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Christopher J. Bayne

The fate of *Schistosoma mansoni* (Trematoda) sporocysts in its molluscan host *Biomphalaria glabrata* (Gastropoda) is determined by circulating phagocytes (hemocytes). When the parasite invades a resistant snail it is destroyed by hemocytes, whereas in a susceptible host it remains unaffected. In our studies we used 2 strains of *B. glabrata*: 13-16-R1 (resistant to the PR-1 strain of *S. mansoni*), and MO (susceptible to PR-1). *S. mansoni* is known to display a variety of potentially immunogenic carbohydrate moieties on its surface. To investigate whether hemocytes from resistant and susceptible *B. glabrata* differ in their ability to recognize carbohydrate structures, we tested 8 different BSA-carbohydrate conjugates for their capacity to elicit an aggressive response in these hemocytes. Three of the carbohydrate structures stimulated hemocyte generation of reactive oxygen species (ROS). The hemocyte responses were similar whether they were derived from susceptible or resistant snails. If the carbohydrate structures we found to stimulate ROS generation are involved in parasite recognition, our results suggest that even susceptible hemocytes may respond to *S. mansoni* sporocysts.

Using an *in vitro* killing assay we assessed the specific role(s) of reactive oxygen and nitrogen species (ROS/RNS), generated by resistant hemocytes, during killing of *S. mansoni*. Inhibition of NADPH oxidase significantly reduced parasite killing, indicating that ROS production is important. Reduction of hydrogen peroxide (H_2O_2) by including catalase in the killing assay also increased parasite viability. Reduction of superoxide (O_2^-), however, by addition of superoxide dismutase, or scavenging of hydroxyl radicals ($^{\cdot}OH$) and hypochlorous acid (HOCl) by addition of hypotaurine did not alter the rate of parasite killing. The nitric oxide synthase (NOS) inhibitor N_ω-nitro-L-arginine methylester (L-NAME) and the nitric oxide (NO) scavenger carboxy-PTIO reduced hemocyte-mediated killing. The peroxynitrite

scavengers uric acid and deferoxamine, however, had no influence on the rate of parasite killing. Combination of the NOS inhibitor L-NAME and catalase reduced average sporocyst mortality to a higher extent than only NOS inhibition by L-NAME. We suggest that NO and H_2O_2 are the only ROS/RNS involved in hemocyte-mediated toxicity against *S. mansoni*, and that NO and H_2O_2 act in a synergistic fashion.

Cytotoxic Responses of *Biomphalaria glabrata* Hemocytes towards the Parasite *Schistosoma mansoni*: The Role of Reactive Oxygen and Nitrogen Species

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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CONTRIBUTION OF AUTHORS

Randall C. Bender was involved in the design and writing of chapter 2 and chapter 3. Dr. Christopher J. Bayne served as my graduate advisor.

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Chapter 1. Introduction

GENERAL BACKGROUND

Like all trematodes, *Schistosoma mansoni* has a parasitic life style, which involves a rather complex life cycle. When *S. mansoni* eggs, released with the feces of a mammalian host, are exposed to freshwater (e.g. are released into lakes or ponds), the fully developed miracidium inside senses the osmotic change in its environment and hatches. The miracidium is a short-lived, free-living life stage, with a ciliated surface that enables it to swim and search for a suitable freshwater mollusc (of the genus *Biomphalaria*) that can serve as an intermediate host. Certain chemical cues help the parasite to identify the presence of a snail. Then the miracidium, with help of cell-lysing enzymes in its penetration glands, penetrates the mollusc's epidermis. Upon entering its intermediate host the miracidium sheds its ciliated plates and transforms into a mother sporocyst, a life stage with limited motility. After 2 to 3 weeks of living in the snail's hemolymph system, the mother sporocyst asexually produces daughter sporocysts, which migrate to the digestive gland, the parasite's final destination in its intermediate host. Here the parasite undergoes several more asexual reproductive cycles, leading to numerous daughter sporocysts, and eventually production of cercariae, which are shed by the snail and released into the surrounding water (reviewed by Basch 1991). Thanks to these asexual reproduction cycles, infection of the snail with only one miracidium can lead to hundreds, if not thousands, of cercariae. The cercaria is the second (short-lived) free-living life stage of *S. mansoni*, and the only one that is infectious to humans. This life stage is also capable of swimming, by use of its long bifurcated tail. Shedding of cercariae by snails is quite often temporally synchronized with the presence of the final hosts (e.g. humans) in the water. Guided by chemical signals the cercaria locates its final host and penetrates the skin by releasing the contents of its penetration glands. It then sheds its tail and undergoes a transformation into a physiologically adapted larval form known as a schistosomulum. These schistosomula rapidly leave the skin and migrate via the blood system to the lungs, through

which they transit over a period of days. Eventually the schistosomula enter the hepatic portal system, where male and female parasites reach sexual maturity 4-6 weeks after infection and enter a stage of permanent copulation. Female parasites begin laying eggs shortly after. Adult worm pairs live for years within the mesenteric veins, where they produce hundreds of eggs daily (reviewed by Basch 1991). While most eggs pass through the intestinal wall, are excreted and continue the life cycle, many become trapped in the liver and the spleen. The granulomata that subsequently form around the eggs are responsible for the pathology of the disease schistosomiasis.

Schistosomiasis, the disease caused by trematodes of the genus *Schistosoma* (the human blood fluke), presents a severe human health problem in tropical regions around the world, with an estimated 200 million people infected, and 500 to 600 million people at risk of infection. Schistosomiasis is a severely debilitating disease causing hepato- and splenomegaly. It accounts for about 20,000 deaths a year, mainly among children. Three species of *Schistosoma* account for the majority of cases: while *Schistosoma japonicum* and *S. haematobium* have decreased in prevalence and distribution over the past 50 years, *S. mansoni* has now become the most widespread of the three species. This is partly due to agricultural and irrigation projects in third world or developing countries, which expand the aquatic habitat of freshwater snails that act as vectors for the disease. Immediate efforts to control the disease include chemical and biological means of reducing or eliminating the vector, extensive surveys to monitor disease distributions, improved diagnostic measures and medical care. While treatment for the disease is available, diagnosis and therapy are expensive and often not available for people living in affected regions. Furthermore, there are indications from laboratory studies that schistosomes may become resistant to praziquantel, which is currently the most effective drug at hand (Fallon and Doenhoff, 1994). A major complication with treatment is that even if successful, it will not prevent reinfection. Development of a vaccine is therefore an important focus of research, and several promising antigen candidates have been identified and are currently under investigation. Recent studies in mice have provided important insights into the specifics of the mammalian immune response against the parasite.

In addition many efforts are under way to improve our understanding of the biology of the parasite. The *Schistosoma* Genome Project to date has identified over 6,000 genes in *S. mansoni*, an estimated 15 to 20% of its entire genome, the majority of which are unique to *S. mansoni* (Franco et al., 2000). cDNA libraries of all life stages of the trematode, except the sporocyst stage, are now available. Attempts are also being made to develop a schistosome

cell line, which could enormously facilitate research of the trematode's biology, because it would eliminate the necessity to maintain the complex and labor intensive parasite life cycle. Just recently the continuous *in vitro* propagation of the intramolluscan life stages of *S. mansoni* has been achieved (Yoshino and Laursen, 1995; Coustau et al., 1997; Ivanchenko et al., 1999; Coustau and Yoshino, 2000).

Trematodes are thought to have evolved from free-living flatworms, which adapted to a parasitic life in molluscs several hundred million years ago. The digenetic (two-host) life cycle that typifies most modern trematodes, and includes infection of a vertebrate host, evolved much later (Basch 1991). The extremely long coevolution of molluscs and trematodes, and the relatively short coexistence of vertebrates and the parasite are reflected in the different degrees of specificity for compatible host-parasite relationships. Infection of the molluscan host is often restricted to only one genus, which suggests that during the course of evolution the host specificity of a parasite increases. *S. mansoni*, for example, can establish a successful infection only in certain individuals of the genus *Biomphalaria*, and is destroyed immediately after penetration in any other freshwater snail. Infection of the vertebrate host, on the other hand is much less restricted, and *S. mansoni* successfully infects mammals as distantly related as mice and humans. Due to the extremely high host specificity, mollusc-parasite associations provide very interesting models for studying survival mechanisms of a particular parasite. Studying the host–parasite interactions in the snail vector may also present new insights into the possibility of interrupting the life cycle during this phase. Natural populations of *Biomphalaria* are very polymorphic with respect to susceptibility to infection with *S. mansoni* (Morand, Manning and Woolhouse, 1996), but the majority of snails is quite often resistant to infection (Mascara et al., 1999). The percentage of infected snails that can be found in natural populations is very low and rarely exceeds 1% (Christie and Upatham, 1977; Sire et al., 1999), which may reflect high levels of resistance in the snail populations. A study conducted in Guadeloupe found that the majority of infected snails harbored parasites that were derived from only a single miracidium and thus, were all the same sex (Sireet al., 1999). Successful infection of a mammal, however, requires both male and female schistosomes. Despite the low number of infected snails and the high number of single sex infections in the vector, over 90% of live-trapped rats in this area were infected with schistosomes, indicating that transmission of the parasite is not dependent on high infection rates in molluscs.

One can observe 3 different phenotypes in *Biomphalaria*, with respect to parasite susceptibility (Lie, Heyneman and Richards, 1977): (1) susceptible individuals provide a

suitable milieu for the parasite, and mount no defensive response; (2) in unsuitable individuals the intramolluscan environment is physiologically unfavorable and does not allow parasite development; (3) resistant individuals, in which the immune system actively responds to invasion of the parasite and destroys it. All 3 phenotypes are genetically determined in the snail (Richards and Merritt, 1972), with both unsuitability and resistance inherited in a dominant Mendelian fashion. Pathology in the snail includes castration for the duration of infection, and massive loss of hemoglobin in the hemolymph (which is ingested by the parasite). Considering that non-susceptibility is inherited in a dominant fashion and that infected susceptible snails suffer a loss in reproductive success compared to uninfected snails, one might reason that the resistant phenotype should have a natural advantage over the susceptible. The resistant phenotype would be expected to soon outnumber the susceptible phenotype in a population, especially if the parasite is present. However, this is not the case. Surprisingly, two studies, determining the reproductive success of susceptible snails versus either incompatible (Minchella and LoVerde, 1983) or resistant snails (Minchella, 1981), show that in the presence of both the parasite and susceptible snails the over all reproductive success of non-susceptible *Biomphalaria* is dramatically impaired. Even though these results are not easily explained they support the hypothesis suggested by Wright (1971) that there must be a disadvantageous character or physiological defect connected with being resistant to *S. mansoni*.

B. glabrata is hermaphroditic and capable of self-fertilization. Selective breeding and interbreeding of *B. glabrata* in the laboratory has led to establishment of strains of snails that display high susceptibility or resistance towards specific strains of *S. mansoni*. Snails of the M-line strain (Newton, 1955) of *B. glabrata* for example show high (over 90%) susceptibility towards a Puerto Rican strain of *S. mansoni* (PR-1). Snails from the 13-16-R1 and 10-R2 strains (Richards and Merritt, 1972), on the other hand, are highly resistant against infection against PR-1. This system provides an excellent means to determine how one strain successfully destroys the pathogen, while the parasite manages to escape destruction in the other strain. Progress in this field has been slow due to the fact that unlike the immune system in mice the internal defense mechanisms in molluscs are still very poorly understood, with only very few proteins characterized or sequenced. And no transgenic snails are at hand, which could be used to study the importance or involvement of specific genes in internal defense responses. On the other hand the immune response in *Biomphalaria* is thought to be much simpler than in mammals, and this might facilitate identification of specific

mechanisms, by which the parasite evades destruction in the host. It is very likely that the parasite employs similar strategies of immune evasion in invertebrates as in mammalian hosts.

The internal defense (immune) systems of molluscs are organized differently from the well-known immune systems of humans and mice. The main difference is the absence in molluscs of both lymphatic tissue and rearranging genes that are needed for development of antigen-specific antibodies and cell surface receptors in vertebrates. Consequently, molluscs do not utilize immunoglobulins for antigen recognition, do not have T-cells or B-cells, and do not have an adaptive immune response in the classical sense (van der Knaap and Loker, 1990). Yet, these animals are exposed to the same pathogens (viruses, bacteria, fungi, protozoan and metazoan parasites) as vertebrates, and despite the lack of an adaptive immune response, manage to efficiently destroy most invaders. Considering that invertebrates greatly outnumber vertebrate individuals and species, and some of them, like lobsters for example, even reach very old age comparable to large mammals, one must conclude that their internal defense systems, although 'simpler', are nevertheless quite competent. Microbes that invade a mollusc are recognized as non-self and then attacked by humoral and cellular components of the immune system.

In the absence of antibodies, lectins are considered to be responsible for self/non-self recognition (Renwrantz, 1986). Lectins are proteins that bind to certain carbohydrate structures with high specificity. In molluscs they occur in either soluble form in the hemolymph, or on the surface of circulating phagocytic defense cells (hemocytes). The surfaces of pathogens quite often display distinct repetitive carbohydrates, such as lipopolysaccharides (LPS) on bacteria, or mannan and glycan structures on yeast cell walls, presenting likely ligands for lectin binding. 'Pattern recognition' (Janeway, 1989) of carbohydrate structures that are associated with typically dangerous invaders allow lectins to bind to and agglutinate pathogens like bacteria, or opsonize them for phagocytosis. The use of lectins for recognition of danger patterns on pathogens still plays an important role in the innate immune systems of modern mammals, in the forms of the mannan-binding lectin and C-reactive protein, for example. There are numerous reports of soluble and hemocyte-associated lectins in molluscs with important immune functions (reviewed by Cheng, Marchalonis and Vasta, 1984; Renwrantz, 1986). *Biomphalaria*, for instance, has lectins that specifically bind to bacteria and to carbohydrate structures on the surfaces of sporocysts (Hertel et al., 1994; Mansour et al., 1995; Negm et al., 1995). Infection of *B. glabrata* with trematodes has been shown to induce expression and elevated concentrations of specific

lectins in the snails' hemolymph (Monroy, Hertel and Loker, 1992; Monroy and Loker, 1993; Loker, Couch and Hertel, 1994). Other humoral factors in the molluscan defense system include fibrinogen-related proteins (Adema et al., 1997), the protease inhibitor α-macroglobulin (Bender, Fryer and Bayne, 1992), probably members of the complement system, and potentially cytokines. There have been several reports of cytokine-like activities in molluscs (Granath, Connors and Tarleton, 1994; Ouwe-Missi-Oukem-Boyer et al., 1994; Ottaviani et al., 1995). Interleukin-1 like activity has been implicated in playing an important role in determining susceptibility and resistance in the *B. glabrata* - *S. mansoni* model (Granath, Connors and Tarleton, 1994; Connors, Buron and Granath, 1995; Connors et al., 1998). However, so far no cytokines have been isolated or sequenced from any invertebrate, and the use of enzyme-linked immunoabsorbance assay (ELISA)-based detection systems to seek evidence of such molecules can be very unreliable in invertebrate systems (Hahn, Fryer and Bayne, 1996).

Even though humoral factors of the snails' immune system may influence susceptibility or resistance towards *S. mansoni* sporocysts, there is good evidence that it is intrinsic differences in hemocytes that determine sporocyst survival in the host (Bayne, Buckley and DeWan, 1980). Hemocytes are professional phagocytic cells, either circulating in the snail's hemolymph, or migrating through connective tissue. Depending on their size, invading microbes are either phagocytosed (bacteria, yeast) or encapsulated by multiple layers of hemocytes (trematode sporocysts) and subsequently destroyed by cell-mediated cytotoxicity.

Cytotoxic mechanisms have been well described for mammalian immune effector cells, and include non-oxidative mechanisms, such as lysosomal enzymes, which are stored in granules in the immune cells and either released into the phagosome after phagocytosis, or secreted during encapsulation of larger targets. These lysosomal enzymes include lysozyme, acid and alkaline phosphatases, esterases, phospholipases and proteases. Macrophages and neutrophils are further capable of killing microbes via generation of reactive oxygen species (ROS). This response is typically receptor-mediated and leads to increased oxygen consumption due to NADPH oxidase activity in the plasma membrane. NADPH oxidase transforms molecular oxygen first into superoxide ($O_2^{\cdot-}$), which is followed by a range of other reactive oxygen species, such as hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\cdot}OH$), and hypochlorous acid (HOCl) (Fig. 1.1.). Cytotoxicity by ROS is exerted through oxidation of lipids, proteins and DNA, which leads to membrane damage, inhibition of enzymes and DNA

strand breaks. Mammalian leukocytes are also capable of generating reactive nitrogen species, by use of the enzyme nitric oxide synthase, which leads to potent oxidizing agents as well (Fig. 1.1.).

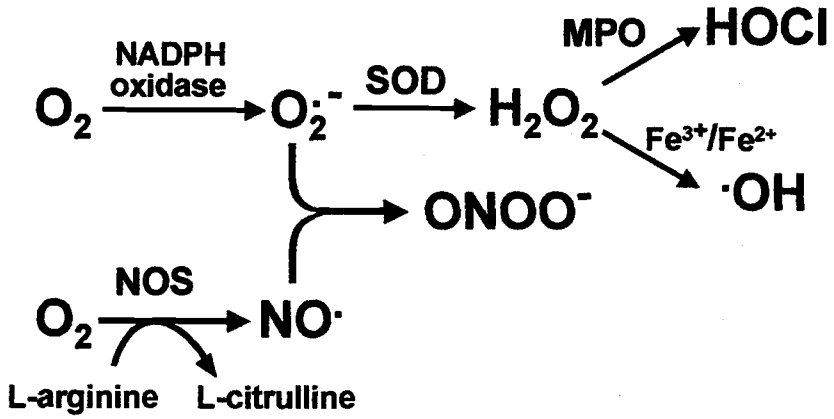

Figure 1.1. Illustration of the events that lead to generation of the major ROS/RNS by phagocytic cells. Membrane-bound NADPH oxidase transforms molecular oxygen (O_2) into superoxide ($O_2^{\cdot-}$). $O_2^{\cdot-}$ can spontaneously dismutates to hydrogen peroxide (H_2O_2), however, this process is greatly accelerated by the enzyme superoxide dismutase (SOD). In the presence of chloride H_2O_2 can be converted into hypochlorous acid (HOCl) by the enzyme myeloperoxidase (MPO). H_2O_2 can also react with reduced iron (Fe^{3+}) to yield hydroxyl radicals (·OH) (Fenton reaction). The enzyme nitric oxide synthase (NOS) utilizes O_2 to deiminate L-arginine to L-citrulline producing the RNS nitric oxide (NO). NO in turn can react with $O_2^{\cdot-}$, from the ROS pathway, to generate highly reactive peroxynitrite ($ONOO^-$).

Molluscan hemocytes seem to share many of the cytotoxic mechanisms with mammalian leukocytes. Lysosomal enzymes present in granules of *B. glabrata* hemocytes are very similar to enzymes found in human macrophages (McKerrow, Jeong and Beckstead, 1985). And like mammalian macrophages, these cells are capable of generating reactive oxygen species via the NADPH oxidase pathway (Adema et al., 1994). There have also been reports that molluscan hemocytes are capable of generating reactive nitrogen species as a cytotoxic response mechanism (Ottaviani et al., 1995; Torreilles and Guerin, 1999).

Even though the population of hemocytes is clearly not homogenous, there are no distinct surface markers known, like 'clusters of differentiation' on mammalian leukocytes, that allow for a characterization of different subpopulations. According to their spreading behavior on glass, the appearance of their cytoplasm, and their morphology, hemocytes are often grouped into hyalinocytes (small, phase-bright, non-spreading cells) and granulocytes

(larger, spreading cells with granular cytoplasm). Smaller, non-spreading hemocytes may, however, simply resemble immature hemocytes (Matricon-Gondran and Letocart, 1999).

All strains of *B. glabrata*, whether they are resistant to *S. mansoni* or not, have a fully competent internal defense system. How does the parasite then manage to avoid destruction? Several strategies have been discussed that may be employed by trematodes to escape the molluscan internal defense system. Among passive evasion mechanisms, molecular mimicry and molecular masking have been suggested. During the course of evolution, schistosomes might have been subject to selection for the expression of surface structures that share similarities with the host. Such molecular mimicry may enable the parasite to prevent immune recognition, and subsequent destruction (Damian, 1987). Lacking a gut, the tegument of sporocysts is the main site of nutrient absorption. When incubated in host plasma, the parasite quickly becomes coated with host molecules (Bayne, Loker and Yui, 1986). Such molecular masking by acquisition of host molecules by sporocysts may also ensure lack of detection by the host's internal defense system. Apart from these passive immune evasion strategies, active interference with the host immune system has also been suggested. Excretory and secretory products of some trematodes have been shown to modulate hemocyte function and behavior, e.g. intracellular biosynthesis (Lodes, Connors and Yoshino, 1991), hemocyte motility (Lodes and Yoshino, 1990), or phagocytosis rates (Fryer and Bayne, 1990). Schistosomes are also not quite defenseless against potential immune attack. *S. mansoni* possesses proteases and enzymes that neutralize ROS (e.g. superoxide dismutase, glutathione peroxidase), which allow them, to a certain extent, to detoxify phagocyte-generated lysosomal enzymes and ROS.

