Cíntia Aparecida de Jesus Pereira

Caracterização da atividade de moléculas solúveis da hemolinfa de *Biomphalaria tenagophila* Taim envolvidos na morte dos esporocistos de *Schistosoma mansoni* cultivados *in vitro*

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Tese apresentada ao programa de Pós-Graduação em Parasitologia do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, como requisito parcial à obtenção do título de Doutor em Parasitologia.

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“ ... O correr da vida embrulha tudo, a vida é assim:
esquenta e esfria,
aperta e daí afrouxa,
sossega e depois desinqueta.
O que ela quer da gente é coragem ... ”

João Guimarães Rosa
Grande Sertão Veredas

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Resumo

As espécies *Biomphalaria glabrata*, *B. tenagophila* e *B. straminea* são Planorbideos de água doce com importância médica relevante no Brasil, pois são os hospedeiros intermediários naturais de *Schistosoma mansoni*. *Biomphalaria tenagophila* oriunda da reseva ecológica do Taim (RS), é uma linhagem completamente resistente a infecção pelo parasito, sendo capaz de destruií-lo poucas horas após a penetração do miracídio no interior do molusco, embora o mecanismo de lesão não sejam conhecido. Apesar dos trabalhos mostrarem que, entre os elementos do sistema interno de defesa dos moluscos, os hemócitos são os principais efetores no processo de eliminação do parasito, trabalhos preliminares demonstraram que a inoculação da fração acelular da hemolinfa de *B. tenagophila* Taim resistente resulta no aumento da resistência de *B. tenagophila* Cabo Frio suscetível a infecção pelo *S. mansoni*, indicado que fatores solúveis da hemolinfa também participam do mecanismo de proteção da resposta protetora desenvolvida pelo molusco. Neste trabalho foi possível confirmar a participação da fração acelular da hemolinfa de *B. tenagophila* Taim na destruição de esporocistos de *S. mansoni* em sistema de cultivo do parasito *in vitro*. Neste sistema, a adição de hemolinfa acelular, especialmente a de Taim, às culturas de esporocisto de *S. mansoni* resulta em aumento da mortalidade dos esporocistos quando comparado às culturas de esporocistos contendo apenas meio CBSS suplementado ou meio e hemolinfa acelular de *B. glabrata*. Alem da ação direta sobre esporocistos, a acombinação da fração acelular de Taim com os hemócitos de *B.tenagophila* de Cabo Frio aumenta significativamente a mortalidade dos esporocistos e o efeito é espécie-especifico. Assim, os dados aqui apresentados indicam que a hemolinfa acelular de caramujos da linhagem Taim atua diretamente na destruição dos esporocistos provocando lesão de tegumento do parasito que permitiu a penetração do

marcador fluorescente, *Hoechst 33258,* e indiretamente auxiliando os granulócitos no reconhecimento do parasito.

O mecanismo pelo qual a fração acelular da hemolinfa de *B .tenagophila* Taim destroe o parasito, sem a aprticipação dos hemócitos, não está definido na literatura. Há evidências da presença de atividade de proteases na hemolinfa de *Biomphalaria,* embora nenhum estudo tenha sido conduzido para evidenciar a participação de aividade protease na interação entre *B. tenagophila* infectado com *S. mansoni*. Neste trabalho também foi avaliado a participação de proteases presentes na hemolinfa de *B.tenagophila* Taim e sua associação no processo de destruição dos esporocistos de *S. mansoni*. Nososo dados mostram atividade proteólitca, especialmente serino- e cisteino-protease na hemolinfa de todas as linhagens de caramujos testadas neste trabalho experiemental, porém a atividade encontrada na hemolinfa de *B.tenagophila* Taim é mais intensa. Além disto a inibição da atividade de serino-protease na hemolinfa de *B.tenagophila* Taim resultou em diminuição significativa da mortalidade dos esporocistos de *S. mansoni* quando comparado com tratamento no qual a atividade proteolítica da fração acelular *B. tenagophila* Taim não foi inibida. O fracionamento da hemolinfa acelular de *B. tenagophila* Taim em coluna de exclusão molecular permitiu identificar que a atividade anti-esporocisto está associada a componentes protéicos da fração 7 e é coincidente com a atividade de serino-protease presente nesta amostra. Separações subseqüentes da fração 7 em coluna de troca iônica, demonstram atividade anti-esporocisto presente na subfração 7.1 e 7.2, sendo que na subfração 7.1 a atividade anti-esporocisto é independente de serino-protease, e na subfração 7.2 a mortalidade e lesão tegumentar de esporocistos de *S. mansoni* coincide com a atividade proteolítica. Os resultados sugerem que a atividade anti-esporocisto presente na hemolinfa de *B.tenagophila* Taim esta associada com a capacidade de lesar diretamente esporocistos de *S. mansoni* na ausência de hemócitos.

Abstract

Biomphalaria glabrata, *B. tenagophila* and *B. straminea* are fresh water Planorbids of great medical relevance as intermediary hosts of *S. mansoni*, a Trematode parasite that causes human Schistosomiasis in Brazil. *Biomphalaria tenagophila* of Taim strain is able to completely destroy *S. mansoni* few hours after parasite penetration, although the mechanism is still not well known. Even though hemocytes have been identified as the main effectors element during *S. mansoni* control inside the snail, we demonstrated that passive transference of cell-free hemolymph from *B. tenagophila* Taim resulted in higher resistance of *B. tenagophila* Cabo Frio to *S. mansoni* infection, indicating that soluble elements of the hemolymph participate in the protective mechanism. In this experimental work we had demonstrated that the protective effect of Taim cell-free hemolymph was also observed during the *in vitro* culture of *S. mansoni* sporocysts. In this system, addition of *B. tenagophila* cell-free hemolymph, especially from Taim strain, was responsible for significant increase in sporocysts mortality compared to *B. glabrata* cell-free hemolymph or culture medium. Moreover, the combination of Taim cell-free hemolymph and Cabo Frio hemocytes increased significantly the mortality of sporocysts and this effect is species specific. The results indicated that Taim cell-free hemolymph would act directly on destruction of *S. mansoni* sporocysts, producing damage to sporocysts tegument that allowed the penetration of *Hoechst 33258* fluorescent marker, and indirectly increasing the parasite recognition by the circulating granulocytes. The mechanism by which cell-free hemolymph from *B. tenagophila* of Taim strain would directly, without the hemocyte participation, mediate the destruction of *S. mansoni* sporocysts is undefined by the literature. There are experimental evidences of

protease activity in hemolymph obtained of *Biomphalaria* species, although no studies had been conducted with *B. tenagophila* infected with *S. mansoni*. Therefore, in this experimental work we evaluated the protease activity in hemolymph of *B. tenagophila* of Taim strain and their association with *S. mansoni* sporocysts destruction. Our data showed proteolitic activity, especially serine- and cisteine-protease activity, in cell-free hemolymph of all snail strains tested in this experimental work; however, the activity was more intense in *B. tenagophila* Taim hemolymph. Moreover, inhibition of serine protease activity in hemolymph of *B. tenagophila* of Taim strain significantly reduced the *S. mansoni* sporocyst mortality induced by the presence of cell-free hemolymph from this snail strain. Fractionation of *B. tenagophila* Taim acellular hemolymph through a supex 75 column resulted in the identification of one fraction (fraction 7) that concentrated the anti-sporocyst activity and the effect on parasite was dependent on serine-protease activity. After re-fractionation of this hemolymph sample (the fraction 7) on Mono Q column we had detected anti-sporocyst activities in two samples, but in one sub-fraction the biological activity was coincident with the proteolitic activity and in the other sub-fraction the anti-sporocyst activity occurred in a serine-protease independent manner. The results suggested that the direct damage on *S. mansoni* sporocysts mediated by the acellular hemolymph of *B. tenagophila* of Taim strain would represent two mechanisms, one of them is independent of serine protease activity and the other is coincident with the proteolitic activity

1- Introdução Geral

Trematódeos Digenéicos do gênero *Schistosoma* estão entre os mais abundantes agentes de infecções em humanos (COELHO, 1995). Dentre as espécies que parasitam vertebrados, *Schistosoma haematobium* (Bilharz, 1852), *Schistosoma mansoni* Sambon, 1907, *Schistosoma japonicum* Katsurada, 1904, *Schistosoma intercalatum* Fisher, 1934 e *Schistosoma mekongi* Voge, Brikener & Bruce 1978 causam infecções de importância médica para o homem. Estima-se que as espécies de *Schistosoma* que completam o desenvolvimento no homem são responsáveis pela infecção de 200 milhões de pessoas em vários países em desenvolvimento, e que 500 a 600 milhões de pessoas vivam em áreas de risco onde ocorre a transmissão desta doença considerada negligenciada (WHO, 2002).

A espécie *Schistosoma mansoni* Sambon, 1907 é o agente da esquistossomose mansoni ou moléstia de Pirajá da Silva, única das infecções causadas pelo gênero *Schistosoma* que é endêmica no Brasil. Estudo de KATZ & PEIXOTO (2000) estimam que aproximadamente oito milhões de pessoas estejam infectados pelo *S. mansoni* no Brasil, que corresponde a 4.6% da população brasileira. Sendo que no Estado de Minas Gerais existem cerca de 1.349.145 (7%) de pessoas infectadas.

Na esquistossomose mansoni existe um tratamento eficaz que apresenta uma redução na incidência de casos graves, mas não tem sido efetivo para diminuir a transmissão da doença (BARBOSA *et al.*, 2008). Não existe relato da extinção de nenhum foco de transmissão em populações que fizeram o tratamento quimioterápico. O controle do parasito é difícil, devido ao habitat e ao modo de reprodução dos moluscos transmissores. Desta maneira, o conhecimento da interação do parasito com os hospedeiros intermediários poderá fornecer subsídios para o desenvolvimento de novas estratégias de controle que,

juntamente com as medidas atualmente adotadas, poderá resultar em maior eficiência no controle da transmissão da esquistossomose mansoni no Brasil.

1.1 - Desenvolvimento de *Schistosoma mansoni* em seus hospedeiros

Schistosoma mansoni é um parasito que para o seu desenvolvimento necessita de um hospedeiro definitivo, o homem ou algumas espécies de mamíferos, e um hospedeiro intermediário, que necessariamente é um molusco do gênero *Biomphalaria*. A evolução do *S. mansoni* no hospedeiro definitivo inicia-se pela penetração ativa das formas infectantes, cercárias, através da pele ou da mucosa do hospedeiro, originando os esquistossômulos (GORDON & GRIFFTHS, 1951; STANDEN, 1951). Os esquistossômulos são levados preferencialmente através da circulação e atingem os pulmões. Dos pulmões, os esquistossômulos chegam assincronicamente aos vasos intra-hepáticos preferencialmente via circulação, passivamente através da aorta para o sistema porta hepático ou ativamente através da veia cava inferior, mas também tem sido descrito que parte dos esquistossômulos possam atingir os vasos intra-hepáticos por migração transdiafragmática (FAUST & HOFFMANN, 1934; COELHO, 1970; PEREIRA *et al*., 1972; LENZI *et al*., 2008). Nos vasos intra-hepáticos os esquistossomulos completam seu desenvolvimento atingindo a maturidade sexual, sendo que o amadurecimento sexual da fêmea depende do contato com o macho. Após o amadurecimento migram para as veias mesentéricas inferiores e em camundongos infectados os vermes acasalados começam a ser recuperados das veias mesentéricas a partir de 27 dias de infecção e iniciam a postura de ovos aproximadamente 30-35 dias após infecção. As fêmeas de *S. mansoni* produzem cerca de 400 ovos por dia, sendo que cerca de 40% destes ovos caem na luz intestinal e saem nas fezes. Outra parte

fica retida no fígado e parede do intestino do hospedeiro, estimulando reações inflamatórias teciduais denominadas de granulomas (BOROS & WARREN, 1970). A reação inflamatória mais comum de ser observada consiste de granulomas periovulares isolados, em várias fases de evolução para a cicatrização. Além de aparecem isolados os granulomas periovulares que caracterizam a forma leve da esquistossomose se formam nas ramificações terminais mais finas da veia porta, sendo que a lesão aparece em pleno parênquima hepático (ANDRADE & PRATA., 1963). Sob a influência de fatores genéticos e imunológicos (CHEEVER *et al*.,1987; DUNNE and PEARCE,1999), intensidade da infecção e exposição à re-infecção (SANTOS *et al.,* 2000; ANDRADE, 2008), a esquistossomose pode evoluir para forma grave, caracterizada pela deposição de ovos na região periportal, provocando expansão fibrosa sistematizada dos espaços porta acompanhada de lesões destrutivas e obstrutivas do sistema da veia porta intra-hepático (ANDRADE, 2008). Desta forma, a fibrose periportal pode provocar alterações hemodinâmicas que afetam a fisiologia do sistema e caracterizam a forma hepatoesplênica da esquistossomose (ANDRADE & PRATA., 1963).

Os ovos maduros de *S. mansoni*, presente nas fezes das pessoas infectadas e eliminadas no ambiente, contêm miracídios que, em contato com a água, eclodem e nadam ativamente a procura do hospedeiro intermediário que são moluscos do gênero *Biomphalaria* (COELHO, 1995; COELHO *et al*., 2008). O contato do miracídio com o tegumento do molusco ocorre inicialmente através do *terebratorium*, órgão localizado na extremidade anterior da larva, que ao contato com o molusco assume a forma de ventosa. Simultaneamente, o conteúdo das glândulas de adesão, localizadas lateralmente ao terebratorium é excretado. A ação combinada de enzimas digestivas das glândulas de penetração e de movimentos contráteis e rotatórios intensos permite a penetração da larva

do parasito pelo epitélio e o seu estabelecimento no tecido subcutâneo do molusco, desenvolvendo-se em esporocisto primário (COELHO, 1995). Em condições ideais de temperatura, entre 25 e 28 ºC, as células germinativas do esporocisto primário originam os esporocistos secundários, a partir do 14º dia após a penetração do miracídio, os quais migram da região cefalopodal para as glândulas digestivas do molusco para completar seu desenvolvimento. Nas glândulas digestivas, os esporocistos secundários sofrem profundas modificações anatômicas e suas células germinativas podem originar as cercárias (COELHO, 1995). Segundo JOURDANE *et al* (1980) alguns esporocistos secundários também podem originar outra geração de esporocistos, que poderão gerar cercárias ou novos esporocistos. O desenvolvimento de *S. mansoni* em *Biomphalaria glabrata*, desde a penetração do miracídio até a produção de cercárias e sua emergência para o meio aquático, pode ocorrer dentro de um período de 27 a 30 dias, em condições ideais de temperatura (cerca de 28 ºC). Um único miracídio pode gerar cerca de 300 mil cercárias (FAUST & HOFFMANN, 1934), sendo que cada miracídio já leva definido o sexo das cercárias que serão produzidas.

1.2 - Suscetibilidade de *Biomphlaria* spp ao *Schistosoma mansoni*

Apesar da grande variedade e complexidade observadas no desenvolvimento de trematódeos digenéicos, a maioria utiliza moluscos gastrópodas como primeiro hospedeiro invertebrado. Uma característica que marca a interação molusco-digenea é o elevado grau de especificidade; tem sido frequentemente relatado que um número restrito de espécies, ou mesmo linhagens geográficas, de *Biomphalaria* são suscetíveis a uma determinada cepa de *S. mansoni*. Segundo PARAENSE (1972), das dez espécies de moluscos do gênero

Biomphalaria descritas no Brasil, apenas três foram encontradas naturalmente infectadas pelo *S. mansoni*: *Biomphalaria glabrata* Say, 1918, *B. tenagophila* Orbigny, 1835 e *B. straminea* Dunker, 1848, sendo estas responsáveis pela transmissão e manutenção do parasito no país. *Biomphalaria amazonica* Paraense, 1966 e *B. peregrina* Orbiny, 1835, apesar de não terem sido encontradas naturalmente infectadas pelo *S. mansoni*, podem ser infectadas experimentalmente, e por isso são consideradas hospedeiras em potencial de *S. mansoni* (CORRÊA& PARAENSE, 1971; PARAENSE, 1973).

A distribuição da esquistossomose pelo território brasileiro coincide com a distribuição geográfica das três espécies de *Biomphalaria* suscetíveis, entretanto, o nível de suscetibilidade de cada espécie do molusco também pode influenciar a prevalência da infecção humana (BARBOSA *et al*., 2008). Em infecções experimentais, SOUZA *et al* (1997) relatam índices de infecção de 75,3 % para *B. glabrata*, 32,5 % para *B. tenagophila* e 11,3 % para *B straminea* expostas à infecção pelo *S. mansoni* da linhagem SJ. Devido a sua ampla distribuição geográfica e alta taxa de suscetibilidade ao *S. mansoni*, *B. glabrata* é a principal espécie transmissora da esquistossomose no Brasil, sendo esta espécie a principal mantenedora da infecção no Estado de Minas Gerais e Bahia (PARAENSE, 1986), onde se concentra o maior número de pessoas infectadas no país. A espécie *B. straminea*, apesar da baixa suscetibilidade à infecção por *S. mansoni*, apresenta alta densidade populacional no Nordeste brasileiro, sendo muito importante do ponto vista epidemiológico, devido a sua ampla distribuição e por estar associada a elevados índices de infecção humana e formas graves da doença. *Biomphalaria tenagophila* é a segunda espécie com importância epidemiológica na transmissão da esquistossomose no Brasil. Inicialmente, esta espécie foi encontrada em uma grande área dos Estados de São Paulo, Paraná e Rio Grande do Sul (PARAENSE,1970). Atualmente, *B. tenagophila* que tem

como localidade-tipo Província de Corrientes na Argentina, é encontrada no Brasil e esta espécie também ocorre na Bolívia, Paraguai, Uruguai, Peru e Argentina (PARAENSE, 2001, 2005). Segundo BORDA & REA (1997, 2007) a possibilidade da esquistossomose se expandir para a Argentina e Paraguai esta associado ao fato de determinadas populações de *B. tenagophila* provenientes destas duas localidades, apresentarem-se suscetíveis à determinadas cepas de *S. mansoni*. No Brasil é a segunda espécie em importância na transmissão da esquistossomose e encontra-se distribuída em uma extensa faixa costeira do Brasil, desde o Sul da Bahia até o Rio grande do Sul (Chuí). É a principal espécie transmissora em extensas áreas do estado de São Paulo e está presente em coleções hídricas de todas as bacias hidrográficas do estado (TELES, 2005). Em Minas Gerais, *B. tenagophila* apresenta-se amplamente distribuída no Vale do Jequitinhonha, Vale do Mucuri, Vale do Rio Doce, Zona da Mata, e na região central do Estado, sendo estas regiões consideradas como endêmicas para a esquistossomose (SOUZA *et al*., 2001). A *B. tenagophila* vem ampliando consideravelmente sua distribuição pelo território brasileiro bem como aumentando a sua importância na transmissão da esquistossomose principalmente nas regiões sul e sudeste do país (PARAENSE & CORRÊA, 1987a).

A compatibilidade entre *S. mansoni* e seu hospedeiro intermediário é influenciada por fatores comportamentais e fisiológicos do molusco. Uma vez encontrado um hospedeiro compatível, o nível de suscetibilidade de *Biomphalaria* ao *S. mansoni* pode ser determinado por diferenças genéticas dos moluscos, bem como pela constituição genética de *Schistosoma* (SOUZA *et al*., 2001). Analisando populações de *B. glabrata* de diferentes localidades do Brasil, PARAENSE & CORRÊA (1963) relataram que as diferentes linhagens apresentavam suscetibilidade variável frente a uma única cepa do *S. mansoni* de

Belo Horizonte – Minas Gerais. Em se tratando *B. tenagophila*, PARAENSE & CORRÊA (1978) demonstraram que esta mesma espécie coletada de 18 localidades do Brasil também apresentava grande variação de susceptibilidade (variando de 0 a 91,5 %) à infecção para uma determinada linhagem de *S. mansoni*. NEWTON (1952) mostrou que a suscetibilidade dos moluscos *B. glabrata* ao *S. mansoni* é condicionada a fatores genéticos. Posteriormente estes resultados foram confirmados por RICHARDS *et al* (1976a, b; 1977; RICHARDS & SHADE 1987) que também demonstraram que o caráter resistência, adquirido na maturidade, é determinado por um único gene dominante, com herança mendeliana. Mais recentemente, a influência genética na determinação do grau de suscetibilidade de *B. tenagophila* também foi experimentalmente demonstrada. Foi verificado que a linhagem de *B.tenagophila* isolada da reserva ecológica do Taim – RS era resistente à infecção por *S. mansoni* de todas as cepas testadas (SANTOS *et al*., 1979; MARTINS-SOUZA *et al.*, 2003; COELHO *et al*., 2004). Cruzamentos realizados entre *B.tenagophila* da linhagem do Taim e *B.tenagophila* da linhagem BH, suscetível à infecção por *S. mansoni*, demonstrou que o caráter da resistência era dominante (SANTOS *et al*., 1979). Posteriormente, ROSA *et al* (2005) estudaram detalhadamente a transmissão genética do caráter de resistência à infecção por *S. mansoni* em cruzamentos realizados entre *B.tenagophila* do Taim e *B.tenagophila* de Joinville (suscetível ao parasito). Os autores demonstraram que 100% dos indivíduos da geração F_1 foram resistentes ao *S.mansoni* e, entre os descendentes da geração F_2, somente 8% dos moluscos produziram cercárias. Os resultados confirmam que a resistência de *B.tenagophila* do Taim à infecção por *S. mansoni* é dominante, sendo influenciada por um gene principal, mas possivelmente com outros genes secundários.

Um dos elementos que influenciam a suscetibilidade e que pode ser geneticamente determinado é a atividade do sistema de defesa interna dos caramujos. Desta forma, a

composição celular da hemolinfa de *Biomphalaria* e a capacidade de ativar estas células durante a infecção por *S. mansoni* poderá definir a sobrevivência do parasito no interior do hospedeiro.

1.3- Sistema interno de defesa dos moluscos:

Os invertebrados precisam responder a todo tipo de agressão, especialmente as produzidas por agentes infecciosos que co-habitam seu ambiente. Desta forma, o sistema interno de defesa dos invertebrados é necessariamente capaz de reconhecer e destruir agentes infecciosos, apesar de não apresentar elementos capazes de gerar a diversidade de reconhecimento observada durante a resposta imune adaptativa dos vertebrados (VAN DER KNNAP & LOKER, 1990; LOKER *et al*. 2004). Estudos recentes têm demonstrado muitas similaridades entre a resposta de defesa inata de vertebrados e o sistema interno de defesa dos invertebrados (HOFFMANN *et al*.,1999; HOFFMANN., 2003), sendo identificados, em diferentes grupos de invertebrados vários componentes da resposta inata, tais como: peptídeos líticos, moléculas de reconhecimento de padrões moleculares altamente conservados em determinados grupos de patógenos, células fagocitárias e produção de metabólicos de oxigênio e nitrogênio altamente tóxicos (LOKER *et al*., 2004). Apesar das semelhanças identificadas no sistema de defesa de diversas formas de invertebrados, é importante ressaltar que estudos genômicos têm indicado mecanismos de defesa bastante diversos em grupos de invertebrados filogeneticamente associados, mas com hábito alimentar e habitat diversos (LOKER *et al*., 2004).

O sistema de defesa interna dos moluscos é composto por elementos celulares, constituídos pelos hemócitos ou amebócitos, e por fatores solúveis presentes na hemolinfa.

Os hemócitos podem estar livres, circulando na hemolinfa, ou fixos nos tecidos. A hemolinfa dos Planorbídeos circula em um sistema semi-aberto, impulsionada pelo coração, de onde parte a artéria aorta, que se ramifica para diversos tecidos, drenando os seios venosos e retornando ao coração pelas veias pulmonar e renal, após ser re-oxigenado na parede pulmonar. O coração, envolto pela membrana do pericárdio, é dividido em duas câmaras, a aurícula, que recebe hemolinfa da cavidade pulmonar, e o ventrículo, que impele a hemolinfa através da aorta. A aorta se divide em duas artérias: a artéria visceral, a qual irriga a parte posterior do corpo do caramujo, incluindo o sistema digestivo e os órgãos genitais, e a artéria cefálica, que atinge a toda região cefalopodal. As artérias se esvaziam nos espaços pseudovasculares dos tecidos, acumulando hemolinfa em três seios venosos: cefalopodal, visceral e sub-renal, retornando para o coração após ser re-oxigenado na a parede pulmonar (BAKER, 1945; PARAENSE, 1970).

A maioria dos estudos indica que os hemócitos são os principais elementos efetores envolvidos na destruição de microorganismos e parasitos que penetram em seus tecidos, incluindo larvas de trematódeos (VAN DER KNAP & LOKER, 1990; NEGRÃO-CORRÊA *et al*., 2007; NEGRÃO-CORRÊA *et al*., 2008). O envolvimento dos hemócitos na destruição de larvas de *S. mansoni* foi inicialmente evidenciado em estudos histológicos de caramujos infectados. Neste sentido, SETA *et al* (1996) relataram que logo após a penetração de miracídios de *S. mansoni* em *B. tenagophila* foi observada uma reação celular em torno do parasito recém penetrado, e após 24h, os esporocistos já apresentavam sinais de degeneração. Os hemócitos apresentam uma origem multi-centrica, sendo observado produção em vários tecidos hematopoéticos (SOUZA & ANDRADE ., 2006). A importância dos hemócitos no mecanismo de destruição de larvas de *S. mansoni* foi demonstrada com o aumento da resistência de linhagens altamente suscetíveis de *B.*

glabrata (VASQUEZ & SULLIVAN, 2001) ou de *B. tenagophila* (BARBOSA *et al*., 2006) que receberam o transplante de um dos órgãos produtores de hemócitos (APO) de caramujos resistentes da linhagem Taim.

Estudos de MARTINS-SOUZA (1999; 2006) têm caracterizado estruturalmente a população de hemócitos circulantes de *B. tenagophila* e sua participação no mecanismo de destruição de *S. mansoni*. Inicialmente, foi verificado que *B. tenagophila* da linhagem Cabo Frio inoculado com sílica apresentou redução do número de hemócitos do tipo granulócito na hemolinfa e na reação tecidual. Esta redução foi associada com aumento de suscetibilidade destes caramujos à infecção por *S. mansoni*, sugerindo a importância destas células no mecanismo de destruição das larvas do parasito (MARTINS-SOUZA *et al*., 2003). Hemócitos circulantes da hemolinfa de *B. tenagophila* e *B. glabrata* também foram analisados por citometria de fluxo (MARTINS-SOUZA *et al*., 2009) sendo revelada a presença de três tipos celulares distintos em relação ao tamanho. Este estudo também mostrou que os três tipos celulares são compostos por células com alta e com baixa granulosidade. Entretanto, somente em *B. tenagophila* do Taim, linhagem de caramujo completamente resistente a infecção por *S. mansoni*, a exposição ao parasito induziu alterações intensas e rápidas no perfil dos hemócitos circulantes.

