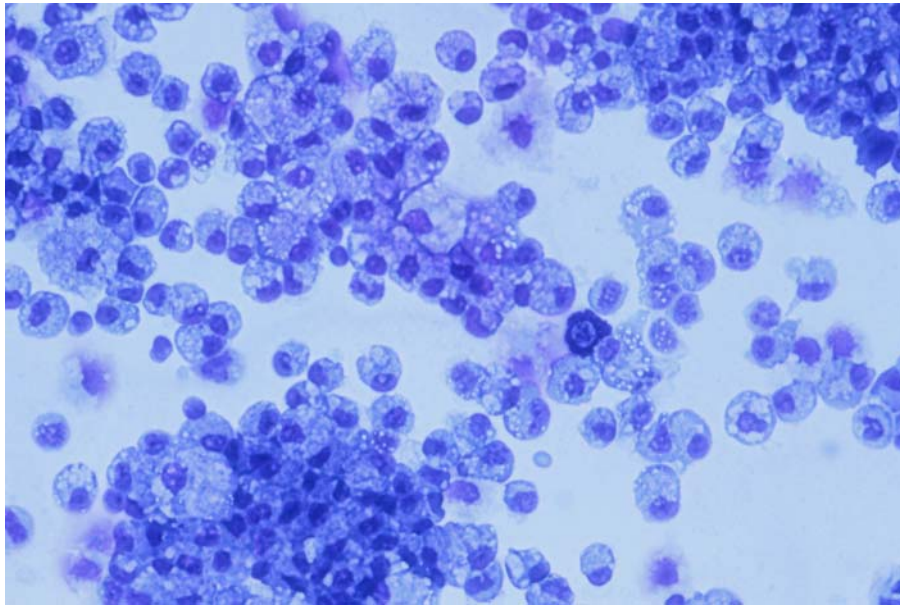
Figure 5.1: Photomicrograph of a Romanowsky's stained (DifQuik™) Cytospin™ slide preparation of mouse peritoneal macrophages collected four days after intraperitoneal injection of 3% thioglycollate (400X).

Bovine Cells

All bovine cells (macrophages and neutrophils) were collected from whole blood as described below and outlined in USDA Experimental Outline, Experiment #5438-32000-022-02. All cattle were from an area in south central Nebraska where no incidence of *F. hepatica* infection has been known to occur.

Bovine Monocyte-Derived Macrophages

Whole blood was obtained by jugular venipuncture from animals (n = 21) within a commercial cattle herd and monocytes were separated using a density gradient procedure as outlined by Clark and Nauseef (1998). The PBMC layer was carefully removed via pipette and brought to a volume of 50 ml with cold PBS then centrifuged at 400 x g at 4°C for 7 minutes to pellet the cells. Any red blood cell contamination was removed by lysis using Red Cell Lysis Solution (Sigma, St. Louis, MO) as per the manufacturer's instructions. The pellet was suspended in 10 ml of cold PBS and an aliquot was removed for differential staining (Figure 5.2) and cell count determination as described above. After counting, cells were transferred into a 24-well microtiter plate at a concentration of 1×10^6 macrophages per well in RPMI 1640 medium plus antibiotics and allowed to adhere to the plate for one hour at 37°C. After this time the medium was removed along with any non-adherent cells and replaced with RPMI 1640 containing 5% fetal bovine serum and antibiotics then returned to 37°C and left undisturbed until the following day.
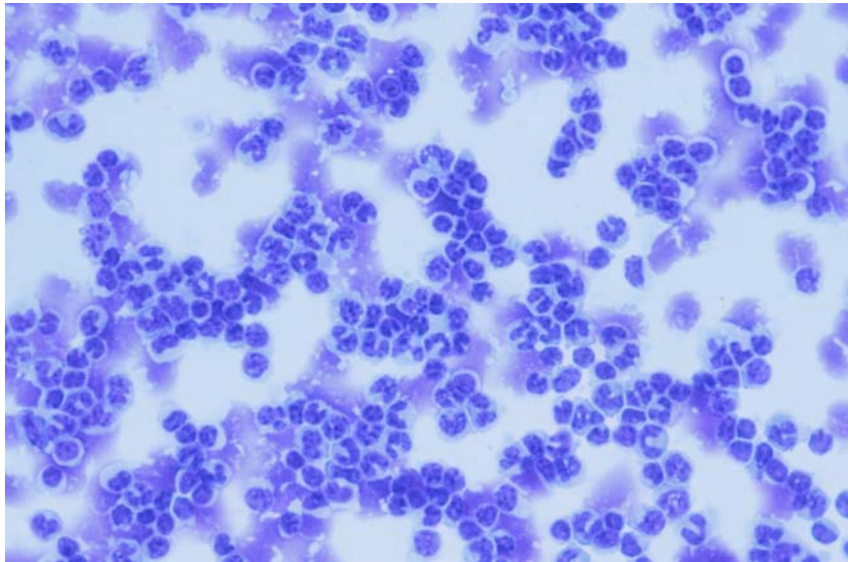
Figure 5.2: Photomicrograph of a Romanowsky's stained (DifQuik™) Cytospin™ slide preparation of bovine peripheral blood mononuclear cells after density gradient concentration and red cell lysis and prior to overnight adherence (400X).