When a *S. mansoni* miracidium penetrates a susceptible host it remains largely unaffected by the host's defense system. In a resistant snail, however, an invading miracidium becomes quickly encapsulated and destroyed by circulating hemocytes in the snail's hemolymph. In such cases invading parasites are killed within 24 to 48 hours (Loker et al., 1982; Jourdane, 1982; Sullivan, Spence and Nunez, 1995; Pan, 1996). In *in vitro* systems, plasma-free hemocytes from *B. glabrata* strain 10-R2 have been shown to damage *S. mansoni* sporocysts (Bayne, Buckley and DeWan, 1980). Furthermore, resistance to *S. mansoni* sporocysts was successfully transferred by transplanting the hemocyte (amoebocyte) producing organ (APO) from resistant into susceptible strains (Sullivan, Spence and Nunez, 1995; Sullivan and Spence, 1999). These findings suggest that humoral factors are not required for killing of the parasite, and are therefore unlikely to be determinants of resistance and susceptibility in this model.

The lack of successful elimination of the parasite in susceptible individuals could be due to either a lack of recognition or to an ineffective immune response by the molluscan hemocytes. The means by which *B. glabrata* hemocytes recognize *S. mansoni* sporocysts as non-self, however, are not fully understood. There is also little known about the mechanisms responsible for killing of *S. mansoni* sporocysts by resistant hemocytes. Due to there being so many unknowns it is very difficult to identify the factor(s) involved in determining susceptibility and resistance in this host-parasite model. Genetic approaches have so far failed to elucidate this problem. Using randomly amplified polymorphic DNA (RAPD) – PCR, two markers were identified that segregated reliably with the resistant phenotype of *B. glabrata* (Knight et al., 1999). Both markers occur as repetitive elements in the snail genome, and one of the markers contains an imperfect open reading frame. However, no homology to immunologically relevant molecules was found. The objective of the following study was therefore first to determine whether resistant and susceptible individuals of *B. glabrata* differ in their basic recognition of non-self, in particular in their recognition of carbohydrate structures that are displayed on the *S. mansoni* sporocyst surface. There are several indications that generation of reactive forms of oxygen by hemocytes plays a crucial role in parasite elimination. *B. glabrata* hemocytes have been shown to produce reactive oxygen species (ROS) when they encounter *S. mansoni* miracidia (Shozawa, Suto and Kumada, 1989) and also use ROS to kill the trematode *Trichobilharzia ocellata* (Adema et al., 1994). Hemocytes from a different snail, *Lymnaea stagnalis*, produce ROS when they encounter *S. mansoni* sporocysts (Dikkeboom et al., 1988) and this oxidative burst was shown to be involved in killing of the parasite (Adema et al., 1994). We therefore focused specifically on differences in ROS generation by *B. glabrata* hemocytes in response to carbohydrate structures. The second objective was to identify the exact mechanisms that are utilized by resistant *B. glabrata* hemocytes to successfully destroy the parasite. Again, we were particularly interested in the role of reactive oxygen species generated by the molluscan immune cells and we also investigated the possible role of generation of reactive nitrogen species in killing of the parasite. Knowledge of the exact mechanisms employed by resistant *B. glabrata* hemocytes, during destruction of *S. mansoni* sporocysts, could then provide the means to investigate why hemocytes from susceptible *B. glabrata* fail to kill.

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Chapter 2.

Production of Reactive Oxygen Species by Hemocytes of *Biomphalaria glabrata*: Carbohydrate-specific Stimulation

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ABSTRACT

Recognition of specific carbohydrate structures, which occur commonly on the surfaces of invading pathogens, is thought to elicit internal defense mechanisms in invertebrates. To investigate the nature of carbohydrates that evoke a defensive response in hemocytes of the gastropod *Biomphalaria glabrata*, we tested 8 different carbohydrates, conjugated to bovine serum albumin (BSA), for generation of reactive oxygen species (ROS). Six of the carbohydrate moieties tested are thought to be present on the *Schistosoma mansoni* sporocyst surface (mannose, galactose, fucose, N-acetyl-glucosamine, N-acetyl-galactosamine, and lactose); the other 2 carbohydrates tested were glucose and melibiose. ROS generation was measured using the fluorescent probe 2',7'-dichlorofluorescein-diacetate (DCFH-DA). Hemocytes were derived from two different strains of *B. glabrata*: one of the strains used (MO) is susceptible to infection by the trematode *S. mansoni* (PR-1 strain), while the other snail strain (13-16-R1) is resistant to infection with PR-1. Three of the BSA-carbohydrate conjugates (BSA-galactose, BSA-mannose, and BSA-fucose) stimulated generation of reactive oxygen species in the molluscan hemocytes. The responses of the hemocytes were similar whether they were derived from susceptible or resistant snails. If the carbohydrate structures we found to stimulate ROS generation are involved in parasite recognition, our results suggest that parasite killing may involve either qualitative differences in production of reactive oxygen species, or additional factors.

Keywords: *Biomphalaria glabrata*, hemocytes, reactive oxygen species, carbohydrate, neoglycoprotein, lectin receptor, *Schistosoma mansoni*

INTRODUCTION

The fate of *Schistosoma mansoni* (Trematoda) in its molluscan host *Biomphalaria glabrata* (Gastropoda) is determined by circulating phagocytes (hemocytes). When the parasite invades a resistant snail, its sporocysts are attacked and destroyed by hemocytes, while in a susceptible host they remain unaffected by the host's defense system. The mechanisms that determine survival of *S. mansoni* sporocysts within a susceptible host are yet

to be elucidated. One possible scenario is that hemocytes in susceptible snails fail to recognize the parasite. However, the specific determinants which elicit defense mechanisms in this particular and many other host-parasite relationships are unknown. Terminal carbohydrate groups are prominent on the surface of all developmental stages of *S. mansoni* [1-4] and some of these carbohydrate structures may play an important role in recognition by the host's defense system. *S. mansoni* expresses glycoconjugates that elicit cellular immune responses in mammalian hosts [5] as well as in one of its molluscan hosts, *Biomphalaria alexandrina* [4]. Thus it is likely that some of the carbohydrates displayed on the sporocyst surface evoke an immune response in *B. glabrata* hemocytes.

The means by which hemocytes from resistant *B. glabrata* manage to kill parasites are not fully understood. However, several studies have indicated that reactive forms of oxygen play a crucial role. Hemocytes from the gastropod, *Lymnaea stagnalis*, produce reactive oxygen species (ROS) when they encounter *S. mansoni* sporocysts [6], and this oxidative burst plays a role in killing of the parasite [7]. In the case of *S. mansoni* and *B. glabrata*, miracidia have been shown to stimulate superoxide production in hemocytes [8]. In this study, we first determined if certain carbohydrates present on the surface of *S. mansoni* sporocysts would elicit production of ROS by resistant *B. glabrata* hemocytes. We then determined whether any of the stimulatory carbohydrates would fail to elicit a response in hemocytes from susceptible snails.

MATERIALS AND METHODS

All chemicals, if not indicated otherwise, were purchased from Sigma (St. Louis, MO).

Animals

Hemocytes of two different strains of *Biomphalaria glabrata* were tested in this study: M-line Oregon (MO), which is susceptible to our PR-1 strain of *Schistosoma mansoni*, and 13-16-R1, which is resistant to this strain. All snails were maintained at 26°C on a 12 hrs light 12 hrs dark cycle, and were fed green leaf lettuce *ad libitum*. Snail hemolymph was retrieved via cardiac puncture and collected on Parafilm to let shell debris settle [9].

Retrieval of hemocytes and incubation with DCFH-DA

Hemoglobin, dissolved in the plasma of planorbid snails, was found to oxidize the fluorogenic probe used to measure reactive oxygen species, which was expected because the probe is known to react with Fe^{2+} [10]. In order to prepare hemoglobin-free hemocytes we separated cells from plasma by spinning them through Ficoll [11]. Hemolymph (500 µl for 13-16-R1 and 800 µl for MO, since this strain has a lower hematocrit) was placed carefully onto 2 ml of a Ficoll solution in 12 x 75 mm siliconized glass tubes, containing a solidified 0.5% agarose gel plug at the bottom. The Ficoll solution consisted of 5% Ficoll (Type 400) in sterile filtered sugar-free (without 0.1% glucose and 0.1% trehalose) Chernin's balanced salt solution (SFCBSS) [12] containing 10 µM 2',7'-dichlorodihydrofluorescein-diacetate (DCFH-DA) (Molecular Probes, Inc., Eugene OR) and 1% dimethyl sulfoxide. Cells were spun through the Ficoll column and onto the agarose plug at 50 x g for 45 min. The plasma fraction remaining on top of the Ficoll and 1.5 ml of the Ficoll solution were aspirated. Using a pipette, the cells were then flushed from the agarose surface. The bottom 500 µl of the Ficoll, containing most of the hemocytes, were transferred to new glass tubes containing 2.5 ml SFCBSS on top of an agarose plug. These tubes were also spun at 50 x g for 30 min at room temperature. This step was included to reduce the amount of Ficoll and non-incorporated fluorescent probe, as recommended by the manufacturer of DCFH-DA (Molecular Probes, Technical Support). The supernatant was aspirated and the remaining 300 µl of cell suspension were distributed into wells of a 96-well plate (Corning; Acton, MA). Each hemocyte pool was divided into 6 different wells (50 µl of cell suspension containing between 5,000 and 15,000 cells).

Assay for measuring ROS production

A microplate assay described by Rosenkranz et al. [13], employing DCFH-DA, was adapted and modified for measuring intracellular ROS in molluscan hemocytes. DCFH-DA is widely used to measure generation of ROS in mammalian neutrophils and macrophages. To our knowledge this is the first study using this fluorescent probe to measure ROS production by invertebrate hemocytes. Oxidation of DCFH-DA by reactive oxygen species leads to a fluorescent product, which can be quantified. DCFH-DA is oxidized by hydrogen peroxide and probably hydroxyl radicals, but not superoxide [10]. It has also been reported to detect nitric oxide (NO) [14]. *B. glabrata* hemocytes incorporated the fluorescent probe during passage through the Ficoll. Fluorescence was measured on a Cyto Fluor 2300 (Millipore; Bedford, MA) fluorescence plate reader, using a 485 nm excitation filter and a 530 nm

emission filter. The plate was incubated at 26°C in a humid chamber in the dark and readings were taken every 20 min. Data for the different treatments were expressed as percent change in fluorescence units (FU) in experimental wells compared to stimulant-free control wells ($FU_{treatment} - FU_{control} / FU_{control} \times 100$).

Stimulation by free sugars

To test whether saccharides will stimulate production of ROS in *B. glabrata* hemocytes, different sugars were dissolved in CBSS and added to the cells at final concentrations of up to 50 mM. ROS production was measured from 0 to 4 hrs after addition of the sugars. The carbohydrates tested were α-D-galactose, L-fucose, α-D-glucose, α-D-mannose, α-methyl-D-mannoside, N-acetyl-β-galactosamine, N-acetyl-β-glucosamine, melibiose, and lactose.

Optimization of stimulant concentration

Bovine-galactosamide albumin (Gal-BSA) was used as a stimulant for ROS production in *B. glabrata* hemocytes. To determine the optimal concentration for stimulation of ROS production, Gal-BSA was diluted in complete Chernin's balanced salt solution (CBSS) and added to the hemocytes at a final concentration of 50, 100, 200, and 400 nM. For each hemocyte pool, one well received 400 nM non-conjugated bovine serum albumin (BSA; fraction V powder, 96-99%) in CBSS as a control for BSA, and a second well for each hemocyte pool received CBSS (without BSA) as a stimulant-free control.

Inhibition of NADPH oxidase

To test whether increase in fluorescence was due to ROS generated by the hemocytes via the NADPH oxidase pathway, we added protocatechuic acid (PCA) to the cells 30 min prior to addition of the stimulant. PCA is a catechol, which after reaction with reactive oxygen species yields quinones that interfere with the assembly of a functional NADPH oxidase enzyme complex from its subunits [15,16]. Adema et al. [17] validated PCA inhibitory effects on NADPH oxidase in molluscan hemocytes. The inhibitor was added at final concentrations of 40, 80, and 160 μM.

BSA-conjugated carbohydrates

The BSA-carbohydrate conjugates consisted of 15-20 sugar molecules per BSA molecule. All conjugates were diluted in CBSS and added to the hemocytes at a final concentration of 200 nM. The conjugates tested were bovine-galactosamide albumin (Gal-BSA), bovine-fucosylamide albumin (Fuc-BSA), bovine-glucosamide albumin (Glc-BSA), bovine-p-aminophenyl-α-D-mannopyranoside albumin, (Man-BSA), bovine-p-aminophenyl-N-acetyl-β-galactosaminide albumin (GalNAc-BSA), bovine-p-aminophenyl-N-β-glucosaminide albumin (GlcNAc-BSA), bovine-lactosyl albumin (lactose-BSA), and bovine-melibiosyl albumin (melibiose-BSA). For each hemocyte pool one well received 200 nM of non-conjugated BSA in CBSS as a control for BSA. A second well for each hemocyte pool received CBSS (without BSA) as a stimulant-free control.

Alternative protocol for testing BSA-conjugated carbohydrates

We were concerned that the small amount of Ficoll, which is a synthetic polymer of sucrose, remaining in the hemocyte suspension during the assay may interfere with possible stimulation of Glc-BSA, lactose-BSA, and melibiose-BSA. These three conjugates were therefore tested using an alternative protocol for preparing the hemocytes without the use of Ficoll. Each well of a 96-well plate received 100 µl of 13-16-R1 (or 150 µl of MO) hemolymph. The cells were allowed to settle for 50 min and then washed 10 x with SFCBSS to remove all hemoglobin. DCFH-DA was added at a final concentration of 10 µM in 1% DMSO and incubated for 20 min before the BSA-conjugated carbohydrates were added. Fluorescence was measured at 30 min intervals.

Competitive inhibition by free sugars

To test whether addition of free carbohydrates would competitively inhibit the production of ROS elicited by carbohydrate-BSA-derivatives, a final concentration of 25 mM of the respective monosaccharide was added to the cells 30 min prior to addition of the carbohydrate-BSA conjugate. Within each hemocyte pool, ROS production was measured after stimulation with Gal-BSA, Man-BSA, and Fuc-BSA, with or without pre-incubation in the respective monosaccharides (i.e. α-D-galactose, α-D-mannose, and L-fucose).

RESULTS

Production of reactive oxygen species

Biomphalaria glabrata hemocytes from both strains were incubated with the fluorogenic probe DCFH-DA, which forms a fluorescent product when it is oxidized by ROS generated by the cells. To test the effects of different carbohydrates (α-D-galactose, L-fucose, α-D-glucose, α-D-mannose, α-methyl-D-mannoside, N-acetyl-β-galactosamine, N-acetyl-β-glucosamine, melibiose, and lactose) these were added to hemocytes and fluorescence was measured. At concentrations of up to 25 mM none of the carbohydrates elicited ROS production by *B. glabrata* hemocytes (data not shown). Carbohydrate concentrations higher than 25 mM posed an osmotic stress upon the cells.

Addition of 200 nM galactose-conjugated BSA resulted in the production of ROS by both MO and 13-16-R1 hemocytes (Figure 2.1.). It is very unlikely that NO was measured in this assay, because generation of detectable NO levels, at least in mammalian leukocytes, typically requires incubation times longer than 6 hrs. The slow increase in fluorescence in control wells containing unstimulated cells is probably due to oxidation of residual (not incorporated) DCFH-DA upon reaction with air. The fluorogenic probe was found to be very sensitive in detecting hydrogen peroxide (H_2O_2) (80 μM of H_2O_2 were still detectable). ROS generated by MO and 13-16-R1 hemocytes after stimulation could be detected even with small numbers of cells (as low as 5,000 per well). The most dramatic increase in relative fluorescence between stimulated and unstimulated hemocytes occurred within 30 min after stimulation. Since oxidation of DCFH-DA and the resulting increase in fluorescent product is cumulative, fluorescence continued to increase over time; however, no significant increase in relative fluorescence occurred after two hours. Therefore the data presented in all subsequent figures are derived from readings taken 120 min after addition of the stimulus.

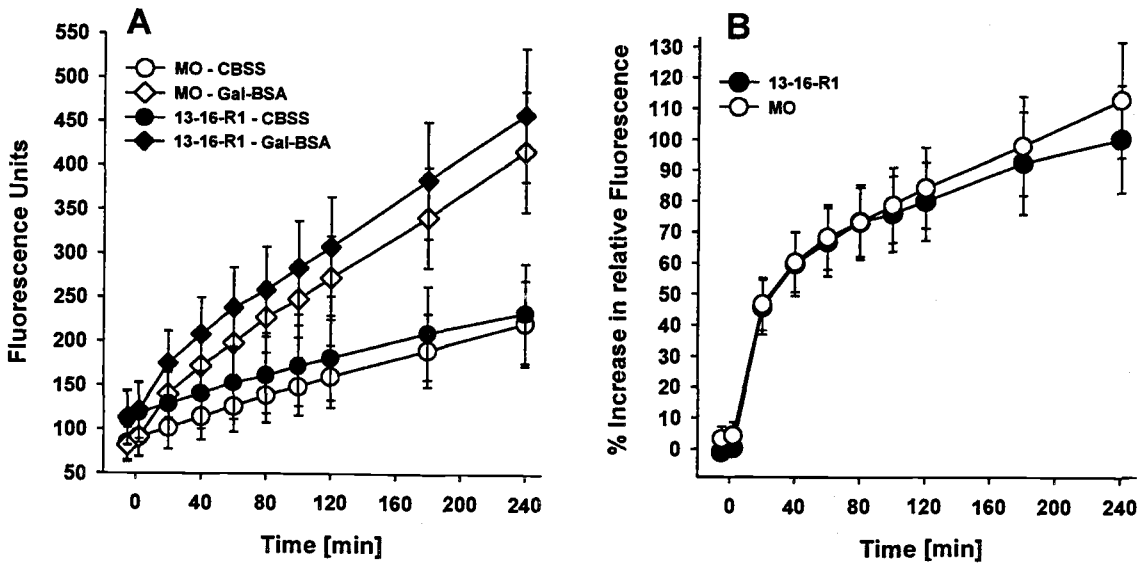

Figure 2.1. Kinetics of the oxidative burst in *Biomphalaria glabrata* hemocytes. Gal-BSA (200 nM) or buffer (CBSS) was added to *B. glabrata* hemocytes at time = 0 min. (A) The graph shows the increase in fluorescence over time for MO and 13-16-R1 hemocyte pools, after addition of Gal-BSA or CBSS. (B) The data points represent percent increase in fluorescence over time compared to unstimulated control cells; (n = 6; error bars = SE).

Figure 2.2. shows that the response to Gal-BSA was concentration-dependent. However, a concentration of 400 nM Gal-BSA compromised hemocyte viability. Therefore, 200 nM of BSA-carbohydrate derivatives was used as the standard dose to optimize stimulation and hemocyte viability.

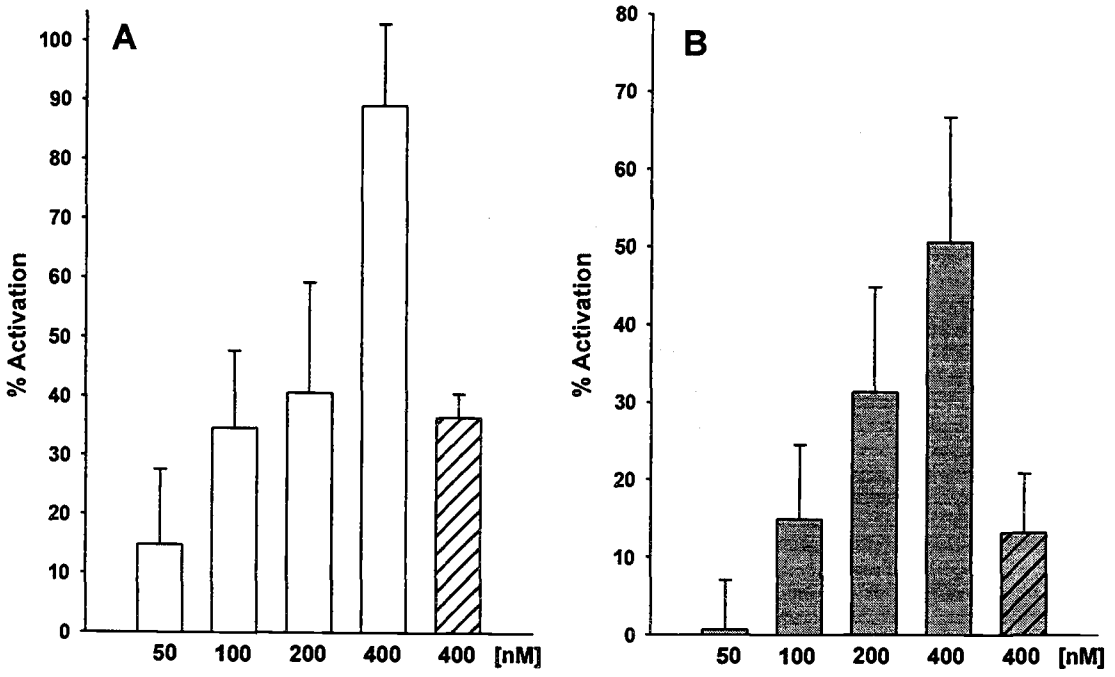

Figure 2.2. Hemocytes were stimulated with different concentrations of Gal-BSA (50 nM, 100 nM, 200 nM, or 400 nM) or 400 nM non-derivatized BSA (hatched bars). The bars represent the increase in fluorescence relative to unstimulated control cells at 120 min after addition of the stimulants. (A) MO hemocyte pools (n = 4; error bars = SE); (B) 13-16-R1 hemocyte pools (n = 3; error bars = SE).