Apesar da presença de tipos celulares morfologicamente semelhantes na hemolinfa de *B. glabrata* e *B. tenagophila*, em *B. tenagophila* Taim, linhagem resistente à infecção pelo parasito, uma grande porcentagem dos hemócitos circulantes de todos os tamanhos apresentaram-se intensamente marcados por lectinas fluorescentes, especialmente WGA-FITC e PNA-FITC (MARTINS-SOUZA *et al*., 2006). Também foi demonstrado que em *B. tenagophila* do Taim a infecção por *S. mansoni* induziu uma redução dos hemócitos marcados por WGA da hemolinfa circulante logo nas primeiras horas, sugerindo que o

estado de ativação e capacidade de resposta dos hemócitos circulantes diferem em linhagens de caramujos com diferente suscetibilidade ao parasito (MARTINS-SOUZA *et al*., 2006). Para testar a relevância funcional deste resultado, esporocistos de *S. mansoni* axenicamente transformados *in vitro* foram incubados com hemócitos circulantes isolados da hemolinfa de *B. tenagoghila* Taim, *B. tenagophila* Cabo Frio ou *B. glabrata* BH na presença de concentrações crescentes de N-acetil galactosamina, monossacarídeo que se liga à WGA (MARTINS-SOUZA *et al*., 2006). Os dados mostraram que a adição de N-acetil galactosamina resultou em redução de mortalidade de esporocistos por hemócitos de *B. tenagophila* Taim e esta redução de mortalidade estava associada à redução da quantidade de células que se ligaram ao esporocisto. Estes dados indicam a importância da ligação lectina-carboidrato no mecanismo de reconhecimento e destruição de larvas de *S. mansoni* por hemócitos (MARTINS-SOUZA, 2006).

A interação entre o parasito e o sistema de defesa dos moluscos é complexa e envolve, além dos hemócitos circulantes e fixos, alguns componentes solúveis da hemolinfa (VAN DER KNAAP,1990). Vários autores (GRANATH AND YOSHINO, 1984; SMINIA & VAN DER KNAAP, 1987; LOKER AND BAYNE, 1986) demonstraram que durante o processo de invasão do parasito, fatores sintetizados pelos hemócitos ou por outros tecidos dos caramujos são encontrados na hemolinfa e podem modificar a atividade dos hemócitos.

BAYNE *et al* (1980 a) foram os primeiros a desenvolver métodos *in vitro* para avaliar o efeito da hemolinfa de *B. glabrata* sobre o esporocisto primário de *S. mansoni* transformado axenicamente *in vitro*. Estes métodos facilitaram o estudo dos mecanismos de resistência na associação parasito-hospedeiro. Nestes estudos os autores mostraram que a hemolinfa livre de células, obtida de linhagens suscetíveis e resistentes de *B. glabrata* não são capazes de alterar visivelmente a morfologia do esporocisto *in vitro*, o mesmo

acontecendo com hemolinfa contendo hemócitos de linhagens suscetíveis. Entretanto, o esporocisto parece ser destruído quando incubado com fatores solúveis da hemolinfa e hemócitos de linhagens resistentes. De maneira mais contundente, a importância de fatores solúveis da hemolinfa foi demonstrada quando os hemócitos de linhagens suscetíveis de *B. glabrata* ativados por fatores solúveis da hemolinfa de linhagens resistentes foram capazes de destruir os esporocistos de *S. mansoni, in vitro*. GRANATH & YOSHINO (1984) confirmaram a importância de fatores solúveis da hemolinfa no processo de destruição de esporocistos de *S. mansoni* em estudos *in vivo*. Estes autores relataram que a transferência dos fatores solúveis da hemolinfa obtidos de linhagens resistentes de *B. glabrata*, previamente infectadas pelo parasito, para linhagens suscetíveis resultou em uma redução da taxa de infecção de linhagens de *B. glabrata* pelo *S. mansoni*.

Entretanto, não são conhecidos quais os componentes da fração solúvel da hemolinfa dos caramujos resistentes são relevantes no processo de transferência de resistência ou mesmo qual o mecanismo é ativado neste processo. LOKER *et al* (1984), RENWRANTZ (1986) detectaram na hemolinfa de moluscos fatores que aglutinam e opsonisam partículas, entre os quais foram identificadas algumas lectinas. Segundo BAYNE (1983), as lectinas presentes na hemolinfa de gastrópodes poderiam mediar o reconhecimento de microorganismos ou parasitos. Neste sentido, JOHNSTON & YOSHINO (1996) identificaram que a maioria dos polipeptídios na superfície dos esporocistos de *S. mansoni* se ligam a lectinas isoladas de *Concanavalia ensiformis* (Con A), *Erythrina corallodendrom* (ECA), *Glycine max* (SBA) , *Tetragonolobus purpureas* (TPA) e *Triticum vulgaris* (WGA). Estes autores sugerem que lectinas semelhantes podem estar presentes na hemolinfa de *B. glabrata* suscetível e resistente, e estas lectinas são capazes de se ligarem à superfície do esporocisto de *S. mansoni*. Nos moluscos, as lectinas

são produzidas e secretadas pelos hemócitos, podendo estar solúveis na hemolinfa ou expressas na superfície dos hemócitos circulantes (RICHARDS & RENWRANTZ, 1991). Segundo BAYNE (1990), a ligação lectina-carboidrato possivelmente leva a uma mudança conformacional do complexo, que pode resultar na ativação do hemócito. Desta maneira, além de ser um fator de reconhecimento expresso pelos hemócitos, também seria um mecanismo de ativação destas células. Mais recentemente, um grupo de proteínas, com homologia ao fibrinogênio e com atividade de lectina, foi identificado na hemolinfa de *B. glabrata*, sendo sua expressão aumentada após a infecção do molusco com *Echinostoma paraensei*, outro trematódeo digenêico. Esta proteína tem a capacidade de precipitar antígenos de excreção/secreção do parasito e os autores sugerem que, além de participar do reconhecimento, estas proteínas poderiam ser importantes inibidores do mecanismo de evasão do parasito (ADEMA *et al*, 1997b).

Além das lectinas, outras proteínas com função homóloga a mediadores celulares já caracterizados em vertebrados e que podem estar envolvidas na ativação dos hemócitos durante uma infecção também têm sido identificadas na hemolinfa de moluscos. A ativação dos hemócitos é um fator fundamental para a destruição de larvas de parasitos, uma vez que caramujos de linhagens susceptíveis à infecção por *S. mansoni* podem produzir o encapsulamento do parasito, mas sem destruição das larvas. OTTAVIANI *et al* (1993) identificaram, através de imunolocalização, proteínas com homologia à citocinas de vertebrados, como interleulina-1 alfa (IL-1 α), IL-1 β, IL-2, IL-6 e Fator de Necrose Tumoral alfa (TNF-α), em hemócitos dos molucos *Planorbarius corneus* e *Viviparus ater*. GRANATH *et al* (1984) identificaram uma proteína semelhante a TNF-α na hemolinfa de *B. glabrata*. Esta proteína também foi detectada na hemolinfa de *B. glabrata* por BOYER (1994), sendo que o seu nível encontra-se diminuído durante a infecção por *S. mansoni*.

Além de TNF-α, a hemolinfa de *B. glabrata* apresenta proteínas com homologia funcional a IL-1, sendo que o aumento da produção desta proteína tem sido associado à ativação e proliferação celular (HUGHES *et al*., 1991; RAFTOS *et al*., 1991 e RAFTOS *et al*., 1992), ao aumento da fagocitose (BURKE & WATKINS, 1991) e com a produção de superóxidos (GRANATH *et al.,* 1984).

Aliado a possível participação no reconhecimento de larvas do parasito e da ativação dos hemócitos, trabalhos ainda preliminares sugerem que a fração solúvel da hemolinfa de *Biomphalaria* também pode apresentar elementos, como proteases e inibidores de proteases, que poderiam lesar diretamente o tegumento do esporocisto de *S. mansoni* ou inativar mecanismos de defesa desenvolvidos pelo hospedeiro. BENDER and BAYNE (1996) purificaram e caracterizaram α-Macroglobulina na hemolinfa de *B. glabrata.* Neste trabalho os autores sugerem que a presença de α-Macroglobulina poderia provocar um efeito de blindagem na hemolinfa, impendindo a ação de proteases. FRYER *et al* (1996) também detectaram α-Macroglobulina na hemolinfa de *Biomphalaria*, sendo que sua presença estava associada a inibição da atividade de cisteíno-protease, no entanto este efeito não estava associado com o nível de susceptibilidade do molusco ao *S. mansoni*. Posteriormente, BAHGAT *et al* (2002) identificaram uma tripsina-like com atividade de serino-protease nos hemócitos de *Biomphalaria glabrata* expostos ao *Schistosoma*. Mais uma vez, não foi possível associar a atividade da protease com a susptibilidade ao trematodeo. Mais recentemente MITTA *et al* (2005) e LOCKYER *et al* (2007) confirmaram a presença de genes homólogos a proteases e inibidores de protease em bibliotecas de cDNA de *B. glabrata*. Apesar de alguns destes genes serem expressos diferencialmente em caramujos infectados por *Echinostoma caproni* (MITTA *et al*., 2005)

ou *S. mansoni* (LOCKYER *et al*., 2007), sua participação no mecanismo de defesa do hospedeiro ou evasão induzida pelo parasito ainda não foi estabelecida.

Baseado nos resultados anteriormente discutidos fica evidente a importância do sistema interno de defesa do molusco, hemócitos e componentes solúveis da hemolinfa, como um dos elementos determinantes da resistência de *Biomphalaria* frente à infecção por larvas de trematódeos, entretanto os mecanismos envolvidos nestes processos ainda não estão estabelecidos. Acreditamos que uma das dificuldades destes estudos tem sido o modelo experimental utilizado, pois grande parte dos trabalhos foi realizada com *B. glabrata*, que não apresentam uma resistência absoluta à infecção por *S. mansoni*. A linhagem de *B. tenagophila* isolada da reserva ecológica do Taim, no Rio Grande do Sul, denominada de *B. tenagophila* Taim, tem se mostrado resistente à infecção frente a todas as cepas de *S. mansoni* testadas e em todas as fases do desenvolvimento do molusco (SANTOS *et al*., 1979; MARTINS-SOUZA *et al*., 2003; COELHO *et al*., 2008). Nesta linhagem de caramujo, os esporocistos de *S. mansoni* penetram no molusco e são rapidamente destruídos pela atuação do sistema interno de defesa deste hospedeiro (NEGRÃO-CORRÊA *et al.*, 2007).

PEREIRA (2005) demonstrou que resistência ao *S. mansoni* observada em *B. tenagophila* Taim pode ser parcialmente transferida para linhagens suscetíveis, como a linhagem Cabo Frio, através da inoculação da fração acelular da hemolinfa (plasma). Este aumento de resistência foi demonstrado in vivo pela redução na taxa de infecção dos caramujos e na produção de esporocistos e cercárias nos caramujos previamente tratados com os fatores solúveis (Plasma) de Taim em relação aos somente infectados por *S. mansoni*. Entretanto, os componentes da fração solúvel da hemolinfa envolvidos no processo de transferência de resistência ou mesmo o mecanismo ativado neste processo não

foi estabelecido. Desta forma, o principal objetivo deste trabalho experimental foi caracterizar componentes da fração solúvel da hemolinfa de *B. tenagophila* Taim envolvidos na destruição de larvas de *S. mansoni*.

2. Justificativa

Considerando a enorme dificuldade de erradicação de *Biomphalaria* em áreas de transmissão de esquistossomose no Brasil e as limitações políticas e socio-econômicas que impedem, em curto prazo, estender o saneamento básico para todas as áreas de transmissão da doença, o controle desta helmintose está baseado no tratamento quimioterápico dos pacientes diagnosticados. Entretanto, o uso de tratamentos quimioterápicos sucessivos, além de ser dispendioso pode resultar na seleção de linhagens resistentes às drogas utilizadas. Estudos realizados em área do Brasil onde o tratamento quimioterápico tem sido utilizado no controle da esquistossomose também indicam que, apesar da grande diminuição no número de casos graves da doença, não existe relatos de completa eliminação de focos de transmissão da doença com o uso de tratamento quimioterápico.

Estes resultados justificam a busca de alternativas que possam resultar em novas estratégias para auxiliar no controle da esquistossomose no Brasil. Apesar dos caramujos constituírem um elo vital para a manutenção do ciclo evolutivo do parasito, relativamente poucos estudos têm sido conduzidos com os mesmos e esta deficiência de estudos se torna mais evidente quando se trata de caramujos transmissores do Brasil.

Trabalhos anteriores realizados em nosso laboratório (MARTINS-SOUZA *et al*., 1999; PEREIRA *et al*., 2005, 2008; NEGRÃO-CORRÊA *et al*., 2007, 2008; ROSA *et al*., 2007) indicam a presença de componentes solúveis na hemolinfa de *B. tenagophila* Taim que atuam na destruição de larvas de *S. mansoni*. Nesta fase do trabalho nosso interesse principal será caracterizar, bioquímica e funcionalmente estes elementos.

3.0 Objetivo

3.1 Objetivo Geral

Caracterizar, bioquímica e funcionalmente, componentes da fração solúvel da hemolinfa de *B. tenagophila* Taim associados à destruição de esporocistos de *S. mansoni*.

3.2 Objetivos Específicos:

1 - Avaliar, in vitro, a capacidade da hemolinfa de *B. tenagophila* Taim livre de hemócitos destruir esporocistos de *S. mansoni*.

2 - Investigar a presença de atividade de proteolítica na hemolinfa de *B. glabrata* BH e *B. tenagophila*, Cabo Frio e Taim.

3 - Fracionar a hemolinfa acelular de *B. tenagophila* Taim e testar, *in vitro*, o efeito das diferentes sub-frações da hemolinfa de *B. tenagophila* Taim sobre viabilidade de esporocistos de *S. mansoni*.

4 - Caracterizar bioquicamente as frações que apresentam atividade contra esporocitos de *S. mansoni*.

Parte I:

Efeito da fração solúvel (plasma) da hemolinfa de *Biomphalaria tenagophila* Taim na sobrevivência de esporocistos recém transformados de *Schistosoma mansoni*

4.1 Introdução

Moluscos do gênero *Biomphalaria*, como outros invertebrados na natureza, estão expostos a uma grande variedade de agentes infecciosos. Entretanto, a maioria dos indivíduos é capaz de conter uma grande parte dos agentes infecciosos, em parte devido à atuação do sistema interno de defesa. Estudos recentes têm demonstrado muitas similaridades entre a resposta de defesa inata de vertebrados e o sistema interno de defesa dos invertebrados (HOFFMANN *et al*., 1999; HOFFMANN, 2003), sendo identificados vários componentes da resposta inata, tais como: peptídeos líticos, moléculas de reconhecimento de padrões moleculares, células fagocitárias e produção de metabólicos de oxigênio e nitrogênio altamente tóxicos (LOKER *et al*., 2004).

O sistema de defesa interna dos caramujos é composto por elementos celulares, constituídos pelos hemócitos ou amebócitos, e por fatores solúveis presentes na hemolinfa. A análise histopatológica (SOUZA *et al*., 1997) e os dados experimentais obtidos com o transplante do órgão hematopoético – APO (SULLIVAN & SPENCER, 1994) demonstram que os hemócitos circulantes são essenciais para o desenvolvimento da resistência de *B. glabrata* contra larvas de *S. mansoni*. Entretanto, existem evidências experimentais que indicam que, além de hemócitos, fatores solúveis também participam do mecanismo de proteção envolvido na destruição de larvas *S. mansoni* por linhagens de caramujos resistentes à infecção. GRANATH & YOSHINO (1984) relataram que a transferência de fatores solúveis da hemolinfa obtido de linhagens resistentes de *B. glabrata* (10-R2) para linhagens suscetíveis (PR) resultou em uma redução da taxa de infecção de linhagens de *B. glabrata* pelo *S. mansoni*. VASQUEZ & SULLIVAN (2001) demonstram que a transferência dos fatores solúveis da hemolinfa de *B. glabrata* suscetível que havia

recebido o órgão produtor de hemócitos (APO) de um caramujo *B. glabrata* resistente foi capaz de diminuir a taxa de infecção pelo *S. mansoni* nos caramujos receptores, sugerindo que os hemócitos do APO são capazes de secretar fatores solúveis que participam no mecanismo de controle do parasito.

Até o momento não houve comprovação experimental dos mecanismos envolvidos neste processo, mas estudos *in vitro* de FRYER & BAYNE (1996) mostram que partículas de poliestireno tratadas com fatores solúveis da hemolinfa de linhagens resistentes de *B. glabrata* são significativamente mais fagocitadas por hemócitos de linhagens suscetíveis do que as partículas não tratadas, sugerindo a participação de elementos da hemolinfa no mecanismo de reconhecimento do parasito.

A maioria dos estudos avaliando as interações entre o *S. mansoni*-caramujos utilizam como modelo experimental *B. glabrata*, embora existam outras espécies de caramujos transmissoras. Entre as espécies transmissoras no Brasil, *B tenagophila* apresenta grande dispersão no território nacional com crescente importância na transmissão da esquistosssomose, entretanto poucos estudos têm sido realizados na tentativa de compreender sua participação na epidemiologia da doença. A linhagem de *B. tenagophila* isolada da reserva ecológica do Taim, no Rio Grande do Sul, denominada de *B. tenagophila* Taim, tem se mostrado resistente à infecção por todas as cepas de *S. mansoni* testadas (SANTOS *et al*., 1979; COELHO *et al*., 2008). Nesta linhagem de caramujo, os esporocistos são rapidamente destruídos pelos hemócitos após a penetração do parasito (NEGRÃO-CORRÊA *et al*., 2007). Apesar dos hemócitos serem confirmadamente essenciais para a resistência de *B. tenagophila* Taim (BARBOSA *et al*., 2006), existem evidencias da participação de fatores solúveis da hemolinfa no mecanismo de proteção desenvolvido nesta linhagem de caramujo (COELHO & BEZERRA, 2006).

PEREIRA (2005) demonstrou que a resistência ao *S. mansoni* observada em *B. tenagophila* Taim pode ser parcialmente transferida para linhagens susceptíveis, como a linhagem Cabo Frio, através da inoculação da fração acelular da hemolinfa (plasma). O aumento de resistência detectado nos caramujos tratados com hemolinfa Taim foi demonstrado *in vivo* pela redução na taxa de infecção dos caramujos e na produção de esporocistos e cercárias nos caramujos previamente tratados com os fatores solúveis (Plasma) de Taim em relação aos somente infectados por *S. mansoni*. Entretanto, os componentes da fração solúvel da hemolinfa de caramujos resistentes envolvidos no processo de transferência de resistência ou mesmo o mecanismo ativado neste processo não é conhecido. Para identificar os possíveis mecanismos envolvidos neste processo pretende-se, inicialmente, estabelecer um teste *in vitro* onde o efeito da hemolinfa possa ser diretamente avaliado.

4.2. Material e Métodos:

4.2.1 Caramujos

Foram utilizadas duas linhagens de *B. tenagophila* para os experimentos: Cabo Frio (CF) e a Taim. Os caramujos da linhagem CF, provenientes da região de Cabo Frio (RJ), são altamente suscetíveis à infecção pelo *S. mansoni* da cepa SJ, mas apresentam taxas de suscetibilidade variando entre 30-40% à infecção pela cepa LE do parasito. A outra linhagem de *B. tenagophila*, denominada Taim, é proveniente da Reserva Biológica do Taim (RS) e apresentam total resistência à infecção pelo *S. mansoni* da cepa LE e SJ (CORRÊA *et al*., 1979; SANTOS *et al*., 1979; COELHO *et al*., 2008).

Ainda no presente trabalho foi utilizada a espécie de *B. glabrata* como um controle positivo para os experimentos. A linhagem de *B. glabrata* utilizada foi proveniente da Lagoa da Pampulha – Belo Horizonte (MG), denominada linhagem BH. Esta linhagem apresenta taxas de suscetibilidade de cerca de 80% a infecção pelo *S. mansoni*.

As espécies de *Biomphalaria* utilizadas neste estudo são rotineiramente mantidas nos moluscários do laboratório de esquistossomose no ICB – UFMG, e antes da realização dos diferentes experimentos os caramujos foram submetidos a um procedimento de limpeza (com solução de dodecil sulfato de sódio (SDS) a 0.1%) e desinfecção (incialmente com solução 0,1 % de Benzoilmetronidazole, seguido por 30 µg/ml Gentamicina e 100 µg/ml Ampicilina).

4.2.2 Parasito

Foi utilizada a cepa LE de *S. mansoni* que vem sendo mantida no laboratório de Esquistossomose - ICB - UFMG em torno de 40 anos. A cepa LE de *S. mansoni* foi isolada do paciente Luis Evangelista de Belo Horizonte – MG e sua manutenção é realizada através de sucessivas passagens em hamsters (*Mesocricetus auratus*) e *B. glabrata* de acordo com a técnica descrita por PELLEGRINO & KATZ (1968). A maior resistência das linhagens de *B. tenagophila* à cepa LE de *S. mansoni* facilita os estudos referentes aos mecanismos que interferem na associação parasito-molusco.

4.2.3 Obtenção de miracídios de *S. mansoni* e dos esporocistos axênicos

Para transformação axênica em esporocistos, Hamsters (*Mesocricetus auratus*) infectados com *S. mansoni* da cepa LE foram sacrificados entre 45 e 50 dias após infecção para obtenção de miracídios (CHAIA, 1956). Os fígados destes animais foram retirados sob condições axênicas, lavados em rapidamente em água desclorada autoclavada e homogeneizados em um triturador de tecidos manual com água desclorada. Esta suspensão foi transferida para um cálice de sedimentação acondicionado a 4 ºC. Esta baixa temperatura é utilizada para evitar a eclosão dos miracídios. Após 60 min, o sobrenadante foi descartado e o sedimento contendo os ovos do parasito foi ressuspenso em água desclorada em temperatura ambiente e exposto à luz artificial para a eclosão dos miracídios. Os miracídios presentes no material foram concentrados sob foco de luz artificial em balão volumétrico recoberto por papel alumínio, recuperados e transferidos para tubos de 15 mL inseridos em banho de gelo. Para a transformação em esporocistos, os miracídios presente no sedimento foram incubados em meio RPMI-1640 suplementado com soro fetal bovino 5% e gentamicina em estufa de CO_2 a 28 ºC por 18 h (SAMUELSON *et al*., 1984).

4.2.4 Obtenção de hemolinfa de *B. tenagophila*

Caramujos não infectados de cada linhagem utilizados no estudo: *B. glabrata* BH, *B. tenagophila* Cabo Frio e *B. tenagophila* Taim, foram sacrificados para a obtenção de hemolinfa utilizada nos diferentes protocolos experimentais. A coleta da hemolinfa foi realizada de acordo com o protocolo de ZELCK *et al* (1995). Resumidamente, a concha de

cada caramujo foi limpa com álcool 70% e seca com papel absorvente. Posteriormente utilizando-se uma seringa plástica de 1 mL (Plastipak) com agulha de 21 G, a concha do molusco foi perfurada na região cardíaca até o extravasamento da hemolinfa que foi coletada e transferida para superfície de um de filme plástico (American National Can) colocado sob banho de gelo e logo em seguida, o sobrenadante foi transferido para tubos de polipropileno (Sarsted) e mantidos à 0 ^{o}C. Vários caramujos tiveram a hemolinfa retirada até a coleta de 1 a 5 mL dependendo do procedimento a ser utilizado. Após a coleta, a hemolinfa total foi centrifugada (80g por 10 min a 4 ºC), sendo recolhido separadamente o sobrenadante e o precipitado. O sobrenadante foi centrifugado novamente por 30 min a 1200 g e 4 ºC para obtenção da fração solúvel da hemolinfa (plasma) utilizada nos ensaios de inativação de esporocistos *in vitro*.

O precipitado de hemócitos foi ressuspendido em CBSS (CHERNIN., 1953) até o volume inicial da hemolinfa total. Os hemócitos presentes na solução foram avaliados quanto à viabilidade e quantificados após adição de azul de tripan (MARTINS-SOUZA *et al*., 2003). Estes foram utilizados em ensaios de mortalidade de esporocitos *in vitro*.

4.2.5 Ensaio de inativação de esporocistos *in vitro*

Para a realização dos ensaios *in vitro,* esporocistos de *S. mansoni* axenicamente transformados (SAMUELSON *et al*., 1984) foram lavados, ressuspendidos em CBSS suplementado [CBSS contendo 10 mM de HEPES, 2 mM glutamina 1 mM de aminoácidos essenciais, 100 U penicilina e 100 U estreptomicina, e 2 % de albumina bovina], quantificados e o volume final ajustado para concentração final de 2000 esporocistos/ml de CBSS. Cinquenta µl de CBSS, contendo cerca de 100 esporocistos, foram plaqueados em

placa de cultura de células de 96 poçoss (Corning-Costar fundo chato). Após a distribuição das amostras nas placas, o número exato de esporocistos presentes em cada poço, no inicio do tratamento, foi contado com auxílio de um microscópio óptico invertido (Olympus IX70).

Com o intuito de testar o efeito da fração acelular de *Biomphalaria* sobre a viabilidade dos esporocistos, 50 µL do plasma recolhido de *B. glabrata* BH, ou de *B. tenagophila* Cabo Frio ou de *B. tenagophila* Taim também foram adicionadas em cada poço contendo os esporocistos e CBSS suplementado foi adicionado para completar o volume de 250 µL/tratamento. Cada amostra foi testada em triplicata e tratamentos contendo esporocistos cultivados em CBSS suplementado, sem adição de hemolinfa, foram utilizados como controle de sobrevivência.

Para avaliar o efeito da adição da fração acelular da hemolinfa sobre a atuação dos hemócitos, a taxa de mortalidade de esporocistos axenicamente transformados também foi avaliada em cultivos contendo 2 x 10^5 hemócitos/tratamento juntamente com 50 µL do plasma recolhido de *B. glabrata* BH, ou de *B. tenagophila* Cabo Frio ou de *B. tenagophila* Taim. Combinações heterólogas, nas quais os esporocitos foram incubados hemócitos de *B. glabrata* ou de *B. tenagophila* Cabo Frio juntamente com a fração acelular isolada de *B. tenagophila* Taim também foram testadas.

As placas contendo os esporocistos foram incubadas a 26 ºC durante 18 h e a viabilidade dos esporocistos nos diferentes tratamentos foi aferida pela observação direta em microscopia óptica após adição de solução contendo 0,4 % de Azul de Tripan (Olympus IX-70). Esporocistos vivos apresentam-se integros, não corados pelo Azul de Tripan e apresentam atividade de células flamas. A mortalidade foi calculada pela somatória do número dos esporocistos corados com Azul de Tripan (tegumento lesado)

com o número de esporocistos completamente destruidos que foi calculado baseado na redução da contagem inicial de cada tratamento.