Bovine Whole Blood Neutrophils

Whole blood was obtained by jugular venipuncture from animals (n = 22) within a commercial cattle herd and neutrophils were separated using a density gradient procedure as outlined in Clark and Nauseef (1998). After centrifugation, all buffer and cell layers above the red blood cell/neutrophil layer were removed via pipette, leaving only the red blood cell/neutrophil pellet. The red cells were removed using cold saline lysis (Clark and Nauseef, 1998). Typically, 2 or 3 lysis steps were required to remove the majority of the red cell contamination, resulting in an essentially pure neutrophil population (Figure 5.3).
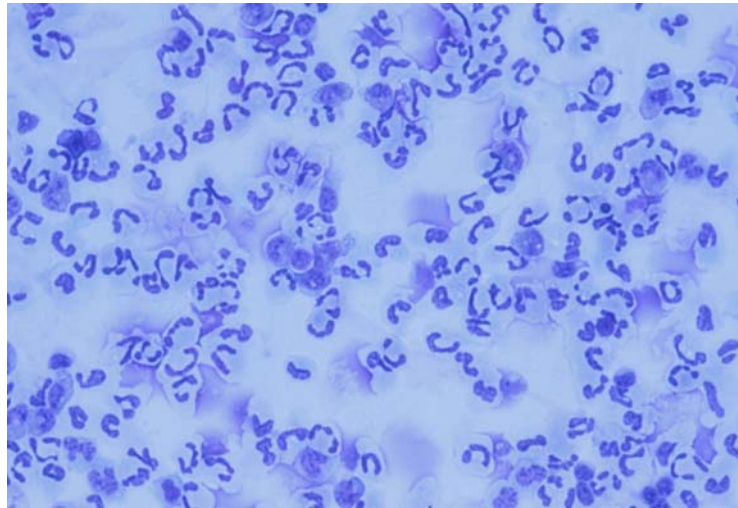
Figure 5.3: Photomicrograph of a Romanowsky's stained (DifQuik™) Cytospin™ slide preparation of bovine neutrophils after density gradient concentration and red cell lysis (400X).

Recombinant Thioredoxin Titration

In order to determine the best concentration of thioredoxin to use in the assays, a titration of different recombinant thioredoxin concentrations was run. Measuring nitric oxide in the supernatants from murine peritoneal macrophages (n = 2) stimulated with four different thioredoxin concentrations (1, 2, 5, and 10 µg/ml), the one determined to be the most stimulatory was used in all subsequent assays, both nitric oxide and superoxide.

Superoxide Assay

The super oxide anion (O_2^-) assay was preformed as described by Pick and Mizel (1981). This assay is a means by which the production of superoxide anion by intact neutrophils and macrophages can be measured spectrophotometrically as a function of

cytochrome C (Fc) reduction. Optical density readings were made at 550 nm using a Microplate Autoreader (Bio-Tek Instruments, Winooski, VT). The control activator or stimulant in this assay was phorbol 12-myristate 13-acetate (PMA) at a stock concentration of 10 µg/ml. Recombinant TRX was used at a stock concentration of 10 µg/ml. Four treatment groups of three wells each were used, with treatments consisting of the following: 1) cells only, 2) cells + PMA, 3) cells + TRX and 4) cells + PMA + TRX. The cell types tested included bovine monocyte-derived macrophages, bovine neutrophils, and mouse peritoneal macrophages.

Briefly, in a flat-bottom 96-well microtiter plate each sample to be tested was plated in triplicate, i.e. 3 wells per sample, to a total final volume of 200 µl. Starting with 150 µl of cell suspension at a concentration of 1×10^6 cells per well, stock reagents were added in the following amounts and order: 20 µl superoxide dismutase (SOD; appropriate test wells), 20 µl Fc (all wells), 2 µl PMA (appropriate test wells) and 10 µl TRX (appropriate test wells). The plate was then incubated at 37°C for 15 minutes, removed and centrifuged at 450 x g for five minutes. The cell-free supernatant was then carefully removed and placed into a new 96-well microtiter plate and kept on ice. The optical density of each well was then determined by reading the plate at 550 nm on a microtiter plate spectrophotometer. The µM concentration of O_2^- was determined by dividing the optical density (OD) reading by 9.5.

Nitric Oxide Assay

This assay, using the Greiss reagent, detects the accumulation of nitric oxide by activated macrophages in cell culture supernatants by measuring the more stable end-

product, nitrite (NO_2^-). This colorimetric assay was performed on bovine monocyte-derived macrophages and murine peritoneal macrophages as described by Chitko-McKown et al. (1991), and was used to determine the amount of NO_2^- present in both stimulated and non-stimulated supernatants. The control stimulus for this assay was different for each cell type. Lipopolysaccharide (LPS) was used on the bovine cells and peptidoglycan (PGN) was used on the murine cells. The reason for this difference is that the C57 Black/10ScN strain of mice are LPS-resistant and were used to eliminate stimulation of the cells by possible LPS contamination since the recombinant protein was generated in bacteria. Treatments were carried out in 24-well microtiter plates with four treatment wells for each animal. The five treatment groups used were 1) cells only, 2) cells + LPS (bovine macrophages) or PGN (murine macrophages), 3) cells + thioredoxin (TRX), 4) cells + TRX + LPS (or PGN) and 5) cells (bovine macrophages) + MBP.

Briefly, serum free RPMI 1640 (Life Technologies, Rockville, MD) containing Penicillin G/Streptomycin Sulfate (Life Technologies, Rockville, MD) and L-Glutamine (Life Technologies, Rockville, MD) was added to all 24 wells of the microtiter plate, then cells were added at a concentration of 1×10^6 cells per well. The plate was then placed in a 37°C, 5% CO_2 incubator and the cells were allowed to adhere for one hour. At the end of the one hour incubation, the spent RPMI 1640 was removed via pipette and was replaced with 1 ml fresh RPMI 1640 plus 5% fetal bovine serum (Life Technologies, Rockville, MD) and antibiotics. At this time, recombinant thioredoxin (10 μg; Shoda et al., 1999), peptidoglycan (20 μg), or both were added to the appropriate test

wells. The cells were then allowed to incubate for 30-36 hours after which the supernatant was collected and frozen at $-80°C$ for later nitric oxide assay.

Results

Endotoxin Assay

The E-Toxate assay for the presence of endotoxin showed there was no endotoxin (LPS) present in the MBP solution used for the cellular assays.