Inhibition of NADPH oxidase

PCA was added to the hemocytes 20 min prior to stimulation with Gal-BSA to test whether the observed increase in fluorescence would be compromised by this NADPH oxidase inhibitor. Preincubation of hemocytes with PCA decreased the response to Gal-BSA significantly in hemocytes from both strains (MO and 13-16-R1) (Figure 2.3.). Data were compared using a paired t-test; p-values < 0.05 were considered significant. Addition of PCA at a final concentration of 40 μM reduced the production of ROS by MO hemocytes by 55%, and by 13-16-R1 hemocytes by 47% on average. PCA at a concentration of 80 μM reduced ROS-levels by an average of 62% and 61% in MO and 13-16-R1 hemocytes, respectively.

PCA at a concentration of 160 μM resulted in an average decrease of ROS production of 60% for MO and 13-16-R1 hemocytes.

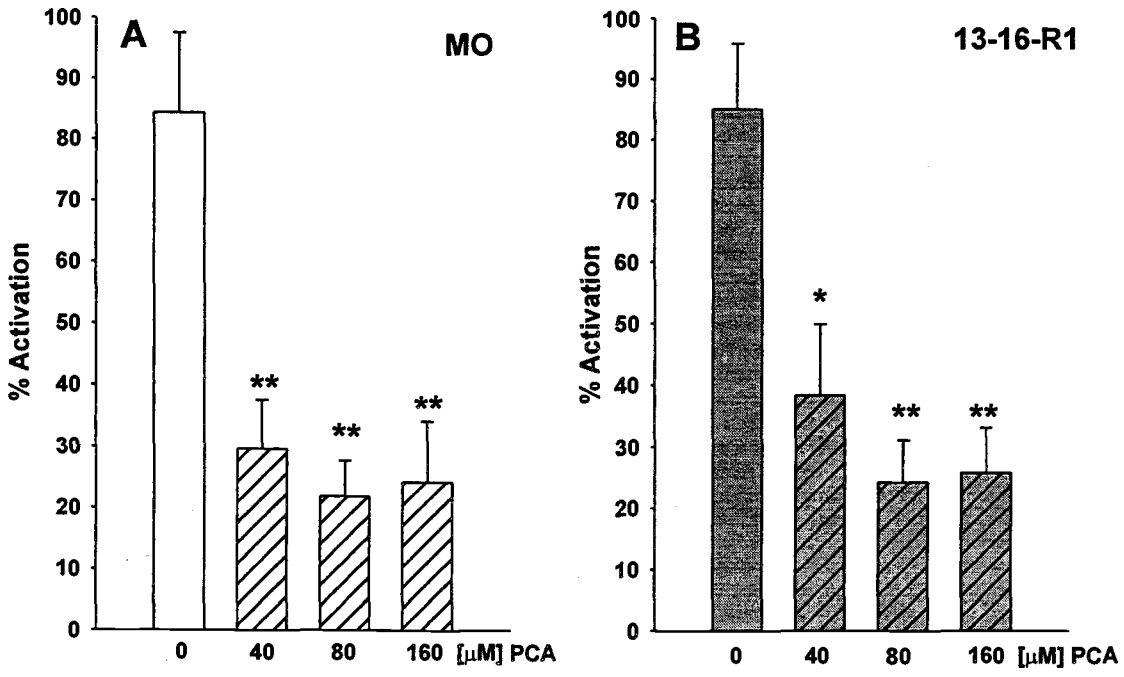

Figure 2.3. The effects of addition of protocatechuic acid (PCA), an NADPH oxidase inhibitor, on the oxidative burst responses of hemocytes stimulated with Gal-BSA. PCA was added to the cells at different concentrations [0μM (unhatched bars), 40, 80, and 160 μM] 30 min prior to addition of the stimulant. The bars represent the relative fluorescence levels at 120 min after addition of Gal-BSA. (A) MO hemocyte pools (n = 6; error bars = SE); (B) 13-16-R1 hemocyte pools (n = 6; error bars = SE). Asterisks indicate significant reductions in the response (*) $p < 0.05$, (**) $p < 0.01$ (using Student's paired t-test).

BSA-conjugated carbohydrates

We tested different carbohydrates, conjugated to BSA, for their ability to stimulate ROS generation by *B. glabrata* hemocytes. All carbohydrate-BSA derivatives were added at a concentration of 200 nM. Only Man-BSA, Fuc-BSA, or Gal-BSA increased relative fluorescence levels significantly above those seen with non-conjugated BSA (Figure 2.4.). Addition of Glc-BSA, GalNAc-BSA, GlcNAc-BSA, lactose-BSA, or melibiose-BSA did not

trigger a cell response different from the response to BSA. No statistically significant differences were seen between the responses of MO hemocytes and 13-16-R1 hemocytes. Assuming base-line fluorescence levels of unstimulated hemocytes are the same for both strains, and all hemocytes in the pools are equivalent in generating ROS, we infer that there are no quantitative differences in the responses of MO and 13-16-R1 hemocytes. Data were compared using a Student's t-test; p-values < 0.05 were considered significant.

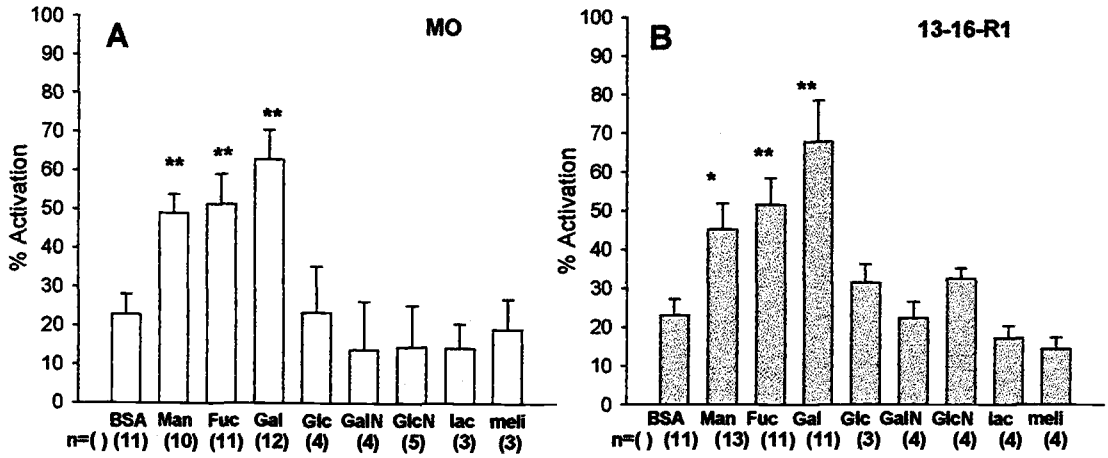

Figure 2.4. The relative increase in fluorescence 120 min after addition of non-derivatized BSA, Man-BSA (Man), Fuc-BSA (Fuc), Gal-BSA (Gal), Glc-BSA (Glc), GalNAc-BSA (GalN), GlcNAc-BSA (GlcN), lactose-BSA (lac), or melibiose-BSA (meli) at final concentrations of 200 nM. (A) MO hemocyte pools; (B) 13-16-R1 hemocyte pools. Asterisks indicate fluorescence levels that are significantly higher than stimulation levels for non-derivatized BSA; (*) $p < 0.05$, (**) $p < 0.01$ (using Student's t-test).

Alternative protocol without Ficoll

To determine if the absence of a response to Glc-BSA, lactose-BSA, or melibiose-BSA was due to the effects of Ficoll (a polymer of glucose and fructose), these BSA-conjugated carbohydrates were also tested separately on cells prepared without Ficoll. The fluorescence in unstimulated control wells was higher in these assays, indicating that the hemocytes were not as 'rested' as hemocytes that were prepared by spinning them through Ficoll. The relative increase in fluorescence in response to the positive control Gal-BSA occurred much more slowly, and continued to increase over 210 min after addition of Gal-BSA. The relative response of 13-16-R1 hemocytes to Gal-BSA using this protocol was

considerably weaker compared to the Gal-BSA response using the Ficoll method but still significantly higher than the response to BSA alone (p-value < 0.001; using a Student's t-test). The response to Glc-BSA, lactose-BSA, and melibiose-BSA was not statistically different from the response to BSA (p-values > 0.05; using a Student's t-test) (Figure 2.5.). MO hemocytes responded similarly (data not shown).

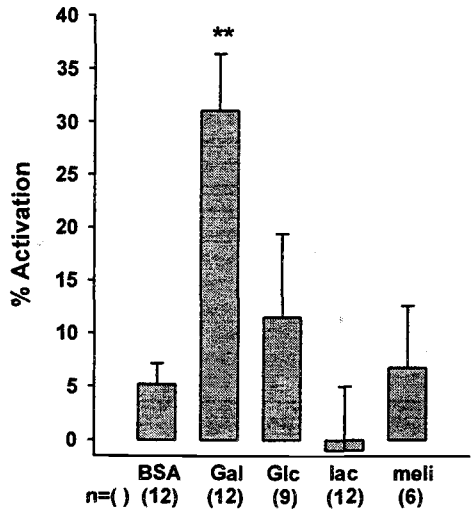

Figure 2.5. The relative increase in fluorescence 210 min after addition of non-derivatized BSA, Gal-BSA (Gal), Glc-BSA (Glc), lactose-BSA (lac), or melibiose-BSA (meli), all at final concentrations of 200 nM, in 13-16-R1 hemocytes prepared without Ficoll. Asterisks indicate fluorescence levels that are significantly higher than stimulation levels for non-derivatized BSA; (**) $p < 0.001$ (using Student's t-test).

Competitive inhibition of cell response

As reported above, concentrations of 25 mM non-conjugated carbohydrates did not elicit a response in hemocytes. In the presence of the respective monosaccharides the responses of MO hemocytes to Man-BSA and Gal-BSA were reduced (on average between 5 and 15%). This reduction, however, was not statistically significant. The response of MO hemocytes to Fuc-BSA in the presence of 25 mM fucose did not change (Figure 2.6.A). The response of 13-16-R1 hemocytes to Gal-BSA was significantly reduced in the presence of 25 mM α-D galactose. The responses to Man-BSA and Fuc-BSA in the presence of 25 mM mannose and fucose, respectively, were reduced (an absolute reduction on average between 10

and 20%); however, this reduction was not statistically significant (Figure 2.6.B). Data were compared using a paired t-test; p-values < 0.05 were considered significant.

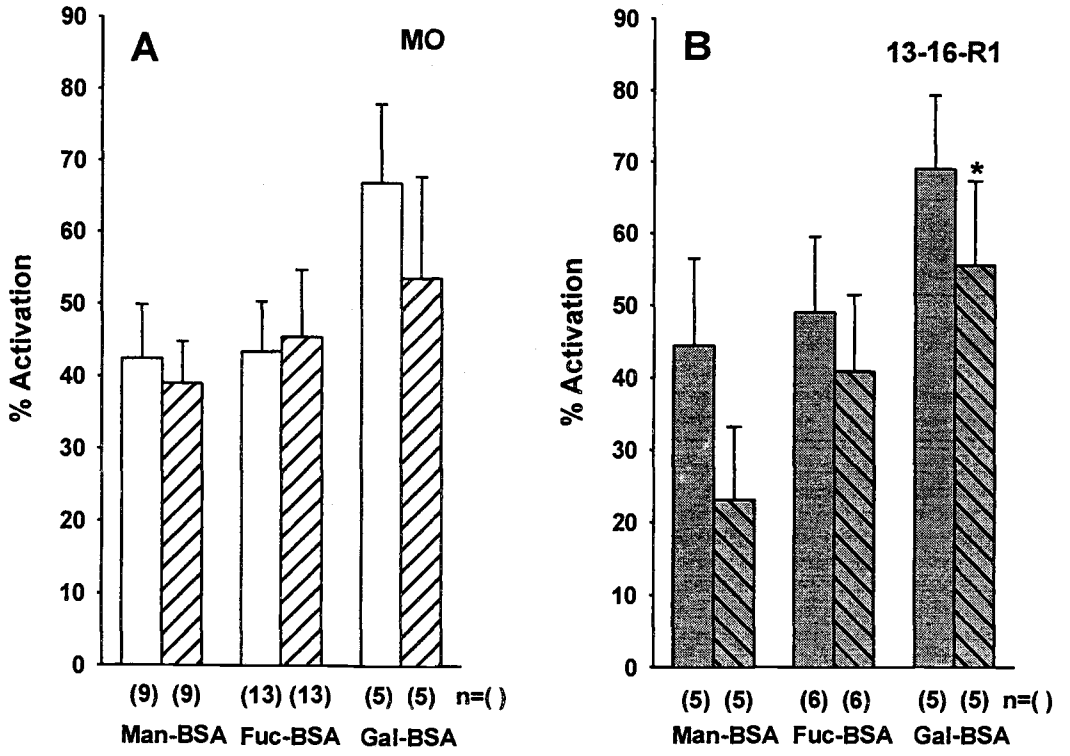

Figure 2.6. Hemocytes were stimulated with 200 nM of either Man-BSA, or Fuc-BSA, or Gal-BSA, with or without 25 mM of the respective monosaccharides (D-mannose, L-fucose, and D-galactose). These monosaccharides were added 30 min prior to stimulation with the BSA-carbohydrate conjugates. The bars show the relative fluorescence levels at 120 min after addition of the stimulants. Unhatched bars, without presence of the respective sugar, hatched bars, in the presence of 25 mM of the respective sugar. A) MO hemocyte pools; B) 13-16-R1 hemocyte pools. An asterisk indicates a statistically significant reduction of fluorescence in the presence of the respective sugar; (*) $p < 0.05$ (using Student's paired t-test).

DISCUSSION

Lectins have important roles in internal defense systems of animals. Many such carbohydrate recognizing molecules are found in vertebrates and invertebrates. Some, for example mannan-binding lectin (MBL) and C-reactive protein, appear to be well conserved in

function throughout the Metazoa [18-21]. Our results strongly suggest the existence of lectin-type receptors on *B. glabrata* hemocytes that mediate a defensive cell response when they bind to specific carbohydrates. The carbohydrates N-acetyl-galactosamine, N-acetyl-glucosamine, glucose, lactose, and melibiose conjugated to BSA did not stimulate the oxidative burst in *B. glabrata* hemocytes. Since residual Ficoll (used to remove hemoglobin) could have interfered with responses to Glc-BSA, lactose-BSA, or melibiose-BSA, these carbohydrate conjugates were tested again on hemocytes prepared without Ficoll. No stimulation was found with these three BSA-conjugates in hemocytes of either strain. BSA-conjugated mannose, fucose, and galactose,
however, did elicit increased production of reactive oxygen species, presumably by sugar-specific binding to lectin-type receptors on the hemocyte surface.

Our findings suggest that glycans containing mannose, fucose and galactose structures might serve as targets for cell surface receptors that trigger defensive responses against pathogens. In particular terminal fucose residues, which are absent on *B. glabrata* plasma proteins and hemocytes [22], are good candidates for self / non-self distinction. In fact, a lectin with specificity for fucosyl-lactose was isolated from *B. alexandrina* and partially characterized [23]. Further, adult *B. alexandrina* hemocytes exhibit increased rates of phagocytosis towards sheep red blood cells coated with *Schistosoma mansoni*-derived glycoproteins containing fucosyl-lactose residues [4]. Zymosan (yeast cell wall particles), which is rich in mannan, is known to elicit superoxide production in *B. glabrata* hemocytes [24], supporting the idea that these cells have receptors specific for mannose-containing structures. Evidence for such a receptor comes also from the fact that internal Ca^{++} levels increase in hemocytes in response to Man-BSA [25]. The carbohydrates which decorate the tegument of *S. mansoni* have been partially characterized by Uchikawa and Loker [26]. They determined that lectins with binding specificities for mannose, galactose, fucose, N-acetyl-galactosamine, and N-acetyl-glucosamine bound to the sporocyst surface. Assuming that ROS play an important role in killing, our study suggests that N-acetyl-galactosamine and N-acetyl-glucosamine are probably not involved in recognition of *S. mansoni* sporocysts by *B. glabrata.* However, mannose, galactose, and fucose did stimulate an oxidative burst in hemocytes from both 13-16-R1 and MO snails. Since we found no net difference in ROS generation between the two strains, these results suggest that either mannose, galactose and fucose are not crucial for parasite recognition, or MO hemocytes are capable of recognizing *S. mansoni.*

Our study did not directly address the capacity of individual hemocytes from the two strains to produce ROS. However, Dikkeboom et al. [24] could not detect differences in superoxide generation by hemocytes derived from susceptible M-line, or resistant 13-16-R1, or 10-R2 snails. Our method also does not account for the possibility that cells within a population of hemocytes may differ with respect to the ability to produce ROS. Connors et al. [27] report a higher percentage of hemocytes generating superoxide during phagocytosis in *S. mansoni*-resistant 10-R2 snails than in parasite-susceptible M-line snails.
Questions about different sub-populations of hemocytes could be best addressed using flow cytometry, in which the capacity for generation of ROS is assessed for individual cells. It is also important to note that DCFH-DA reacts only with specific ROS (hydrogen peroxide and hydroxyl radicals) [10]. Therefore this method will not detect differences in generation of other reactive oxygen species (i.e. superoxide, hypochlorite, singlet oxygen, etc.) that may be important for parasite killing.

Concentrations up to 25 mM of mono- and disaccharides did not stimulate ROS production in *B. glabrata* hemocytes. Furthermore, 25 mM non-conjugated carbohydrates failed to significantly reduce the production of ROS by hemocytes, which were stimulated with the respective BSA-conjugated carbohydrate. This apparent lack of competition for receptor sites by non-conjugated sugar suggests that the receptors involved may be polyvalent and that activation of the receptors requires binding of more than one ligand per receptor. Carbohydrates conjugated to BSA present such multiple ligand arrays. The necessity for simultaneous binding of multiple sites in order to achieve biological function has been reported for other lectins. Human MBL, for example, exhibits only weak affinity towards oligosaccharides; however, the cluster-array of multiple binding sites can achieve overall higher affinity binding when the lectin binds to surfaces with highly repetitive sugar groups [28]. In our study, inhibition and/or activation by non-conjugated carbohydrates was not achieved at sugar concentrations of 25 mM, possibly because multiple binding on single receptors does not occur at these concentrations. Alternatively, cell activation by BSA-conjugated carbohydrates may be achieved by cross-linking of neighboring receptors. Assuming receptor activation occurs by multiple ligand binding, higher concentrations of carbohydrates might have led to successful competitive inhibition of the response. However, concentrations above 25 mM posed an osmotic stress on the hemocytes, and it was not possible to determine the concentrations required to inhibit receptor activation by BSA-

carbohydrate conjugates. Because of this we were also unable to determine the exact sugar specificities of the responsive receptors.

In summary, our findings show that certain specific carbohydrate structures will elicit a defensive response in *B. glabrata* hemocytes, while other carbohydrates presented in the same context (i.e. linkage to a BSA molecule) will not evoke such a response. In order to activate the receptor, it seems essential that these carbohydrate structures are displayed in a cluster fashion that offers multiple binding sites. This type of recognition is ideally suited for receptors initiating an immune response, since the surfaces of many microorganisms, such as yeast and bacteria, possess repetitive sugar groups.

ACKNOWLEDGMENTS

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Chapter 3.

Killing of *Schistosoma mansoni* Sporocysts by Hemocytes from resistant *Biomphalaria glabrata*: Role of Reactive Oxygen Species

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ABSTRACT

The fate of *Schistosoma mansoni* (Trematoda) sporocysts in its molluscan host *Biomphalaria glabrata* (Gastropoda) is determined by circulating phagocytes (named hemocytes). When the parasite invades a resistant snail it is attacked and destroyed by hemocytes, whereas in a susceptible host it remains unaffected. In our studies we used 3 inbred strains of *B. glabrata*: 13-16-R1 and 10-R2, which are resistant to the PR-1 strain of *S. mansoni*, and MO, which is susceptible to PR-1. In an in vitro killing assay using plasma-free hemocytes from these strains the rate of parasite killing corresponded closely to the rate by which *S. mansoni* sporocysts are killed in vivo. Hemocytes from resistant snails killed more than 80% of *S. mansoni* sporocysts within 48 hr, whereas hemocytes from susceptible snails killed less than 10%. Using this *in vitro* assay we assessed the involvement of reactive oxygen species (ROS) produced by resistant hemocytes, during killing of *S. mansoni* sporocysts. Inhibition of NADPH oxidase significantly reduced sporocyst killing by 13-16-R1 hemocytes, indicating that ROS play an important role in normal killing. Reduction of hydrogen peroxide (H_2O_2) by including catalase in the killing assay increased parasite viability as well. Reduction of superoxide (O_2^-), however, by addition of superoxide dismutase, or scavenging of hydroxyl radicals ($^{\cdot}OH$) and hypochlorous acid (HOCl) by addition of hypotaurine did not alter the rate of sporocyst killing by resistant hemocytes. We conclude that H_2O_2 is the ROS mainly responsible for killing.