Para identificar possiveis lesões nos esporocistos que estavam em processo de destruição foram utilizados um marcador fluorescente (*Hoechst 33258* - Sigma) capaz de penetrar no parasito somente quando há lesões no tegumento. Esta marcação tem sido utilizada para verificar a integridade do tegumento dos vermes adultos de *S. mansoni* (OLIVEIRA *et al*., 2006) e lesão provocada no tegumento de esporocistos de *S. mansoni* (MATTOS et al., 2006) expostos ao tratamento com praziquantel. Em nossos experimentos, os esporocistos de *S. mansoni* axenicamente transformados foram cultivados por 18 h em CBSS suplementados, contendo ou não 20 % da hemolinfa acelular das diferentes linhagens de *Biomphalaria*, conforme detalhado acima. Após o período da interação *in vitro*, foi acrescentado 5 µl da sonda *Hoechst 33258* (2 µg/µl) a cada tratamento e, após 10 min, a integridade do tegumento de esporocistos foi avaliada em microscopia de Fluorescência (Olympus IX70 filtro WU). Para tanto, esporocistos presentes em cada tratamento foram fotografados com Câmera digital Optronics modelo DEI-470 acoplada ao microscópio, sendo avaliada a presença de marcas fluorecentes no interior do parasito nos diferentes tratamentos.

4.2.6 Analise estatística

Os resultados obtidos em cada tratamento foram estatisticamente comparados, utilizando-se análise de variância para dados paramétricos (ANOVA, post teste Turkey). Valores de $p \leq 0.05$ foram considerados estatisticamente diferentes

4.3. Resultados:

A mortalidade dos esporocistos de *S. mansoni* em condições da cultura *in vitro* utilizadas nos experimentos foi muito baixa (<1%). Ao adicionarmos fatores solúveis da hemolinfa de *B.glabrata* (BH) não infectado também não foi detectado aumento significativo na mortalidade dos esporocistos. Diferente dos dados relatados com *B. glabrata*, a fração acelular da hemolinfa de *B.tenagophila* (Taim e de Cabo Frio) aumenta significativamente a mortalidade dos esporocistos em comparação aos grupos controles. De maneira interessante, a mortalidade de esporocistos induzida pela adição da fração acelular da hemolinfa de *B.tenagophila* Taim foi duas vezes maior que a mortalidade induzida pela adição de fatores solúveis da hemolinfa de *B.tenagophila* Cabo Frio ($p \leq 0,001$). Estes resultados indicam que os fatores solúveis da hemolinfa, e especialmente da linhagem Taim, podem diretamente induzir mortalidade no esporocisto recém-transformado (figura 1).

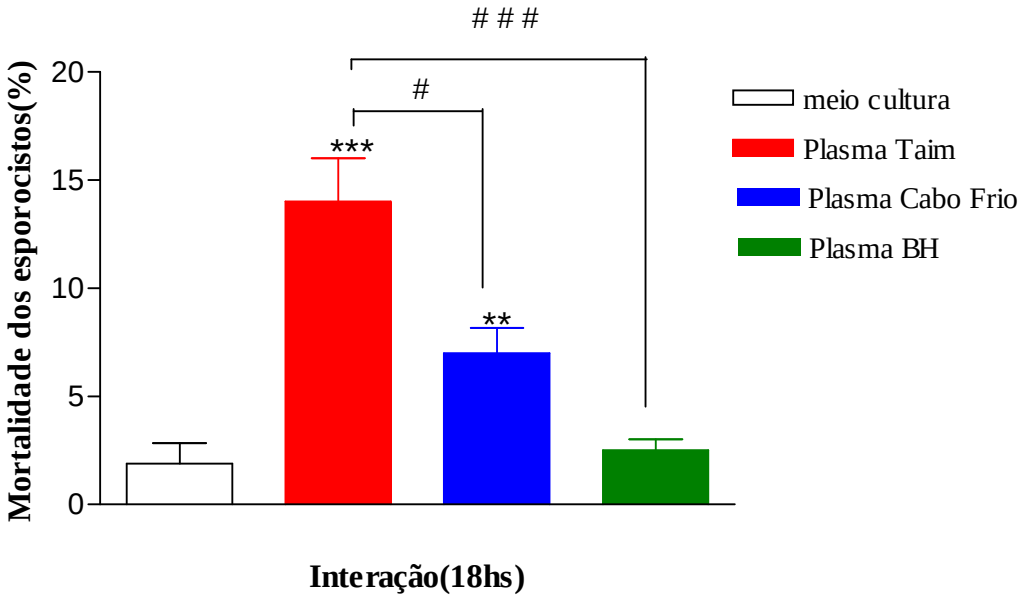

Figura 1: **Efeito da hemolinfa livre de células de *B. glabrata* da linhagem BH (BH), *B. tenagophila* Taim(Taim) ou *B. tenagophila* Cabo Frio (Cabo Frio) não infectados na mortalidade de esporocistos de *S.mansoni* transformados axenicamente.** Cerca de 100 esporocistos foram incubados, em triplicata, com CBSS suplementado contendo ou não hemolinfa acelular de caramujos de BH, Cabo Frio ou Taim por 18 h. A porcentagem de esporocistos mortos foi calculada para cada tratamento e o resultado demonstrado em média±desvio padrão de três experimentos independentes. Diferenças estatisticamente significantes entre a mortalidade dos esporocistos nos tratamentos que contem a hemolinfa livre de células comparado ao controle (esporocistos cultivados somente em meio de cultura) estão indicadas por *** $p \leq 0.001$, ** $p \leq 0.01$; e entre cada tratamento indicado por # $p \leq 0,05$ ou ### $p \leq 0,001$.

A utilização da sonda fluorescente *Hoechst 33258* confirmaram os dados de mortalidade contabilizados com a utilização de azul de tripan. Desta forma, esporocistos incubados com meio de cultura ou com meio contendo hemolinfa acelular recolhida de *B. glabrata* da linhagem BH não apresentaram fluorescência após a adição da sonda *Hoechst 33258* (figura 2A e B), confirmando a integridade do tegumento destas larvas. Em contraste, nas culturas que receberam hemolinfa acelular de *B. tenagophila*, especialmente quando proveniente de caramujos da linhagem Taim, alguns esporocistos apresentaram fluorescência característica da incorporação da sonda *Hoechst 33258* (figura 2E e F), sugerindo que a mortalidade do esporocisto seja conseqüência de lesões tegumentares provocadas por elementos presentes na hemolinfa destes hospedeiros.

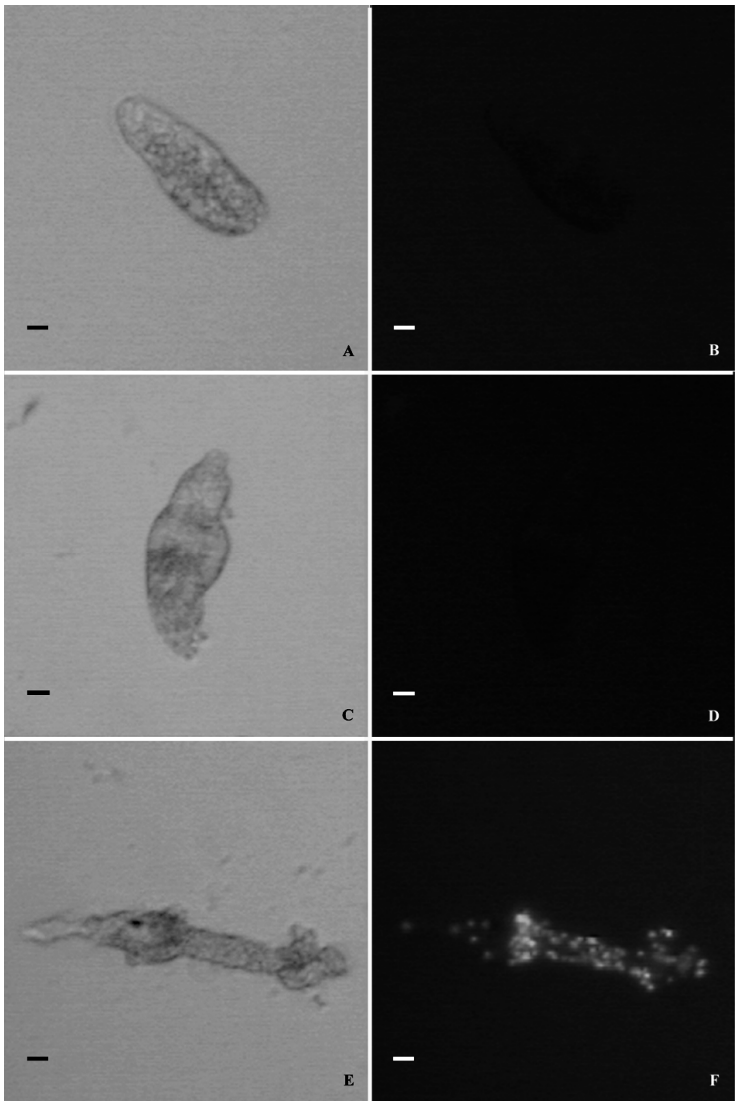

Figura 2: Padrão de marcação dos esporocistos de *S. mansoni*, após incubação com o marcador de fluorescência *Hoechst 33258*. Em A, C e E esporocitos cultivados *in vitro* por 6 h foram fotografados em campo claro e em B, D e F o mesmo material foi fotografado com filtro para fluorescencia. Em A e B representa um esporocisto incubado com meio de cultura (CBSS suplementado), mostrando esporocistos integros e ausência de incorporação da sonda. Padrão semelhante também foi observado na presença de hemolinfa acelular de *B. glabrata*. Em C, D E e F foi documento esporocistos cultivados *in vitro* na presença de hemolinfa acelular de *B. tenagophila* da linhagem Taim, sendo observado esporocistos integros e não fluorescentes (C e D)

O efeito da adição combinada de hemócitos e plasma dos caramujos na viabilidade dos esporocistos de *S. mansoni* axenicamente transformados foi demonstrado na figura 3. Os resultados mostram que esporocistos de *S. mansoni* incubados com CBSS suplementado apresentam alta viabilidade durante o período de exame e a adição de hemócitos e fração acelular de *B. glabrata* ao cultivo não foi capaz de alterar significativamente a taxa de mortalidade dos esporocistos. Contrariamente, a adição de hemócitos e da hemolinfa acelular de *B. tenagophila*, Taim ou Cabo Frio, ao meio de cultura resultou em aumento significativo na mortalidade dos esporocistos. Foi estimado que a adição de hemócitos e fatores solúveis de *B.tenagophila* Taim resultaram na destruição de 37 ± 6 % dos esporocistos, no caso da adição de hemócitos e fatores solúveis de *B.tenagophila* Cabo Frio houve 16 ± 6 % de mortalidade, sendo a mortalidade nestes grupos estatisticamente maior ($p>0,001$) quando comparados ao grupo controle ou ao grupo contendo hemócitos e a hemolinfa acelular de *B. glabrata* (figura 3). A destruição de esporocistos induzida na presença de fração solúvel da hemolinfa e hemócitos das diferentes linhagens de caramujo nos testes in vitro refletem a suscetibilidade destes caramujos nos experimentos realizados *in vivo*. Hemócitos juntamente com a hemolinfa da linhagem Taim, caramujo resistente induziu a mortalidade mais elevada de esporocistos *in vitro*, enquanto nenhuma destruição significante de esporocistos foi observada com adição de hemócitos com a hemolinfa livre de células de *B.glabrata* BH, uma cepa altamente suscetível ao parasito

Nas combinações heterólogas, a adição de hemócitos das linhagens suscetíveis de *Biomphalaria*, *B. glabrata* BH e *B. tenagophila* Cabo Frio, com fração acelular de *B. tenagophila* Taim resultou em aumento de mortalidade de esporocistos somente nas associações com a mesma espécie. Assim, a adição de hemócitos de *B. tenagophila* de Cabo Frio e hemolinfa acelular de *B. tenagophila* do Taim a cultura de esporocistos

resultou em um aumento significante no número de parasitos mortos, alcançando níveis de mortalidade semelhantes ao observado em cultura que contém hemócitos mais hemolinfa livre de células de *B. tenagophila* do Taim, um caramujo resistente ao parasito resistente (Figura 3). Entretanto, a mortalidade dos esporocistos obtida quando hemócitos de *B. glabrata* foram acrescentados a hemolinfa livre de células do Taim não foi estatisticamente maior que o detectado em culturas que contém somente elementos de *B. glabrata*.

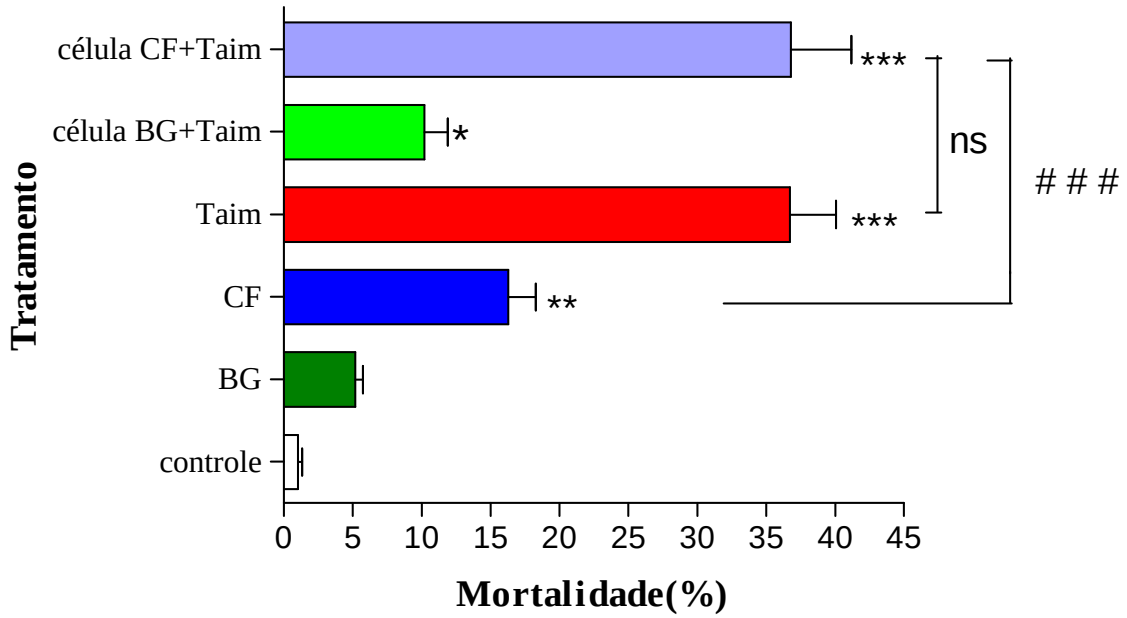

Figura 3: **Taxa de mortalidade dos esporocistos de *S. mansoni* cultivados *in vitro* na presença ou ausencia de hemolinfa e hemócitos recuperados de *B. glabrata* e *B. tenagophila.*** Tratamentos: **Controle** representa os esporocistos cultivados somente na presença de CBSS suplementado; **BG** – esporocistos cultivados somente na presença de CBSS suplementado contendo hemócitos *B. glabrata* BH não infectados e livres de hemolinfa, **CF** - esporocistos cultivados somente na presença de CBSS suplementado contendo hemócitos *B. tenagophila* Cabo Frio não infectados e livres de hemolinfa, **Taim** - esporocistos cultivados somente na presença de CBSS suplementado contendo hemócitos *B. tenagophila* Taim não infectados e livres de hemolinfa; **BG+Taim** - esporocistos cultivados na presença de CBSS suplementado contendo hemócitos *B. glabrata* BH não infectados e hemolinfa de *B. tenagophila* Taim não infectado ; **CF+Taim** - esporocistos cultivados na presença de CBSS suplementado contendo hemócitos *B. tenagophila* Cabo Frio não infectados e hemolinfa de *B. tenagophila* Taim não infectado . A porcentagem de esporocistos mortos foi calculada para cada tratamento realizado em triplicata e os resultados demonstrados em média ± desvio padrão de três experimentos independentes. As diferenças estatisticamente significantes quando comparamos os controles versus os tratamentos estão indicados por *** p≤ 0.001, ** p≤ 0,01; e entre cada tratamento estão indicados por # p≤ 0,05 ou ### p≤ 0,001. ns - não significativo

Este trabalho avaliou a participação da fração acelular da hemolinfa de *B. tenagophila* da linhagem Taim, linhgem de caramujo completamente resistente à infecção experimental com *S. mansoni*, no mecanismo de destruição das larvas do parasito *in vitro*. Nossos dados claramente demonstraram que a fração acelular da hemolinfa de *B. tenagophila* Taim participa direta e indiretamente do mecanismo de destruição de esporocistos de *S. mansoni*, através da indução de lesão no tegumento do parasito e do aumento a capacidade de reconhecimento e destruição mediada por hemocitos. Efeito similar não foi detectado na presença da fração acelular da hemolinfa de *B. glabrata* da linhagem BH.

A importância dos fatores solúveis da hemolinfa de *Biomphalaria* no mecanismo de proteção contra o *S. mansoni* foi demonstrado, *in vivo*, por GRANATH & YOSHINO (1984) e por COELHO & BEZERRA (2006). Os autores verificaram uma diminuição em *S. mansoni* infecção taxa de de *B. glabrata* de PR e *B. tenagophila* Cabo Frio do que foi inoculado simultaneamente com hemolinfa acelular do caramujo resistente. No caso de *B. glabrata* (GRANATH & YOSHINO, 1984) somente a inoculação da fração acelular da hemolinfa de um caramujo infectado diminui a taxa de infecção no caramujo receptor; além disto, o número de cercárias recuperadas nos caramujos receptores eram muito semelhantes aos dos moluscos não tratados. Trabalhos preliminares realizados em nosso laboratório (PEREIRA *et al*., 2008) também demonstram que a transferência de hemolinfa acelular de Taim recuperada de caramujos não infetados é suficiente para aumentar resistência de *B. tenagophila* de linhagem suscetível durante interação *S. mansoni*. Nós também demonstramos que além da redução no número de caramujos infectados, a transferência de hemolinfa acelular de Taim para moluscos suscetíveis também resultou em redução significativa de carga parasitária (esporocistos e cercárias) durante todo o período avaliado.

Estudos bioquímicos realizados na tentativa de identificar possíveis fatores da hemolinfa associados com níveis diferenciados de suscetibilidade das diferentes linhagens de caramujos (GRANATH & SPRAY, 1987; SPRAY & GRANATH, 1990) identificaram diferenças na composiçao de proteínas da hemolinfa de *B. glabrata* resistente e suscetível, mas nenhuma correlação entre tal diferencia e resistência foi descrita.

Apesar da grande relevância biológica dos estudos *in vivo*, estes trabalhos não esclarecem a participação da fração acelular da hemolinfa de *Biomphalaria* no mecanismo de destruição de larvas de *S. mansoni*. Com este intuito, BAYNE *et al.* (1980) avaliaram *in vitro* o efeito da hemolinfa de *B. glabrata* sobre esporocistos primários de *S. mansoni* transformados axenicamente. Nestes estudos, os autores demonstraram que a hemolinfa acelular, obtida de *B. glabrata* suscetível ou resistente não podem alterar a morfologia do esporocistos *in vitro*, o mesmo acontece com hemolinfa contendo hemócitos de caramujos suscetível. Resultados semelhantes também foram obtidos neste trabalho experimental, não sendo detectado aumento significante em mortalidade de esporocistos com a adição da hemolinfa acelular ou hemolinfa acelular mais hemócitos obtido de *B. glabrata* BH . Ao contrário, nos caramujos *B. tenagophila* a possibilidade de um efeito direto de hemolinfa acelular em esporocistos de *S. mansoni* deve ser considerada, desde que a hemolinfa acelular pode matar 8 a 16% das larvas do parasito na ausência hemócitos. Alguns mecanismos de lesão direta de patógenos, como produção de peptídeos antimicrobianos (MITTA *et al*., 2000) foi descrito em *Mytilus galloprovincialis*, mas não há nenhuma confirmação experimental da participação deste mecanismo na destruição de *S. mansoni* em *Biomphalaria* spp. BAYNE *et al*. (2001) detectaram níveis elevados de espécies reativas de oxigênio ou de nitrogênio na hemolinfa acelular de *B. glabrata*, sendo que a produção destes metabólicos está associada com a destruição de esporocistos nos sistemas *in vitro* e

na presença de hemócitos. Entretanto, o envolvimento destes elementos reativos no controle do parasito observado durante a transferência *in vivo* da hemolinfa acelular do Taim ou ensaios *in vitro* na ausência de hemócitos ainda não está confirmado, especialmente devido a rápida metabolização destes componentes.

Outro elemento que pode estar associado com controle de patógenos é a presença de proteínas com ação proteolítica que são ativadas pelo parasito. O envolvimento da ativação da cascata enzimática que participam da coagulação, melanização ou da ativação de proteínas com funções semelhantes ao sistema de complemento tem sido caracterizado em alguns invertebrados como nos insetos, sendo que este processo culmina com o formação de melanina e a destruição dos patógenos (LEE *et al*., 2000; BLANDIN *et al*., 2004). BAHGAT *et al*, (2002) observou atividade de serino protease e fenoloxidase na hemolinfa e no lisado de hemócitos de *Biomphalaria glabrata*, no entanto não foi possível associar a atividade da protease com a suscetibilidade do molusco ao *S. mansoni*.

Além do efeito direto de alguns fatores solúveis do sistema de defesa dos moluscos, elementos da hemolinfa acelular poderiam agir indiretamente, facilitando o reconhecimento do parasito e/ou ativando os hemócitos. Evidências experimentais de um possível efeito indireto da hemolinfa acelular foram evidenciadas na interação entre *B. glabrata* - *S. mansoni* onde os hemócitos do molusco associados com fatores solúveis da hemolinfa de *B. glabrata* resistentes adquirem a capacidade de destruir os esporocistos de *S. mansoni* (FRYER & BAYNE, 1996; SPRAY & GRANATH, 1990; BAYNE, 1990). Em nosso sistema *in vitro*, o efeito indireto da hemolinfa acelular recolhida de *B. tenagophila* da linhagem Taim foi confirmado pela alta taxa de mortalidade de esporocistos obtida com a adição da hemolinfa acelular de *B. tenagophila* Taim aos hemócitos de *B. tenagophila* de Cabo Frio, sugerindo que hemolinfa acelular de Taim pode aumentar o reconhecimento

do parasito por hemócitos de linhagens susceptíveis do caramujo, como é o caso da linhagem Cabo Frio. Alternativamente o aumento da mortalidade de esporocistos do parasito na presença de hemócitos de linhagens susceptiveis de caramujos e fração acelular da hemolinfa de Taim pode ser consequência de aumento da ativação celular por elementos presentes na hemolinfa. Muitos estudos demonstraram que carboidratos associados ao tegumento dos esporocistos de *S. mansoni* podem ligar-se a lectinas expressas hemócitos e/ou solúveis na hemolinfa acelular de *B. glabrata*, então lectinas poderiam mediar reconhecimento do parasito (LOKER *et al*., 2004, LOKER *et al*., 1989; JOHNSTON & YOSHINO, 1996; ADEMA *et al*., 1997; CASTILHO *et al*., 2002). O envolvimento de lectinas no reconhecimento dos esporocistos de *S. mansoni* pelo *B. tenagophila* do Taim tem sido investigação em nosso laboratório. O efeito cooperativo de hemócitos e hemolinfa acelular não foi detectado entre espécies de caramujos diferentes, pois não foi observado nenhuma mudança significante na mortalidade de esporocistos cultivados na presença de hemócitos de *B. glabrata* BH e hemolinfa acelular de Taim comparados a taxa de mortalidade observado com a presença somente da hemolinfa livre de células do Taim. Mais recentemente, MARTINS-SOUZA *et al*. (2006), usando o mesmo caramujo e linhagem do parasito demonstraram que em *B. glabrata* o padrão de marcação dos hemócitos circulantes incubados com lectinas fluorescentes foi muito diferente do observado com *B. tenagophila* (Taim ou Cabo Frio), sugerindo diferenças qualitativas e quantitativas na expressão de glicoproteínas na membrana dos hemócitos, que podem justificar a ausência de efeito indireto da hemolinfa acelular de *B. tenagophila* nos hemócitos de *B. glabrata*. Embora os mecanismos de defesa empregados por *B. tenagophila* para controlar infecções pelo trematódeo estão longe de ser completamente compreendido, a participação da hemolinfa livre de células do caramujo resistente tem sido

demonstrado por nossos experimentos. Nossos dados também sugerem que componentes solúvel da hemolinfa podem danificar diretamente as larvas de *S. mansoni* e também podem mediar reconhecimento do parasito feito pelos hemócitos circulantes.

PARTE II

Identificação de atividade proteolítica na fração solúvel de *B. glabrata* e *B. tenagophila*

5.1. Introdução

Historicamente, proteases estão associadas com degradação de proteínas, e muito cedo atraíram a atenção de pesquisadores que se interessavam pelo processo de digestão protéica em animais e no homem. As classes mais conhecidas são as serino-proteases, as metalo-protesases, as cisteino-proteases e as aspartil-proteases. A inativação destas proteases pode ocorrer por dois mecanismos distintos: por degradação proteolítica ou pelo bloqueio com inibidores específicos. Assim como em vertebrados, proteases estão envolvidas nos processos de digestão protéica de invertebrados. VALE *et al* (2007) mostraram que algumas proteases como tripsina e quimotripsinas são importantes nos processos digestivos de *Lutzomya longipalpis*. ALVES *et al* (2007) evidenciaram a presença de catepsinas D na glândula digestiva de *B. glabrata* (BH), sugerindo que esta enzima participe na degradação de nutrientes. Entretanto, alguns estudos (MUÑOZ *et al*., 2003; MCKERROW *et al*., 2006; LEMAITRE AND HOFFMANN, 2007) têm indicado que proteases e inibidores de proteases produzidos e liberados pelos invertebrados podem também participar de mecanismos de defesa induzidos durante a interação com os hospedeiros, como por exemplo, a ativação da cascata enzimática que participam da coagulação e melanização, que juntamente com a fagocitose participam destruindo o patógeno de maneira direta ou indireta. Neste sentido, LEMAITRE *et al* (1996) sugerem que a indução de genes que codificam proteases endógenas em *Drosophila* podem participar do mecanismo de defesa anti-fúngica destes insetos. Mais recentemente, ALTINCICEK *et al* (2007) verificaram a importância da expressão gênica de metalo-protease durante a invasão de fungos e bactérias em lepidópteros do gênero *Galleria*. Metalo-proteases endógenas ou induzidas por produto microbiano são liberadas na

hemolinfa deste inseto e provocam a hidrólise de proteínas da hemolinfa, gerando pequenos fragmentos de 3 KDa, que participam na ativação e regulação de lisozimas, e peptídeos antimicrobianos, que participam do controle destes microrganismos (ALTINCICEK *et al*., 2007).

A importância da ativação de proteases na resposta protetora do hospedeiro também tem sido documentada na interação entre *Anopheles gambiae* e *Plasmodium*. Nesta interação, os hemócitos do mosquito infectado produzem e secretam na hemolinfa glicoproteínas semelhantes a proteínas do complemento dos vertebrados, designadas proteínas da família tioester (TEP) e também proteínas que participam da cascata de melanização (PPO). Em vetores suscetíveis ou refratários à infecção por *Plasmodium* têm sido detectado ativação de TEP, gerando fragmentos que se ligam a superfície do protozoário e formam poros na membrana de *Plasmodium*. Nas linhagens resistentes, além de lise de membrana do protozoário, ocorre ativação de enzimas da hemolinfa do inseto responsáveis pela formação de uma cápsula de melanina em torno do oocineto (BLANDIN *et al*., 2004).