Insulin Reduction Assay

The biological activity of the recombinant TRX was determined by the reduction of insulin in the presence of the reducing agent DTT. In the negative control treatment in the absence of TRX, minimal reduction was seen over the assay period. However, in the presence of recombinant TRX, a significant increase ($p < 0.05$) in insulin reduction was observed. MBP and LPS had no effect on insulin reduction above that of the non-enzymatic control (Figure 5.4).

Recombinant Thioredoxin Titration

Four different recombinant TRX concentrations (1, 2, 5 and 10 µg/ml) were tested using murine ($n = 2$) peritoneal macrophage production of nitric oxide. The µg/ml concentrations of nitric oxide produced at each thioredoxin concentration were as follows: 1µg/ml, 0.004 µg/ml; 2 µg/ml, 0.008 µg/ml; 5 µg/ml, 0.0175 µg/ml; 10µg/ml, 0.029 µg/ml. From the assay results it was determined that 10 µg/ml recombinant TRX was the optimum concentration to be used in all of the cellular assays.
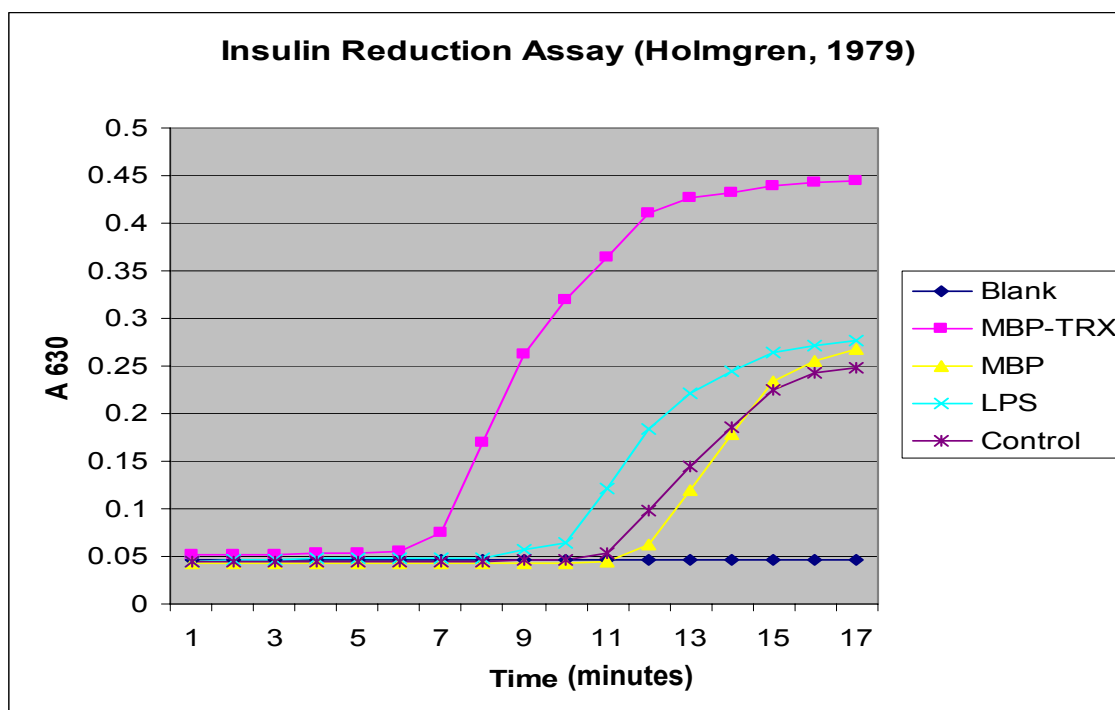
Figure 5.4: Results of the insulin reduction assay used to determine the biological activity of recombinant *Fasciola hepatica* thioredoxin.

Superoxide Assay

With significance set at $p \leq 0.05$, an analysis of variance test revealed no significant differences between the cell activator (PMA) and the activator plus TRX in any of the cell types tested (Figures 5.5 - 5.7).

Effect of TRX on Murine Peritoneal Macrophage Superoxide Production (n = 10)
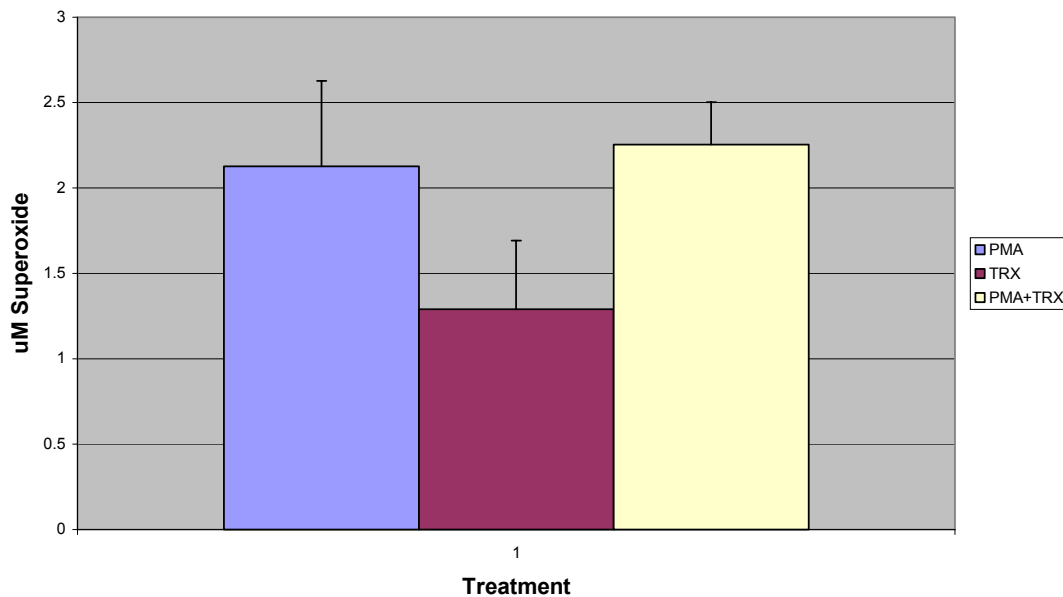
Figure 5.5: Effects of phorbol 12-myristate 13-acetate (PMA) and recombinant *Fasciola hepatica* thioredoxin (TRX) on the production of superoxide anion (O_2^-) on activated mouse peritoneal macrophages after 15 minutes exposure and measured spectrophotometrically by reduction of cytochrome c (Fc) at 550 nm (n = 10; PMA, 2 µl of 10 µg/ml stock per well; TRX, 10 µl of 10 µg/ml stock per well). After initial analysis, in order to normalize all values, the control value was subtracted from the treatment values in order to have all represented values greater than zero.