INTRODUCTION

Within populations of *Biomphalaria glabrata* (Gastropoda), different genetically determined phenotypes exist with respect to susceptibility to *Schistosoma mansoni* (Trematoda) (Richards and Merritt, 1972). In resistant snails, the internal defense system recognizes the invading parasite as a pathogen, which is then quickly attacked and destroyed by hemocytes (circulating phagocytic cells). In susceptible snails, however, the parasite remains totally unaffected by the snails' hemocytes. Humoral factors of the snail's immune system have been reported to be involved in determining resistance towards the trematode (Bayne et al., 1980; Granath and Yoshino, 1984), possibly by enhancing hemocyte toxicity (Connors et al., 1995). However, for some specific cases, it has been shown that hemocytes

can kill sporocysts in the absence of humoral factors. For example, plasma-free hemocytes from the 10-R2 strain of *B. glabrata* destroyed sporocysts from the PR-1 strain of *S. mansoni* (Bayne et al., 1980). This suggests intrinsic differences in hemocytes from resistant and susceptible strains. Differences in killing may be due to the failure of the susceptible host to recognize the parasite, or the mounting of an inappropriate (ineffective) response against the parasite. The objective of the present study was to elucidate the mechanism(s) by which hemocytes from resistant *B. glabrata* manage to kill PR-1 sporocysts. This knowledge could then provide the means to investigate on a more specific level why hemocytes from susceptible snails fail to kill.

Generation of reactive oxygen species (ROS) by phagocytic defense cells is one main constituent of their microbicidal activity. ROS are typically generated to kill pathogens after phagocytosis. Encapsulations of larger pathogens too big to be taken up by a defensive cell, such as in the case of sporocysts and hemocytes, have been considered a 'frustrated phagocytosis'. In such encounters, processes at the hemocyte membrane might actually be similar to processes at the phagosomal membrane. Killing of *S. mansoni* schistosomula by human neutrophils, for example, was found to correlate with hydrogen peroxide production by these cells (Kazura et al., 1981). The parasite, however, is not entirely defenseless against cytotoxicity mediated by ROS. All life-stages of *S. mansoni* are known to possess enzymes that detoxify ROS (Simurda et al., 1988; Nare et al., 1990; Connors et al., 1991; Mei et al., 1996; LoVerde, 1998). Endogenous levels of these enzymes increase during maturation from schistosomula to the adult worm, and are closely correlated with the worms' susceptibility to oxidant killing (Mkoji et al., 1988; Nare et al., 1990). The larval stages of *S. mansoni,* and other trematodes, have been shown to induce an oxidative burst in *B. glabrata* hemocytes (Shozawa et al., 1989). And there is evidence that NADPH oxidase activity plays a role in killing of trematode sporocysts by hemocytes from incompatible snails (Dikkeboom et al., 1988a; Adema et al., 1994). We therefore explored the involvement of ROS produced by 13-16-R1 hemocytes in the successful destruction of PR-1 sporocysts.

A cellular oxidative burst is initiated by assembly of an NADPH enzyme complex at the phagocyte's membrane and a dramatic increase of oxygen consumption. Figure 3.1. presents an overview of reactions that generate ROS. Electrons are transferred from cytoplasmic NADPH to oxygen generating $O_2^{\cdot}$. Important ROS include superoxide (O_2^{-}), and its products hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\cdot}OH$), singlet oxygen ($^1O_2$) and hypochlorous acid (HOCl). The enzyme superoxide dismutase (SOD) facilitates dismutation

of O_2^- into H_2O_2. Hydrogen peroxide can then, upon reaction with reduced iron or copper salts, produce ·OH (Fenton reaction), or react with HOCl to produce $^1O_2$, both of which are highly reactive with a number of biological molecules. Hydrogen peroxide can also be converted into HOCl, a strong nonradical oxidant, by the enzyme myeloperoxidase. In the present study, we added selective inhibitors and scavengers of critical ROS to test for their involvement in killing of *S. mansoni* sporocysts by 13-16-R1 hemocytes in vitro.

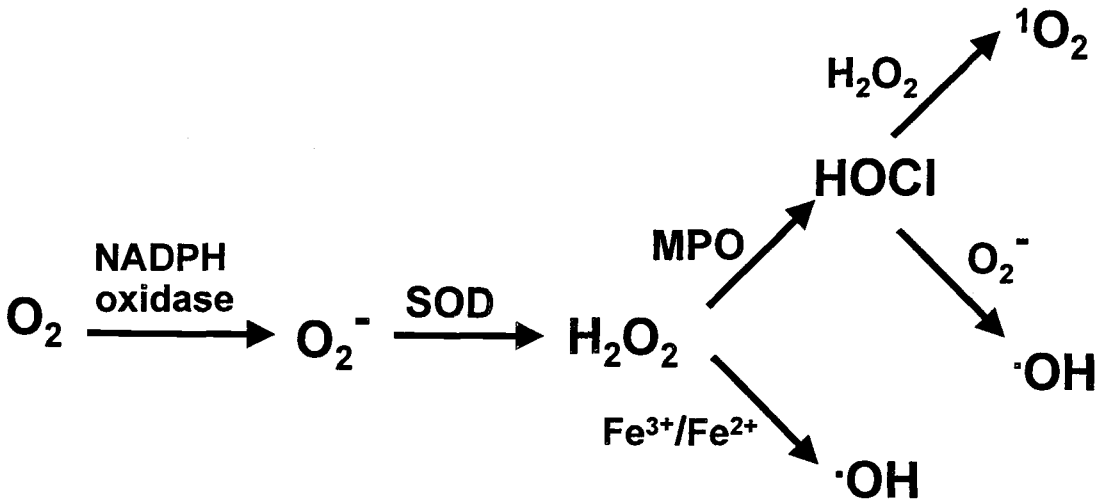

Figure 3.1. Possible pathways for the generation of ROS during an oxidative burst in cytotoxic cells. SOD, superoxide dismutase; MPO, myeloperoxidase

MATERIALS AND METHODS

All chemicals, if not indicated otherwise, were purchased from Sigma, St. Louis, Missouri.

Media and buffer

The culture medium used in the cell-mediated cytotoxicity (CMC) assay (CMC Medium) was a modified version of Bge medium (Hansen, 1976), containing 22% Schneider's Drosophila Medium (Gibco #172), 7 mM α-D-glucose, 24 mM NaCl, and 20 μg/ml gentamicin (Gibco BRL, Grand Island, New York) at pH 7.40 with an osmolality of 120 mOsm. Test compounds were dissolved in Chernin's Balanced Salt Solution (CBSS) (Chernin, 1963) containing 1 mM HEPES (pH 7.4) and 0.005% phenol red. All treatments

were added at 4 times the final concentration. The final 'media' was, therefore, 75% CMC Medium and 25% CBSS.

S. mansoni sporocysts

The PR-1 strain of *S. mansoni* was used in this study. S. *mansoni* sporocysts were obtained by transforming miracidia overnight at 26 C in medium F base (Stibbs et al., 1979) containing 1% bovine serum albumin and 20 μg/ml gentamicin. The next day sporocysts were washed 3 times in CMC Medium to remove ciliated plates, before they were used in the CMC assay.

Sterile collection of hemolymph

Snails were kept in temperature controlled rooms at 26 C on a 12 hr light 12 hr dark cycle. The animals were fed green leaf lettuce ad libitum. For collection of sterile hemolymph we followed the protocol described by Fryer and Bayne (1995) . Sterile techniques were used throughout. In brief, the snails' shells were swabbed using cotton swabs soaked in 70% ethanol. The snails were then soaked in sterilized beakers containing 2% antibiotic/antimycotic solution (Sigma A-7292, Sigma Chemical Co., St. Louis, Missouri; 10,000 units penicillin, 10 mg streptomycin and 25 μg amphotericin B per ml in 0.9% sodium chloride) in artificial spring water (ASW) (Ulmer, 1970) for 30 to 40 min. Not more than 18 snails were soaked in each beaker containing 75ml ASW. Then snails were dried with tissue, again swabbed with 70% ethanol, and bled by cardiac puncture. Hemolymph was pooled on Parafilm to allow shell debris to settle before it was transferred into 96-well plates.

Cell mediated cytotoxicity assay

The assay was based on the protocol described by Boehmler, Fryer and Bayne (1996) with some changes. Sterile, 96-well tissue culture plates (Corning, Acton, Massachusetts) were treated with poly-L-lysine as previously described (Boehmler, Fryer and Bayne, 1996). Poly-L-lysine treatment has been shown to increase hemocyte mobility on the plastic surface and is essential for good hemocyte-sporocyst interactions. Two-hundred μl of sterile hemolymph from 13-16-R1 or 10-R2 snails (both strains are resistant to PR-1) or 400 μl hemolymph from M-line Oregon (MO) snails (susceptible to PR-1) were added to each well. Twice as much susceptible hemolymph was used on account of the lower hematocrit in this susceptible strain. Hemocytes were spun down at 10 g for 10 min at room temperature and the

supernatant (plasma) was aspirated. The hemocytes were then very carefully washed twice with CMC Medium. Hemocytes will not attach to poly-L-lysine coated plastic and have a tendency to clump together in a corner of the plate if washed too vigorously. Pipetting over autoclaved Styrofoam balls (about 5 mm in diameter before autoclaving, about 1.5 mm in diameter after autoclaving) floating on top of the hemolymph has been shown to be useful, because the cells experience less turbulences. After washing residual plasma was less than 1%. At this concentration, no plasma toxicity towards *S. mansoni* could be observed for the duration of the assay (C. J. Bayne, unpubl. obs.). About 75 to 100 sporocysts per well were added to the washed hemocytes, and immediately counted using an inverted phase-contrast microscope (to determine their exact number per well), because hemocytes start to encapsulate the parasites very quickly (within minutes). Propidium iodide (PI) was added to a final concentration of 10 μg/ml. After 1 hr, sporocysts were examined using an inverted fluorescence microscope, to determine initial sporocyst viability. Hemocyte-mediated mortality of the parasite does not occur within the first hr of interaction. Parasite death was determined by PI-stained nuclei. Dead sporocysts were typically counted at 6, 18, 22, 26, 42, and 47 hr after the start of the assay. Percent cumulative sporocyst mortality was calculated using the formula $[(d_x-d_1)/(T-d_1) \times 100]$, where d_x = number of dead sporocysts at time x, d_1 = number of dead sporocysts after 1 hr, and T = total number of sporocysts per well.

Individual pools of hemolymph were divided into 2 to 3 wells, depending on the number of treatments. To assess potential toxicity of the individual test compounds to the parasite, each assay included control wells of sporocysts without hemocytes. The person scoring sporocyst mortality was unaware which treatment was added to the wells.

Inhibition of NADPH oxidase

To test whether reactive oxygen species, produced by resistant *B. glabrata* hemocytes, are involved in killing of *S. mansoni* sporocysts, we added protocatechuic acid (PCA), an inhibitor of mammalian NADPH oxidase that does not interfere with phagocytosis (t'Hart et al., 1990). PCA has also been shown to inhibit NADPH oxidase activity in molluscan hemocytes (Adema et al., 1993; Hahn, Bender and Bayne, 2000). PCA (Sigma P-5630) in CBSS, at a final concentration of 160 μM, was added to washed 13-16-R1 hemocytes immediately before sporocysts were added. Cells in control wells received CBSS. To assess PCA toxicity at this concentration towards sporocysts we included wells without 13-16-R1 hemocytes.

Effects of superoxide dismutase and catalase

To scavenge reactive oxygen species, superoxide dismutase (SOD) and catalase were added to the CMC assay. SOD converts O_2^- to H_2O_2, which is then converted by catalase to the non-toxic products H_2O and O_2. Both enzymes were dissolved in CBSS and added to washed 13-16-R1 hemocytes in treatment wells immediately before sporocysts were added. SOD (Sigma S-2515) was added to a final concentration of 2,500 units/ml; catalase (Sigma C-40) was added to a final concentration of 14,000 units/ml. SOD at a concentration of 2,500 units/ml (in combination with catalase) has been shown to protect *S. mansoni* sporocysts from damage by artificially generated superoxide (Adema et al., 1994). Catalase, which had no toxic effects towards either hemocytes or sporocysts, was used at the highest, still soluble concentration. Control wells, containing hemocytes derived from the same pools of hemolymph as the treatment groups, received CBSS.

To test whether the protective effect of SOD and catalase are due to the O_2^- scavenging properties of SOD or to the detoxifying of H_2O_2 by catalase, we divided hemocytes from the same pool of 13-16-R1 into 3 wells for the different treatments: addition of SOD at a final concentration of 2,500 units/ml; addition of SOD (final concentration of 2,500 units/ml) and catalase (final concentration of 14,000 units/ml) and the control treatment of CBSS.

To determine whether removal of H_2O_2 alone has an effect on sporocyst killing by 13-16-R1 hemocytes, we added catalase at a final concentration of 14,000 units/ml. Second sets of wells with hemocytes from the same pool of hemolymph received both SOD (2,500 units/ml) and catalase (14,000units/ml). Control wells received CBSS. All treatments were added immediately before sporocysts were added to the hemocytes.

Hypotaurine protection

Hypotaurine is an efficient and specific scavenger for hypochlorous acid (HOCl) and hydroxyl radicals ($OH^·$) (Aruoma et al., 1988; Shi et al., 1997) and is also known to protect proteins from damage caused by oxygen radicals. To determine if hypotaurine protects sporocysts from damage by HOCl, we incubated parasites with 0, 0.5, 1, 2, 6, and 10 mM hypotaurine (Sigma H-1384) in the presence of various concentrations of HOCl. HOCl was prepared as follows: NaOCl (Acros Organics, Pittsburgh, Pennsylvania) was diluted with CBSS and the pH readjusted to 7.4 with 1 M H_2SO_4. At this pH, the solution will contain HOCl and OCl^- at a ratio of approximately 1:1 (Vissers et al., 1994). The solution was made

immediately prior to addition to the sporocysts. In the CMC assays, 6 mM of hypotaurine were added to the 13-16-R1 hemocytes prior to adding the sporocysts.

HOCl and H_2O_2 toxicity

To assess the relative toxicity of HOCl and H_2O_2, *S. mansoni* sporocysts were incubated with various concentrations of HOCl (prepared as described above) and H_2O_2 (Sigma H-1009). The concentrations ranged from 25 to 300 μM. Sporocyst mortality was determined by propidium iodide staining and scored using an inverted fluorescence microscope.

RESULTS

Plasma-free CMC

Even though complete encapsulation of sporocysts by hemocytes from all strains, resistant and susceptible, occurred very quickly (within 1 to 2 hr); no parasite mortality could be observed during the first 6 hr of the assay. Starting at 18 hr sporocyst mortality was significantly higher when incubated with hemocytes from resistant snails (13-16-R1 or 10-R2) than when incubated with hemocytes from susceptible snails ($P < 0.0001$). Data were compared using Student's *t*-test; *P*-values < 0.05 were considered significant. After 47 hr, 13-16-R1 hemocytes killed 87% of the sporocysts and 10-R2 hemocytes killed 84% on average (Fig. 3.2.). When sporocysts were incubated with M-line hemocytes, mortality was at no time point significantly different from control mortality of sporocysts incubated in media without hemocytes. After 47 hr, parasite mortality in media averaged 6.6%; in co-culture with MO hemocytes, it averaged 7.4%.

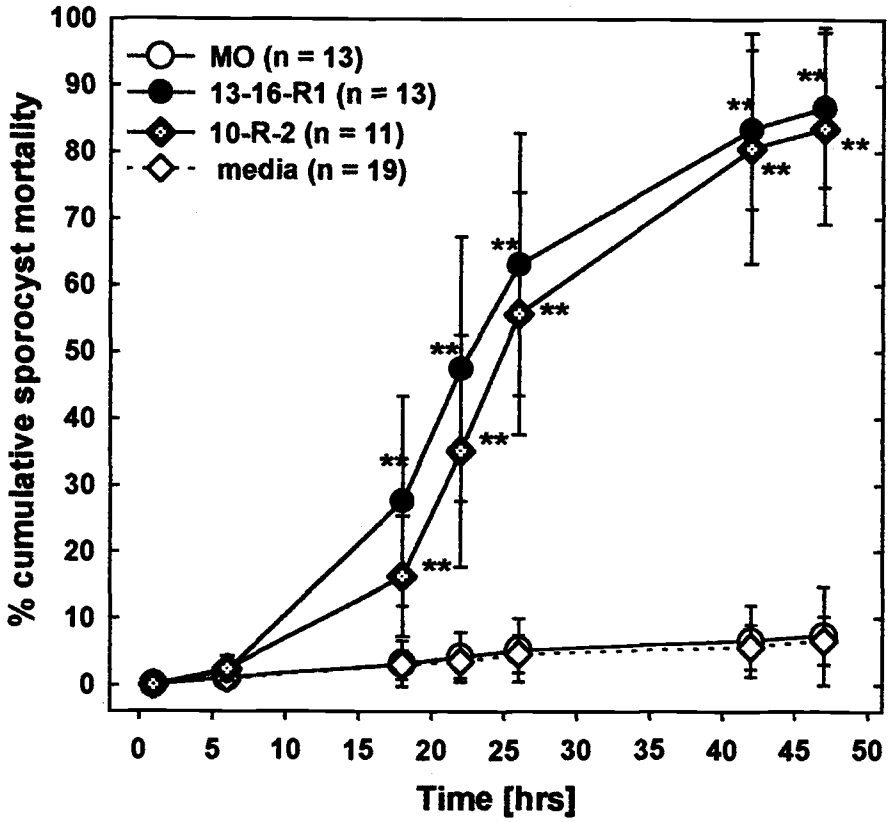

Figure 3.2. Time course of parasite killing by washed hemocytes derived from 1 susceptible (MO) and 2 resistant (13-16-R1, 10-R2) strains of *Biomphalaria glabrata,* and mortality of control sporocysts in media without hemocytes. The numbers in parentheses indicate the number of replicates. The symbols represent mean values ± standard deviation. The asterisks (**) indicate values that are significantly higher than sporocyst mortality values obtained with MO hemocytes ($P < 0.001$, using Student's *t*-test).

Inhibition of NADPH oxidase

Inhibition of NADPH oxidase by addition of 160 μM PCA to the killing assay reduced sporocyst mortality significantly compared to mortality in control wells (Fig. 3.3.). Statistically significant differences were seen first at 18 hr. Data were compared using a paired *t*-test. After 46 hr, an average of 87% of the sporocysts in control wells were killed, whereas average sporocyst mortality in PCA treated wells was only 59% (32% reduction in killing). Treatment with PCA had no influence on sporocyst viability in hemocyte-free control wells,

compared to their survival in media alone (approximately 10% mortality for both treatments after 47 hr).

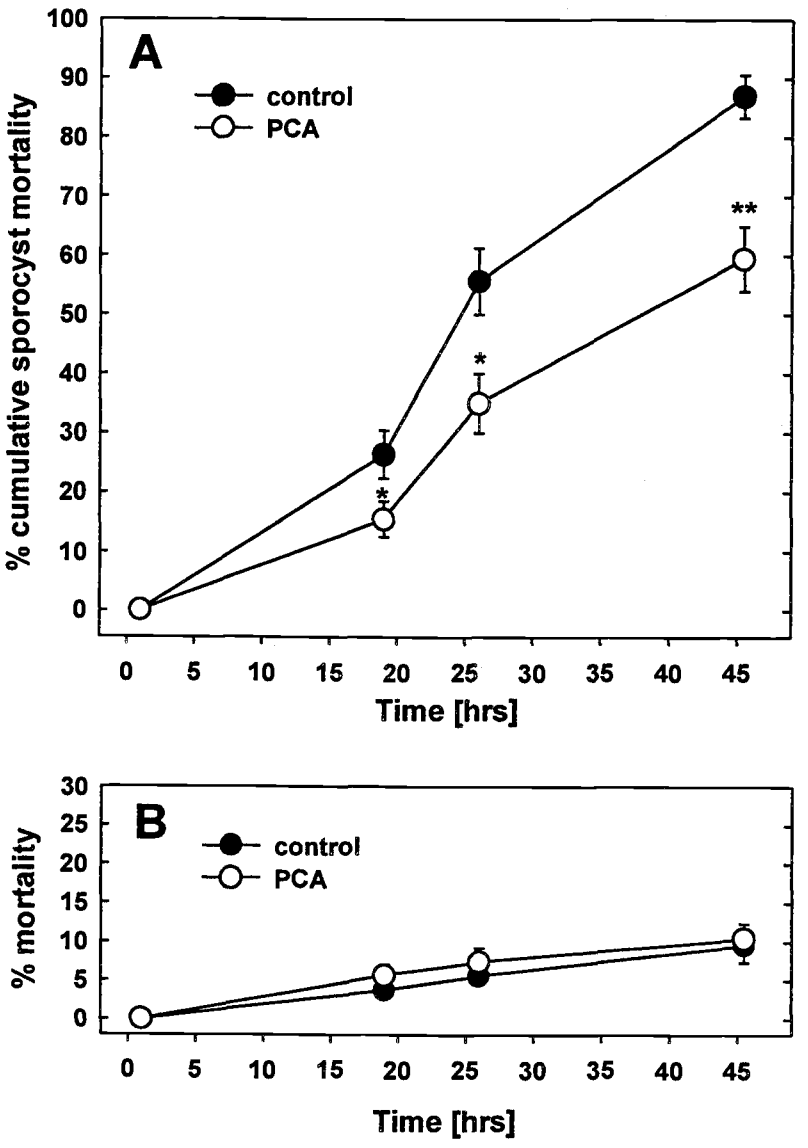

Figure 3.3. (A) Effect of the NAPDH oxidase inhibitor protocatechuic acid (PCA) on parasite killing by washed 13-16-R1 hemocytes. Represented are the mean values of 9 replicates ± standard error. (•) control killing (CBSS added), (o) killing in the presence of 160 μM protocatechuic acid (PCA). The asterisks indicate a significant reduction of killing compared to control killing (* = $P < 0.01$, ** = $P < 0.001$; using a paired *t*-test). (B) The effect of 160 μM PCA on cumulative sporocyst mortality in control wells without hemocytes. Represented are mean values of 9 replicates ± standard error.