Alguns trabalhos têm identificado a presença de inibidores de proteases, como α-Macroglobulina (BENDER AND BAYNE, 1996) e proteínas com atividade de proteases semelhante à tripsina (BAHGAT *et al*., 2002) na hemolinfa e no lisado de hemócitos de *Biomphalaria glabrata*, respectivamente. No entanto não foi possível associar a atividade da protease com a suscetibilidade do molusco ao *S. mansoni*. Mais recentemente, MITTA *et al* (2005), avaliando uma biblioteca de cDNA de hemócitos de *B. glabrata* não infectados, identificaram a presença de genes homólogos a proteases e inibidores de protease. Posteriormente, LOCKYER *et al* (2007), tendo como o modelo experimental

B.glabrata infectado por *S. mansoni* e utilizando a técnica de hibridização subtrativa supressiva (SSH) construída com cDNA do órgão produtor de hemócitos (APO) de caramujos expostos e não expostos ao *S. mansoni*, descreveram a presença de uma seqüência gênica com homologia a uma serino-protease nos caramujos resistentes e não expostos ao parasito. Apesar de muitos trabalhos demonstrarem a presença de proteases na hemolinfa de moluscos, a possível participação deste grupo de proteínas em mecanismos de defesa destes hospedeiros ainda não foi devidamente demonstrada. Neste trabalho temos como objetivo analisar comparativamente a atividade de protease na hemolinfa de *B. glabrata* e de *B. tenagophila* (Taim e de Cabo Frio) e verificar o efeito desta atividade na viabilidade de esporocistos de *S. mansoni* em testes *in vitro*.

5.2 Material e métodos

5.2.1 Caramujos

Como citado na primeira parte deste trabalho, os caramujos utilizados pertencem às espécies *B.glabrata* e *B.tenagophila*, ambas mantidas no moluscário do laboratório de Esquistossomose ICB – UFMG. A linhagem de *B. glabrata*, utilizada é proveniente da Lagoa da Pampulha – Belo Horizonte (MG) e denominada de *B. glabrata* da linhagem BH. Duas linhagens de *B. tenagophila* foram utilizadas nos experimentos, uma proveniente de Cabo Frio – RJ, denominada de *B. tenagophila* da linhagem Cabo Frio, e outra da reserva ecológica do Taim – RS, denominada de *B. tenagophila* da linhagem Taim.

Antes da coleta da hemolinfa os caramujos foram submetidos a um procedimento de limpeza (com solução de detergente SDS 0,1%) e desinfecção (inicialmente com solução 0,1 % de Benzoilmetronidazole, seguido por 30 μg/ml Gentamicina e 100 μg/ml Ampicilina).

5.2.2. Determinação do pH da hemolinfa

Os caramujos foram devidamente anestesiados em solução aquosa contendo 0,4 mg/ml pentabarbitol sódico por 8 h (MARTINS-SOUZA *et al*., 2001), após o completo relaxamento e exposição da região cefalopodal foi verificado o pH da hemolinfa utilizando a técnica descrita por SOARES *et al*., (2006). Resumidamente, um tubo capilar (tubos Ø = 1,1 mm) contendo 30 μl de solução indicadora de pH (0,5% Azul de bromomotimol pH 7,5) que foi introduzido delicadamente no pé dos moluscos, permitindo o contato da hemolinfa com a solução ainda no interior do caramujo. A cor desenvolvida na interface da hemolinfa com a solução indicadora foi comparada com soluções padrões preparadas em diferentes unidades de pH entre 5,0 e 8,0. Para estimativa do pH da hemolinfa dos moluscos foram observados 5 caramujos de cada uma das espécies ou linhagens estudadas.

5.2.3. Coleta de hemolinfa para análise de atividade proteolítica

Para utilização nos testes de atividade enzimática, hemolinfa de *B. glabrata* BH e de *B. tenagophila*, das linhagens Taim e Cabo Frio, não expostos ao parasito, foi coletada por punção na região cardíaca conforme descrito por ZELCK *et al* (1995) e detalhado anteriormente. Para cada linhagem de caramujo testada, foi coletado hemolinfa de 9

caramujos na presença ou ausência de cada inibidores de proteases testado. Nos experimentos foram testados os inibidores 4-(2-Aminoethyl)-benzeneesulfonyl fluoride hydrocloride (**AEBSF)**, **Pepstatina-A,** transepoxysuccinyl-L-leucylamido 4-guanidino butane (**E64)**, Ac-Leu-Leu-argininal x 1/2 H_2S0_4 (**Leupeptin)**, N-α-tosyl-L-phenylalanine chloromethyl ketone (**TPCK)**, N-α-tosyl-L-lysine chloromethyl ketone (**TLCK)**, Phenylmethylsulfonyl fluoride (**PMSF**) e um coquetel de inibidor de protease da Amersham Biosciences. As especificações de cada inibidor testado e a concentração utilizada estão detalhadas no Quadro I.

Após a coleta individual, a hemolinfa de 3 caramujos de um mesmo grupo experimental foi agrupada para obtenção de volume necessário para os testes, sendo utilizada 3 amostras contendo hemolinfa total de 3 caramujos para cada cada grupo experimental de cada linhagem de caramujo.s. A hemolinfa foi imediatamente transferida para superfície de parafilm em banho de gelo e o sobrenadante foi então transferido para tubos de polipropileno de 1,5mL e centrifugado 180×g for 10 min at 4 ºC. O sobrenadante, designado de fração solúvel ou acelular, foi retirado e utilizado para eletroforese, ensaios enzimáticos e na determinação de da concentração de proteínas pelo método de LOWRY *et al* (1951).

Quadro I: Painel de Inibidores de Proteases utilizados para inibir a atividade proteolítica presente na hemolinfa de *B. glabrata* BH, *B. tenagophila* Cabo Frio e *B. tenagophila* Taim, mostrando a marca utilizada, a especificidade do inibidor, o veículo de diluição da solução estoque e a concentração final utilizada no ensaio.

Inibidor (Marca)	Especificidade do inibidor	Dissolução	Concentração final
PMSF (Amersham)	serino protease (r)	etanol absoluto*	6mM
TLCK (Amersham)	tripsina (i)	água-HCl pH 3,0*	135µM
TPCK (Amersham)	quimotripsina(i)	metanol	284µl
AEBSF (Amersham)	serino protease (i)	Tris-HCl pH 8,0	6mM
PepstatinA (Amersham)	aspartil protease(r)	DMSO	5µM
Coquetel (Sigma)	aspartil-, serino e cisteíno proteases (r)	água	3X
E-64 (Sigma)	Cisteino protease(r)	água	2.8µM
Leupeptin (Amersham)	serino- e aspartil-protease(r)	água	1µM
EDTA (Gibco)	Metalo-protease(r)	Tris-HCl pH 8,0	1,3mM

(i)interação irreversível com a proteína.
(r) interação reversível com a proteína.
* preparo feito imediatamente antes da utilização.

5.2.4. Eletroforese da Hemolinfa

As proteínas da fração acelular da hemolinfa dos diferentes grupos experimentais foram separadas por eletroforese em gel de poliacrilamida (LAEMMLI., 1970), utilizando o sistema mini-VE (Hoefer Pharmacia Biotech, San Francisco, CA, USA). Para tanto, 5 µl

da amostra da fração acelular da hemolinfa de cada tratamento foi diluída (1:2) em tampão Tris-HCl 0,125 M, pH 6,8, contendo 2% SDS e 10% de glicerol 0,1% de bromofenol e incubada a 100 ºC por 3 min. Para referência de peso molecular foi aplicado o padrão de pesos moleculares SDS6H2 (Sigma). Cada amostra foi aplicada em uma canaleta do gel de poliacrilamida (SDS-PAGE) descontínuo (empacotamento 4% e resolução 10%) e a separação eletroforetica das proteínas foi realizada em 15 mA (60V). Após a separação das proteínas, o gel foi fixado em metanol 50% e corado com Azul de Coomassie (solução contendo 0,1% de Azul de Coomassie G-250, 25% de metanol, 5 % de ácido acético) e descorado em uma solução de 10% de metanol e 5% de ácido acético.

5.2.5.Ensaio de atividade proteolítica

A atividade proteolítica da fração acelular de cada hemolinfa foi avaliada utilizando o substrato caseína e/ou gelatina. No caso do ensaio utilizando a caseína seguimos a técnica descrita por KUNITZ (1945). Resumidamente 20 µl de cada amostra de hemolinfa foi adicionada a 0,5 mL de tampão Tris 0,5M pH 7,2 contendo 1 % de caseína (Sigma St Louis, MO) e a mistura foi incubada em banho-maria a 28 ºC por 3 h. Após este período, 0,5 mL de solução de ácido perclórico a 7 % foi adicionado a cada tubo para parar a reação e precipitar a caseína não digerida. Após 15 min de incubação com ácido perclórico, cada tubo foi centrifugado a 10.000g/5min/4 ºC. O sobrenadante de cada tratamento foi transferido para um tubo de polipropileno de 2 mL e o nível de peptídeos, resultantes da digestão da caseína pela hemolinfa, foi estimado através da leitura da absorbância de cada amostra em espectrofotômetro (Shimatzu) a 280nm. O controle negativo foi realizado substituindo a

amostra de hemolinfa por tampão CBSS e no controle positivo foi adicionado 0,1 mg/mL de quimotripsina à solução de caseína. A atividade total foi expressa em densidade óptica / hora / concentração de proteína (DO / h / mg proteína).

A outra técnica utilizada para estimar a atividade proteolítica nas amostras de hemolinfa foi a digestão de gelatina co-polimerizada em gel de poliacrilamida, segundo técnica originalmente descrita por HEUSSEN AND DOWDLE (1980). Para tanto, o gel contendo 10% de acrilamida/bisacrilamida e 1% de SDS foi co-polimerizado com 0,1% gelatina (sigma) e as amostras de hemolinfa foram diluídas em tampão Tris-HCl 0,125 M, pH 6,8 sem SDS e também sem adição de agentes redutores e sem aquecimento. As amostras de hemolinfa foram aplicadas ao gel e eletroforeticamente separadas utilizando o sistema mini-VE (Hoefer Pharmacia Biotech) e corrente de 80 V a 4 ºC. Juntamente com as amostras, foi aplicado a uma canaleta o padrão de pesos moleculares composto de proteínas pré-coradas (Biolabs), permitindo identificação e marcação da posição aproximada de cada banda do padrão após a separação eletroforética com um pequeno orifício.

Após a separação das proteínas, o gel foi lavado com água contendo 2,5% Triton X-100 por 1 h, para a retirada do SDS, e incubado em tampão Tris (30 mM Tris-HCl, 60 mM NaCl, 0,05% NaN_3, pH 7,2) por 18 h a temperatura ambiente. Após incubação, o gel foi fixado e corado com azul de Coomassie, conforme detalhado anteriormente. A gelatina que compõem o gel é homogeneamente corada pelo Coomassie, enquanto as áreas claras demonstram a presença de atividade proteolítica da amostra.

Para confirmação da atividade proteolítica, amostras de hemolinfa foram coletadas na presença dos inibidores anteriormente descritos e utilizadas para realização dos testes de atividade.

5.2.6.Obtenção de miracídios de *S. mansoni* e dos esporocistos axênicos

Conforme detalhado anteriormente (**no item 4.2.3**), miracídios de *S. mansoni* foram obtidos em condições estéreis, a partir de homogenato de fígado de hamsters infectados (45-50 dias após infecção) conforme técnica descrita por CHAIA (1956). Os miracídios recolhidos foram axenicamente transformados em esporocistos em meio RPMI contendo 5 % de soro fetal bovino por 18 h a 26 ºC (SAMUELSON *et al*., 1984). Os esporocistos transformados foram lavados 5 vezes em CBSS estéril, quantificados e ressuspensos em CBSS suplementado [CBSS contendo 10 mM de HEPES, 2 mM glutamina 1 mM de aminoácidos essenciais, 100 U penicilina e 100 U estreptomicina, e 2 % de albumina bovina] onde permaneciam até serem utilizados nos respectivos protocolos.

5.2.7 Ensaio de inativação de esporocistos *in vitro*

A fração acelular da hemolinfa recolhida de *B. glabrata* BH e *B. tenagophila*, Taim e Cabo Frio, coletada com e sem a presença de inibidores de proteases, também foi avaliada na sua habilidade de destruir esporocistos de *S.mansoni* axenicamente transformados. Para tanto foi utilizada metodologia detalhada anteriormente (**no item 4.2.5**; PEREIRA *et al*., 2008), onde aproximadamente 50 esporocistos de *S. mansoni* foram cultivados, por 6 h a 26 ºC, em meio CBSS suplementado na presença de 20% de fração acelular da hemolinfa dos caramujos de diferentes grupos, coletados na presença ou ausência de inibidor de protease (AEBSF). Após incubação foi adicionado a cada tratamento solução contendo 0,4 % de Azul de Tripan e a viabilidade dos esporocistos foi aferida pela contagem de parasitos íntegros não corados em microscopia óptica.

5.2.8 Analise estatística

Os resultados obtidos em cada tratamento foram estatisticamente comparados, utilizando-se análise de variância para dados paramétricos (ANOVA, post teste Tukey). Valores de $P \leq 0,05$ foram considerados estatisticamente significativos.

5.3 Resultados:

As amostras de hemolinfa coletadas de *B. glabrata* ou *B. tenagophila* das diferentes linhagens testadas não apresentaram diferenças estatisticamente significantes na quantidade de proteínas e no pH. Em todas as amostras de hemolinfa das diferentes espécies ou linhagens de *Biomphalaria* testadas o pH variou de 6,5 a 7,0. Foi quantificado 11,2 ± 0,0208 mg de proteína/mL de hemolinfa de *B. glabrata,* enquanto que *B.tenagophila* Taim e Cabo Frio apresentam 10.56 ± 0,082 e 11.36± 0,021 mg/mL respectivamente. Nas diferentes espécies ou linhagens de *Biomphalaria* utilizadas nestes estudos foi verificado que proteínas de alto peso molecular, possivelmente hemoglobina, são predominantes na hemolinfa acelular, dificultando a identificação de outras proteínas (figura 4A). Com a ultracentrifugação da hemolinfa acelular (100.000g/3h/4 ºC), foi possível visualizar melhor as demais proteínas e observar que existe muita semelhança entre o perfil eletroforético das

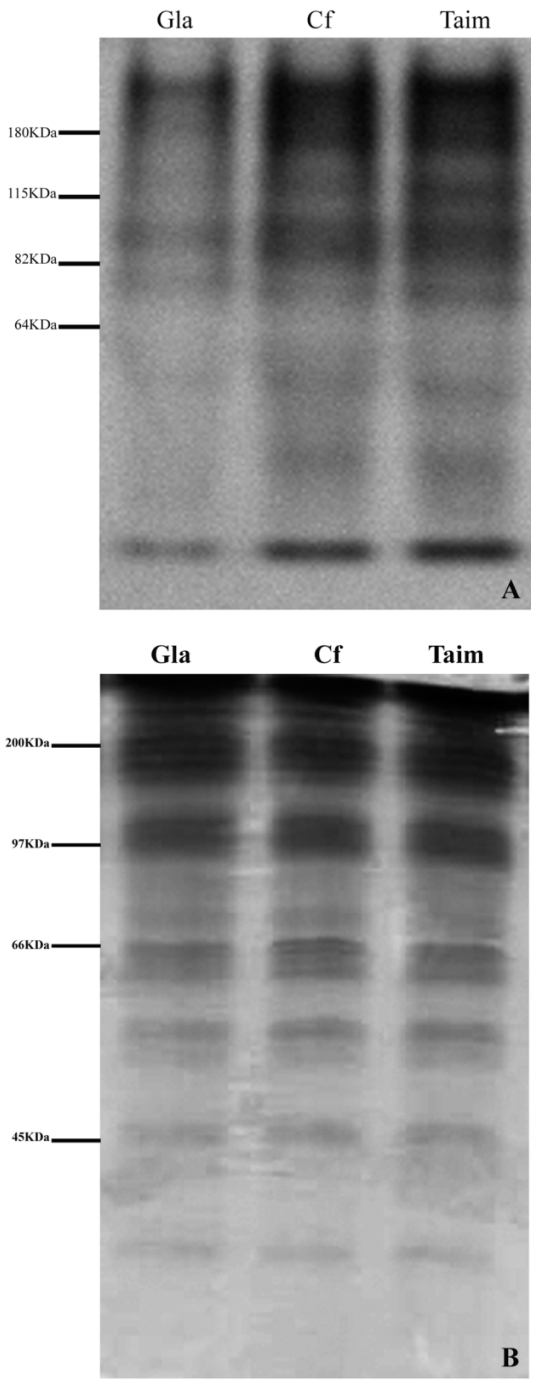

Figura 4: Separação das proteínas da fração solúvel da hemolinfa de *B. glabrata* BH (Gla), *B.tenagophila* Cabo Frio (Cf), e *B.tenagophila* Taim (Taim) por SDS-PAGE em condições desnaturantes. Números à esquerda dos géis indicam padrões de massa molar (KDa).

proteínas solúveis da hemolinfa nas espécies de *B. glabrata* BH, *B.tenagophila* Cabo Frio, e *B.tenagophila* Taim (figura 4B).Apesar do perfil eletroforético semelhante, foram detectadas variações estatisticamente significativas na atividade proteolítica da fração acelular coletada das diferentes espécies e linhagens de caramujos hospedeiros intermediários de *S. mansoni* e incubada na presença de caseína (figura 5). Nas condições experimentais utilizadas, a adição de quimotripsina (0,1mg/mL), controle positivo, gerou digestão de caseína em níveis mensuráveis pelo teste e estatisticamente superior ao detectado no controle negativo, contendo apenas CBSS ($p<0,0001$). A adição da fração solúvel de *B. glabrata* à solução contendo caseína gerou aumento significativo da atividade proteolítica em relação ao controle com CBSS, mas muito menor que o detectado no controle positivo. Em contraste, a adição de uma amostra da fração acelular de *B. tenagophila*, tanto da linhagem Taim como Cabo Frio, resultou em aumento altamente estatístico da atividade proteolíca sobre caseína, sendo superior ao detectado com a hemolinfa acelular de *B. glabrata*. De maneira interessante, a atividade observada com adição da fração acelular de *B. tenagophila* Taim foi estatisticamente maior ($p<0,05$) quando comparado com o valor detectado para *B.tenagophila* Cabo Frio (figura 5)

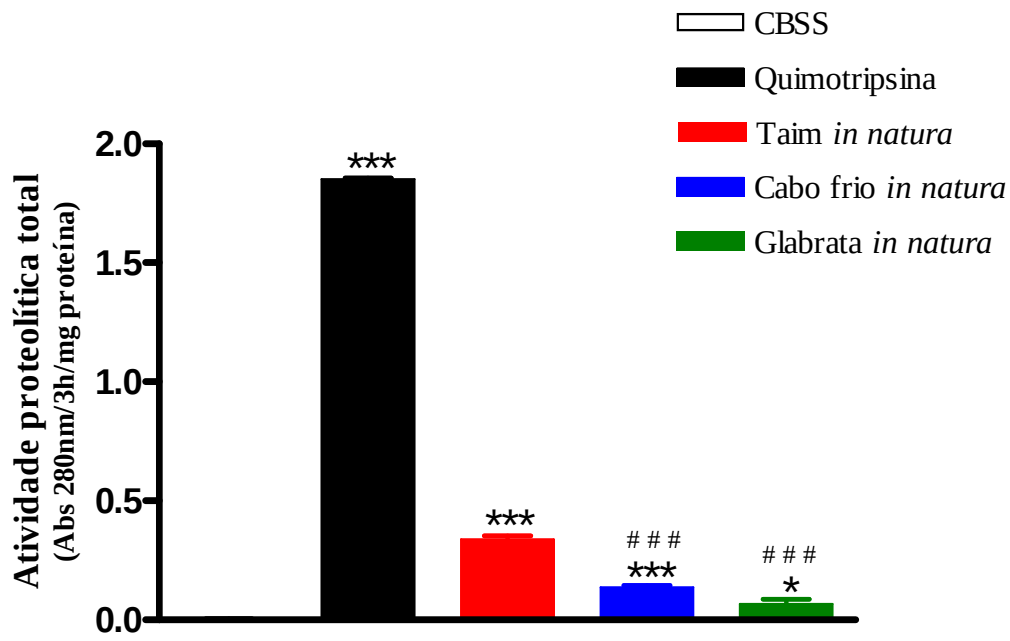

Figura 5: **Atividade proteolítica da fração acelular da hemolinfa de *B.glabrata, B.tenagophila* Taim e *B.tenagophila* Cabo Frio sobre solução de Caseína.** Atividade proteolítica estimada pela leitura de absorbância à 280 nm da solução de caseína após incubação com a fração solúvel da hemolinfa dos caramujos dos diferentes grupos experimentais, ou seus controles, por 3 h seguido de precipitação protéica. *** p≤ 0,001 em relação ao controle negativo contendo apenas CBSS; diferenças estatisticamente significantes entre a atividade detectada com na presença de hemolinfa de Taim e outros tratamento contendo hemolinfa estão indicados por ### p≤ 0,001.

A utilização de inibidores específicos para grupos de proteases durante o teste de avaliação da atividade proteolítica da fração acelular da hemolinfa dos caramujos, tendo a caseína como substrato, permitiu caracterizar a atividade proteolítica da hemolinfa das diferentes espécies e linhagens de caramujos testadas (figura 6). A baixa atividade proteolítica da fração solúvel de *B. glabrata* foi praticamente abolida pela adição de E64 (cisteino-protease), Leupeptin (serino- e aspartil-protease), AEBSF (serino-protease) e Pepstatina A (aspartil-protease). *Biomphalaria tenagophila*, Cabo Frio e Taim, apresentaram atividade proteolítica maior que *B. glabrata* e esta atividade foi apenas parcialmente reduzida, mas não bloqueada, pela adição dos inibidores E64, Leupeptin e AEBSF à hemolinfa, sendo que a redução foi maior para a atividade da hemolinfa de *B. tenagophila* da linhagem Cabo Frio. Ao contrário do observado com hemolinfa de *B. glabrata*, a adição de Pepstatina A não afetou a atividade proteolítica da hemolinfa de *B. tenagophila* Cabo Frio ou Taim. Também foram testados os inibidores TPCK (inibidor de quimotripsina), e TLCK (inibidor de tripsina), sendo que os mesmos reduziram a atividade proteolítica da hemolinfa dos caramujos estudados ao nível semelhante ao detectado com adição de AEBSF. Mesmo com a adição do coquetel de inibidores comercialmente disponivel (Sigma), não foi possível abolir a atividade proteolítica detectada na hemolinfa acelular de *B. tenagophila*. Vale ressaltar que a adição de AEBSF, inibidor de serino-proteases, produziu os maiores índices de inibição da atividade proteolítica, sendo que esta redução foi estatisticamente significante para amostras de hemolinfa das diferentes espécies de *Biomphalaria* estudada e em todos os testes realizados.

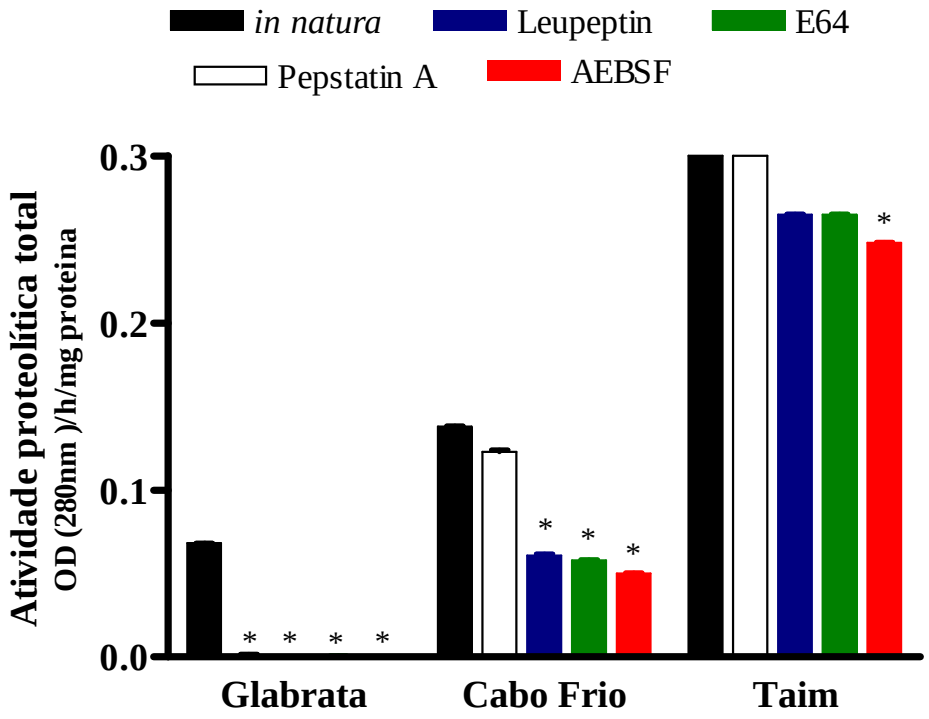

Figura 6: Atividade proteolítica específica da fração acelular da hemolinfa de *B. glabrata* (Glabrata), *B. tenagophila* Cabo Frio (Cabo Frio) e *B. tenagophila* Taim (Taim) na ausência e na presença de inibidores específicos para diferentes classes de proteases. As proteases foram ensaiadas ***in natura***, hemolinfa coletada sem inibidor ou com hemolinfa coletada na presença de **Pepstatina, Leupeptin, E64 ou AEBSF.** A atividade proteolítica específica era calculada para cada tratamento, realizado em triplicata, e os resultados demonstrados em média ± desvio padrão de quatro experimentos independentes. Diferenças estatisticamente significantes em relação a atividade detectada com a hemolinfa in natura do mesmo grupo experimental estão indicadas por * p□≤ 0,05

A atividade proteolítica da fração acelular da hemolinfa dos diferentes grupos experimentais também foi verificada quando a fração solúvel in natura foi aplicada ao gel co-polimerizado com gelatina (figura 7). Áreas não coradas, sugestivas de degradação da gelatina pela amostra de hemolinfa, foram detectadas nas canaletas contendo fração acelular de hemolinfa de *B. glabrata* BH e de *B. tenagophila*, tanto da linhagem Cabo Frio como Taim. Apesar de ter sido detectada degradação de gelatina com hemolinfa acelular de todos os caramujos estudados, a degradação produzida por *B. tenagophila* Taim foi mais intensa e extensa. (figura 7A). Diferentemente do observado com caseína, a atividade proteolítica da fração acelular da hemolinfa de caramujos tendo como substrato gelatina não foi bloqueada pela adição de E64, pepstatina A, ou leupeptin (dado não mostrado). Os únicos inibidores capazes de reduzir a atividade proteolítica da hemolinfa em gel contendo gelatina foram os inibidores de serino-proteases, o PMSF e o AEBSF, sendo que AEBSF foi mais eficiente na redução da atividade proteolítica (figura 7b).