**Effects of TRX on Bovine Monocyte Derived Macrophage
Superoxide Production (n = 21)**
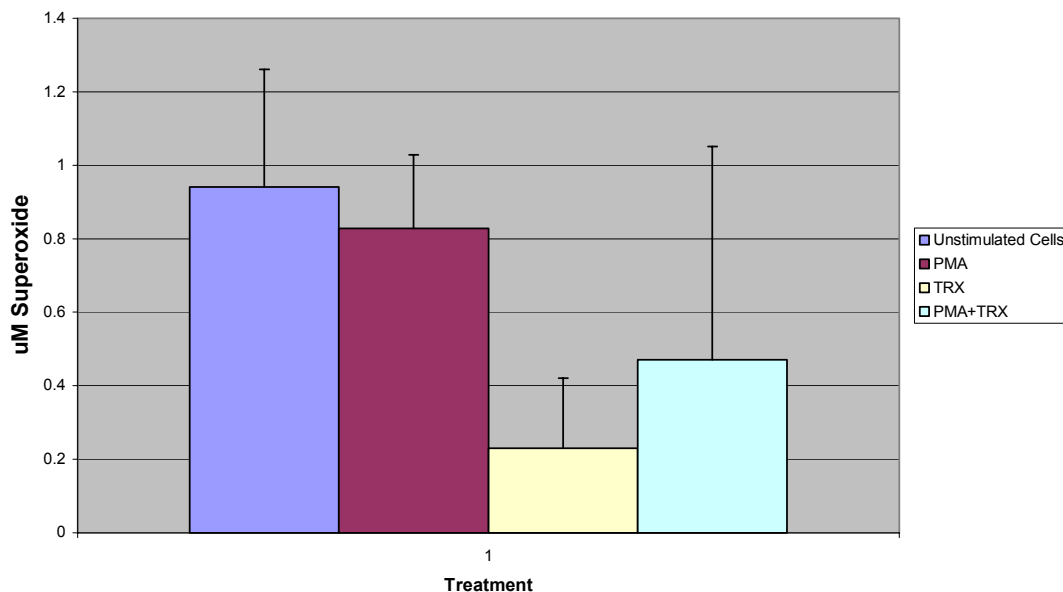
Figure 5.6: Effects of phorbol 12-myristate 13-acetate (PMA) and recombinant *Fasciola hepatica* thioredoxin (TRX) on the production of superoxide anion (O_2^-) on bovine monocyte-derived macrophages after 15 minutes exposure and measured spectrophotometrically by reduction of cytochrome c (Fc) at 550 nm (n = 21; PMA, 2 µl of 10 µg/ml stock per well; TRX, 10 µl of 10 µg/ml stock per well).

**Effects of TRX on Bovine Neutrophil
Superoxide Production (n = 22)**
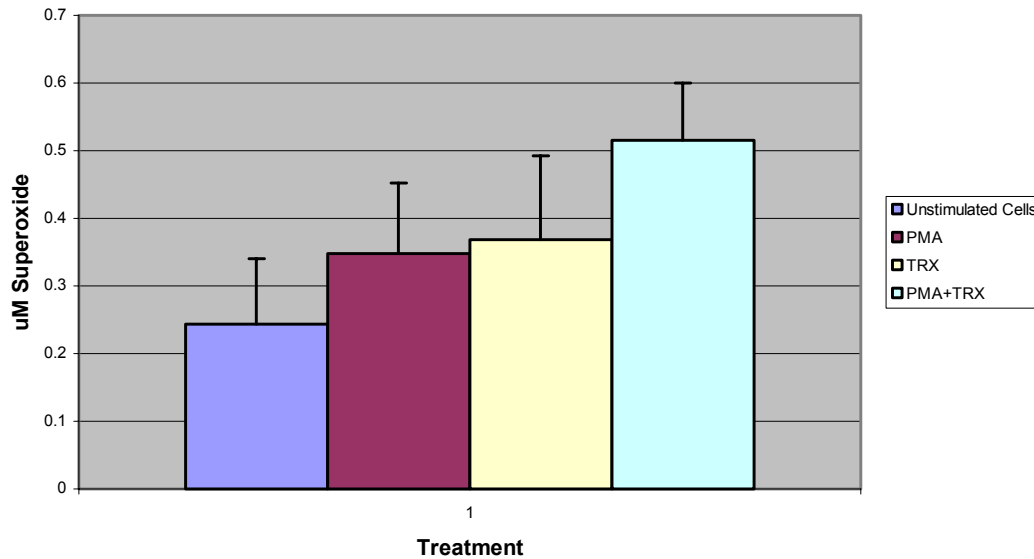
Figure 5.7: Effects of phorbol 12-myristate 13-acetate (PMA) and recombinant *Fasciola hepatica* thioredoxin (TRX) on the production of superoxide anion (O_2^-) on bovine neutrophils after 15 minutes exposure and measured spectrophotometrically by reduction of cytochrome c (Fc) at 550 nm (n = 22; PMA, 2 µl of 10 µg/ml stock per well; TRX, 10 µl of 10 µg/ml stock per well).