Effects of SOD and catalase

Scavenging reactive oxygen intermediates (O_2^- and H_2O_2) by addition of SOD and catalase to the CMC assay significantly reduced killing of *S. mansoni* sporocysts; this was first evident at 18 hr after the beginning of the assay (Fig. 3.4.). Cumulative sporocyst mortality after 47 hr in CBSS control wells was 89% on average, whereas addition of both SOD and catalase reduced killing to 52% on average (42% reduction in killing).

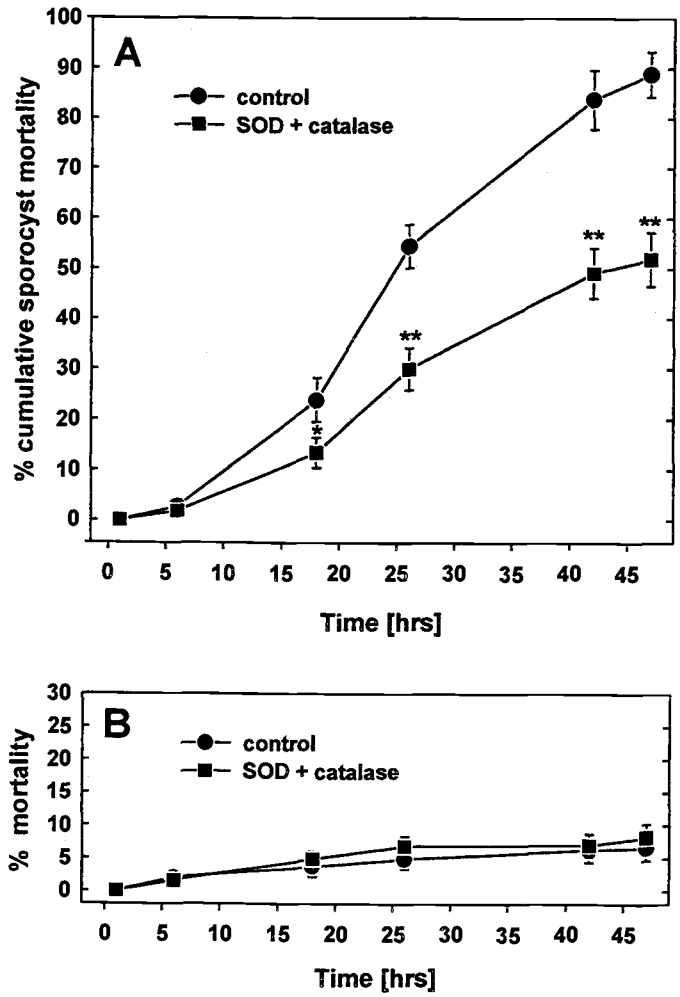

Figure 3.4. (A) The effect of the reactive oxygen scavenging enzymes superoxide dismutase (SOD) and catalase on parasite killing by washed 13-16-R1 hemocytes. Represented are the mean values of 9 replicates ± standard error. (•) control killing, (■) killing in the presence of 2,500 units/ml SOD and 14,000 units/ml catalase. The asterisks indicate a statistically significant reduction of killing compared to control killing (* $P < 0.01$, ** $P < 0.001$; using a paired *t*-test). (B) The effect of 2,500 units/ml SOD and 14,000 units/ml catalase on sporocyst mortality in control wells without hemocytes (n = 9, error bars = SE).

To test whether O_2^- is directly involved in sporocyst killing, we added SOD, which quickly converts O_2^- into H_2O_2. If O_2^- produced by hemocytes were directly toxic to the parasite, a reduction in sporocyst mortality would be expected. Addition of SOD neither reduced nor enhanced the rate of killing compared to CBSS controls. On the other hand, addition of SOD and catalase to the same pools of hemocytes significantly reduced sporocyst mortality between 18 and 47 hr, as seen in previous assays (Fig. 3.5.). Therefore, catalase, which converts H_2O_2 into O_2 and H_2O, was responsible for protection of the parasite. In fact, addition of only catalase significantly reduced sporocyst mortality between 18 and 47 hr compared to mortality in CBSS control wells (Fig. 3.6.). The reduction in parasite mortality was not different from the reduction seen with the combination of SOD and catalase. SOD or catalase, or a combination of both enzymes did not affect viability in control wells without hemocytes. The mean sporocyst mortality was generally around 10%.

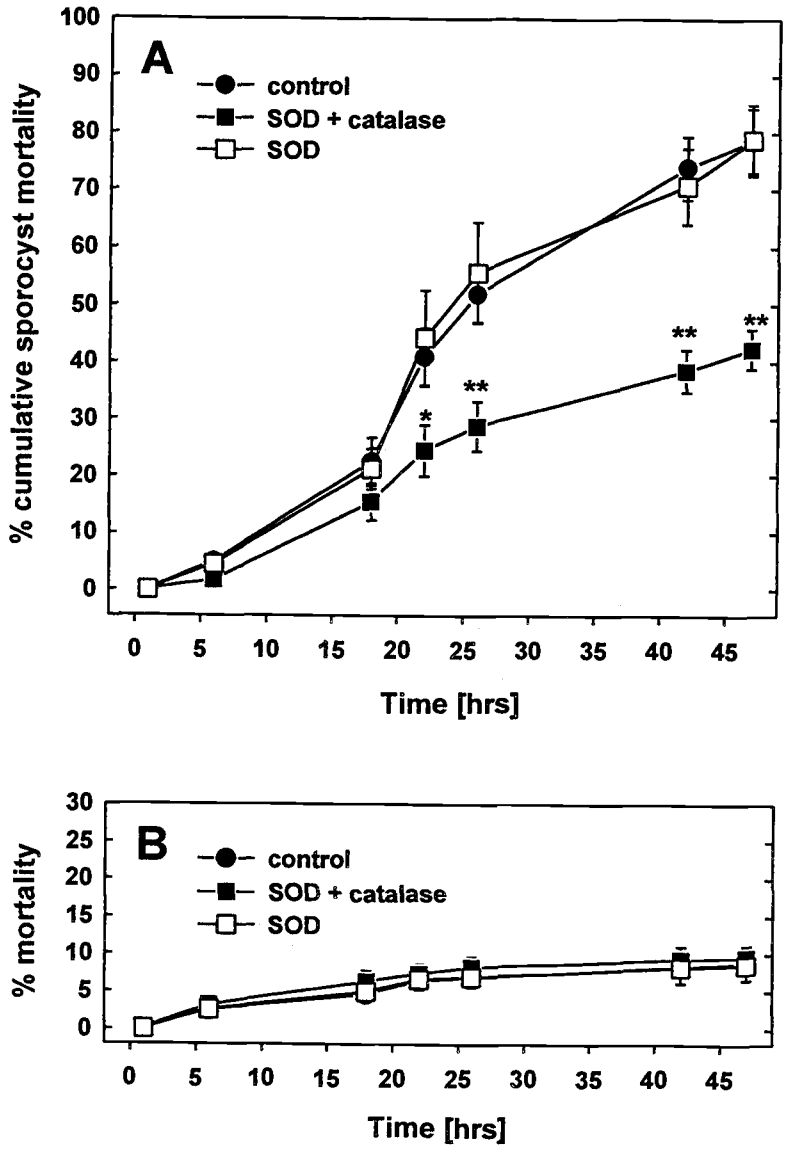

Figure 3.5. (A) Effects of scavenging superoxide (addition of SOD) compared to scavenging of superoxide and hydrogen peroxide (addition of SOD and catalase) on parasite killing by 13-16-R1 hemocytes. (•) control killing, (■) killing in the presence of 2,500 units/ml SOD and 14,000 units/ml catalase, (□) killing in the presence of 2,500 units/ml SOD. Asterisks indicate a significant reduction of killing compared to control levels (* $P < 0.01$, ** $P < 0.001$), (n = 8; error bars = SE). (B) Effects of SOD, or the combination of SOD and catalase on sporocyst mortality in control wells without hemocytes (n = 9; error bars = SE).

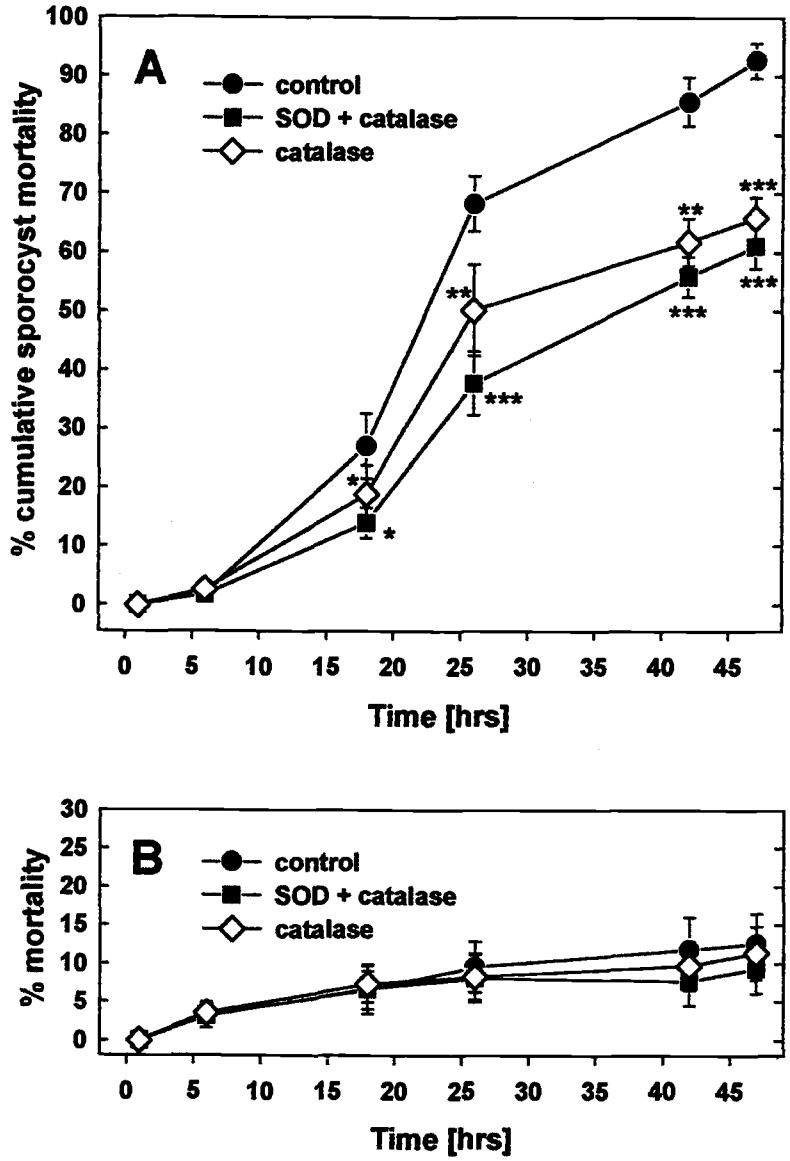

Figure 3.6. (**A**) Scavenging of hydrogen peroxide (addition of catalase), and its effects on parasite killing by 13-16-R1 hemocytes. (•) control killing by 13-16-R1 hemocytes, (■) killing in the presence of 2,500 units/ml SOD and 14,000 units/ml catalase, (◊) killing in the presence of 14,000 units/ml catalase (n = 7; error bars = SE). The asterisks indicate values that are significantly reduced compared to control killing (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; using a paired *t*-test). (**B**) Effects SOD and catalase, and catalase alone, on sporocyst mortality in control wells without hemocytes (n = 7, error bars = SE).

Effects of hypotaurine

Hypotaurine is an efficient and specific scavenger of HOCl and OH^- and does not react with H_2O_2 at the concentrations we used (Aruoma et al., 1988). In sporocyst viability assays (without hemocytes), addition of 250 μM HOCl killed ca. 80% of the sporocysts within 4 hr, and ca. 90% after 23 hr. Addition of hypotaurine protected sporocysts from HOCl damage (Fig. 3.7.). Lower concentrations of hypotaurine (0.5 to 2 mM) significantly increased short-term survival in the presence of HOCl, but only higher concentrations (6 mM and 10 mM) significantly reduced mortality over a long period of time (up to 113 hr). Protection due to hypotaurine was not significantly different between 6 mM and 10 mM. Therefore, 6 mM hypotaurine was the concentration included in killing assays. Compared to events in CBSS control wells, no significant reduction in parasite killing by 13-16-R1 hemocytes could be observed by inclusion of 6 mM hypotaurine (Fig. 3.8.). Hypotaurine (6 mM) had no effect on sporocyst viability in hemocyte-free control wells.

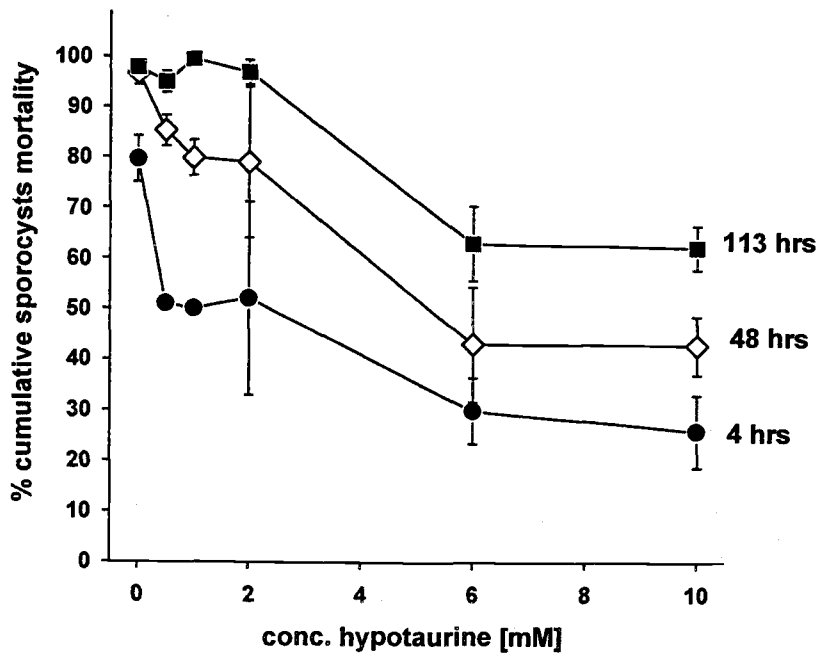

Figure 3.7. Protection of sporocysts against toxicity of hypochlorous acid (HOCl) by different concentrations of hypotaurine. Sporocysts were incubated with 250 μM HOCl in the presence of different concentrations of hypotaurine (0 to 10 mM). Each line represents cumulative sporocyst mortality at different time points. (•) 4 hr, (◊) 48 hr, and (■) 113 hr after addition of HOCl (n = 3; error bars = SE).

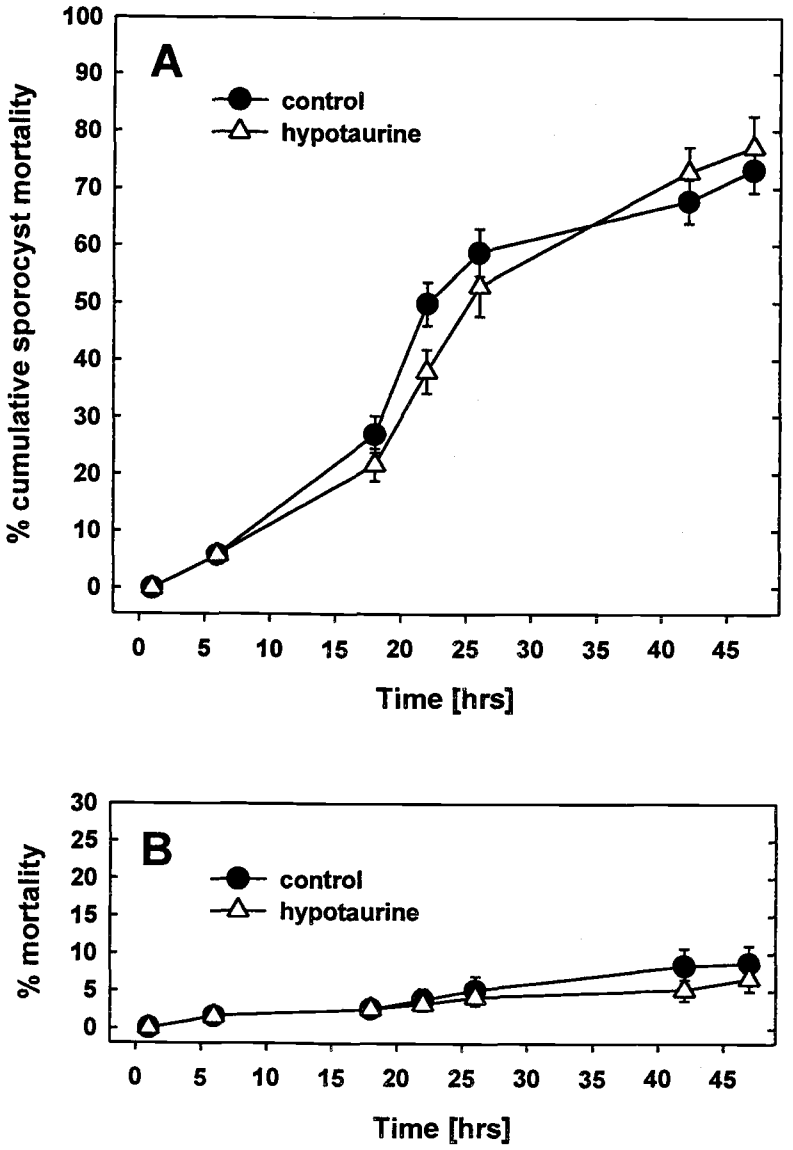

Figure 3.8. (A) The effect of hypotaurine, which scavenges hypochlorous acid and hydroxyl radicals, on parasite killing by washed 13-16-R1 hemocytes. (•) control killing, (Δ) killing in the presence of 6 mM hypotaurine (n = 9; error bars = SE). (B) The effect of 6 mM hypotaurine on sporocyst survival in control wells without hemocytes (n = 9; error bars = SE).

HOCl and H_2O_2 toxicity

Results from CMC assays suggest that H_2O_2, but not HOCl is directly involved in parasite killing by 13-16-R1 hemocytes. We, therefore, assessed the relative toxicity of H_2O_2 and HOCl to *S. mansoni*

sporocysts. On a mole-to-mole basis, H_2O_2 was much more toxic to sporocysts than HOCl. Whereas a concentration of only 100 μmM H_2O_2 killed 100% of sporocysts within 24 hr, 300 μM HOCl was required to kill all sporocysts within 24 hr (Fig. 3.9.). HOCl toxicity increased linearly with regard to concentration and time. For H_2O_2 toxicity, on the other hand, there appeared to be a distinct threshold. Lower concentrations (25 to 50 μM) had no toxic effects for up to 50 hr, whereas higher concentrations (≥ 100 μM) killed the parasites within 24 hr.

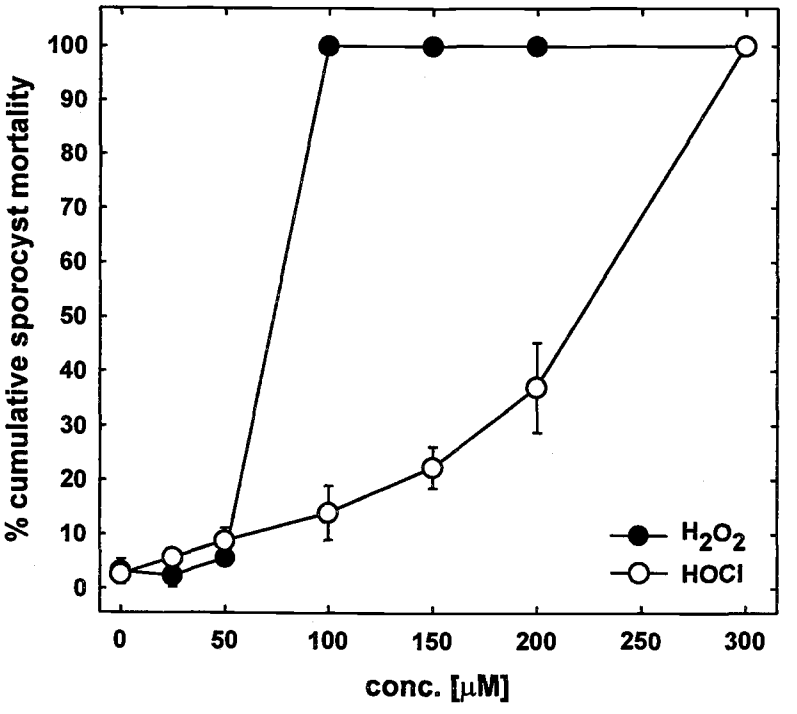

Figure 3.9. Effects of H_2O_2 (•) and HOCl (o) on sporocyst viability. Sporocysts were incubated with different concentrations (25 to 300 μM) of either H_2O_2 or HOCl, and mortality was determined by propidium iodide staining at 24 hr (n = 3, error bars = SD).