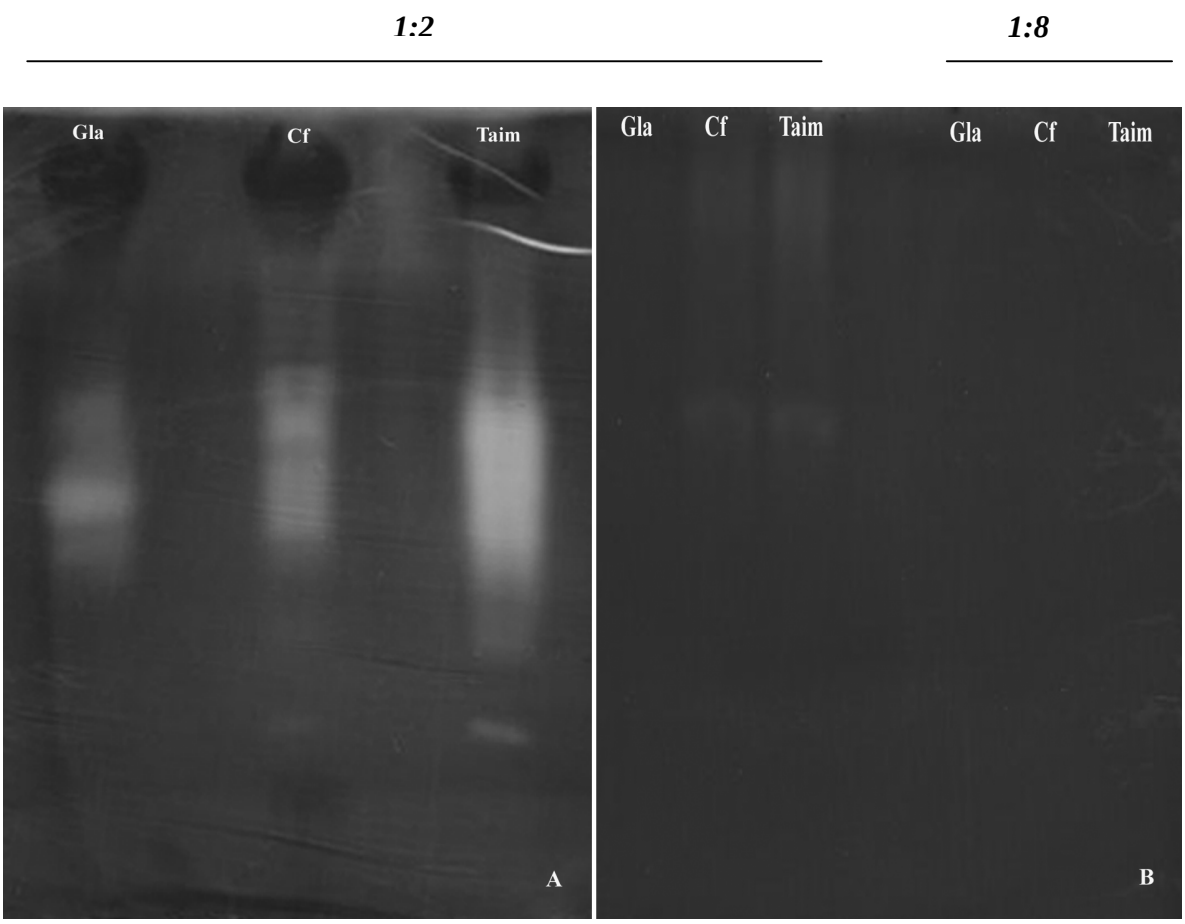

Figura 7: Atividade proteolíca da hemolinfa acelular de *B. glabrata* da linhagem BH (Gla) e *B. tenagophila* das linhagens Cabo Frio (CF) e Taim (Taim) em gel de poliacrilamida contendo 1% de gelatina. Em (A) as amostras de hemolinfa acelular foram coletas, mantidas em gelo, diluídas 1:2 em tampão não denaturante e aplicada no gel contendo 1 % de gelatina. Em (B) a hemolinfa foi coletada na presença de 6 mM do inibidor de serino-proteases – AEBSF e processada conforme descrito para A (1:2) ou foi coletada e diluída 1:4 em CBSS contendo 6 mM de AEBSF antes de ser processada e aplicada no gel (1:8). As áreas claras represenda locais onde a gelatina co-polimerizada no gel foi digerida por enzimas presentes na fração acelular da hemolinfa.

Uma vez que a adição de AEBSF, inibidor específico de serino-proteases, resultou em redução da atividade proteolítica da fração acelular da hemolinfa de *Biomphalaria* nos ensaios realizados e que este inibidor é irreversível e pouco tóxico para as células de mamíferos, o efeito funcional da adição deste inibidor foi avaliado nos ensaios in vitro contendo esporocistos e hemolinfa acelular (figura 8). Esporocistos de *S. mansoni* cultivados por 24 h em meio de cultura na presença ou ausência de 6mM de AEBSF apresentaram baixa mortalidade não sendo estatisticamente diferente entre estes grupos. A adição da hemolinfa acelular de *B. glabrata* ao meio de cultura resultou em pequena mortalidade dos esporocistos, mas significantemente maior que a observada nos grupos controles, que não foi alterada na presença de hemolinfa coletada com AEBSF (figura 8A). Confirmando os resultados anteriores (figura 1), a adição da fração acelular de *B. tenagophila*, das linhagens Taim e Cabo Frio, induz aumento estatístico da mortalidade de esporocistos, sendo que a mortalidade do parasito foi maior na presença de hemolinfa da linhagem Taim, naturalmente resistente à infecção pelo parasito (figura 8B e 8C). A adição de hemolinfa acelular de *B. tenagophila*, de ambas as linhagens, coletada na presença de AEBSF reduziu significativamente a mortalidade dos esporocistos (figura 8B e 8C).

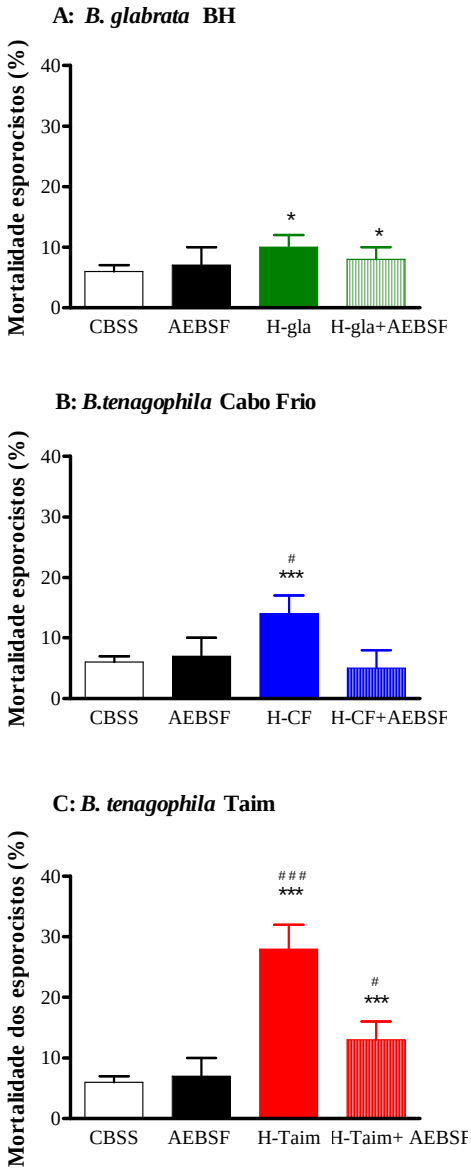

Figura 8: **Taxa de mortalidade dos esporocistos de *S. mansoni* cultivados na presença de fração acelular da hemolinfa de *B. glabrata* (A), *B. tenagophila* Cabo Frio (B) ou *B. tenagophila* Taim (C) coletadas na presença ou ausência de inibidor de protease (AEBSF).** Tratamentos Controles: **CBSS**: representa os esporocistos cultivados em CBSS suplementado; **AEBSF**– esporocistos cultivados em CBSS suplementado e 6mM de AEBSF (inibidor de serino-protease); **HTaim** - esporocistos cultivados em CBSS suplementado contendo 20% de fração acelular da hemolinfa *B. tenagophila* Taim; **HTaim+ AEBSF** - esporocistos cultivados em CBSS suplementado contendo 20% de fração acelular da hemolinfa *B. tenagophila* Taim e 6mM de AEBSF; **HCabo Frio** - esporocistos cultivados em CBSS suplementado contendo hemolinfa de *B. tenagophila* do Cabo Frio; **HCabo frio + AEBSF** - esporocistos cultivados em CBSS suplementado contendo hemolinfa de *B. tenagophila* Cabo Frio e 6mM de AEBSF. **Hgla** - esporocistos cultivados em CBSS suplementado contendo hemolinfa de *B. glabrata*; **Hgla + AEBSF** - esporocistos cultivados em CBSS suplementado contendo hemolinfa de *B. glabrata* e 6mM de AEBSF. A porcentagem de esporocistos mortos foi calculada para cada tratamento realizado em triplicata e os resultados demonstrados em média ± desvio padrão de três experimentos independentes. As diferenças estatisticamente significantes quando comparamos os controles versus os tratamentos estão indicados por *** $p \leq 0,001$; ** $p \leq 0,01$; e # $p \leq 0,05$ ou ### $p \leq 0,001$ indica diferenças estatísticas entre grupo contendo hemolinfa e hemolinfa com inibidor.

Discussão:

Pouco se conhece da composição da hemolinfa acelular de *Biomphalaria* e da participação destes elementos na interação parasito-hospedeiro. Durante a realização da primeira parte do nosso estudo (PEREIRA *et al*., 2008), foi possível demonstrar que a transferência da fração solúvel da hemolinfa de *B. tenagophila* da linhagem Taim confere proteção contra *S. mansoni* à linhagem suscetível de *B. tenagophila*; sendo detectado redução no número de esporocistos sobreviventes nos desafios *in vitro* e *in vivo*. Os ensaios *in vitro* (parte I) permitiram identificar que a destruição dos esporocistos de *S. mansoni* pode ser mediada diretamente por elementos presentes na fração acelular da hemolinfa de *B. tenagophila*, provavelmente por lesão do tegumento do parasito. Desde os primeiros estudos publicados por NEWTON (1952) foi evidenciado histologicamente que alguns esporocistos eram destruídos no interior do hospedeiro intermediário, *B. glabrata*, mesmo na ausência de encapsulamento por hemócitos, indicando a possibilidade de um mecanismo controle do parasito sem ação direta dos componentes celulares. Recentemente, SAAP & LOKER (2000) demonstraram que na maioria das interações de gastropoda-digenéicos incompatíveis, fatores solúveis da hemolinfa, não relacionados a osmolaridade, matam o esporocisto após 6 h de incubação *in vitro*, mas a natureza destes fatores não foi estabelecida. Assim, apesar de várias evidências experimentais da participação direta de elementos da hemolinfa acelular de *Biomphalaria* na destruição de esporocistos de *S. mansoni*, a natureza desta atividade ainda não foi identificada.

Alguns trabalhos (GRANATH & SPRAY, 1987; SPRAY *et al*, 1988) objetivaram identificar diferenças, através de eletroforese ou Western blot, na composição das proteínas da hemolinfa de linhagens resistentes e suscetíveis de *B. glabrata* que justificassem a

proteção; entretanto, apesar de algumas diferenças serem relatadas na concentração total de proteínas solúveis e no perfil dos polipeptídios examinados, não foi possível relacionar estas diferenças com o mecanismo de proteção.

Com o intuito de identicar genes associados com o controle da infecção por larvas de trematódeos em *Biomphalaria* estudos recentes focam no sequenciamento de ESTs ("expression sequence tag") em biblioteca gênicas construidas a partir de cDNA de hemócitos circulantes, que são considerados os principais efetores do sistema interno de defesa dos moluscos. Alguns genes com produção aumentada durante a infecção por trematódeos foram identificados e pertencem a diferentes grupos funcionais, sendo detectadas sequências com homologia a integrinas, lectinas, peptídeos anti-microbianos, proteínas associadas ao estresse oxidativo, glicosidases, proteases e inibidores de proteases (MITTA *et al*., 2005; GUILHOU *et al*., 2007; LOCKYER *et al* ., 2007; ZHANG *et al*., 2004). Entretanto, a participação da maioria destes grupos funcionais de genes no controle da infecção por larvas de trematódeos dependem da ação associada dos hemócitos e não poderiam justificar a efeito direto da hemolinfa acelular na mortalidade dos esporocistos verificada *in vitro*. Assim, nesta etapa dos experimentos verificamos a atividade proteolítica da hemolinfa acelular das diferentes espécies e linhagens de *Biomphalaria,* desde que este grupo de proteínas poderia ter um efeito direto no tegumento do esporocisto, como já demonstrado durante a infecção (BLANDIN *et al*., 2004)

De acordo com nossos resultados foi verificado que na fração acelular da hemolinfa de *B. glabrata* foi detectada pequena atividade protelítica frente a diferentes substrados e esta atividade foi bloqueada na presença de inibidores de cisteino-, aspartil- e serino- proteases, sugerindo a atividade destas proteases possam estar associadas. Em contraste, a hemolinfa acelular de *B. tenagophila,* Cabo Frio e Taim, apresentou atividade

proteolítica estatisticamente superior a atividade especifica detectada em *B. glabrata*, que foi sobrou apenas redução parcial na presença de inibidores de cisteíno- e serino-proteases, mas não de aspartil-proteases. Estes dados claramente indicam que a atividade protelíca da hemolinfa acelular de *B. tenagophila* e *B. glabrata* é qualitativa e quantitativamente diversa. Além disto, nossos dados também sugerem que a atividade proteolíca da hemolinfa acelular de *B. tenagophila* Taim, linhagem resistente à infecção natural por *S. mansoni*, é qualitativamente superior à detectada em *B. tenagophila* Cabo Frio.

Além da produção pelo hospedeiro, vários autores (LODES & YOSHINO, 1989; YOSHINO *et al*., 1993; ZELCK & VON JANOWISKY, 2004) relataram atividade proteolítica, especialmente cisteíno-protease, nos produtos excretados e secretados de *S. mansoni*, que estariam envolvidos na penetração e transformação do miracídio de *S. mansoni* e na aquisição de nutrientes pelo parasito. A produção de proteases e inibidores de proteases pelo parasito também pode estar associada a supressão do sistema interno de defesa do molusco (YOSHINO *et al*., 1993).

Espécies do gênero *Biomphalaria* possuem sistema circulatório aberto, assim as diferentes classes de proteases, cuja atividade foi detectada na hemolinfa, podem ter sido produzidas em diferentes locais do corpo do caramujo e por diferentes células ou tecidos, não necessáriamente estando associada com o controle de parasito. Desta forma, parte da atividade de cisteíno-protease detectada na fração acelular da hemolinfa de *B. glabrata* e de *B. tenagophila* pode ser devido a catepsinas, que são produzidas em abundância na região das glândulas digestivas (ALVES *et al*., 2007; MYERS *et al*., 2008), e atingem a hemolinfa circulante (ALVES *et al*., 2007). MYERS *et al*. (2008) reportaram que a atividade proteolítica desta catepsina foi maior na glândula digestiva (hepatopâncreas) de *B. glabrata*

da linhagem BS-90 (resistente ao parasito) que na linhagem M-line, que é altamente suscetível à infecção por *S. mansoni*.

Diferença no nível de atividade proteolíca na hemolinfa também pode estar associada com a produção de inibidores de proteases. Em *B. glabrata* FRYER *et al*. (1996), verificaram que a atividade de cisteíno-protease presente na hemolinfa circulante estava associada com a presença de α-Macroglobulina, que inibia a atividade de cisteíno-protease. Posteriormente, VERGOTE *et al* (2004) demonstraram que o perfil protéico obtido nos estudos de proteoma apresentavam pequenas diferenças entre linhagens de *B. glabrata* resistentes e suscetíveis ao trematódeo *E. caproni*, sendo que inibidores de cisteíno-protease e enzimas glicolícas foram identicadas entre as proteinas que apresentavam-se diferentemente expressas na hemolinfa dos diferentes moluscos.

Nossos dados mostraram que a atividade proteolítica da hemolinfa de *B. glabrata* foi menor que em *B. tenagophila* e não interefere na sobrevivência dos esporocistos de *S. mansoni* cultivados *in vitro*. Em contraste, a atividade proteolítica detectada na hemolinfa de *B. tenagophila* foi maior e associada com a suscetibilidade das linhagens à infecção por *S. mansoni*, sendo mais intensa na linhagem Taim. Interessantemente, o uso de inibidores de serino-protease bloqueou a mortalidade de esporocistos induzida diretamente por hemolinfa acelular.

BAHGAT *et al*., (2002) relatam detectaram atividade de uma serino protease no lisado de hemócitos de *B.glabrata* resistente e suscetível à infecção por *S. mansoni*, embora não tenha sido possível verificar diferenças na atividade proteolica entre as espécies de *B.glabrata* suscetível e resistente, não sendo possivel associar esta atividade com controle do parasito. MITTA *et al* (2005), avaliando gene relacionados a resposta de *B. glabrata*

frente a infecção por *E. caproni* em biblioteca de cDNA de hemócitos também identificaram gnes com homologia a enzimas envolvidas na cascata de coagulação de mamíferos, metalo-proteases e inibidores de serino-proteases, alem de lisozimas, cuja atividade já havia sido demonstrada na fração solúvel da hemolinfa e nos grânulos dos granulócitos de caramujos desafiados com patógenos, inclusive *S. mansoni* (CHENG *et al.*,1978). Usando a técnica de hidrização subtrativa de bibliotecas de cDNA obtida de *B. glabrata* suscetivel e resistante e exposto e não exposto a *S. mansoni*, LOCKYER *et al* (2007) identificaram um serino-protease (HtrA2) associada a resposta de estresse oxidativo, que apresentou elevados níveis de transcritos em caramujo resistentes e expostos ao parasito.

Desta forma, os resultados aqui apresentados sugerem que a atividade proteolítica da hemolinfa de *B. tenagophila* pode estar associada com mecanismos de destruição de esporocistos de *S. mansoni* por moluscos de linhagens resistentes e serão mais bem avaliados neste estudo.

Parte III

Fracionamento e caracterização da hemolinfa de *B. tenagophila* Taim e sua atividade sobre esporocistos de *S. mansoni*

6.1. Introdução

A linhagem de *B. tenagophila* isolada da reserva do Taim no Rio Grande do Sul (*B. tenagophila* Taim) tem se mostrado completamente resistente à infecção por diferentes cepas de *S. mansoni* (SANTOS *et al*., 1979; COELHO *et al*., 2008). Apesar de não terem sido recuperados esporocistos secundários ou cercárias de *B. tenagophila* Taim experimentalmente infectado por *S. mansoni* (MARTINS-SOUZA *et al*., 1999; 2003; PEREIRA *et al*., 2008), as análises histológicas mostram que o parasito é capaz de penetrar nesta linhagem de caramujo, induzindo uma intensa resposta celular que resulta na sua completa destruição poucas horas após a infecção (MARTINS-SOUZA, 1999; NEGRÃO-CORRÊA *et al.*, 2007).

A transferência da região pericárdica, local de formação de hemócitos (APO – amebocite producing Organ), de *B. tenagophila* da linhagem resistente do Taim para a linhagem suscetível de Cabo Frio, resultou em completa proteção contra infecção por *S. mansoni* nos receptores Cabo Frio, quando o transplante teve sucesso, demonstrando a importância dos hemócitos no mecanismo de proteção (BARBOSA *et al*., 2006). Além de hemócitos, a inoculação da fração acelular da hemolinfa de *B. tenagophila* Taim em *B. tenagophila* Cabo Frio resultou em aumento de resistência dos receptores à infecção por *S. mansoni*, indicando que fatores solúveis presentes na hemolinfa de *B. tenagophila* Taim também participam do mecanismo de proteção (PEREIRA, 2005; COELHO & BEZERRA, 2006; PEREIRA *et al*., 2008). Estudo funcional realizado *in vitro* revelou que componentes presentes na fração acelular da hemolinfa de *B. tenagophila* Taim podem diretamente lesar o tegumento de esporocistos axenicamente transformados provocando a

morte de alguns parasitos (PEREIRA *et al*., 2008; Parte I da tese). Os resultados anteriormente apresentados (Parte II) indicam que a lesão direta no tegumento de esporocistos de *S. mansoni* esta associada à atividade de serino-proteases presentes na fração acelular de *B. tenagophila* Taim. Baseado nestes dados apresentados anteriormente, nesta fase do trabalho pretende-se fracionar a hemolinfa de *B. tenagophila* Taim e caracterizar bioquímica e funcionalmente as diversas frações que atuam sobre esporocistos recém-transformados de *S. mansoni*.

6.2. Material e métodos

6.2.1 Caramujos e Parasito

Nesta etapa do trabalho foram utilizados exemplares de *B. tenagophila* da linhagem do Taim para obtenção da fração acelular de hemolinfa e *B. tenagophila* da linhagem Cabo Frio para obtenção de hemócitos utilizados em interações *in vitro*. Os caramujos são mantidos nos moluscários por mais de 40 anos no laboratório de Esquistossomose ICB – UFMG conforme procedimento descrito por PELLEGRINO & KATZ (1968). Os exemplares utilizados nos experimentos apresentavam 12-14 mm de diâmetro e foram inicialmente submetidos a um procedimento de limpeza (com solução de detergente 0,1 %) e desinfecção (inicialmente com solução 0,1 % de Benzoilmetronidazole, seguido por 30 µg/ml Gentamicina e 100 µg/ml Ampicilina).

Nos testes de interação *in vitro* foram utilizados esporocistos de *S. mansoni* da cepa LE, isolada de um paciente, Luis Evangelista, de Belo Horizonte – MG, e vem sendo mantida em condições laboratoriais através de sucessivas passagens em hamsters

(*Mesocricetus auratus*) e *B. glabrata* de acordo com a técnica descrita por PELLEGRINO & KATZ (1968).

6.2.2 Coleta de hemolinfa para análise

A hemolinfa de *B. tenagophila* Taim foi coletada por punção na região cardíaca, de acordo com o protocolo de ZELCK *et al* (1995) como detalhada anteriormente. Para os experimentos realizados nesta fase a hemolinfa foi coletada de 60 caramujos até completar um volume total de 5 mL de hemolinfa total. A hemolinfa coletada de cada animal foi imediatamente transferida para tubos de polipropileno mantidos em banho de gelo. Para alguns experimentos a hemolinfa coletada foi transferida na presença de AESBF, na concentração final de 6mM. Após a coleta, a hemolinfa total foi centrifugada a 80 x g, por 10 min e 4 ºC e o sobrenadante, designado como fração acelular, foi utilizado para fracionamento e testes biológicos.

6.2.3 Processamento da hemolinfa

Ultracentrifugação:

A fração acelular foi ultracentrifugada a 100.000 x g, por 2h e 30 min a 4 ºC para a separação da hemoglobina ZELCK *et al* (1995). O sobrenadante resultante da ultracentrifugação foi separado e o precipitado, contendo principalmente hemoglobina, foi ressuspendido em CBSS até o volume inicial de 5 mL. As amostras foram mantidas a 4 ºC até serem utilizadas.

Precipitação com sulfato de amônio:

A fração solúvel da hemolinfa de *B. tenagophila* Taim após ultracentrifugação bem como as diferentes frações recolhidas após aplicação de hemolinfa à coluna de exclusão molecular (Superdex 75 HR 10/30) foi precipitada com sulfato amônio a 80% de saturação segundo JANSON AND RYDÉN (1998). Resumidamente, o sal foi adicionado aos poucos sobre o volume conhecido da amostra, em banho de gelo, e homogeneizado levemente com auxílio de um agitador magnético. Após a completa dissolução do sal, o material permaneceu em repouso por 4 h a 4 ºC, logo em seguida foi centrifugado por 1 h a 5000g e o sobrenadante transferido para um membrana de diálise (poros de exclusão de 12000 Da; Sigma São Louis). A proteína contida no precipitado após a preciptação com o sulfato de amônio foram ressuspendidas em 1 mL de CBSS e também transferido para uma membrana de diálise. As membranas contendo as frações proteícas e não protéicas de cada amostra de hemolinfa foram dialisadas contra 2 L de tampão CBSS pH 7,2 por 24 h a 4 ºC e mantidas sob agitação magnética, com troca do tampão de 12 em 12 h. As amostras processadas da hemolinfa acelular foram recolhidas do interior da membrana de diálise com o auxílio de uma seringa de 1 mL estéril e imediatamente testadas em ensaios *in vitro* .

Dosagem de carboidratos:

Após precipitar com sulfato de amônio a fração ultracentrifugada da hemolinfa de *B. tenagophila* Taim, a presença de carboidratos no sobrenadante desta amostra foi determinada pelo método de Antrona (YEMM & WILLIS., 1954). Resumidamente, um tubo de vidro tipo pirex (15mL) contendo 2 mL da solução de 0,2% de antrona diluída em ácido sulfúrico foi

acondicionado em banho de gelo por 5 min. Logo após este período, cerca de 1 mL da amostra foi adicionada ao tubo sob leve agitação manual seguida da incubação a 100 ºC por 20 minutos até o desenvolvimento da cor verde. A reação de antrona se baseia na ação hidrolítica e desidratante do acido sulfúrico concentrado sobre os carboidratos. Quando a reação é levada a efeito na presença de carboidratos com ligações glicosídicas, estas são hidrolisadas e os açucares simples desidratados para furfural ou hidroximetilfurfural que condensam com o antrona (9,10-dihidro-9-oxoantraceno), gerando um produto de coloração azul petróleo. Os açucares totais foram quantificados por espectofometria (620nm), utilizando uma curva padrão de glicose (100µg /mL) de intervalo de 20-100µg.

Fracionamento da hemolinfa acelular de *B. tenagophila* Taim

Inicialmente, a fração acelular da hemolinfa *B. tenagophila* Taim ultracentrifugada foi separada em coluna de exclusão molecular, utilizando gel de filtração Superdex 75 10/30 HR (Amershn biosciences). Para tanto uma coluna de foi montada acoplada ao sistema FPLC (equipamento de cromatografia líquida de alto rendimento (Farmacia). A coluna foi equilibrada com tampão CBSS, pH 7,2 e uma amostra de 1 mL de hemolinfa ultracentrifugada foi aplicada à coluna. A eluição das proteínas foi realizada com adição de tampão CBSS, pH 7,2 e as frações que absorveram a 280nm foram coletadas em um fluxo de 0.5 mL/min, a 4 ºC. As frações que apresentaram leitura de absorbância a 280nm foram então utilizados nos testes *in vitro* contra os esporocistos de *S. mansoni*. A fração da hemolinfa coletada da separação em coluna de exclusão e que apresentou atividade biológica foi em seguida aplicada a uma coluna de troca iônica. Para esta etapa foi utilizada uma coluna MONO-Q Sepharose HR 5/5, que foi equilibrada com

tampão CBSS pH 7,2 e acoplada ao FPLC com um fluxo de 1mL /min. Após a aplicação da amostra a eluição foi realizada com tampão CBSS, pH, 7,4 com gradiente de 0 a 1mol/L de NaCl, a 4 ºC. Mais uma vez, as subfrações foram novamente testadas contra os esporocistos de *S. mansoni*.