Nitric Oxide Assay

Results of the nitric oxide assay showed that TRX had no significant effects (p = 0.12) on the suppression of NO formation by murine peritoneal macrophages exposed to PGN. (Figure 5.8)

Figure 5.8: Effects of peptidoglycan (PGN) and recombinant *Fasciola hepatica* thioredoxin (TRX) on the nitric oxide production of activated mouse peritoneal macrophages as assayed by use of the Greiss reagent and measured spectrophotometrically at 550 nm. (n = 10; PGN, 20 μg; TRX, 10 μg).

The results of the nitric oxide assay showed all treatments were stimulatory to bovine monocyte-derived macrophages compared to control values. With significance set at $p \leq 0.05$ an analysis of variance test (ANOVA) showed that the differences seen between treatment groups were significant ($p = 0.02$) when compared to the non-treated control group (Figure 5.9).
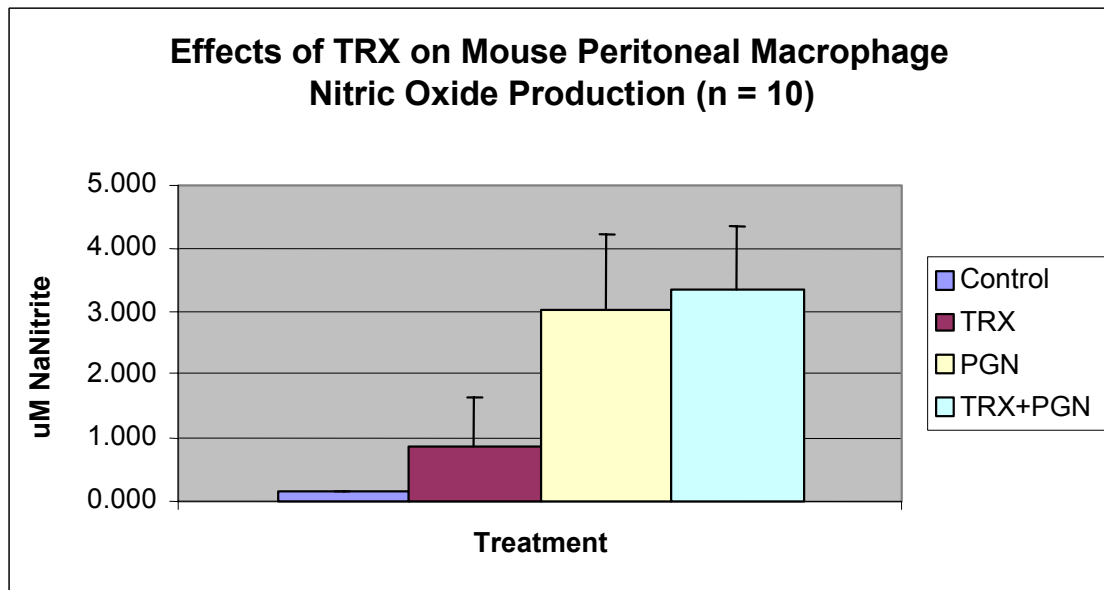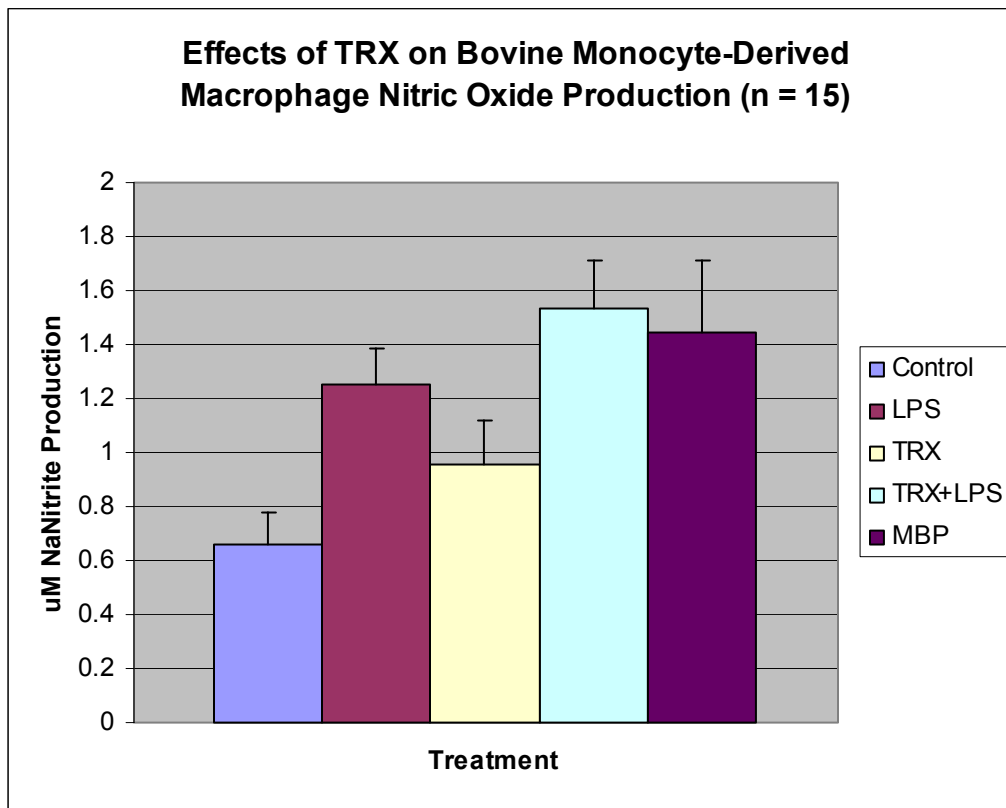

Figure 5.9: Effects of lipopolysaccharide (LPS) and recombinant *Fasciola hepatica* thioredoxin (TRX) on the nitric oxide production of bovine monocyte-derived macrophages as assayed by use of the Greiss reagent and measured spectrophotometrically at 550 nm. (n = 15; LPS, 20 µg; TRX, 10 µg).