DISCUSSION

Hemocytes from 2 strains of *B. glabrata* that are constitutively resistant to *S. mansoni* killed sporocysts efficiently under plasma-free conditions; no cytotoxic effect towards the parasite was found by hemocytes from susceptible snails. These results confirm that humoral

factors, even though they may play a role in the snails' immune response, are not required for parasite killing by the snail. Hemocytes from 10-R2 snails have been shown previously to damage *S. mansoni* sporocysts under plasma-free conditions (Bayne et al., 1980). Our findings suggest that intrinsic differences exist between hemocytes from resistant and susceptible strains. The notion that it is the cellular component of the snails' immune system that determines resistance to the parasite is supported by reports of resistance transfer by transplanting the hemocyte (amoebocyte)-producing organ (APO) from resistant *B. glabrata* into susceptible snails (Sullivan et al., 1995, 1998). However, in the APO transplant studies, successful transfer of resistance was restricted to donors that had previously been exposed to *S. mansoni*, and failed when unexposed resistant snails were used as donors. In contrast, our study was done using naïve snails, and indicates that there is no need to 'prime' hemocytes to achieve efficient killing of the parasite.

Relative to the high survival rate, we report for *S. mansoni* sporocysts cultured with washed MO hemocytes, higher parasite mortalities have been previously reported for incubation in whole hemolymph from the PR-1 susceptible strains MO and MRLc (> 30 % in MO and >40 % in MRLc after 49 hr) (Boehmler, Fryer and Bayne, 1996). Mortality in susceptible whole hemolymph is probably an in vitro artifact, due to plasma components becoming toxic upon oxidation. The plasma-free killing assay used in the present study reflects more faithfully the situation in vivo, where we know that sporocysts in MO snails survive and thrive.

The involvement of reactive oxygen species (ROS) in incompatible snail-trematode combinations was first demonstrated for *Lymnaea stagnalis* and *S. mansoni*, as well as for *B. glabrata* and *Trichobilharzia ocellata* (Adema et al., 1994). In both incompatible combinations, survival of the sporocysts was prolonged by addition of the NADPH oxidase inhibitor protocatechuic acid (PCA). In our experiments, inhibition of NADPH oxidase by PCA significantly reduced sporocyst killing by 13-16-R1 hemocytes, indicating that ROS produced via the NADPH oxidase pathway are required for normal killing in this snail-trematode system as well. We then attempted to determine which of the ROS produced by the molluscan hemocytes are responsible for toxicity. Elimination of superoxide (O_2^-) by addition of superoxide dismutase (SOD) neither reduced nor enhanced parasite killing by the hemocytes. These results suggest first that O_2^- is not directly involved in the killing process, and second that SOD is produced in sufficient amounts by either hemocytes or sporocysts (or both). If SOD were limiting in the system, additional SOD should

increase the rate of production of hydrogen peroxide (H_2O_2). As H_2O_2 was found to be highly toxic to sporocysts, an increase in the rate of killing would have been expected.

Scavenging of hypochlorous acid (HOCl) and hydroxyl radicals ($^{\cdot}OH$) by addition of hypotaurine to the killing assay had no effect on the rate of parasite killing. These data suggest that neither HOCl, nor singlet oxygen ($^1O_2$) (which is 'downstream' of HOCl) or $^{\cdot}OH$, are involved in sporocyst killing by 13-16-R1 hemocytes. This result was unexpected because HOCl is typically thought to be the reactive oxygen species mainly employed by phagocytic cells to kill microorganisms. However, we also determined that, on a mole-to-mole basis, H_2O_2 is much more efficient than HOCl at killing sporocysts. There appears to be a distinct concentration threshold for H_2O_2 toxicity, which has also been reported for H_2O_2-dependent killing for *S. mansoni* schistosomula (Smith et al., 1989). Scavenging of H_2O_2 by addition of catalase did significantly reduce parasite killing by 13-16-R1 hemocytes, indicating that H_2O_2 is the reactive oxygen species mainly responsible for killing.

Addition of protocatechuic acid (PCA) or catalase significantly reduced killing of *S. mansoni* sporocysts by 13-16-R1 hemocytes, but killing was never completely eliminated. This lack of complete inhibition could be due to several factors. First, PCA is known to reduce ROS generation by *B. glabrata* hemocytes; however, it does not entirely inhibit NADPH oxidase activity (Adema et al., 1994; Hahn, Bender and Bayne, 2000). Another reason for the lack of total inhibition of parasite killing could be because catalase, even though a potent enzyme for converting H_2O_2, may, due to its large size, not have been able to efficiently act at the interspace between hemocytes and sporocysts during the encapsulation. Catalase also requires reaction with two H_2O_2 molecules simultaneously, which makes the enzyme less efficient at low concentrations of H_2O_2. Finally, it is very likely that 13-16-R1 hemocytes employ additional killing mechanisms, other than generation of ROS, to kill *S. mansoni* sporocysts. Potential additional cytotoxic factors could include the release of lysozyme or other lytic enzymes, or possibly the generation of reactive nitrogen species.

Hemocytes from all strains of *B. glabrata*, whether they are resistant or susceptible *to S. mansoni*, are fully immunocompetent, and MO hemocytes are known to be adequately capable of generating ROS (Dikkeboom et al., 1988b; Connors and Yoshino, 1990; Hahn, Bender and Bayne, 2000). Our findings, however, emphasize the importance of generating the right species of reactive oxidants to achieve successful elimination of the parasite. Insights from the present study provide the means to investigate on a more specific level why hemocytes from susceptible snails fail to kill. For example, do susceptible hemocytes generate

ROS in response to the parasite, indicating effective recognition? Even if the recognition system functions normally, it is possible that susceptible hemocytes mount ineffective or inappropriate responses. Myeloperoxidase (MPO), which converts H_2O_2 into HOCl, is often considered the cornerstone of ROS mediated cytotoxicity, and there is evidence that molluscan hemocytes possess MPO activity similar to that found mammalian phagocytes (Schlenk et al., 1991; Torreilles et al., 1997). In this particular host-parasite relationship, however, high MPO activity may actually be beneficial to parasite survival, and might even render the host susceptible.

In summary, our results show that in vitro killing of *S. mansoni* sporocysts by naive *B. glabrata* hemocytes occurs independently of the presence of snail plasma. We infer that resistance and susceptibility towards *S. mansoni* is reflected by intrinsic differences in the hemocytes of the molluscan host. Whereas reactive oxygen species are important for sporocyst killing by 13-16-R1 hemocytes, among all ROS tested only reduction of H_2O_2 protected the parasite from cell-mediated cytotoxicity. Further, *S. mansoni* sporocysts seem to be more susceptible to killing by H_2O_2 than HOCl.

ACKNOWLEDGMENTS

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Chapter 4.

Involvement of Nitric Oxide in Killing of *Schistosoma mansoni* by Hemocytes from resistant *Biomphalaria glabrata*

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In Review: *Journal of Parasitology*

ABSTRACT

In the hemolymph of individuals of *Biomphalaria glabrata* (Gastropoda), which are resistant to infection with *Schistosoma mansoni* (Trematoda), phagocytic cells (hemocytes) are responsible for elimination of the intramolluscan life stages of the parasite (sporocysts). We investigated the role of reactive nitrogen species in in vitro interactions between hemocytes, derived from the *S. mansoni*-resistant 13-16-R1 strain of *B. glabrata*, and the parasite. The nitric oxide synthase (NOS) inhibitor N_{ω}-nitro-L-arginine methylester (L-NAME) and the nitric oxide (NO) scavenger 2-(4-carboxyphenyl)-4,4,5,5,-tretramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) reduced cell-mediated killing of *S. mansoni* sporocysts. The peroxynitrite scavengers uric acid and deferoxamine, however, had no influence on the rate of parasite killing, indicating that NO is directly responsible for mediating cytotoxicity, but peroxynitrite is not. Combination of the NOS inhibitor L-NAME and catalase, an enzyme which detoxifies hydrogen peroxide, reduced average sporocyst mortality to a higher extent than only NOS inhibition by L-NAME. We suggest that NO and hydrogen peroxide play a synergistic role in hemocyte-mediated toxicity of 13-16-R1 *B. glabrata* against *S. mansoni* sporocysts.

INTRODUCTION

The freshwater snail *Biomphalaria glabrata* (Gastropoda) serves as a natural intermediate host of the human blood fluke *Schistosoma mansoni* (Trematoda). Yet, natural populations of *Biomphalaria* are very polymorphic regarding their compatibility with *S. mansoni* (Basch, 1976; Morand, Manning and Woolhouse, 1996), and often high percentages of snails in a population are resistant to infection with the parasite (Mascara et al., 1999). Resistance in the mollusc is genetically determined (Richards, Knight and Lewis, 1992), and inbred strains of *B. glabrata* with selective resistance or susceptibility towards specific strains of *S. mansoni* are available. The snail strain used in this study (13-16-R1), for example, is resistant towards the PR-1 strain of *S. mansoni*. Upon entering an individual snail of this strain, *S. mansoni* sporocysts are quickly attacked by circulating phagocytic cells in the snail's hemolymph. These so called hemocytes will encapsulate the parasite and kill it within a few days (Loker et al., 1982; Jourdane, 1982; Sullivan, Spence and Nunez, 1995). This cell-

mediated killing of the parasite can be demonstrated in in vitro killing assays (Bayne, Buckley and DeWan, 1980; Fryer and Bayne, 1995; Boehmler, Fryer and Bayne, 1996; Hahn, Bender and Bayne, accepted for publication), which present valuable tools for determining the exact mechanisms that underlie successful destruction of *S. mansoni* sporocysts. For example generation of reactive oxygen species has been shown to play a role in sporocyst killing by molluscan hemocytes in incompatible snail-trematode combinations (Adema et al., 1994; Hahn, Bender and Bayne, accepted for publication). Studies on interactions between *B. glabrata* 13-16-R1 hemocytes and *S. mansoni* PR-1 sporocysts in particular has determined that among all reactive oxygen species produced by the resistant hemocytes, it is H_2O_2 specifically that is responsible for mediating cytotoxicity towards the parasite (Hahn, Bender and Bayne, accepted for publication). Inhibition of NADPH oxidase, the enzyme responsible for generation of reactive oxygen species, or detoxifying H_2O_2 by catalase reduced parasite killing by 13-16-R1 hemocytes, however, did not completely inhibit it. These findings suggest that reactive oxygen species are not solely responsible for hemocyte-mediated cytotoxicity, but there may be additional mechanisms involved in parasite destruction by molluscan hemocytes.

Pathogens that induce generation of reactive oxygen species in phagocytes quite often also induce generation of nitric oxide (NO) (MacMicking, Xie and Nathan, 1997). NO production results from enzymatic oxidative deimination of L-arginine to L-citrulline. The enzyme responsible for this reaction is nitric oxide synthase (NOS). For mammalian phagocytes it has been well documented how the antimicrobial and cytotoxic actions of reactive oxygen species are enhanced by nitric oxide (NO) that is generated by high out-put nitric oxide synthases. Superoxide, for example, will react with NO to produce the highly toxic peroxynitrite, and NO has been shown to enhance cytotoxic effects of hydrogen peroxide by inhibiting peroxidases, which are often used by pathogens to protect themselves against oxidative damage (Farias-Eisner et al., 1996). NO, a labile highly reactive gas, has been implicated as an important cytotoxic effector molecule against an array of pathogens, including bacteria, fungi, and both protozoan and helminth parasites. Its importance against parasitic diseases, including schistosomiasis, has been reviewed by James (1995).

Inducible NOS activity has also been reported in invertebrates (Radomski, Martin and Moncada, 1992; Conte and Ottaviani, 1995; Luckhart et al., 1998; Torreilles and Guerin, 1999). It therefore seemed worthwhile to investigate whether, in addition to reactive oxygen species, the generation of reactive nitrogen species contributes to the killing of *S. mansoni*

sporocysts by resistant *B. glabrata* hemocytes. The present study was designed to determine whether generation of reactive nitrogen species (nitric oxide, and/or peroxynitrite) is involved in elimination of *S. mansoni* PR-1 sporocysts by resistant hemocytes from the 13-16-R1 strain of *B. glabrata*. Employing an in vitro killing assay, we assessed the influence of the NOS inhibitor L-NAME and the NO scavenger carboxy-PTIO on cell-mediated killing of the parasite. Using specific scavengers for peroxynitrite, we further examined whether NO is directly responsible for cytotoxicity, or whether formation of peroxynitrite is required to mediate toxicity. Hydrogen peroxide has previously been reported to be in involved in killing of *S. mansoni* sporocysts by resistant hemocytes. We therefore attempted to define the relative contribution of NO generation and generation of hydrogen peroxide during parasite killing by resistant *B. glabrata* hemocytes.

MATERIALS AND METHODS

Most chemicals were purchased from Sigma, St. Louis, Missouri. Other sources are specified.

Animals

The PR-1 strain of *S. mansoni* was used in this study. *S. mansoni* eggs were recovered from the livers of patently infected hamsters. Miracidia were hatched in sterile artificial spring water (Ulmer, 1970) and collected. Sporocysts were obtained by overnight in vitro transformation of (10,000 to 30,000) miracidia in 2.5 ml medium F base (Stibbs et al., 1979) containing 1% bovine serum albumin (Sigma A-3912, Sigma Chemicals Co., St. Louis, Missouri) and 20 μg/ml gentamicin (Gibco BRL, Grand Island, New York). The next day sporocysts were washed with CMC Medium (Hahn, Bender and Bayne, accepted for publication).

The snails used in this study were from the 13-16-R1 strain of *B. glabrata*, which is resistant to infection with *S. mansoni* PR-1. Snails were kept in 100 L plastic aquaria at 26 C and fed green leaf lettuce ad libitum.

Cell-mediated cytotoxicity assay

The cell-mediated cytotoxicity (CMC) assay was conducted as described earlier (Hahn, Bender and Bayne, accepted for publication). In brief, sterile hemolymph from 8 to15

adult snails (12 to 15 mm in diameter) was obtained and divided into 2 or 3 200 µl aliquots, depending on the number of different treatments called for in the particular experiment. The hemolymph was added to the wells of a 96-well tissue culture plate (Corning, Acton, Massachusetts), which had been pretreated with poly-L-lysine (Boehmler, Fryer and Bayne, 1996). The hemocytes were then spun down (10 x g for 10 min at room temperature), and washed twice with CMC Medium. Then 100 µl CMC Medium was added to each well. At this point 50 µl of the test compound were added to the cells. All test compounds were diluted in Chernin's Balanced Salt Solution [CBSS; (Chernin, 1963)], containing 1 mM HEPES buffer (Sigma, H-7006 and H-9136) at pH 7.4. Immediately after the addition of test compounds or CBSS (controls), 75 to 100 sporocysts were added, in a volume of 50 µl, to each well containing hemocytes and immediately counted using an inverted phase contrast microscope, to determine their exact number. Then 15 µl propidium iodide (PI; Sigma, P-4170) in CBSS was added to a final concentration of 10 µg/ml. Sporocyst mortality was determined by PI incorporation into nuclei. Tissue culture plates were incubated at 26 C in the dark. Dead parasites were counted under a fluorescence microscope at several intervals over a period of 47 hr.

Effects of NOS inhibition on sporocyst killing

In order to test for the involvement of nitric oxide (NO) in killing of *S. mansoni* sporocysts by resistant hemocytes, we added the competitive nitric oxide synthase (NOS) inhibitor N_{ω}-nitro-L-arginine methylester (L-NAME; Sigma N-5751) to CMC assays. L-NAME was dissolved in CBSS and added to a final concentration of 1mM. Concentrations higher than I mM were found to be toxic to sporocysts. The control treatment included the same concentration of its D-isoform, Nω-nitro-D-arginine methylester (D-NAME; Sigma N-4770), which does not interfere with NOS activity. L-NAME or D-NAME was added to washed 13-16-R1 hemocytes, derived from the same pool of snails, immediately before addition of sporocysts to the cells. To evaluate the potential direct toxicity of the test compounds, sporocysts without hemocytes were incubated in 1 mM L-NAME, or 1mM D-NAME.

Effects of NO scavenging by carboxy-PTIO

PTIO is an efficient scavenger for NO (Akaike et al., 1993; Maeda et al., 1994). It oxidizes NO to form nitrite, before NO can react with O_2^- to yield highly reactive $ONOO^-$. We

employed PTIO to determine whether NO is directly involved in hemocyte-mediated cytotoxicity towards *S. mansoni* sporocysts. In our study we used the more water soluble form 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO; Dojindo Laboratories, Kumamoto, Japan). Certain cytotoxic effects of carboxy-PTIO have been implicated (Amano and Noda, 1995). We therefore screened the chemical for potential toxicity towards *B. glabrata* hemocytes and sporocysts. While hemocytes showed no signs of impaired health with carboxy-PTIO concentrations of up to 1 mM, concentrations above 0.1 mM were toxic to sporocysts. To assess the effects of hemocyte-mediated killing of *S. mansoni* sporocysts we therefore included 0.1 mM of carboxy-PTIO into CMC assays. Carboxy-PTIO is relatively stable, but sensitive to heat and UV light. When decomposing, it may emit carbon monoxide and nitrogen oxides. To minimize its exposure to UV light, the number of counts in the CMC assays were reduced.

Protection from peroxynitrite toxicity with uric acid

Uric acid specifically reacts with peroxynitrite ($ONOO^-$), detoxifying it, but it will not react with NO (Hooper et al., 1998). In order to determine whether addition of uric acid will protect sporocysts from damage caused by $ONOO^-$, sporocysts were incubated in various concentrations of 3-morpholinosydnonimine (SIN-1; Sigma M-5793), with or without the presence of 1 mM uric acid. SIN-1 is a spontaneous NO donor, which also releases superoxide (O_2^-). NO and O_2^- are released at the same rate and will quickly react to produce $ONOO^-$. SIN-1 generates $ONOO^-$ at a rate roughly equivalent to 1% of its concentration per minute. Its decomposition is essentially complete after 3 hr (Darley-Usmar et al., 1992). A 20 mM stock solution of uric acid (Sigma, U-0881) was prepared by dissolving it in 0.1 M potassium hydroxide, then the pH was adjusted to 7.4 using 2 M hydrochloric acid. This stock solution of uric acid was then diluted in CBSS and added to the sporocysts to a final concentration of 1 mM, prior to addition of the $ONOO^-$ donor SIN-1. SIN-1 was stored at -70°C. It was weighed under dim light and dissolved in CBSS just minutes before adding it to sporocysts. Control wells received CBSS. SIN-1 was added at final concentrations of 0.5, 1, 2, and 5 mM. Sporocyst mortality was determined by PI staining.

To determine whether NOS-dependent hemocyte toxicity towards *S. mansoni* sporocysts is in part due to formation of $ONOO^-$, 1 mM of uric acid in CBSS was included in CMC assays. Control wells received CBSS. As a control for direct toxicity of uric acid to the

parasite, separate wells with sporocysts but no hemocytes were incubated with 1 mM uric acid or CBSS.

Protection from peroxynitrite toxicity with deferoxamine

Deferoxamine is a potent scavenger for $ONOO^-$ and hydoxyl radicals ($^{\cdot}OH$). To determine the optimum concentration for protection of sporocysts from $ONOO^-$ toxicity, we co-incubated parasites in 1mM of the $ONOO^-$ donor SIN-1 together with different concentrations of deferoxamine mesylate (Sigma D-9533) ranging from 0.5 to 10 mM. Sporocyst viability was assessed by PI exclusion. In order to determine the effect of deferoxamine in hemocyte-mediated killing of *S. mansoni* sporocysts, deferoxamine was diluted in CBSS and included into CMC assays at a final concentration of 2 mM. Control wells received CBSS.

Effect of reducing NO and H_2O_2 in CMC assays

Previous studies have shown that hydrogen peroxide (H_2O_2), generated by 13-16-R1 hemocytes, is involved in cell-mediated toxicity against *S. mansoni* sporocysts (Hahn, Bender and Bayne, accepted for publication). In order to assess the contribution of NO and H_2O_2 during 13-16-R1 hemocyte killing of *S. mansoni* sporocysts, we included the NOS inhibitor L-NAME, and catalase (which detoxifies H_2O_2) into CMC assays. Hemocytes derived from the same pool of hemolymph received either 1 mM L-NAME, or a combination of 1 mM L-NAME and 14,000 units/ml catalase (Sigma, C-40). Hemocytes in control wells received CBSS.