6.2.4 Perfil eletroforético e atividade proteolítica das frações da hemolinfa de *B. tenagophila* Taim

Paralelamente aos ensaios funcionais, uma alíquota das frações obtida com a separação da hemolinfa acelular em coluna de exclusão molecular e posteriormente em coluna de troca iônica também foi submetida à separação em gel de poliacrilamida a 10% (LAEMMLI, 1970). A atividade proteolítica destas mesmas frações foi avaliada através da digestão de gelatina co-polimerizada em gel poliacrilamida, segundo técnica originalmente descrita por HEUSSEN AND DOWDLE (1980) e detalhada anteriormente (item 5. 2.5.). Para tanto, uma amostra de 30µl de cada fração da hemolinfa foi diluída em tampão Tris-HCl 0,125 M, pH 6,8 sem adição de agentes redutores e aplicada ao gel (SDS_PAGE) co-polimerizado com 0.1% gelatina. A separação eletroforética foi realizada em sistema mini-VE (Hoefer Pharmacia Biotech, San Francisco, CA, USA) e corrente de 80 V. Após a retirada do SDS, o gel foi fixado e corado com azul de Coomassie para evidenciar áreas claras indicativas de digestão enzimática.

6.2.5 Avaliação funcional das frações da hemolinfa de *B. tenagophila* Taim

Durante todo processo de fracionamento da fração acelular da hemolinfa de *B. tenagophila* Taim a atividade dos componentes envolvidos no mecanismo de destruição de larvas de *S. mansoni* foi avaliada por meio de ensaio de viabilidade de esporocistos *in vitro* detalhado anteriormente no item 4.2.3 (PEREIRA *et al*., 2008). Para tanto, miracídios obtidos de ovos coletados do fígado de hamsters infectados foram axenicamente transformados em esporocistos (SAMUELSON *et al*., 1984). Os esporocistos obtidos foram lavados em tampão CBSS, concentrados e quantificados para os ensaios *in vitro*. Os ensaios foram realizados, em triplicatas, em placas de 96 poços contendo 50 esporocistos de *S. mansoni* cultivados em 250 µl de CBSS suplementado, na presença ou ausência de hemolinfa acelular ou de amostras obtidas a partir do fracionamento da hemolinfa acelular de *B. tenagophila* Taim (20% v/v). Após 6 - 24 h de incubação à 26 ºC em estufa de CO_2 a 5 ºC, foi adicionado a cada tratamento solução de Azul de Tripan e a viabilidade das larvas foi estimada pela contagem de esporocistos íntegros e não corados. Para confirmar a atividade de algumas subfrações da hemolinfa acelular de *B. tenagophila* do Taim sob o tegumento de esporocistos de *S. mansoni* cultivados foi acrescentado 5 µl da sonda *Hoechst 33258* (2 µg/µl) a cada tratamento e, após 10 min, a integridade do tegumento de esporocistos foi avaliada em microscopia de Fluorescência (Olympus IX70 filtro WU), conforme descrito no item 4.2.3.

6.2.6. Análise estatística

Os resultados obtidos em cada tratamento foram estatisticamente comparados, utilizando-se análise de variância para dados paramétricos (ANOVA, post teste Tukey). Valores de $P \leq 0.05$ foram considerados estatisticamente diferentes.

6.3 Resultados

A primeira etapa do fracionamento da hemolinfa acelular foi a ultracentrifugação e a avaliação da atividade funcional destas frações. Após a ultracentrifugação, a concentração total de proteínas solúveis presentes na hemolinfa de *B. tenagophila* da linhagem Taim é reduzida de 10,1 mg/mL para 4,5 mg/mL, indicando que a hemoglobina representa a maior parte das proteínas na hemolinfa destes moluscos.

Conforme mostrado na figura 9, a adição de 20 % do sobrenadante da hemolinfa acelular após a ultracentrifugação induziu aumento significativo da mortalidade de esporocistos axenicamente transformados em relação ao controle contendo apenas CBSS suplementado, sendo que a mortalidade neste grupo foi semelhante à induzida pela adição de hemolinfa total. Posteriormente, a hemolinfa ultracentrifugada foi submetida à precipitação protéica com sulfato de amônio seguido de diálise contra tampão CBSS para retirada do excesso de sal, que é tóxico ao esporocisto. A adição do precipitado protéico da fração ultracentrifugada da hemolinfa de *B. tenagophila* Taim às culturas de esporocistos resultou em aumento significativo de mortalidade do parasito, enquanto que nas culturas que receberam amostras de sobrenandante não protéico da hemolinfa apresentaram índices de

sobrevivência de esporocistos semelhantes aos controles mantidos em CBSS suplementado (figura 9). Estes dados indicam que a fração protéica sem hemoglobina da hemolinfa de *B. tenagophila* Taim é responsável pela destruição de esporocistos de *S. mansoni*.

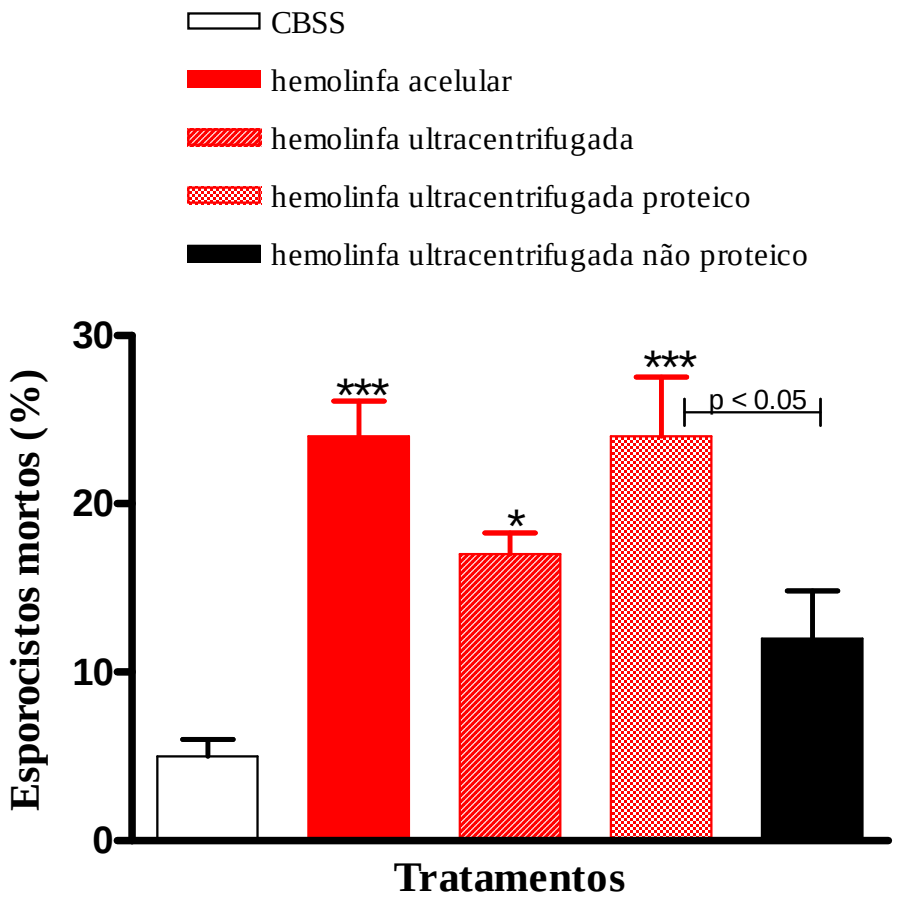

Figura 9: Taxa de mortalidade dos esporocistos de *S. mansoni* cultivados em CBSS suplementado contendo ou não hemolinfa acelular de *B. tenagophila* da linhagem Taim, submetidas a diferentes tratamentos. Tratamentos: Controles: **CBSS-** representa os esporocistos cultivados somente na presença de CBSS suplementado; **Hemolinfa acelular** - esporocistos cultivados somente na presença de CBSS suplementado contendo 20% (v/v) da amostra de hemolinfa total acelular; **Hemolinfa ultracentrifugada** - esporocistos cultivados somente na presença de CBSS suplementado contendo 20% (v/v) da amostra de hemolinfa total acelular ultracentrifugada; **Hemolinfa ultracentrifugada protéico** - esporocistos cultivados somente na presença de CBSS suplementado contendo 20% (v/v) da amostra protéica da hemolinfa ultracentrifugada; **Hemolinfa ultracentrifugada não protéico** - esporocistos cultivados somente na presença de CBSS suplementado contendo 20% (v/v) da amostra não protéica da hemolinfa ultracentrifugada. A porcentagem de esporocistos mortos era calculada para cada tratamento após 18 h de incubação, baseado no número de esporocitos viaveis no início e no final da incubação. Diferenças estatísticas na taxa de mortalidade dos esporocistos nos diferentes tratamentos em relação ao controle cultivado em CBSS estão indicadas por * p≤ 0,05 ou *** p≤ 0,001.

Na etapa seguinte, a hemolinfa ultra-centrifugada foi fracionada em cromatografia de exclusão por peso molecular (figura 10). As proteínas da hemolinfa ultracentrifugada foram recuperadas entre as frações 5 a 15, sendo que as frações 6 e 7 apresentaram maior concentração protéica (0,5 mg/mL).

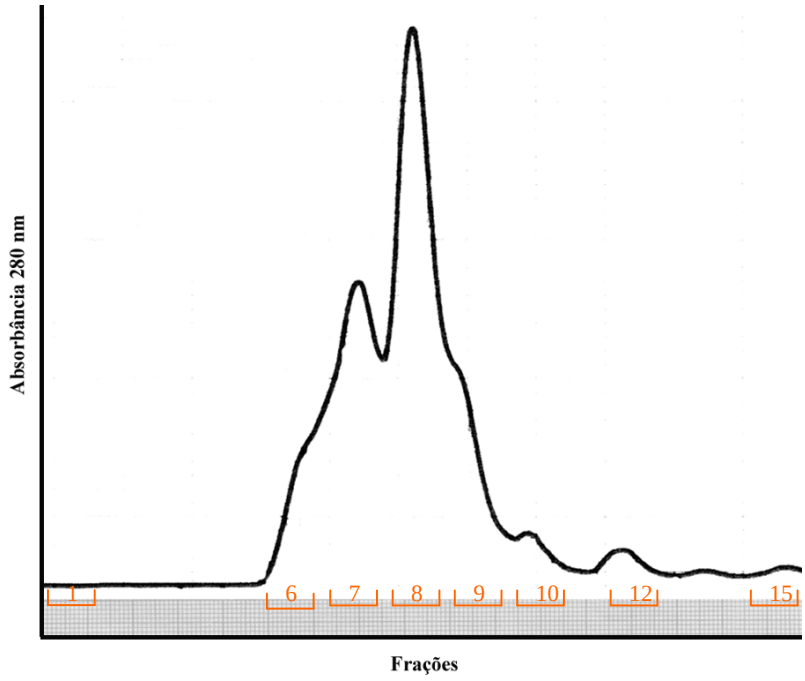

Figura 10: Curva de eluição da hemolinfa ultracentrifugada de *B.tenagophila* do Taim durante o fracionamento em cromatografia de exclusão molecular, em coluna superdex 75. Utilizamos o tampão CBSS e um fluxo de 1mL/min sendo que o procedimento foi conduzido a 4 ºC. A linha do gráfico representa a leitura obtida de cada fração recolhida à 280nm.

As diferentes frações recuperadas da coluna de exclusão molecular e que apresentaram leitura de absorbância em 280 nm, juntamente com uma amostra de tampão recolhida no inicio da eluição (fração1) foram avaliadas funcionalmente sob esporocistos de *S. mansoni* cultivados *in vitro* (figura 11). Esporocistos de *S. mansoni* cultivados por 24 h em CBSS suplementado contendo 20% da amostra coletada na fração 7, fração 9 ou fração 12 apresentaram taxa de mortalidade significativamente aumentada em relação ao controle contendo somente CBSS suplementado. Todas as demais frações examinadas não alteraram a taxa de mortalidade dos esporocistos cultivados *in vitro*. Apesar das frações 9 e 12 resultarem em aumento de mortalidade de esporocistos, a maior atividade estava concentrada na fração 7 (figura 11). A precipitação dos componentes protéicos presentes na hemolinfa ultracentrifugada de *B. tenagophila* Taim e nas diferentes frações obtidas da cromatografia de exclusão molecular confirmaram que a capacidade de lesar os esporocistos de *S. mansoni* foi recuperada com a adição dos componentes protéicos da fração 7 (figura 12). É importante ressaltar que mesmo contendo cerca de um décimo das proteínas totais da hemolinfa ultracentrifugada (4,5 mg proteínas/mL na hemolinfa ultracentrifugada e 0,5 mg/mL na fração 7), as proteínas recuperadas na fração 7 induziram taxa de mortalidade de esporocistos similares à detectada no tratamento contendo os componentes protéicos da hemolinfa ultracentrifugada (figura12).

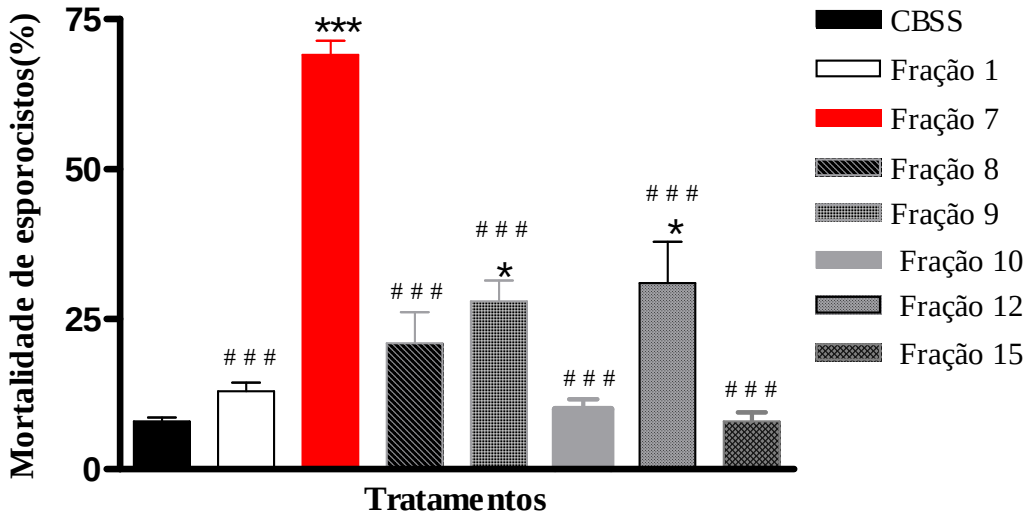

Figura 11: **Taxa de mortalidade dos esporocistos de *S. mansoni* cultivados *in vitro* em CBSS suplementado na presença ou ausência de sub-frações da hemolinfa de *B.tenagophila* do Taim obtidas da coluna de exclusão molecular. Tratamentos: Controle**s: **CBSS-** representa os esporocistos cultivados somente na presença de CBSS suplementado; **, Fração 1** - esporocistos cultivados somente na presença de CBSS suplementado contendo o tampão utilizado no equilíbrio da coluna, **Fração 7 a 15-** esporocistos cultivados somente na presença de CBSS suplementado contendo 20% (v/v) da amostra de hemolinfa coletada na fração de numero correspondente. A porcentagem de esporocistos mortos era calculada para cada tratamento realizado em triplicata e os resultados demonstrados em média ± desvio padrão de três experimentos independentes. Diferenças estatísticas na taxa de mortalidade dos esporocistos nos diferentes tratamentos em relação ao controle cultivado em CBSS estão indicadas por * $p \leq 0,05$ ou *** $p \leq 0,001$; e entre **Fração 7** com os outros tratamentos com as frações estão indicados por # $p \leq 0,05$.

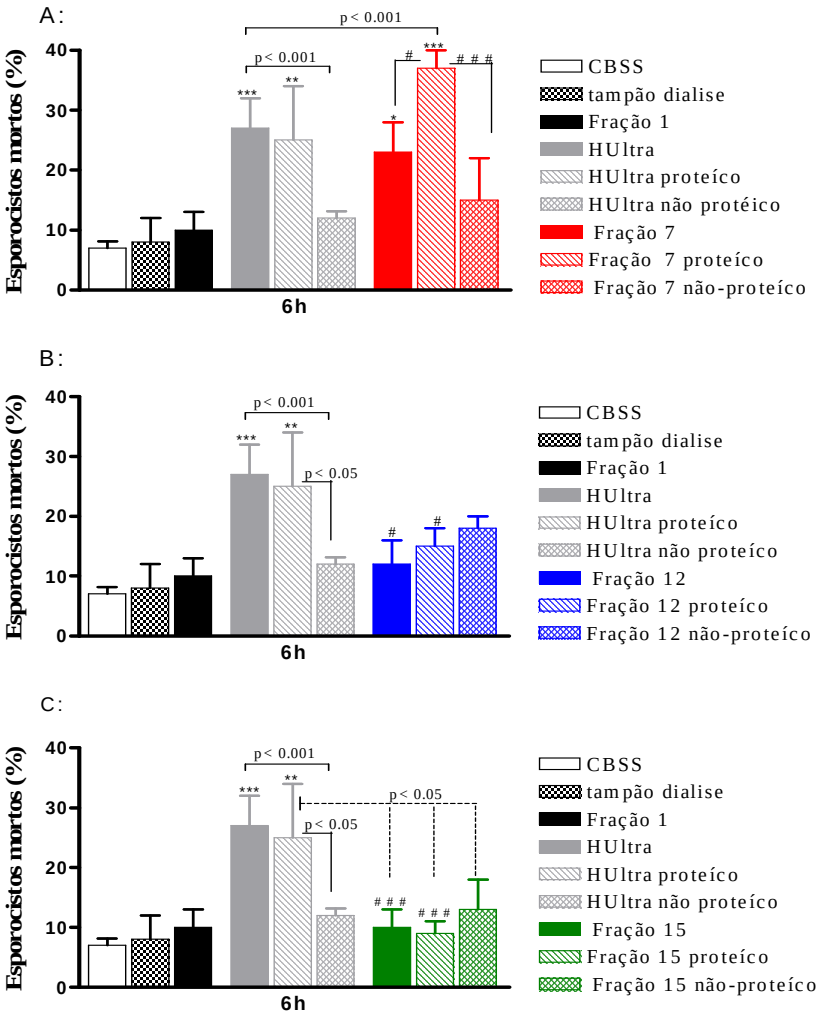

Figura 12: **Taxa de mortalidade dos esporocistos de *S. mansoni* cultivados *in vitro* em CBSS suplementado na presença das sub-frações 7 (A), 12 (B), 15 (C) da hemolinfa acelular de *B.tenagophila* do Taim obtidas da coluna de exclusão molecular e precipitadas com sulfato de amônio. CBSS**- representa os esporocistos cultivados somente na presença de CBSS suplementado; **Fração 1** - esporocistos cultivados com CBSS e tampão utilizado no equilíbrio da coluna, **Hultra** - esporocistos cultivados com CBSS e hemolinfa ultracentrifugada de *B.tenagophila* do Taim, **HUltra protéica** - esporocistos cultivados com CBSS e proteínas de Hultra precipatadas com sulfato de amônio, **HUltra não protéica** - esporocistos cultivados com CBSS e sobrenadante da hemolinfa ultracentrifugada após precipitação com sulfato de amônio, **Fração 7, 12 e 15** - esporocistos cultivados em CBSS suplementado contendo 20% (v/v) da amostra de hemolinfa coletada na fração correspondente após separação em coluna de exclusão molecular, **Fração 7, 12 e 15 protéica** - esporocistos cultivados com CBSS suplementado e precipitado protéico destas frações, **Fração 7, 12 e 15 não protéica** - esporocistos cultivados com CBSS suplementado e sobrenadante destas frações após após precipitação com sulfato de amônio. A porcentagem de esporocistos mortos foi calculada para cada tratamento realizado em triplicata e os resultados demonstrados em média ± desvio padrão de três experimentos independentes. As diferenças estatisticamente significantes quando comparamos o controle **CBSS, tampão diálise, fração 1** - versus o tratamentos e estão indicados por *** p≤ 0,001; e entre **Frao 7** com os outros tratamentos com as frações estão indicados por # p≤ 0,05.

As mesmas frações recuperadas da coluna de exclusão molecular foram avaliadas quanto à atividade proteolítica. Conforme verificado na figura 14, a degradação da gelatina co-polimerizada em gel de acrilamida foi observada na canaleta 2 contendo a fração 7. As demais frações obtidas na cromatografia de exclusão molecular não apresentaram atividade proteolítica neste teste. Mais uma vez a atividade proteolíca detectada na fração 7 foi inibida quando a hemolinfa foi coletada na presença de 6 mM de AEBSF, inibidor de serino-protease.

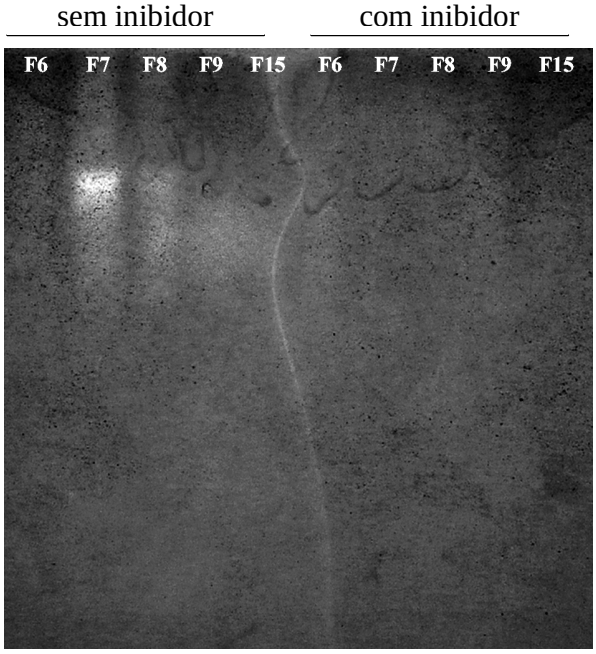

Figura 13: Padrão de digestão enzimática das frações de hemolinfa acelular e ultracentrifugada de *B.tenagophila* do Taim obtidas após fracionamento em coluna de exclusão molecular. F6, F7, F8, F9, F15 corresponde a frações de hemolinfa acelular de número correspondente coletada após separação em coluna de exclusão molecular. As amostras, inicialmente coletadas na presença ou ausência de 6 mM de AEBSF, foram fracionadas, diluídas 1:2 em tampão de amostra sem adição de agente redutor e aplicadas em gel SDS-PAGE co-plimerizado com 1 % de gelatina. Condições de corrida e revelação da digestão foram realizadas conforme descrito em Material e Método.

Como foi constatado que os componentes protéicos da fração 7 recuperada da cromatografia de exclusão da hemolinfa ultracentrifugada concentravam a atividade proteolítica e atividade anti-esporocisto contida em *B. tenagophila* Taim foi decidido testar a associação destas funções. Para tanto, hemolinfa de *B. tenagophila* Taim foi coletada, na ausência e na presença de inibidor de protease (AEBSF), fracionada em cromatografia de exclusão molecular. As subfrações obtidas de hemolinfa coletada com e sem inibidor foram simultaneamente testadas em culturas de esporocistos de *S. mansoni*. A figura 14 confirma dados anteriores que mostram que a adição da fração 7, mas não da fração 8 e 9, induziu aumento significativo da mortalidade de esporocistos cultivados em CBSS. Entretanto, o aumento de mortalidade de esporocistos induzido pela adição da fração 7 da hemolinfa não aconteceu quando a hemolinfa foi coletada na presença de inibidor de serino-protease (AEBSF) (figura 14). Estes dados confirmam os resultados anteriores (figura 8) onde foi detectado que o efeito lesivo ao esporocisto de *S. mansoni* ocasionado pela hemolinfa acelular não fracionada de *B. tenagophila* era reduzido na presença de inibidor de serino-protease.

A fração 7, que apresenta atividade proteolítica e lesiva ao tegumento do esporocisto foi coletada e re-fracionada em cromatografia de troca iônica, utilizando coluna MONO Q HR 5/5. Na figura 15 está representado o perfil cromatográfico da fração 7 após separação em cromatografia de troca iônica. Em amostra de hemolinfa coletada na ausência de inibidor de protease, o re-fracionamento da fração 7 resultou em 3 picos protéicos, denominados de fração 7.1, 7.2 e 7.3 (figura 15A) enquanto que hemolinfa coletada com inibidor de serino-protease (AEBSF) o perfil protéico foi diferente.

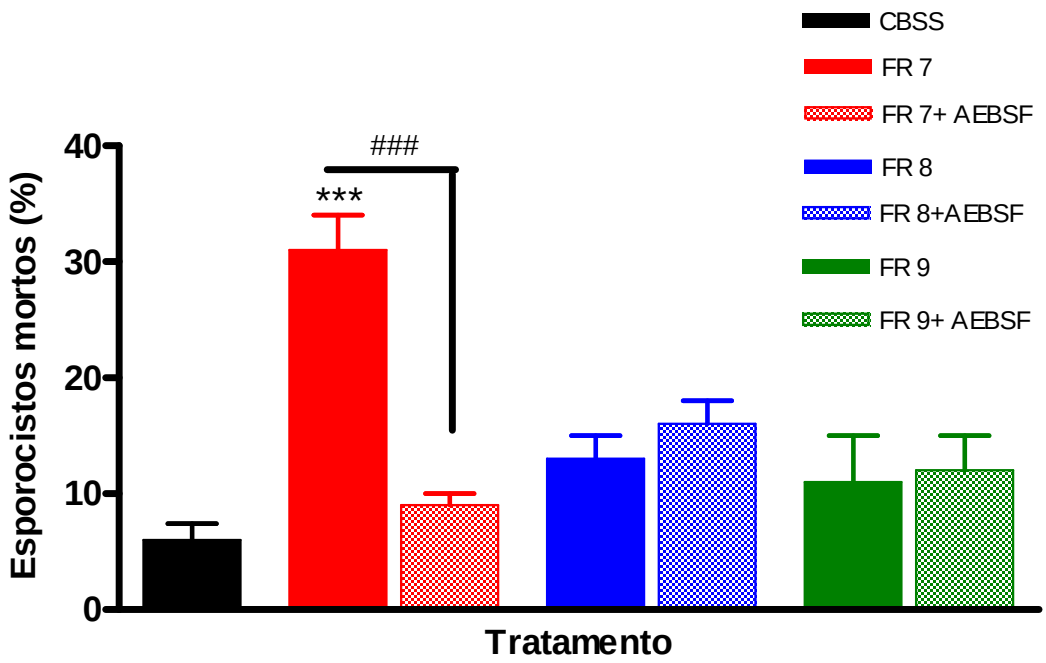

Figura 14: **Taxa de mortalidade dos esporocistos de *S. mansoni* cultivados em CBSS na presença ou ausência de sub-frações da hemolinfa de *B.tenagophila* do Taim coletada na presença ou ausência de AEBSF e fracionadas por cromatografia de exclusão molecular.** **CBSS**- representa os esporocistos cultivados somente na presença de CBSS suplementado; **Fração 7, 8 e 9** - esporocistos cultivados na presença de CBSS suplementado contendo 20% (v/v) da amostra de hemolinfa coletada na fração correspondente após separação em coluna de exclusão molecular , **fração 7, 8 e 9 + AEBSF**- esporocistos cultivados na presença de CBSS suplementado contendo 6mM de AEBSF contendo 20% (v/v) da amostra de hemolinfa coletada na fração de numero correspondente após separação em coluna de exclusão molecular. A porcentagem de esporocistos mortos era calculada para cada tratamento realizado em triplicata e os resultados demonstrados em média ± desvio padrão de três experimentos independentes. As diferenças estatisticamente significantes quando comparamos o controle **CBSS, tampão diálise, fração 1** - versus o tratamentos e estão indicados por *** $p \leq 0,001$; e entre **Frao 7** com os outros tratamentos com as frações estão indicados por # # # $p \leq 0,001$.