Discussion

When considering the generic phrase "functions of thioredoxin" as it relates to parasitic infections, there appears to be some glaring differences dependent upon whether one is speaking about the host thioredoxin or that of the infecting parasite. Human thioredoxin has been shown, among numerous other functions, to provide cells with protection from oxidative stresses, the effects of hydrogen peroxide and the activities of stimulated neutrophils (Follmann and Haberlein, 1995; McGonigle et al., 1997; Nakamura et al., 1997; Rahlfs et al., 2002). While protecting the host from such events, all of which can and do occur during parasite invasion, thioredoxin has at the same time been shown to be a strongly chemoattractive to neutrophils, monocytes and lymphocytes (Bertini et al., 1999) as well as eosinophils (Hori et al., 1993). All of which are involved in the release of the very compounds needed in the defense against a parasitic infection.

Components of parasite thioredoxin systems such as the peroxiredoxins, if not thioredoxin itself, have been shown, by the use of electrons derived from the system, to reduce hydrogen peroxide produced by host cells (Henkle-Duhrsen and Kampkotter, 2001). It has also been suggested that in parasitic nematodes the peroxiredoxins are an essential component in the parasite's defense against reactive oxygen species generated by the macrophages, neutrophils and eosinophils of the infected host. They may also be of particular importance to those parasites responsible for chronic infections as seen with some nematode and trematode (*Schistosoma* and *Fasciola*) species of veterinary and human importance (Henkle-Duhrsen and Kampkotter, 2001). Another host protective mechanism is the production of immunoglobulins in response to various infections be they parasitic, bacterial, etc. In the case of *F. hepatica*, both Smith et al. (1992) and van

Milligen et al. (1998) demonstrated that immunoglobulins, in particular IgG, coated the

surface of NEJs. It was also shown that these antibody-coated flukes were attacked and

damaged by host eosinophils, neutrophils and macrophages, suggesting that the NEJs are

killed by an antibody-dependent cell-mediated cytotoxic response involving IgG. Using

Paragonimus westermani, Shin (2000) determined that neutrophils and eosinophils

express cell surface receptors for IgG and that a cell's efficiency at phagocytosis is

increased when specific IgG is bound to the worm.

In the late 1990s, various laboratories studying thioredoxin showed that human

IgG was a suitable substrate for thioredoxin at physiological concentrations typically

found within the tissues (Magnusson et al., 1997). Differences were also demonstrated in

the susceptibility of different IgG isotypes to cleavage by thioredoxin, with IgG2

showing no cleavage, but IgG1, IgG3 and IgG4 readily cleaved into heavy and light

chains (Berasain et al., 2000; Kerblat et al., 1999). Interestingly, it has been shown in

both naive and chronically *F. hepatica*-infected cattle and rats, that IgM, IgG1 and IgG2

are the most commonly detected immunoglobulins, with IgG1 being dominant (Clery et

al., 1996; Poitou et al., 1993; van Milligen et al., 1999). Whether the same cleavage

action against the various IgGs can be attributed to *Fasciola* thioredoxin is unknown.

It would appear that thioredoxin functions in ways that may be either beneficial or

detrimental to an invading parasite. Prior to this study, the only specific evidence of the

effects of *Fasciola* thioredoxin on host (cattle) immune function is the work of Shoda et

al., (1999) and el-Ghaysh et al., (1999). Shoda et al., (1999) determined that bovine T-

cell clones responded specifically, but weakly, to recombinant *F. hepatica* thioredoxin. Appearing to be only weakly antigenic, *F. hepatica* thioredoxin would thus most likely be a poor candidate for inducing an adaptive immune response in the host to infection. Similarly, el-Ghaysh et al. (1999) showed that both somatic extracts (which possibly contained thioredoxin even though this was not mentioned specifically) as well as excretory-secretory products from *Fasciola gigantica*, a species very closely related to *F. hepatica,* actually inhibited the production of superoxide anion by PMA-stimulated sheep neutrophils. Taken together, these two studies suggest a possible protective role for fluke thioredoxin against the host immune response.

The current study did not support the hypothesis and conclusions as demonstrated by el-Ghaysh et al. (1999) with *F. gigantica* or those of Jefferies et al. (1997) with *F. hepatica;* in that the production of O_2^- was not significant in the presence of recombinant *F. hepatica* thioredoxin unlike that seen with somatic extracts used in the other studies. The inhibition of O_2^- by somatic extracts in both studies was shown to be dose-dependent with greater concentrations causing more inhibition. However, this inhibition was only seen in those cells stimulated with PMA and not in resting cell populations. No such inhibition occurred in PMA-stimulated cells in the presence of TRX alone in the current study. The differences seen could be explained by the actions other compounds found in the extracts, and while the composition of the extracts was not determined, the amount or concentration of thioredoxin, if present at all, is unknown.

The purpose of this study was to determine the effects of *Fasciola* recombinant TRX on bovine immune cell types. When the results of the current study were taken into

account the factor that must be considered was demonstrated in Figure 5.9, which showed

the results of nitric oxide production by bovine monocyte-derived macrophages.

Included in this particular assay was the use of an MBP control to determine its effects on

cellular NO production. When compared to the control group, consisting of cells only,

all other treatment groups were stimulatory and increased the amount of NO produced to

a significant level ($p = 0.02$). A further breakdown of the data gave a clearer picture of

the results. When the effects of recombinant TRX were compared to the control, there

was a significant ($p = 0.01$) increase in the amount of NO produced. At the same time it

must be taken into account that the recombinant TRX was bound to an MBP moiety that

could also be having an effect on NO production. When this was considered, and the NO

production of the control, the recombinant and MBP alone were analyzed, there was a

significant ($p = 0.04$) increase in NO production, but not to as high a level. This indicated

that MBP stimulated bovine monocyte-derived macrophages to produce NO.