RESULTS

Effects of NOS inhibition on sporocyst killing

In order to determine if nitric oxide is involved in killing of *S. mansoni* sporocysts by 13-16-R1 hemocytes, N_ω-nitro-L-arginine methylester (L-NAME) and N_ω-nitro-D-arginine methylester (D-NAME) were added to CMC assays. L-NAME inhibits both inducible and constitutive nitric oxide synthases (NOS), by out-competing their natural substrate L-arginine. Its isoform (D-NAME) has no influence on NOS activity. Addition of 1 mM D-NAME into a CMC assay with 13-16-R1 hemocytes and *S. mansoni* sporocysts had no effect on the time

course of killing, when compared to controls in which buffer only was added (see killing with CBSS in Figures 4.2.,4.4., 4.6., and 4.7.). Addition of L-NAME at a concentration of 1mM into the assay significantly reduced sporocyst mortality compared to mortality in the presence of 1 mM D-NAME. This reduction was first significant 18 hr into the assay. At 47 hr the average reduction of sporocyst killing by L-NAME treated hemocytes was ca. 30% (Fig. 4.1.A). The presence of 1 mM D-NAME or L-NAME had no apparent influence on sporocyst viability in control wells without hemocytes (Fig. 4.1.B)

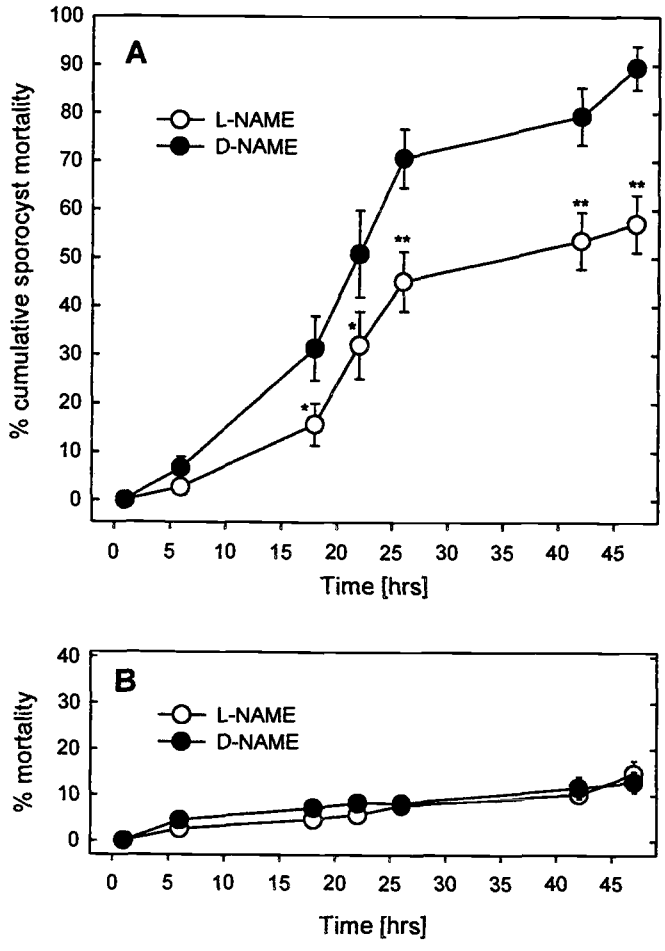

Figure 4.1. Effect of NOS inhibition on sporocyst killing (PI positive) by 13-16-R1 hemocytes. (**A**) *S. mansoni* sporocysts and *B. glabrata* hemocytes were cultured in the presence of either 1 mM of the NOS inhibitor L-NAME (o), or 1 mM D-NAME (control killing) (•). (**B**) Sporocysts without hemocytes in the presence of either 1 mM L-NAME (o), or 1 mM D-NAME (•). Each data point represents the mean of 8 replicates, ± standard error. Asterisks represent time points with significantly lower sporocyst mortality compared to control killing using a paired *t*-test; (*) $P < 0.05$, (**) $P < 0.01$.

Effects of NO scavenging by carboxy-PTIO

In order to determine whether NO is directly responsible for cell-mediated sporocyst killing we included the NO scavenger carboxy-PTIO in CMC assays. The presence of 0.1 mM carboxy-PTIO

significantly reduced sporocyst killing by 13-16-R1 hemcytes (Fig. 4.2.A). This reduction was evident as early as 18 hr into the assay. At 47 hrs the average reduction in sporocyst mortality in the presence of carboxy-PTIO was ca. 20% compared to CBSS controls. A concentration of 0.1 mM carboxy-PTIO had no apparent effect on sporocyst mortality in control wells without hemocytes (Fig. 4.2.B).

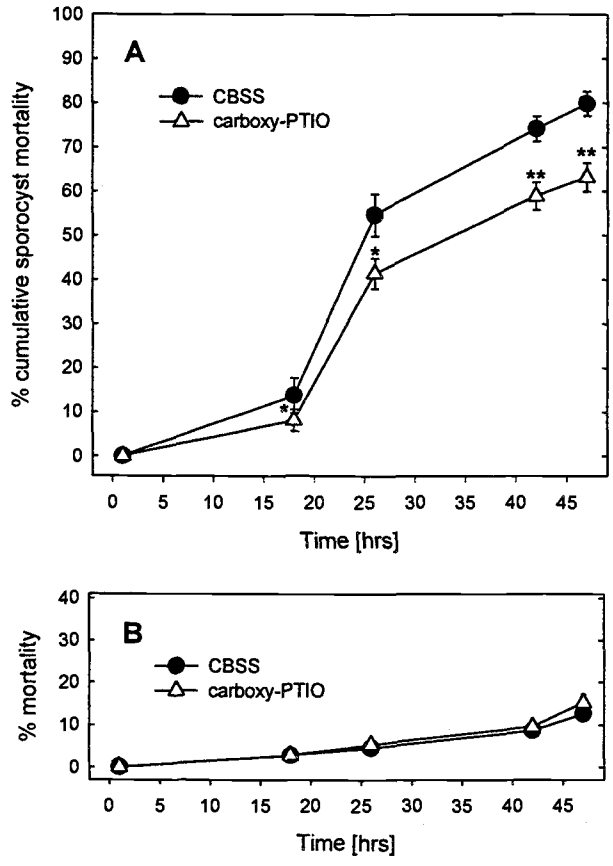

Figure 4.2. (A) Effect of the NO scavenger carboxy-PTIO on sporocyst killing by 13-16-R1 hemocytes. *S. mansoni* sporocysts and *B. glabrata* hemocytes were incubated with 0.1 mM carboxy-PTIO (Δ), or buffer (•). **B)** Control wells without hemocytes to assess sporocyst viability in the presence of 0.1 mM carboxy-PTIO (Δ), or buffer (•). Each data point represents the mean of 10 replicates ± standard error. Asterisks indicate time points with significantly lower sporocyst mortality compared to control (CBSS) killing (using a paired *t*-test); (*) $P < 0.05$, (**) $P < 0.01$.

Protection from peroxynitrite toxicity with uric acid

The ability of uric acid to protect *S. mansoni* from damage by artificially generated peroxynitrite was assessed. Sporocysts were incubated in different concentrations (0.5 to 5 mM) of the spontaneous peroxynitrite donor SIN-1, in the presence of 1 mM uric acid, or buffer. Uric acid prolonged parasite survival significantly at all concentrations of SIN-1 (Fig. 4.3.).

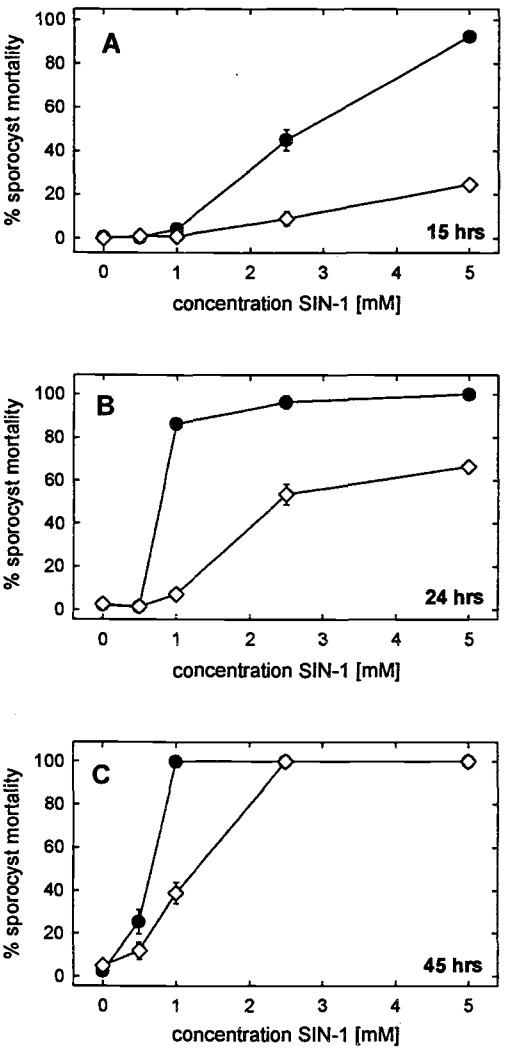

Figure 4.3. Effect of the peroxynitrite scavenger uric acid on sporocyst viability in the presence of different concentrations of peroxynitrite. *S. mansoni* sporocysts were incubated in 0.5 to 5 mM of SIN-1 (peroxynitrite donor), in the presence of 1 mM uric acid (◊), or buffer (•). Mortality was determined by PI-staining at different time points: (**A**) 15 hr, (**B**) 24 hr, and (**C**) 45 hr. Each data point represents the mean of 3 replicates ± standard error.

In order to test for the involvement of peroxynitrite ($ONOO^-$) in hemocyte-mediated toxicity towards *S. mansoni* sporocysts, uric acid at a final concentration of 1 mM was included in CMC assays. Uric acid is a selective scavenger of $ONOO^-$, but does not react with NO. Uric acid at a concentration of 1 mM failed to protect sporocysts from killing by 13-16-R1 hemocytes. Killing in the presence of uric acid was at no time point different from control killing in which only buffer was added (Fig. 4.4.A). As shown in control wells without hemocytes, the presence of 1 mM uric acid had no effect on sporocyst viability compared to addition of only CBSS (Fig. 4.4.B)

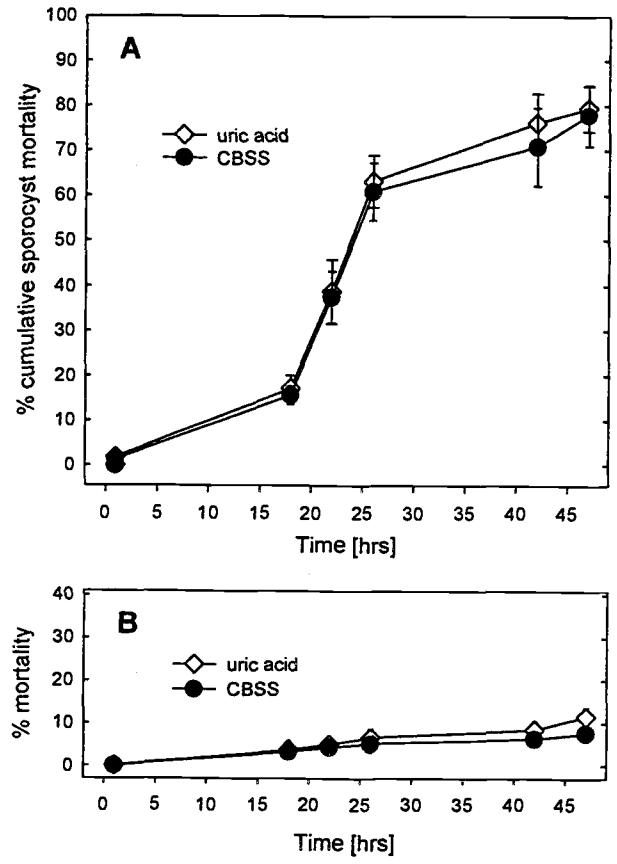

Figure 4.4. Effect of scavenging peroxynitrite on killing of *S. mansoni* sporocysts by 13-16-R1 hemocytes. (**A**) Sporocysts and hemocytes were incubated with either 1 mM of the peroxynitrite scavenger uric acid (◊), or CBSS (control killing) (•). (**B**) Control wells with sporocysts without hemocytes incubated with either 1 mM uric acid (◊), or CBSS (•). Each data point represents the mean of 6 replicates, ± standard error.

Protection from peroxynitrite toxicity with deferoxamine

Deferoxamine is a potent scavenger of $^{\cdot}OH$ and $ONOO^{-}$. All tested concentrations of deferoxamine protected sporocysts to some extent from damage by $ONOO^{-}$ (produced by SIN-1) (Fig. 4.5.). However, only concentrations ≥ 2 mM offered long-term protection (up to 68 hr). Sporocyst viability in the presence of 10 mM was not significantly higher than in the presence of 2 mM. We therefore included deferoxamine at a final concentration of 2 mM in CMC assays to test for the possible involvement of $ONOO^{-}$ or $^{\cdot}OH$ in the cell-mediated killing process. *S. mansoni* killing by 13-16-R1 hemocytes was not altered in the presence of 2 mM deferoxamine (Fig.4.6.).

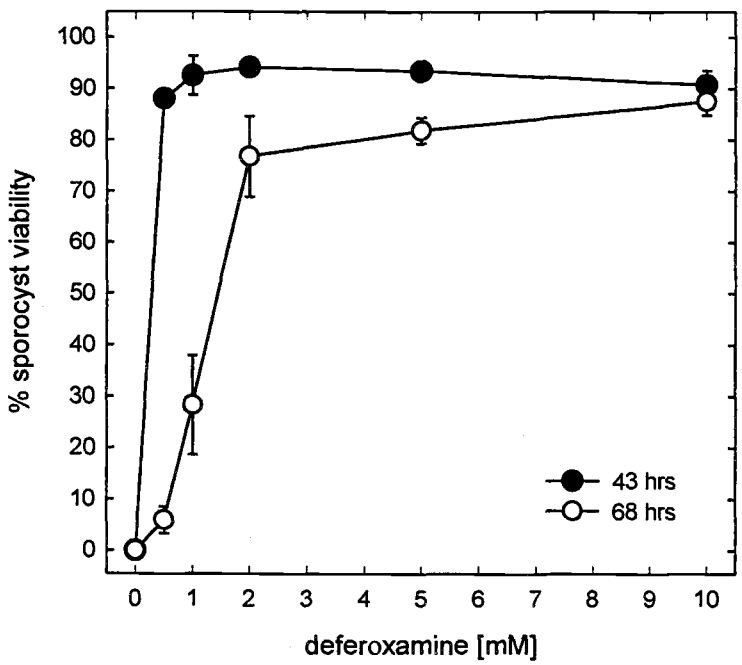

Figure 4.5. Protective effect of different concentrations of deferoxamine against $ONOO^{-}$ damage. Sporocysts were incubated in 1 mM SIN-1 in the presence of different concentrations of deferoxamine (0, 0.1, 1, 2, 5, and 10 mM). Sporocysts viability was determined by PI exclusion. Viability was assessed after 43 hr (•), and 68 hr (o), (n = 3, error bars = SE).

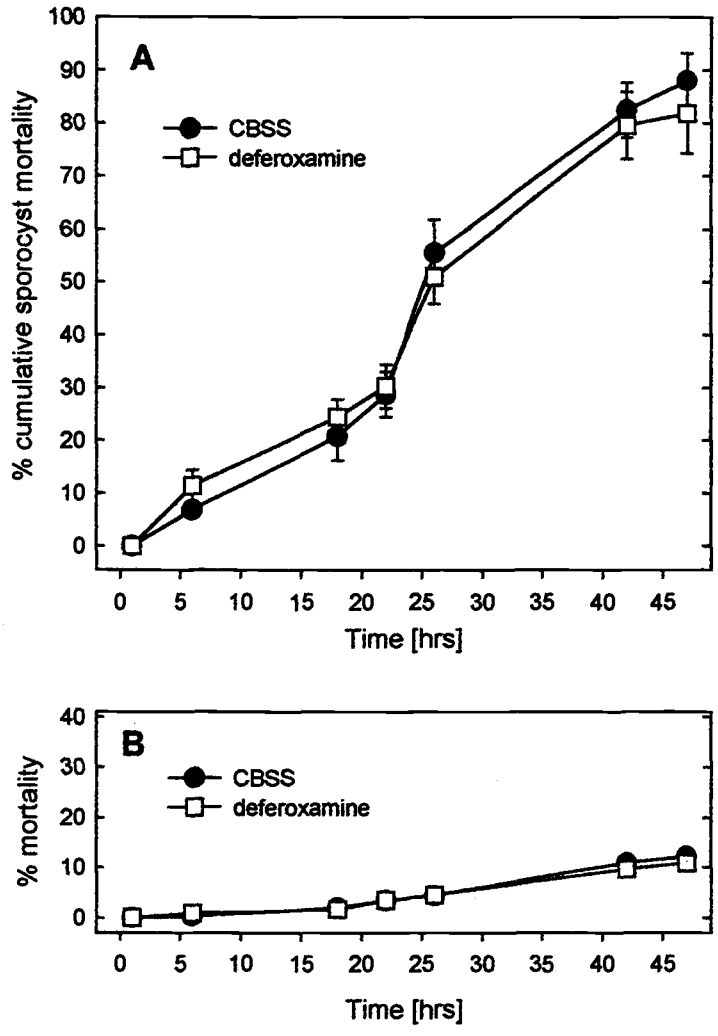

Figure 4.6. Effect of scavenging $ONOO^-$ and $^{\cdot}OH$ on hemocyte-mediated killing of sporocysts. (A) Sporocysts and 13-16-R1 hemocytes were incubated with either 2 mM of the $ONOO^-$ and $^{\cdot}OH$ scavenger deferoxamine (□), or CBSS (•) (control killing). (**B**) Sporocysts without hemocytes in the presence of either 2 mM deferoxamine (□) or CBSS (•). Each data point represents the mean of 5 replicates ± standard error.

Effect of reducing both NO and H_2O_2 in CMC assays

Both NOS inhibition by L-NAME (Fig. 4.1.) and H_2O_2 scavenging by catalase (Hahn, Bender and Bayne, accepted for publication) reduced sporocyst killing by 13-16-R1 hemocytes. In order to determine whether NO and H_2O_2 act in a synergistic manner, CMC assays including L-NAME and catalase were performed. Addition of both catalase (final

concentration 14,000 units/ml) and L-NAME (final concentration 1mM) to CMC assays reduced average sporocyst killing compared to addition of only L-NAME. However, this reduction was statistically significant (P-value < 0.05; using a paired t-test) only at 42 hr (Fig. 4.7.).

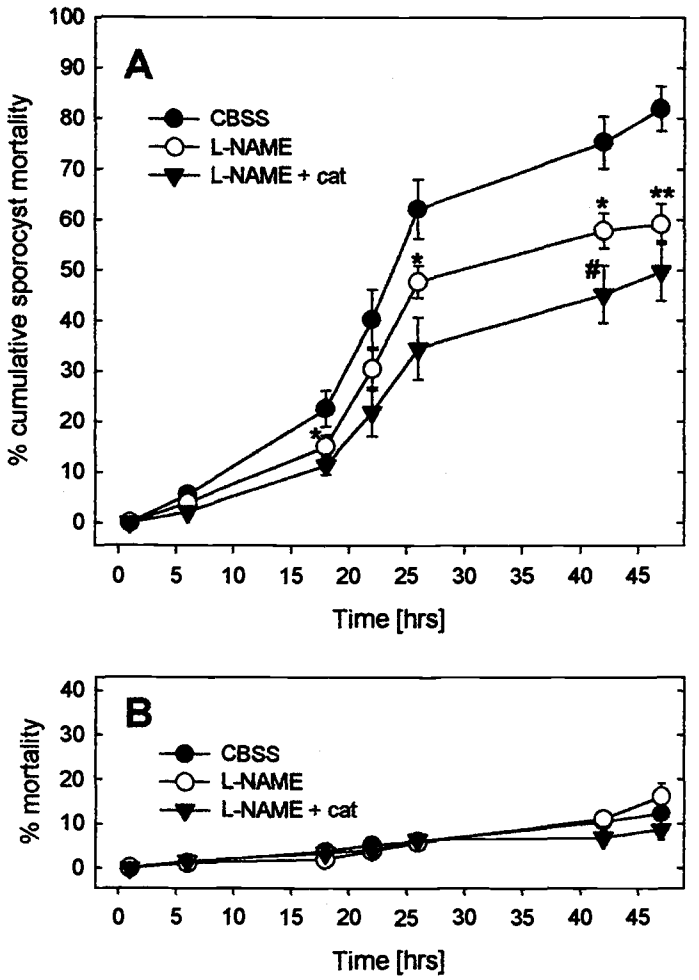

Figure 4.7. Scavenging H_2O_2 (with catalase) and inhibition of NO generation (with L-NAME) reduces killing of parasites by resistant hemocytes. (**A**) *S. mansoni* sporocysts and 13-16-R1 hemocytes were incubated with either CBSS (control killing) (•), 1 mM of the NOS inhibitor L-NAME (o), or a combination of 14,000 units/ml catalase and 1 mM L-NAME (▼). **B**) Control wells with sporocysts (without hemocytes) incubated in either CBSS (•), 1 mM L-NAME (o), or a combination of 1 mM L-NAME and 14,000 units/ml catalase (▼). Each data point represents the mean of 9 replicates ± standard error. Asterisks represent data points significantly lower than control killing; (*) $P < 0.05$, (**) $P < 0.01$; (#) represents data points significantly lower than killing in the presence of L-NAME ($P < 0.05$). Data were compared using a paired t-test.