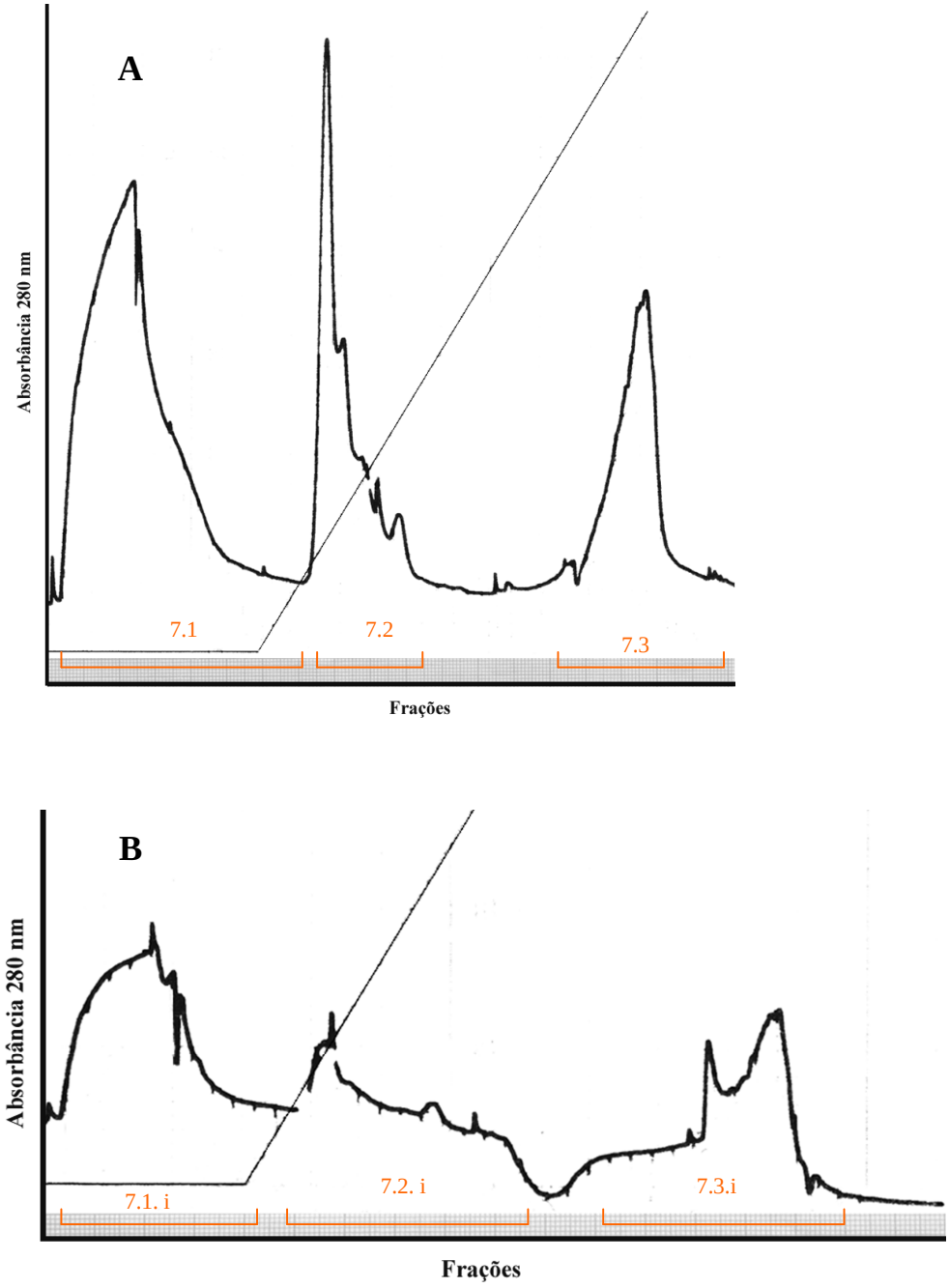

Figura 15: Cromatografia de troca iônica, em resina mono Q, da fração 7 proveniente hemolinfa acelular de *Biomphalaria tenagophila* do Taim. Em 15A a fração 7 foi obtida na ausência de inibidor e 15B na presença de AEBSF. A cromatografia foi conduzida a 4 ºC o monitoramento das frações foi feito a 280nm.

O perfil eletroforético e a atividade proteolítica da fração 7 e das sub-frações 7.1, 7.2 e 7.3 da hemolinfa acelular de *B. tenagophila* Taim estão representados na **Figura 17,** sendo possível verificar que a atividade proteolítica presente na fração 7 foi recuperada na sub-fração 7.2 e a adição de 6 mM de AEBSF mais uma vez inibiu esta atividade

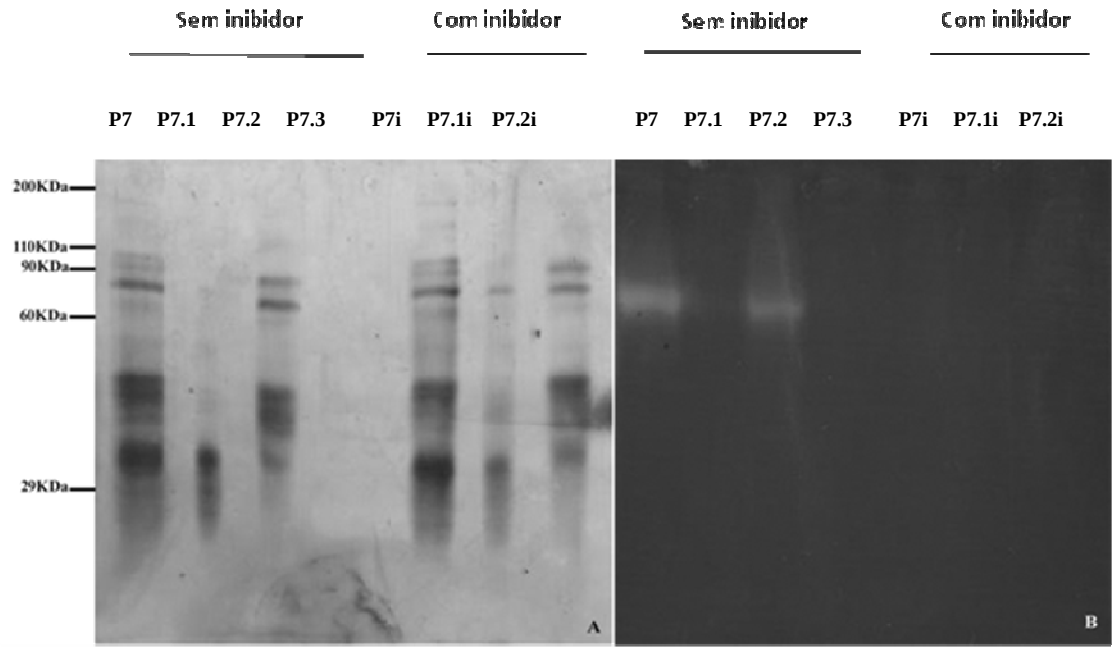

Figura 16: Perfil protéico (A) e atividade proteolíca (B) das proteínas da fração 7 da hemolinfa acelular de *B.tenagophila* Taim e das subfrações obtidas após separação em coluna de troca iônica. Em **A:** proteínas presentes na fração 7 e nas sub-frações 7.1, 7.2 e 7.3 da hemolinfa acelular de *B. tenagophila* coletada na presença ou ausência de 6mM de AEBSF foram eletroforeticamente separadas em SDS-PAGE e coradas com nitrato de prata. Os números à esquerda correspondem ao peso molecular do padrão utilizado. Em **B:** proteínas presentes na fração 7 e nas sub-frações 7.1, 7.2 da hemolinfa acelular de *B. tenagophila* coletada na presença ou ausência de 6mM de AEBSF foram eletroforeticamente separadas em SDS-PAGE co-polimerizado com 1% de gelatina sob condições não denaturantes. Condições de corrida e revelação da digestão foram realizadas conforme descrito em Material e Método. As áreas claras correspondem a locais onde a gelatina contida no gel foi digerida por proteínas com ação proteolíca presente na amostra aplicada.

A figura 17 mostra o efeito da adição da fração 7 e das subfrações 7.1, 7.2, 7.3 de hemolinfa acelular de *B. tenagophila* Taim coletada na ausência ou presença de inibidor de serino protease sobre a mortalidade de *S. mansoni* cultivado *in vitro*. É possível verificar que o aumento de mortalidade de esporocistos de *S. mansoni* induzido pela adição da fração 7 da hemolinfa acelular de *B. tenagophila* Taim foi também detectado nos tratamento contendo as subfrações 7.1 e 7.2 e uma mistura delas, mas não na subfração 7.3 (figura 17 A). É importante salientar a indução de mortalidade de esporocistos nos tratamentos contendo fração 7.1 de hemolinfa acelular sem inibidor, desde que esta amostra não apresenta atividade proteolíca (figura 16B). A presença de inibidor de serino-protease na fração 7 e na mistura de frações obtidas do refracionamento da hemolinfa impede o aumento de mortalidade de esporocistos de *S. mansoni in vitro*. Entretanto, mesmo na presença do inibidor de serino-protease foi possível detectar aumento significativo de mortalidade de esporocistos de *S. mansoni* cultivados na presença das sub-frações 7.1 e 7.2 (figura 17B), sugerindo que a hemolinfa de *B. tenagophila* Taim também apresente elementos independentes da atividade de serino-protease lesivos ao parasito.

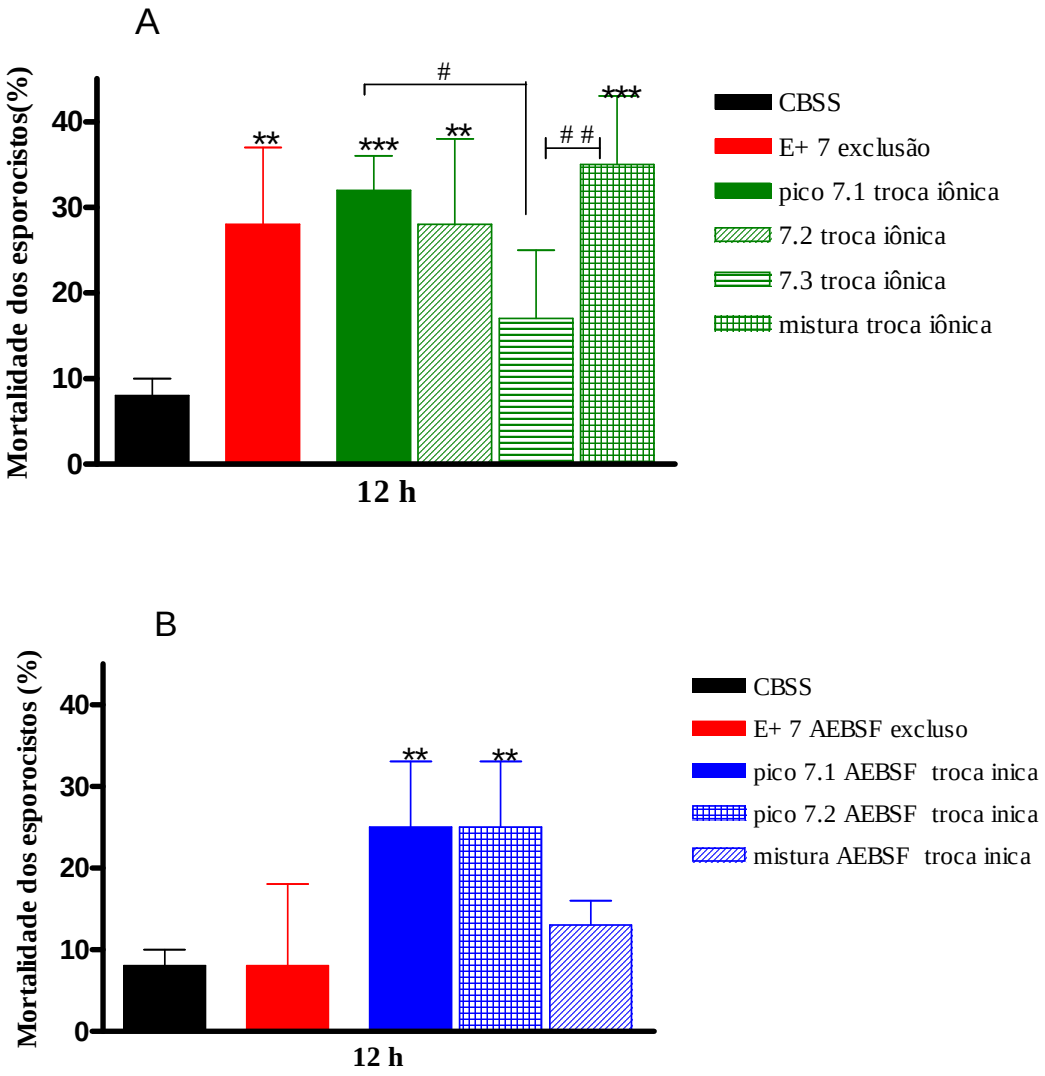

Figura 17: **Taxa de mortalidade dos esporocistos de *S. mansoni* cultivados na presença da fração 7 obtida no fracionamento da hemolinfa acelular de *B. tenagophila* Taim utilizando a cromatografia de exclusão molecular e das sub-frações da fração 7 obtidas pela cromatografia de exclusão molecular.** Em **A** os ensaios foram realizados com frações de hemolinfa coletada na ausência de inibidor de protease. **.** Em **B** os ensaios foram realizados com frações de hemolinfa coletada na presença 6 mM de ABESF. **CBSS**- representa os esporocistos cultivados somente na presença de CBSS suplementado; **Frao 7, 7.1, 7.2 e 7.3**- esporocistos cultivados na presença de CBSS suplementado contendo 20% (v/v) da amostra de hemolinfa coletada na fração de numero correspondente. A porcentagem de esporocistos mortos era calculada para cada tratamento realizado em triplicata e os resultados demonstrados em média ± desvio padrão de três experimentos independentes. As diferenças estatisticamente significantes quando comparamos o controle **CBSS** - **Frao 7** versus o tratamentos e estão indicados por ** $p \leq 0,01$; e entre **Frao 7.** com os outros tratamentos com as frações estão indicados por # # $p \leq 0,01$ # # # $p \leq 0,001$.

A utilização da sonda fluorescente *Hoechst 33258* confirmaram a lesão de tegumento de esporocistos cultivados na presença de frações da hemolinfa acelular de *B. tenagophila* Taim que apresentam atividade proteolítica (figura 18). Desta forma, esporocistos incubados com meio de cultura não apresentaram fluorescência após a adição da sonda *Hoechst 33258* (figura 18A e B), confirmando a integridade do tegumento destas larvas. Em contraste, nas culturas que receberam a fração ou as subfrações da hemolinfa de *B. tenagophila*, proveniente de caramujos da linhagem Taim, alguns esporocistos apresentaram fluorescência característica da incorporação da *sonda Hoechst 33258* (figura 18 C, D, E e F), sugerindo mais uma vez a mortalidade do esporocisto seja conseqüência de lesões tegumentares provocadas por elementos presentes na hemolinfa destes hospedeiros.

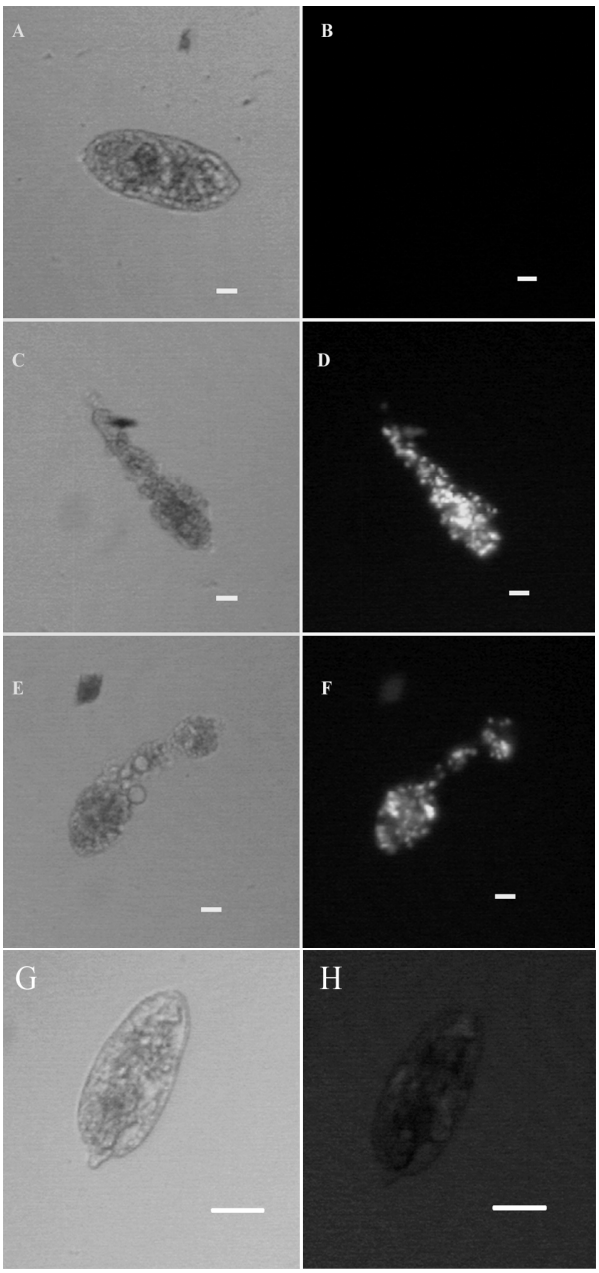

Figura 18: Padrão de marcação dos esporocistos de *S. mansoni*, após incubação com o marcador de fluorescência Hoechst 33258. Em A, C e E esporocitos cultivados *in vitro* por 6 h foram fotografados em campo claro e em B, D e F o mesmo material foi fotografado com filtro para fluorescencia. Em A e B representa um esporocisto incubado com meio de cultura (CBSS suplementado), mostrando esporocistos integros e ausência de incorporação da sonda. Em C, D, foi documentado esporocistos cultivados in vitro na presença de fração 7 de *B. tenagophila* da linhagem Taim, sendo observado esporocistos em processo de desintegração e fluorescentes e em E e F representa esporocistos cultivados *in vitro* na presença da mistura das sub-fração 7.1,7.2,7.3 oriundas do fracionamento da fração 7 de *B. tenagophila* da linhagem Taim. Em G, H, foi documentado esporocistos cultivados *in vitro* na presença de fração 7.1 de *B. tenagophila* da linhagem Taim, sendo observado esporocistos integros e ausência de incorporação da sonda

Discussão:

Estudo funcional realizado *in vitro* revelou que componentes presentes na fração acelular da hemolinfa de *B. tenagophila* Taim podem diretamente lesar o tegumento de esporocistos axenicamente transformados provocando a morte de alguns parasitos (PEREIRA *et al.*, 2008; Parte I da tese). Os resultados anteriormente apresentados (Parte II) indicam que a lesão direta no tegumento de esporocistos de *S. mansoni* esta associada à atividade de serino-proteases da hemolinfa acelular de *B. tenagophila* Taim.

Nosso próximo passo foi fracionar a hemolinfa acelular de *B. tenagophila* Taim com o intuito de caracterizar e purificar elementos associados à destruição dos esporocistos de *S. mansoni*. Nossos dados mostraram que a atividade lesiva da hemolinfa acelular de *B. tenagophila* Taim está presente na fração protéica após ultracentrifugação. Em moluscos a hemoglobina apresenta uma ocorrência isolada nos Planobideos e acredita-se que a hemoglobina de *Biomphalaria* evolui de uma mioglobina de moluscos pulmonatos quando ocorreu provavelmente uma substituição de uma hemocianina por uma globina (LIEB *et al*., 2006). Além da hemoglobina LIEB *et al* (2006) descrevem a expressão de hemocianina na hemolinfa circulante de *B. glabrata*. Segundo JIANG *et al*., (2007) proteínas respiratórias como a hemoglobina (Hb) associada ao ferro, e as hemocianinas (HMC), dependentes de cobre, além da função nas trocas gasosoas podem produzir derivados de oxigênio (ROI) com capacidade para destruir patógenos, através de mecanismos indenpendentes de células do sistema imune. Entretanto, nosso estudo indica que hemoglobina ou hemocianina da hemolinfa não são essenciais para destruição direta de esporocitos de *S. mansoni* pela hemolinfa acelular de *B. tenagophila* Taim, uma vez que a

hemolinfa acelular ultracentrifugada, onde grande parte destas proteínas não está presente, continua induzindo aumento de mortalidade dos esporocistos.

O fracionamento da hemolinfa acelular de *B. tenagophila* Taim em coluna de exclusão molecular permitiu identificar que a atividade anti-esporocisto está na parte protéica da fração 7 e é dependente da atividade de serino-protease presente nesta amostra. Além disso, foi possível confirmar que parte dos esporocistos cultivado na presença de frações de hemolinfa com atividade proteolíca apresentam lesão tegumentar, sugerindo que o mecanismo de destruição. Apesar de não ter sido caracterizado o mecanismo de lesão mediado por serino-proteases

O padrão de fluorescência observado em esporocistos de *S. mansoni* lesados após incubação com a sonda *Hoechst 33258* sugere a possibilidade da lesão no tegumento da larva ser mediada pela ativação de proteínas formadoras de poros. Estas proteínas têm sido identificadas em vários sistemas da natureza, sendo encontradas desde em procariotas até em organismos vertebrados. Elas constituem um grupo de moléculas membranolíticas que são capazes de interagir com a camada lipídica, formando canais transmembrana (BHAKDI & TRANUM-JENSEN, 1983b). Um crescente número de moléculas formadoras de poros tem sido indentificado em bactérias (BERNHEIMER & RUDY, 1996), em eucariotas como fungos, protozoários, insetos e vertebrados. Também têm sido relatado pequenos peptídeos capazes de formar poros em membranas, como as metilinas, as cecropinas e defensinas (BERNHEIMER & RUDY, 1996; OJCIUS & YOUNG, 1991; YOUNG & COHN, 1987). Em mamíferos, os dois exemplos clássicos de proteínas, que sob ativação induzem a formação de poros em células alvos são as perforinas, enzimas presentes em granulos de linfócitos T citotóxicos e células NK, e o complexo de ataque a membrana do sistema do complemento (YOUNG & COHN, 1987).

Em protozoários a primeira proteína formadora de poros foi descrita em *Entamoeba histolytica* (LYNCH *et al*., 1982), que apresenta homologia a metilina (LEIPPE et al., 1991). Em tripanosomatídeos, como *Trypanossoma cruzi* foi identificado TC-TOX, o parasito não é capaz de se multiplicar em ambiente ácido, a proteina em condições de pH ácido provoca poros na membrana do vacúolo parasitoforo auxiliando o escape de *T.cruzi* para o citoplasma onde ocorre então a sua multiplicação (ANDREWS, 1995), e em *Leishmania amazonensis* foi identificado e caracterizado uma proteína formadora de poros (NORONHA *et al*., 1996). Em muitos modelos de formação de poro tem sido identificada a de proteases, principalmente as serino-proteases, na ativação em cascata de proteínas solúveis que termina com lesar de membranas (MCKERROW *et al*., 2006).

A participação de proteases na resposta protetora de hospedeiros invertebrados tem sido bem documentada na interação entre *Anopheles gambiae* e *Plasmodium*. Nesta interação, os hemócitos do mosquito infectado produzem e secretam na hemolinfa glicoproteínas semelhantes a proteínas do complemento dos vertebrados, designadas proteínas da família tioester (TEP) e também proteínas que participam da cascata de melanização (PPO). Em vetores suscetíveis ou refratários à infecção por *Plasmodium* têm sido detectado ativação de TEP, gerando fragmentos que se ligam a superfície do protozoário e formam poros na membrana de *Plasmodium.* Nas linhagens resistentes, além de lise de membrana do protozoário, ocorre ativação de enzimas da hemolinfa do inseto, com a participação efetiva de serino-proteases, responsáveis pela formação de uma cápsula de melanina em torno do oocineto (BLANDIN *et al*., 2004).

Na tentativa de isolar e caracterizar bioquímicamente a proteina da hemolinfa de *B. tenagophila* capaz de lesar esporocistos de *S. mansoni* a fração 7 foi re-fracionada. Interessantemente, as sub-fração 7.1 e 7.2 apresentaram atividade anti-esporocisto que é

parcialmente independente da atividade de serino protease. Uma melhor caracterização do efeito anti-esporocisto, dependente e independente da atividade de serino-proteases, demanda a identificação bioquímica das proteínas envolvidas neste processo. Entretanto, as subfrações obtidas e testadas funcionalmente neste trabalho experimental ainda apresentam uma grande mistura protéica que dificulta uma análise por espectrometria de massa. Esta analise será o foco do nosso estudo posterior.