Further speculation can be made about the effects of MBP and TRX together and

separately. From these results it would appear that the MBP is stimulatory to the bovine

cells. Since the concentration of MBP in both the recombinant and the MBP alone were

the same, and if the MBP was stimulatory as it appeared to be, then one would expect

that the increase in NO production in both would be close to the same. This is in fact

proven statistically when the two are compared and no significant ($p = 0.39$) differences

in NO production were seen.

In order to see what effects recombinant TRX has in its ability to affect immune

cells of a given host, it would appear that TRX would have to be cleaved from the MBP

so that it could be tested alone and not in combination with the fusion protein. After

many attempts at cleaving the two, the decision was made to test the recombinant in the

uncleaved form (TRX•MBP). Further review of the literature dealing with recombinant

TRX (Alger et al., 2002; Salazar-Calderón et al., 2001; Shoda et al., 1999) and

consultation with the primary author of one of these papers (David L. Williams, personal

communication), it was found that all work done on trematode recombinant TRX has

been performed using the recombinant attached to its fusion protein. In all cases

including the present study, it was impossible to separate the two portions of the

recombinant protein and test the TRX alone. In the previous studies where cleavage was

attempted, each used a recombinant construct other than MBP, Salazar-Chalderón et al.,

used a glutathione S transferase (GST) carrier while Alger et al., used a histidine carrier,

with neither able to obtain sufficient cleavage and separation of the carrier from the

thioredoxin to test the recombinant thioredoxin alone. Only by using purified native

protein isolated from the parasite or until such time as the separation of the recombinant

from its fusion protein can be accomplished, will the true effects of recombinant *Fasciola*

TRX on host immune cells become clear.

CHAPTER VI

SUMMARY AND CONCLUSIONS

While fascioliasis and its causative agent *Fasciola hepatica* have been studied extensively, comparatively little of this research has been performed on the protein biochemistry of this parasite. The early work in this area has predominantly described the isolation and characterization of *Fasciola* proteins (Lammas et al., 1985; Rege et al., 1989) with few examples of actually localizing the proteins anatomically within the parasite (Hanna and Trudgett, 1983; Hanna et al., 1988; Zurita et al., 1989). In recent years however, the localization of proteins using various methods such as immunohistochemistry (Zurita et al., 1989), immunofluorescence (Marin et al., 1992; Stitt et al., 1992a), and immunogold labeling (Hanna et al., 1988; Marks et al., 1995; Smith et al., 1993), have become more routine in the study of *Fasciola* as well as numerous other helminth species (Bogers et al., 1995; Havercroft et al., 1991; Tuan et al., 1991).Protein biochemical studies to date primarily consist of protein isolation and localization in adult parasites (Marks et al., 1995; Rege et al., 1989; Stitt et al., 1992b; Waite and Rice-Ficht, 1989). Some work has been carried out on newly excysted juvenile flukes (NEJs) or those immatures still found in the liver parenchyma (Carmona et al., 1994; Lammas et al., 1985; Stitt et al., 1992b). However, very little has been done regarding the isolation and characterization of larval stage proteins or localization or occurrence of adult proteins in larval stages, such as sporocysts and redia. The paucity of such studies is most likely due to the complexity of the parasite life cycle and the inherent difficulty of obtaining the various life cycle stages for study.

One specific protein isolated from adult *F. hepatica* is thioredoxin (Richardson, 1994; Salazar-Calderon et al., 2001). Since its original isolation from the bacteria *Escherichia coli* (Laurent et al., 1964), thioredoxin has been found in diverse groups of both prokaryotic and eukaryotic organisms from plants, viruses, and bacteria, up to and including higher mammals. Within these species, thioredoxin performs or participates in a wide variety of biochemical processes, including protein disulfide reduction, ribonucleotide reduction, light regulation of chloroplast enzymes, sulfur metabolism, signal transduction and cytokine-like effects. Consisting of a group of small redox proteins with molecular masses of approximately 12 kD and showing functional and species diversity among the various thioredoxins, the active site sequence of Cys-Gly-Pro-Cys is conserved among all species, whether bacterial, viral, plant, or animal.

Recently thioredoxin has been shown to have protective activity against some host cellular immune functions (Follmann and Haberlein, 1995; Henkle-Duhrsen and Kampkotter, 2001; Magnusson et al., 1997; Nakamura et al., 1997). It exhibits protective action against the effects of tumor necrosis factor (TNF; Matsuda et al., 1991), as well as acting as an inhibitor of nuclear factor kappa B (NF-kB; Flohe et al., 1997). Another study, Fernando et al. (1992) demonstrated that thioredoxin is a component in the regeneration of proteins that have been inactivated by oxidative stress in endothelial cells.

It is well known that reactive oxygen species are an effective host defense mechanism against both intracellular and extracellular parasites (Abo-Shousha et al., 1999; Burnet, 2001; Callahan et al., 1988; James, 1995; Otsuka et al., 2001; Smith et al., 1992). Phagocytic cells such as eosinophils and neutrophils have been shown to kill parasites by undergoing a respiratory burst and releasing such reactive oxygen species as

hydrogen peroxide (H_2O_2) and superoxide anions (O_2^-) as well as reactive nitrogen species such as nitric oxide (NO). With the discovery of thioredoxin in several parasite genera such as *Fasciola* (Richardson, 1994), *Schistosoma* (Finken-Eigen and Kunz, 1997), *Echinococcus* (Chalar et al., 1999), *Plasmodium* (Muller et al., 2001), and *Trypanosoma* (Reckenfelderbaumer et al., 2000), and the growing evidence that thioredoxin can act as a redox-regulating molecule in the maintenance of cellular redox status (Nakamura et al., 1997), it was reasonable to speculate that thioredoxin may play a role in the protection of the parasite against the host immune response.