DICUSSION

Previous work in this laboratory focused on the roles of reactive oxygen species in killing of *S. mansoni* sporocysts by resistant *B. glabrata* hemocytes under in vitro conditions. Among all individual reactive oxygen species tested, only hydrogen peroxide (H_2O_2) was found to be responsible for mediating cytotoxicity against the parasite (Hahn, Bender and Bayne accepted for publication). The focus of the present study was first to assess the contribution of reactive nitrogen species in hemocyte-mediated killing of *S. mansoni*, secondly to identify the reactive nitrogen species that is/are involved in cell-mediated toxicity, and lastly to assess the combined effects of reactive nitrogen and oxygen species in hemocyte-mediated killing of the parasite. Addition of N_ω-nitro-L-arginine methylester (L-NAME), which competitively inhibits nitric oxide synthase activity, reduced killing of *S. mansoni* sporocysts by resistant *B. glabrata* hemocytes. Addition of its non-inhibiting isoform N_ω-nitro-D-methylester, on the other hand, had no effect on the time course of killing. These results imply that hemocyte-mediated killing of the parasite involves an arginine-dependent mechanism, most likely generation of nitric oxide (NO) by the molluscan hemocytes. Nitric oxide synthase (NOS) activity in molluscan hemocytes has been reported earlier (Conte and Ottaviani, 1995; Torreilles and Guerin, 1999), and inducible NOS activity in the mosquito *Anopheles stephensi* has been implicated in the retardation of the development of its malaria parasite (Luckhartet al., 1998). However, to our knowledge this is the first report in which NOS activity in a mollusc is correlated with elimination of a pathogen.

To confirm that reduction of hemocyte-mediated toxicity by L-NAME is due to interference with the generation of nitric oxide (NO), we tested the specific NO scavenger carboxy-PTIO for its effects in in vitro killing assays. Scavenging of NO by carboxy-PTIO reduced parasite killing by resistant hemocytes. This reduction was not as pronounced as the reduction seen with L-NAME; it was, however, statistically significant at all time points, confirming that NO generation plays a role in destruction of the parasite. It is important to keep in mind that hemoglobin, which accounts for the majority of protein in the plasma of *B. glabrata*, is also an excellent scavenger of NO. Even in our in vitro killing assays, in which most of the plasma was removed and hemocytes were washed, there was still residual hemoglobin in the medium. It seems therefore inconsistent to assume that NO could possibly be utilized by hemocytes as a cytotoxic agent. Why would the NO scavenging properties of hemoglobin not interfere with killing, while NO scavenging by carboxy-PTIO reduces killing

of the parasite? This apparent contradiction might be explained by the structure of the two molecules. There is electron-microscopical evidence that during encapsulation of sporocysts by molluscan hemocytes there is close contact between the phagocytes' membranes and the parasite's tegument (Loker et al., 1982). A large protein like hemoglobin may not be able to reach this interspace. Carboxy-PTIO, on the other hand, is a small lipid-soluble compound, which may actually penetrate membranes and thus manage to protect the sporocyst from NO damage.

Nitric oxide itself is highly reactive and can exert cytotoxicity by inactivating iron/sulfur centers in key metabolic enzymes. Its main antimicrobial function, however, is by enhancing the toxic effects of reactive oxygen species (Hurst and Lymar, 1997). For example reaction of NO with superoxide (O_2^-), produced by NADPH oxidase during an oxidative burst, will lead to formation of peroxynitrite ($ONOO^-$), a short lived strong oxidant, which hydroxylates and nitrates aromatic compounds. Peroxynitrite can also quickly decay releasing highly reactive hydroxyl radicals (Beckman et al., 1990), which are extremely reactive with most biological molecules, causing lipid peroxidation, DNA strand breaks, and enzyme inactivation. In order to determine whether *S. mansoni* killing by resistant *B. glabrata* hemocytes is due in part to formation of $ONOO^-$, we monitored killing in the presence of the $ONOO^-$ scavengers uric acid and deferoxamine. Uric acid specifically scavenges $ONOO^-$, but does not react with NO (Hooper et al., 1998). Deferoxamine will detoxify $ONOO^-$ and hydroxyl radicals, but is not thought to react with NO. Both uric acid and deferoxamine were found to prolong *S. mansoni* sporocyst viability in the presence of various concentrations of the $ONOO^-$ donor 3-morpholinosydnonimine (SIN-1). However, inclusion of either uric acid or deferoxamine into CMC assays failed to protect sporocysts from hemocyte-mediated toxicity. These results imply that formation of $ONOO^-$ is not required for successful elimination of the parasite, and NO is directly involved in cytotoxicity. Even though this finding was surprising, evidence that $ONOO^-$ does not play a role in sporocyst killing was also obtained earlier. Addition of superoxide dismutase to CMC assays, which scavenges O_2^- thus reducing $ONOO^-$ formation, failed to protect the parasites (Hahn, Bender and Bayne accepted for publication). Further, NO mediated killing of *S. mansoni* schistosomula by mouse macrophages does not require O_2^-, indicating that in the mammalian host as well, generation of $ONOO^-$ is not involved in destruction of the parasite (Fouad Ahmed et al., 1997). Therefore NO probably acts directly on the parasite target. The principal targets in the schistosome might be enzymes containing a catalytically active iron/sulfur center, such as aconitase, a key

enzyme in the parasites' electron transport chain. Inactivation of this enzyme by various inhibitors shows similarities to NO toxicity in schistosomula (Fouad et al., 1994). An alternative mechanism by which NO may be detrimental to pathogens is by inactivation of peroxidases, which are often used by microbes to protect themselves against the damaging effects of hydrogen peroxide (H_2O_2). Adult stages of *S. mansoni* are known to have high glutathione peroxidase activity (Mei et al., 1996), which aids them in resisting oxidative damage (Mkoji, Smith and Prichard, 1988a; Mkoji, Smith and Prichard, 1988b; Nare, Smith and Prichard, 1990). Further, H_2O_2 has been implicated as an important factor in *S. mansoni* sporocyst elimination in resistant *B. glabrata*, and inhibition of the parasites' glutathione peroxidase activity by NO would render them more susceptible to H_2O_2.

In order to asses whether NO and H_2O_2 supplement each other in their toxic effect towards *S. mansoni* during hemocyte-mediated killing of *S. mansoni* sporocysts, we compared the reduction of sporocyst killing in the presence of L-NAME to the reduction seen with a combination of L-NAME and catalase. When resistant *B. glabrata* hemocytes and *S. mansoni* sporocysts were incubated in the presence of catalase, which efficiently detoxifies H_2O_2, and the NOS inhibitor L-NAME, average sporocyst mortality was reduced compared to killing in the presence of only L-NAME. This reduction, however, was statistically significant only at 42 hr. These data confirm that in hemocyte-mediated parasite killing NO and H_2O_2 enhance each other's toxic effects.

In summary, inhibition of nitric oxide synthase activity or scavenging of NO by carboxy-PTIO reduced killing of *S. mansoni* sporocysts by resistant *B. glabrata* hemocytes, indicating that generation of NO contributes to cell-mediated toxicity towards the parasite. Cytotoxicity is not due to formation of $ONOO^-$, because specific scavenging of peroxynitrite by uric acid failed to protect sporocysts from hemocyte killing. These results suggest that NO is directly involved in cytotoxicity. Inclusion of catalase together with inhibition of NOS activity reduced hemocyte-mediated killing even more than inhibition of only NOS activity, indicating that both H_2O_2 and NO are involved in schistosome killing. It is still unclear by what mechanism NO exerts its toxic effects towards *S. mansoni* sporocysts. One possibility is that it nitrosylates important enzymes, which contain iron/sulfur centers (e.g. acotinase); another possibility is that NO inactivates parasite peroxidases thereby ensuring higher sustained levels of toxic H_2O_2. Our findings suggest that, for parasite elimination, NO and H_2O_2 are more important than the highly reactive but short-lived reactive oxygen/nitrogen species such as hydroxyl radicals or $ONOO^-$. This suggestion is reasonable considering that *S.*

mansoni sporocysts have a dynamic, protective tegument, which may be able to recover from much of the oxidative damage caused by more labile reactive oxygen and nitrogen species. The combination of membrane permeability and relative longevity (seconds) of NO and H_2O_2, however, perhaps ensures their ability to pass through the tegumental layer, enabling them to cause damage within the parasite.

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Chapter 5. Conclusions

The studies described in this thesis were designed to determine the role(s) of reactive oxygen and nitrogen species in the cytotoxic response of *Biomphalaria glabrata* hemocytes towards *Schistosoma mansoni* sporocysts. We first investigated whether resistant and susceptible individuals of *B. glabrata* differ in their basic recognition of carbohydrate structures that are displayed on the *S. mansoni* sporocyst surface. *B. glabrata* hemocytes generate reactive oxygen species (ROS) in response to certain carbohydrate structures, when these are presented as multiple-ligand arrays, such as multiple sugars conjugated to bovine serum albumin (BSA). This defensive response was found to be specific to the nature of the carbohydrates that are presented. Mannose, fucose, and galactose residues elicited ROS generation, whereas N-acetyl-galactosamine, N-acetyl-glucosamine, glucose, lactose, and melibiose structures did not stimulate an oxidative burst. These results indicate the presence of lectin receptors on the hemocyte surface, and are consistent with the notion that carbohydrate recognition by lectins plays an important role in pathogen recognition by the molluscan internal defense system. However, we did not find differences in the responses of hemocytes derived from *Schistosoma mansoni*-resistant or *S. mansoni*-susceptible *B. glabrata*. Hemocytes from both snail strains responded in a similar fashion to all carbohydrate-BSA conjugates that were tested. If the carbohydrate structures we found to stimulate ROS generation are involved in recognition of the parasite, our results suggest that killing of the parasite may involve either qualitative differences in production of ROS by hemocytes from the two strains, or the involvement of additional factors. However, we tested only six monosaccharides and two disaccharides, and the carbohydrate moieties displayed on the *S. mansoni* sporocyst surface include far more complex structures. Differential recognition of the parasite by resistant and susceptible hemocytes can, therefore, not be ruled out as the basis of host susceptibility or resistance.

To resolve whether the lack of killing by susceptible hemocytes is in fact due to a lack of recognition, or to an ineffective response by these cells, requires elucidation of the mechanisms used by resistant hemocytes that lead to successful destruction of the parasite. We therefore investigated processes that occur during sporocyst killing, and focused in particular on the role(s) of reactive oxygen and nitrogen species (ROS/RNS). By addition of specific inhibitors of ROS/RNS production to in vitro killing assays with 13-16-R1 (resistant) *B. glabrata* hemocytes and *S. mansoni* sporocysts, we determined that both ROS and RNS are

involved in hemocyte-mediated toxicity against the parasite. Alterations in the rate of killing after addition of selective scavengers for specific ROS/RNS brought us to the conclusion that it is mainly hydrogen peroxide (H_2O_2) and nitric oxide (NO) that mediate cytotoxicity. Both H_2O_2 and NO are relatively weak oxidants, compared to hypochlorous acid (HOCl), hydroxyl radicals ($^{\cdot}OH$), or peroxynitrite ($ONOO^-$). They are, however, relatively long-lived, have high diffusion rates and, unlike superoxide (O_2^-), excellent membrane-permeability, which could explain their importance for killing of *S. mansoni* sporocysts. Whereas short-lived, highly reactive oxidants would react only with the surface of the sporocyst, NO and H_2O_2 may be able to pass through the parasite's tegument and cause considerable damage within the trematode. NO may also react with endogenous O_2^- inside the sporocyst to generate more reactive $ONOO^-$, and H_2O_2 might react with reduced iron to form $^{\cdot}OH$, which can be very damaging to the parasite (Fig. 5.1.). The highly reactive, or not membrane-permeable ROS/RNS, on the other hand, might not be able to penetrate the parasite tegument and would damage the sporocysts only superficially. It is possible that the *S. mansoni* tegument possesses high antioxidant activities or good repair mechanisms for oxidative damage. This would explain why ROS/RNS, which are unable to penetrate the sporocyst, are also unable to kill it.

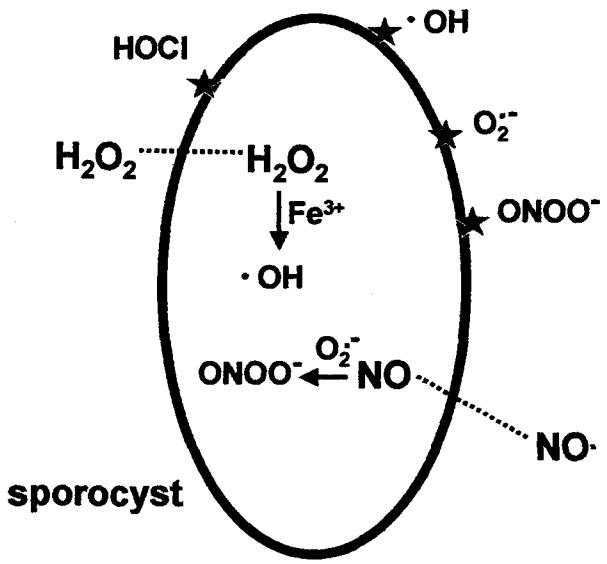

Figure 5.1. Illustration of possible interactions of ROS/RNS with the sporocyst. Highly reactive, but short-lived ROS/RNS, like hypochlorous acid (HOCl), hydroxyl radicals ($^{\cdot}OH$), and peroxynitrite ($ONOO^-$), or membrane-impermeable ROS like superoxide (O_2^-) may damage the parasite only superficially. Longer-lived ROS/RNS with good diffusion rates, like hydrogen peroxide (H_2O_2) and (NO), on the other hand, manage to penetrate the sporocyst tegument and cause oxidative damage within the parasite. The damaging effects might be augmented by reaction of H_2O_2 with endogenous reduced iron (Fe^{3+}) to form $^{\cdot}OH$ radicals, or reaction of NO with endogenous O_2^- to generate $ONOO^-$.

Our findings correlate well with what is known about *S. mansoni* killing by human or murine leukocytes. In vitro killing of *S. mansoni* schistosomula is linked to the levels of H_2O_2 that are produced by human monocytes (Lehn et al., 1991), granulocytes (Kazura et al., 1981), or murine leukocytes (Smith, Mkoji and Prichard, 1989) and, as in our studies in the invertebrate host, the rate of killing was reduced by addition of catalase (Kazura et al., 1981; Lehn et al., 1991), indicating that H_2O_2 is involved in cytotoxicity. Granulocyte-mediated toxicity to miracidia in *S. mansoni* eggs was also linked directly to H_2O_2 as being the toxic agent (Kazura et al., 1985). In this study addition of inhibitors of myeloperoxidase, which convert H_2O_2 into hypohalides, actually reduced cell-mediated toxicity. All life-stages of *S. mansoni* are known to possess enzymes such as superoxide dismutases and glutathione peroxidase that detoxify ROS (Simurda et al., 1988; Nare, Smith and Prichard, 1990; Connors, Lodes and Yoshino, 1991; Mei et al., 1996; LoVerde, 1998), and endogenous levels of these enzymes appear to be correlated with the worms' susceptibility to oxidant killing (Mkoji, Smith and Prichard, 1988; Nare, Smith and Prichard, 1990). During maturation of schistosomes the expression levels of these antioxidant enzymes increase several fold, which explains their increased resistance to oxidative damage caused by immune cells (LoVerde, 1998). Antioxidant levels in the parasite have also been linked to the mechanisms of action of anti-schistosomal drugs. In adult *S. mansoni*, which are usually quite resistant to oxidative killing, the compound oltipraz decreases levels of reduced glutathione, which is vitally important for protection against oxidative damage. These lowered glutathione levels render the worms more susceptible to killing by mouse leukocytes (Mkoji, Smith and Prichard, 1990).

Involvement of NO in elimination of schistosomula by mouse macrophages was first suggested by James (James and Glaven, 1989) and later confirmed by several additional studies (reviewed by James, 1995). NO-mediated killing by mouse macrophages does not require superoxide, indicating that in the mammalian host as well, generation of peroxynitrite is not involved in destruction of the parasite, but NO most likely acts directly on the parasite target (Fouad-Ahmed et al., 1997). In the mammalian host only early lung-stage larvae are susceptible to killing by the immune system, whereas adult worms are resistant to attack. Due to their complexity, it has been notoriously difficult to develop vaccines against metazoan parasites. Some protection can be achieved by exposing mice to irradiation-attenuated *S. mansoni* cercariae. Upon reinfection with normal cercariae these 'vaccinated' mice acquire a reduced parasite load compared to control mice. Studies on knock-out mice, lacking inducible

nitric oxide synthase (iNOS), revealed that iNOS activity is not required for this vaccination effect (Coulson et al., 1998; James et al., 1998). However, iNOS knock-out mice had much higher parasite loads (vaccinated and non-vaccinated) than wild type control mice, which indicates the importance of NO for controlling the parasitic infection.

We have found that NO is involved in sporocyst killing by resistant *B. glabrata* hemocytes, and there is a possibility that there is an intrinsic difference between resistant and susceptible *B. glabrata* hemocytes regarding NO production, or regarding NO production in response to the parasite. In in vitro studies we found that sublethal concentrations NO combined with sublethal concentrations of H_2O_2 result in sporocyst mortality (Bender, unpublished data). This synergistic toxicity suggests the possibility that resistant hemocytes produce relatively low levels of H_2O_2 and NO, and it is the combination of the two effects that leads to a lethal result. Furthermore, the lack of a single component in a multi-component killing mechanism may result in a much lower killing efficiency, or it may eliminate killing altogether. It would therefore be worthwhile to investigate whether susceptible *B. glabrata* hemocytes generate NO in response to *S. mansoni* sporocysts.

Our results emphasize that for successful elimination of a pathogen it is not enough to mount an aggressive response, but it is further critically important to generate the qualitatively appropriate response. Most of the H_2O_2 generated, in mammalian leukocytes at least, is consumed by myeloperoxidase (MPO). MPO readily oxidizes chloride ions to the strong nonradical oxidant HOCl. Hypochlorous acid is the most bactericidal oxidant known to be produced by leukocytes, potentiating the microbicidal activity of H_2O_2 by nearly 50-fold (Hampton, Kettle and Winterbourn, 1998). For the specific case of *S. mansoni* sporocysts, however, we found that in vitro the parasite is much more susceptible to damage mediated by H_2O_2 than HOCl. We also found no indication that HOCl is involved in sporocyst killing by resistant hemocytes. High MPO activity in this case, therefore, may inhibit parasite killing rather than enhance it. Lack of a functional NADPH oxidase enzyme complex, which prevents all generation of ROS by leukocytes, is a severe immuno-compromising condition that can occur in humans. It is known as chronic granulomatous disease (CGD). MPO-deficiency, on the other hand, has no clinical implications (in humans and MPO knock-out mice), except for a higher susceptibility to infections with *Candida* (Malech and Nauseef, 1997). In fact MPO-deficiency occurs quite frequently in the human population with prevalences as high as 1 in 2,000 individuals (Kutter, Al-Haidarik and Thomas, 1994). The reason why MPO-deficient individuals are not immuno-compromised may be the higher availability of H_2O_2 in

leukocytes, which takes over antimicrobial activities (Klebanoff and Pincus, 1971). For elimination of *S. mansoni* sporocysts, MPO-deficiency may actually be advantageous, because of the high susceptibility of the parasite to H_2O_2.

Hemocytes from *S. mansoni*-susceptible *B. glabrata* are fully immuno-competent, and readily phagocytose and destroy bacteria, fungi, or incompatible trematode larvae. They are also fully capable of mounting an oxidative burst against pathogens, even though the specific nature of the reactive oxygen species generated by these cells has not been determined. Whether the lack of killing of *S. mansoni* sporocysts by susceptible hemocytes is due to a lack of recognition of this particular parasite, or due to an ineffective response, remains elusive. However, the present study identified the components necessary for successful killing of the parasite, and this makes it possible to test susceptible hemocytes for specific responses, that we know are involved in cytotoxicity. For example, failure to kill could be due to higher MPO activity in susceptible hemocytes, which would lead to increased levels of ineffective HOCl, and reduced levels of H_2O_2. On the other hand, resistant *B. glabrata* hemocytes might be MPO-deficient, thus requiring higher metabolic effort to eliminate most pathogens, however, ensuring high levels of H_2O_2 that are helpful for killing *S. mansoni* sporocysts. This scenario is in agreement with the notion that there is a disadvantageous character or physiological defect connected with being resistant to *S. mansoni* (Wright, 1971).

Identification of the mechanisms that enable resistant *B. glabrata* to eliminate *S. mansoni* sporocysts will facilitate further investigations of survival strategies used by this parasite. The obvious parallels in the way schistosomes are killed in mammalian hosts and molluscan hosts suggest that cytotoxic responses involved in successful elimination of these parasites are well conserved. Immune evasion mechanisms exploited by *S. mansoni* in the snail might be utilized in the vertebrate host as well. Further studies of the *B. glabrata-S. mansoni* model may, therefore, be able to shed light on fundamental anti-parasite strategies, with possible relevance to the mammalian host-parasite relationship.

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