By utilizing an organism such as *F. hepatica* with its complex life cycle to study a protein like thioredoxin, it was deemed possible to determine if the protein was expressed throughout all stages of the life cycle or expressed selectively in some stages and not in others. Also, it was felt important to determine if its presence was consistent in the same organ systems or tissues between the various stages. Since some protective functions have been attributed to thioredoxin in other organisms, it was deemed pertinent to determine if it provided any protection against the host immune response to *Fasciola* infection.

Based on previous reports of the expression of thioredoxin in adult *F. hepatica* (Richardson, 1994; Salazar-Calderon et al., 2001), Western blot and immunohistochemical techniques were used to determine the presence and localization of this protein in the various life cycle stages of the parasite. Western blotting showed that thioredoxin was present not only in adult flukes, but also in redia and cercaria; however, it was not detected in eggs, miracidia, or sporocysts. Immunohistochemical studies using rabbit anti-*Fasciola* thioredoxin antibodies on paraffin embedded sections showed the

thioredoxin to be located within the tegument and cecal epithelium of the migrating juvenile, adult, redia, tegument of cercaria, and sperm within the uterus. The Western results obtained in this study were very similar to those of Alger et al. (2002), and their work with the thioredoxin of *Schistosoma mansoni* that described its location in cercaria, 3-hour schistosomula and adults, both males and females.

A BLAST 2 sequence comparison of the 560-base pair sequence of *Fasciola* thioredoxin (Fh2020.A) as described by Richardson (1994), to various other mammalian thioredoxins (mouse, rat, ovine, bovine, and human) resulted in no significant sequence similarities. While previous work and database searches have shown that numerous potential hosts of *F. hepatica* produce thioredoxin, the current immunohistochemical study convincingly showed the specificity of the primary antibody to *Fasciola* thioredoxin and not to that of the host. When sections of mouse liver containing migrating flukes where stained, distinct positive staining of fluke structures were demonstrated but no staining of the host tissues occurred.

Along with immunohistochemistry, *in situ* hybridization studies have shown that the two previously described isotypes of *Fasciola* thioredoxin are processed in what appears to be the same anatomical locations as where the protein itself is located. In addition, part of the previous work of Richardson (1994) was confirmed via *in situ* hybridization in that there appears to be variation in the expression of the two isotypes with Fh2020.A having a much greater staining intensity than does Fh2020.SL, which could demonstrate its greater production and abundance.

Because of the localization of thioredoxin within the different stages, its location in various tissues of the fluke and its known redox functions in other species, it was

deemed logical that *Fasciola* thioredoxin could have a possible role in the protection of the parasite against the host's response to infection. This hypothesis was tested using different blood cell types typically present in parasitic infections i.e. neutrophils, peripheral blood mononuclear cells, and monocyte-derived macrophages from cattle and peritoneal macrophages from mice. In the presence of recombinant *Fasciola* thioredoxin, the production of either superoxide anion or nitric oxide was determined against that of unexposed cells as well as cells exposed to a known stimulant as controls.

No significant effects were seen in superoxide production in any cell type tested ($p > 0.05$) and no significant effects were seen in nitric oxide production in mouse peritoneal macrophages ($p = 0.12$). However, in bovine monocyte-derived macrophage cultures, all treatments groups including an MBP control, showed significant differences in nitric oxide production ($p = 0.002$), when compared to the control group. When this finding is contemplated further, it must be remembered that the *Fasciola* recombinant thioredoxin is still bound to the MBP fusion protein. When the two treatment groups consisting of recombinant thioredoxin and TRX•MBP were compared, there was no significant difference in nitric oxide production ($p = 0.39$). Thus, these results must be viewed in the context of the role of MBP as a stimulant to nitric oxide production, at least in bovine monocyte-derived macrophages. While test results confirmed the stimulatory effects on that one cell type, whether this can be applied to the other cell types studied, remains to be seen. Intuitively, it seems likely that this effect would occur in the other cells as well. Thus, more work involving either native *Fasciola* thioredoxin or recombinant thioredoxin cleaved from the MBP will have to be performed in order to state with any certainty what the true effects of thioredoxin are on host immune cells.

As with most studies, in the process of answering the proposed questions, new questions have been generated. The presence and location of thioredoxin within the various stages of the parasite have been addressed in both the Western blotting studies and the immunohistochemical work. More detailed electron microscopy utilizing other immunostaining techniques (immunogold) could further isolate the cellular location of thioredoxin within the fluke and whether or not multiple cytoplasmic or mitochondrial systems are at work (Rahlfs et al., 2002). Such information could enhance understanding of its function as well. However, to date the function of *Fasciola* thioredoxin within the parasite remains to be fully elucidated. From an immunological standpoint, much remains to be worked out, for example, does recombinant thioredoxin, as used in the current study, have the same effects on host cellular activity as would the native protein? Previous work has shown the cleavage effects of human thioredoxin-1 on various IgG subtypes (Kerblat et al., 1999; Magnusson et al., 1997). Does *Fasciola* thioredoxin have the same effects? The location of the protein would seem to indicate that it could.

Since thioredoxin was first discovered and isolated from the bacteria *Escherichia coli* (Laurent et al., 1964), a tremendous amount of research has been directed towards revealing the functions of this small but multi-functional protein in a wide variety of organisms. From a parasitological viewpoint it has been shown that various parasites and their host organisms possess forms of thioredoxin with similar sequence homologies. But only recently have endeavors been directed towards a better understanding of the relationships between thioredoxin and how both the hosts and the parasites infecting them utilize this protein in their protection and defense against each other. With current

technology, the answers to these and many other questions are within reach and could

contribute greatly to our understanding of the functions of this highly important protein.

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