7.0 Conclusões

A fração acelular de *B. glabrata* BH não induz mortalidade de esporocisto de *S. mansoni* cultivados *in vitro*

A fração acelular de *B. tenagophila*, Taim e Cabo frio, induz mortalidade dos esporocistos de *S.mansoni* cultivados *in vitro*, indicando um efeito direto da hemolinfa sobre as larvas do parasito;

A mortalidade dos esporocistos de *S.mansoni* na presença da fração acelular de *B. tenagophila* é acompanhado por lesão de tegumento;

A mortalidade dos esporocistos de *S.mansoni* foi estatisticamente maior na presença da fração acelular de *B. tenagophila* Taim que de Cabo frio;

A fração acelular de *B. tenagophila* Taim também aumenta a capacidade de hemócitos de *B. tenagophila* Cabo Frio (suscetivel) destruir esporocistos de *S.mansoni*, sugerindo um efeito indireto da hemolinfa no reconhecimento e/ou ativação dos hemócitos;

A hemolinfa acelular de *B. glabrata* BH apresenta atividade de aspartil-, serino- e de cisteíno-proteases frente a diferentes substratos, que foram completamente bloqueadas com a adição de inibidores de proteases;

A hemolinfa acelular de *B. tenagophila*, Taim e Cabo Frio, apresentaram atividade de serino- e cisteíno-proteases que foram estatisticamente mais intensa que a observada em *B. glabrata* e apenas parcialmente bloqueada pela adição de inibidores de proteases;

A atividade de serino-protease da hemolinfa acelular de *B. tenagophila* foi associada com a indução de lesão do tegumento de esporocistos de *S. mansoni* e aumento de mortalidade do parasito cultivado *in vitro*;

A atividade anti-esporocisto da hemolinfa acelular de *B. tenagophila* Taim está presente na fração protéica após ultracentrifugação, indicando que hemoglobina ou hemocianina da hemolinfa não participam na destruição direta de esporocitos de *S. mansoni;*

O fracionamento da hemolinfa acelular de *B. tenagophila* Taim em coluna de exclusão molecular permitiu identificar que a atividade anti-esporocisto está associada a componentes protéicos da fração 7 e é coincidente com a atividade de serino-protease presente nesta amostra;

Separações subseqüentes da fração 7 em coluna de troca iônica, demonstram atividade anti-esporocisto presente na subfração 7.1 e 7.2. Sendo que na subfração 7.1 atividade anti-esporocisto é independente de serino-protease, e na subfração 7.2 a mortalidade e lesão tegumentar de esporocistos de *S. mansoni* coincidem com a atividade proteolítica;

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9.0 Anexos

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REVIEW

Molluscan response to parasite: *Biomphalaria* and *Schistosoma mansoni* interaction

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Abstract

Digenetic trematodes use molluscs, almost always a Gastropoda, in their evolutive cycle, as intermediary hosts. The genus *Schistosoma*, with three main species that infect humans - *S. mansoni, S. japonicum*, and *S. haematobium* – shows a prevalence of 200 million patients in various countries worldwide, and 600 million people are still at risk of infection. *S. mansoni* is the most prevalent species, and *Biomphalaria* snails are its intermediary hosts. Although the campaigns of schistosomiasis control based on chemotherapy have reduced the morbidity and prevalence of this disease, transmission continues in almost all the areas submitted to intervention. One of the factors that has influence on the susceptibility of *Biomphalaria* to *S. mansoni* infection is ability of the host internal defense system (IDS) to recognize and destroy the parasite. In *Biomphalaria,* the IDS is composed of cellular elements named hemocytes that act jointly with soluble components present in hemolymph, which could affect directly the larvae, or act in the recognition of the parasite, and activation of hemocytes. The susceptibility level of the mollusc has been attributed to the hemocyte capacity of involving and destroying the parasite, and this will be the centre of interest of this review.

The study of *S. mansoni* and *Biomphalaria* interaction in resistant snail strains is important not only due to the academic-scientific value of this fascinating research area, but also to the potentially possible alternatives for the control of this endemia.

Key words: *Schistosoma mansoni* sporocysts*; Biomphalaria glabrata; Biomphalaria tenagophila*; circulating hemocytes; soluble factors of hemolymph

Introduction

Although the great majority of the living beings is represented by invertebrates, up to now the publications in mass dealing with the defense mechanisms against pathogens is practically restricted to interactions between pathogens of vertebrate animals. The invertebrate animals must necessarily reckon upon their defense system to recognize and destroy infectious agents, although this system are not able to generate the diversity of recognition observed during the adaptative immune response of vertebrates (van der Knaap and Loker, 1990; Loker *et al.*, 2004).

Recent studies have demonstrated many similarities between the innate defense response of vertebrates and the internal defense system of invertebrates (Hoffman *et al.*, 1999; Hoffman, 2003), being identified in various invertebrates organisms production of complement-like proteins, anti-microbial peptides, pattern-recognition receptors (PRRs) such as toll-like receptor and C-type lectins, phagocytic cells, production of highly toxic metabolites of oxygen and nitrogen (Loker *et al.*, 2004). Although many similarities were identified in the defense system of several groups of invertebrates, it is important to note that genomic studies have indicated varied defense mechanisms in invertebrate groups phylogenetically associated,

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but with very different alimentary habits and habitat (Loker *et al.*, 2004).

Several groups of invertebrates, such as insects and molluscs, are important intermediary hosts of parasites species that are transmitted to humans and domestic animals. As an example we can mention the necessity of Diptera insects genus *Lutzomya* in the development of different species of *Leishmania*, the anophelins in the transmission of Malaria or the anophelins and culicides for the development of lymphatic filariosis. Among the molluscs, Gastropoda are obligatorily intermediary hosts in the development of the majority of digenetic trematode species, such as *S. mansoni* and *Fasciola hepatica.* Thus, it is of the utmost importance to get a better understanding of the effector mechanisms used by the internal defense system (IDS) of these invertebrates for the development of new strategies related to the control of these parasite infections. These studies have also contributed significantly to a better knowledge about the innate response of vertebrates. This review will focus primarily on the response of *Biomphalaria* during infection with *S. mansoni*.

Schistosoma mansoni infection in *Biomphalaria*

Schistosomiasis is an important health problem that affects over 200 million people worldwide. Among the schistosomes species that infect human beings, *S. mansoni* is transmitted by *Biomphalaria* snails and causes intestinal and hepatic schistosomiasis in Africa, Arabian Peninsula, and South America (Gryseels *et al.*, 2006). Recent estimative indicates that 6-7 million people are infected by *S. mansoni* only in Brazil (Katz and Peixoto, 2000).

S. mansoni infects *Biomphalaria* by means of active penetration of the parasite´s ciliated larvae, named miracidia, at any site of the snail's exposed parts, frequently the base of the antennae and cephalopodal mass. In the process of penetration, the parasite undergoes morphological and physiological changes, being transformed into primary sporocyst that remains in the fibro-muscular tissue of the host´s cephalopodal region near the penetration site. The primary sporocysts generate the secondary ones, which migrate from the cephalopodal musculature to the digestive glands or hepatopancreas of the mollusc, where they undergo profound anatomic changes and their germinative cells can generate the cercariae (Maldonado and Acosta-Matienzo, 1947; Pan, 1965; Pereira *et al.*, 1984).

In Brazil, out of ten species of molluscs genus *Biomphalaria* described, only three were found naturally infected by *S. mansoni: B. glabrata, B. tenagophila* and *B. straminea* (Paraense, 2001). The susceptibility level of these different species of *Biomphalaria* to infection with the same lineage of *S. mansoni* is much diversified, and *B. glabrata* may present up to 75.3 % of susceptibility in experimental infections, *B. tenagophila* 32.6 %, and *B. straminea* 11.3 %, as demonstrated by Souza *et al.* (1997). Besides the difference in susceptibility observed among *Biomphalaria* species compatible with the parasite, some lineages or geographic isolates of a same species of *Biomphalaria* also present a great variation of susceptibility to the parasite. As far as *B. tenagophila* is concerned, the geographic lineage isolated at the Biological Reservoir in Taim (Rio Grande do Sul, Brazil) was found to be completely resistant to the development of all *S. mansoni* isolates already tested. The character of resistance of this *B. tenagophila* lineage has been explored at our laboratory, aiming at studying the possible mechanisms of the parasite's destruction, representing a potential model for the control of transmission in endemic areas, where *B. tenagophila* is the unique transmitter agent of the disease (Coelho *et al.*, 2004).

Genetic control of resistance of *Biomphalaria* to *S. mansoni* infection

The compatibility between *S. mansoni* and its intermediary host is influenced by behavioral and physiological factors of the mollusc. Once found a suitable host, the susceptibility level of *Biomphalaria* to *S. mansoni* can be determined by the genetic differences of the molluscs, as well as by the genetic constitution of *Schistosoma* (Basch, 1976). Newton (1952, 1953) demonstrated for the first time that the susceptibility of *B. glabrata* snail to *S. mansoni* depends largely upon genetic factors. Later, these results were corroborated by Richards (1970), who demonstrated that the resistance character, acquired at the maturity phase, is determined by a single dominant gene, with mendelian inheritance. Nevertheless, in *B. glabrata*, age is a determinant factor of the snail susceptibility; juvenile snails being more susceptible to infection even in lineages where the adult snail is resistant to *S. mansoni* infection. Thus, the susceptibility of juvenile *B. glabrata* to *S. mansoni* infection is also regulated by genetic factors, being estimated that four or more genes affect this character (Richards, 1977).

B. tenagophila Taim is a lineage that presents absolute resistance to infection by all *S. mansoni* strains tested at any phase of the mollusc development (Santos *et al.*, 1979; Martins-Souza *et al.*, 2003; Coelho *et al.*, 2004). Crossbreedings between *B. tenagophila* Taim and *B. tenagophila* BH (susceptible lineage) showed that the character of resistance to *S. mansoni* infection is dominant (Santos *et al.*, 1979). The dominance of the resistance character of *B. tenagophila* Taim was confirmed in crossbreedings with the susceptible Joinville lineage (Rosa *et al.*, 2005), showing that 100 % of the F_1 offspring were resistant and only 8 % of the F_2 offspring were susceptible to infection by the parasite (Table 1). This study suggests that at least two dominant genes would be responsible for the resistance to *S. mansoni* observed in *B. tenagophila* Taim, one of them being the most important since it was expressed in all F_1 offspring (Rosa *et al.*, 2005). The dominance of the resistance character in *B. tenagophila* Taim added to the reproductive success of this lineage in the presence of susceptible snails of the same species (Rosa *et al*., 2006), led us to suggest the use of this lineage as an alternative for biological control in

Table 1 Susceptibility rates of *B. tenagophila* Joinville, *B. tenagophila* Taim, F1 and F2 when submitted to infection with 25 miracidia of LE strain of *S. mansoni*

Experiment	Group	Number of snails exposed to *S. mansoni*	Number of surviving snails	Number of infected snails eliminanting cercariae (%)
1	Taim	64	60	0 (0)
	Joinvillle	35	12	7 (58.3)
	F1	170	150	1 (0.6)
	F2	110	87	7 (8)
2	Taim	30	25	0 (0)
	Joinville	30	15	9 (60)
	F1	50	44	0 (0)
	F2	50	38	2 (5.3)

some areas of schistosome transmission (Coelho *et al.*, 2004).

One of the factors that influence the susceptibility and may be genetically determined is the activity of the snails IDS. Experimental infections in *B. tenagophila* Taim have shown that *S. mansoni* miracidia are able to penetrate this snail lineage, however the parasites induce an intense cellular infiltration and are rapidly destroyed, suggesting an important participation of the IDS on determination of resistance to *S. mansoni* in *B. tenagophila* Taim.

Internal defense system (IDS) of the mollusc

The IDS of snails is composed of cellular elements constituted by hemocytes, and by soluble factors present in hemolymph. The hemocytes may be circulating in hemolymph or fixed in tissues. In Planorbids the hemolymph circulates in a semi-open system impelled by the heart. The hemoymph leaves the heart through the aorta reaching the tissues, draining in the venous sinus and returning to the heart via the pulmonary and renal veins, after being re-oxygenated in the pulmonar wall (Baker, 1945). The heart, enclosed by the pericardium membrane, is divided into two chambers, the auricula, which receives hemolymph from the pulmonary cavity, and the ventricule that impels the hemolymph through the aorta. The aorta is divided into two arteries: the visceral artery, which irrigates the posterior part of the snail´s body, including the digestive and genital systems, and the cephalic artery, that reachs all the cephalopodal region. The arteries are exhausted in the pseudovascular spaces of the tissues, accumulating hemolymph in three venous sinuses: cephalopodal, visceral and sub-renal, returning to the heart after circulating through the kidney and lung (Baker, 1945; Paraense, 2001).

In *B. glabrata* and *Bulinus sp* a well defined region, located between the pericardium and the posterior epithelium of the mantle cavity (Fig. 1A), also called amebocyte producing organ (APO), was identified as the main site for the production of hemocytes (Lie, 1976). Recent observations (Sullivan *et al.*, 2004; Sullivan and Castro, 2005) showed an increase of mitoses in the cells of this region, ranging from 24-72 h after inoculation of antigens of *S. mansoni* miracidia or cercariae, this being more evident in resistant lineage of *B. glabrata*. Nevertheless, some authors (Matricon-Gondran, 1990; Souza and Andrade, 2006) demonstrated that *B. glabrata* hemocytes may present multi-centric origin, and sites with proliferation of hemocytes were detected also at the saccular portion of the renal tubules and in the ventricular cavity of the heart (Fig. 1B).

The circulating hemocytes of different species of molluscs present morphological and functional heterogeneity. According to Ottaviani (1992; 2006), the population of circulating hemocytes of the majority of gastropod molluscs is constituted by two cellular types: the starry hemocytes that emit pseudopodes, and the roundish hemocytes. In *Planorbarius corneus*, the starry hemocytes are cells that present phagocytic activity, adhere to glass and express proteins that are recognized by pro-inflammatory anti-cytokine antibodies of vertebrates. On the other hand, the roundish hemocytes are not endowed with phagocytic activity, they are not able to adhere to glass, and besides they proliferate in the presence of phytoagglutinin (Ottaviani, 1992; Ottaviani *et al.*, 1993). Similarly to *P. corneus*, the majority of the authors (Harris, 1975; Lie *et al.*, 1987; Barraco *et al.*, 1993; Borges and Andrade, 2003) also distinguish two sub-populations of circulating hemocytes in hemolymph of *B. glabrata.* These sub-populations are called granulocytes, *i.e.*, the hemocytes that emit pseudopodes and produce phagocytosis, and hyalinocytes that are the small and roundish hemocytes (Fig. 2A). Granulocytes can be easily identified by the uptake of neutral red stain into the cell vesicles (Fig. 2B), showing that *S. mansoni* infection induce cellular proliferation (Martins-Souza *et al.*, 2003). However, ultra-structural studies (Matricon-Gondran and Letorcart, 1999 a,b), analyses of distribution and abundance of lysosomal enzymes (Granath and Yoshino, 1983), as well as of expression of lectin-ligants on the cellular surface (Joky *et al.*, 1983; Martins-Souza *et al.*, 2006) suggest that the circulating hemocytes of *Biomphalaria* constitute a cellular population significantly more heterogenous than that previously described (Fig. 2B). The phenotypical and functional definition of *Biomphalaria* hemocytes is of fundamental importance to understand the participation of these cells, or of any cellular sub-population, in the destruction mechanism of *S. mansoni* larvae or other parasites.

Even though hemocytes are the main component of the mollusc IDS, there are some experimental

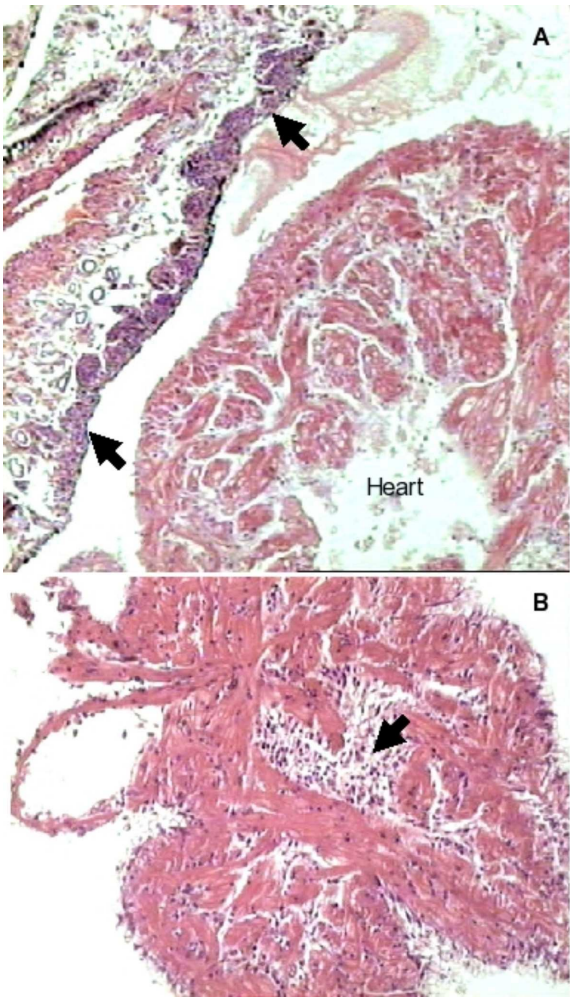

Fig. 1 Photomicrographs of *Biomphalaria glabrata* heart region. **(A)** Snail heart tissue in close contact with the pericardial membrane. The arrows indicate the amebocyte-forming organ (APO), a narrow and long band of epithelial-like cell along the pericardial membrane (100x). **(B)** Heart tissue with a dense collection of hemocytes (Arrow) (200x). Hematoxylin-Eosin stain

evidences indicating that soluble elements of the hemolymph would participate in the protective mechanism against pathogens. Soluble components of the hemolymph of molluscs can directly interact with pathogenic agents, by means of production of toxic substances or lytic peptides, or indirectly through mediator molecules for recognition of the pathogen or hemocyte activators. Peptides with anti-microbial function, called mytilines, are produced and stored in hemocyte granules, and they are secreted in hemolymph of *Mytilus galloprovincialis* (Mitta *et al.*, 2000) notwithstanding the participation of these peptides in the destruction of bacterial infections, there are no evidences of the participation of these peptides in the interaction of mollucs with metazoan parasites.

Fryer and Bayne (1996) show that particles of polystireno treated with soluble factors of hemolymph of *B. glabrata* are significantly more phagocyted by hemocytes than the untreated particles, demonstrating that soluble factors of hemolymph may participate of the recognition mechanism and opsonization of particles by hemocytes. Johnston and Yoshino (1996) demonstrate that lectins similar to those of *Conavalia ensiformis* (ConA), *Erythrina corallodendrom* (ECA), *Glycine max* (SBA), *Tetragonolobus purpureas* (TPA), and *Triticum vulgaris* (WGA) are present in hemolymph of *B. glabrata*. In molluscs, lectins are synthesized by hemocytes and released in hemolymph, where they immobilize the material particularized by agglutination, or are expressed at the surface of circulating hemocytes, where apparently they act as cytophylic receptors (Richards and Renwrantz, 1991; Fryer and Bayne, 1989).

Besides the lectins, other proteins with homologous function to cellular mediators, and already characterized in vertebrates, have been identified in hemolymph of molluscs and may be involved in the activation of hemocytes during infection by digenetic trematodes (Ottaviani *et al.*, 1993, 1995). Ottaviani and co-workers (1993) reported the presence of a variety of proteins similar to pro-inflammatory cytokines of vertebrates, including interleukin-1 alpha (IL-1α), interleukin-1 beta (IL-1β), interleukin-2 (IL-2), interleukin-6 (IL-6), and alpha Tumoral Necrosis Factor (TNF-α) in hemocytes of two species of molluscs, *Planorbarius corneus* and *Viviparus ater,* being present only in hemocytes with phagocytic activity. In further studies, Ottaviani *et al.* (1995), relate the production of homologous proteins to cytokine with an increase of phagocytic activity and induction of nitric oxide synthase (NOS) of mollusc hemocytes. These results suggest that cytokines-like may participate in the activation of hemocytes.

Functional mechanisms in parasite-mollusc interaction

In the last years, many aspects of the interaction between the digenetic trematode larvae and the internal defense system of molluscs have been elucidated. Nevertheless, the possible mechanisms responsible for destruction of the majority of larvae in resistant snails remain to be totally understood. The results reported up to now suggest that the hemocyte could be the effector element in the destruction mechanism of trematodes, being directly involved in the death of some encapsulated parasites (van der Knaap and Loker, 1990; Bayne *et al.*, 2001) or in the production of soluble factors which could be cytotoxic (Connors *et al.*, 1995). The majority of the authors (Connors *et al.*, 1995; Bayne *et al.*, 2001; Martins-Souza, 2003) agree that the snails´ defense generally occurs by means of destruction, total or partial, of the primary sporocyst at the first few hours following the penetration of the miracidium.

The existence of a cellular defense mechanism deployed by molluscs against trematode infection was initially suggested by the finding of histological reactions around parasite sporocysts (Newton, 1952). Further studies have shown that hemocytes infiltration around parasite larvae in *S. mansoni*-

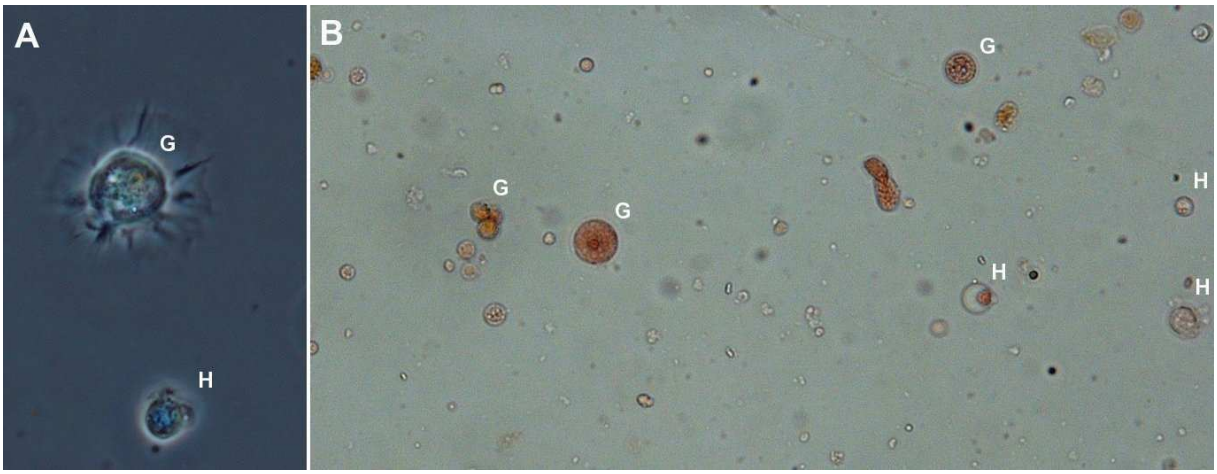

Fig. 2 Morphological aspects of circulating hemocytes from *Biomphalaria tenagophila*. (**A**) Phase contrast photomicrography showing a granulocyte (G) and a hyalinocytes circulating in *B. tenagophila* hemolymph (400x). (**B**) Bright field photomicrography of *B. tenagophila* circulating hemocytes after addition of neutral red staining, showing the heterogeneous granulocyte population stained in red (G) and the non-stained cells designated as hyalinocytes (H) (200x)

infected *Biomphalaria* was stronger in snail species that are more resistant to parasite infection, such as *B. tenagophila* and *B. straminea* (Souza *et al*., 1997). In highly susceptible *B. glabrata*, confirmed by the great quantity of cercariae eliminated during a long period of time, sporocysts and cercariae at different developmental stages are found in abundance in the inner of the host, resulting in compression of the host´s structures, mainly in the interstice of the digestive glands, ovotestis and renal tubules. However, the presence of a great number of parasites did not induce cellular reaction in the parasite susceptible snail strains (Godoy *et al*., 1997). On the opposite extreme appear the resistant snails, which shed a few cercariae and show an extensive infiltration in tissues with numerous hemocytes, frequently placed around the parasite structures in disintegration. The focal reactions frequently assumed a granuloma-like appearance (Godoy *et al*., 1997). In our experimental model, *S. mansoni* infection in *B. tenagophila* of Taim strain resulted in intense, diffuse or granuloma-like cellular infiltration in the infection site, mainly in the connective tissue of the snail cephalopodal region and antennae (Fig. 3). The cellular infiltration was detected few hours after the parasite infection and no viable sporocysts were recovered from these infected snails. Direct evidence of the hemocyte participation in *S. mansoni* infection control was obtained with experiments that transferred the APO from resistant to susceptible snail strains. In *B. glabrata*, the transplantation of APO from miracidia-exposed resistant strain to susceptible NIH snails resulted in significantly more killed sporocysts than as observed during *S. mansoni* infection in NIH snails (Sullivan and Spencer, 1994). Recently, Barbosa *et al.* (2006) showed in *B. tenagophila* that transplantation of the hematopoietic organ from Taim lineage (totally resistant) to a susceptible *S. mansoni* strain resulted in an absolute resistance in the receptors whose transplant was successful. Moreover, inoculation of silica particles in *B. tenagophila* Cabo Frio resulted in transitory reduction of a macrophage-like cell population of circulating hemocytes. The cellular depletion induced by silica-treatment in *B. tenagophila* Cabo Frio was accomplished by enhanced susceptibility to *S. mansoni* infection, shortening the intramolluscan phase of the parasite and increasing the number of sporocysts and cercariae produced (Martins-Souza *et al.*, 2003).

The process of destruction of *S. mansoni* larvae by hemocytes initiates with the recognition and encapsulation of the newly-penetrated sporocyst. Bayne and co-workers (1980b) demonstrated that cell-free hemolymph obtained from susceptible and resistant *B. glabrata* strains are unable to change visibly the morphology of *S. mansoni in vitro,* the same occurring with hemolymph containing hemocytes of susceptible lineages. However, hemocytes of susceptible strains associated with soluble factors of hemolymph of resistant *B. glabrata* lineages acquire the ability to destroy *S. mansoni* sporocysts. The importance of the soluble fraction of *Biomphalaria* hemolymph in the destruction mechanism of *S. mansoni* sporocysts was also confirmed *in vivo* in studies dealing with the transference of this fraction obtained from resistant *B. glabrata* snails to other ones susceptible to the parasite (Granath and Yoshino, 1984). Recent results obtained with *B. tenagophila* Taim also showed that addition of the cell-free hemolymph of this resistant snail strain significantly increased the ability of hemocytes from susceptible strain of *B. tenagophila* (Cabo Frio or Joinville) to destroy *S. mansoni* sporocysts, *in vitro*. Moreover, the increased mortality of sporocysts was associated with higher number of hemocytes on the parasite tegument (Fig. 4), suggesting that cell-free hemolymph from resistant snail strain increased parasite recognition by hemocytes. However, in contrast with *B. glabrata* (Bayne *et al.*, 1980), cell-free

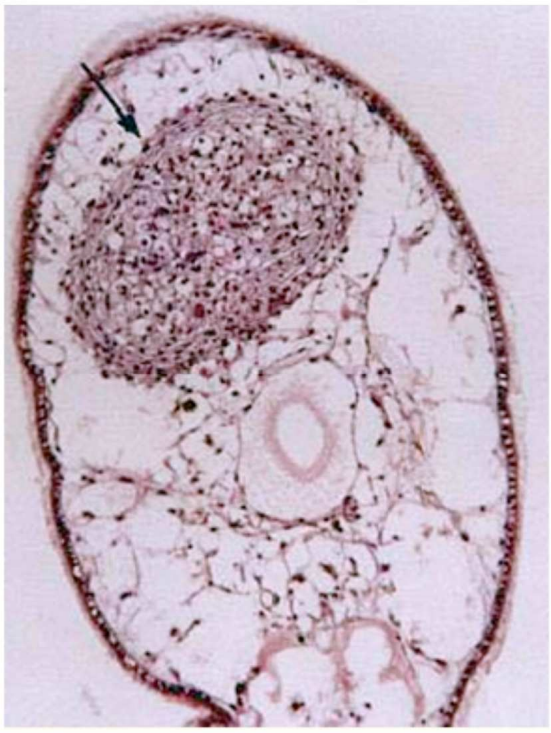

Fig. 3 Intense hemocyte infiltration in *Schistosoma mansoni* infected *Biomphalaria tenagophila* Taim, a parasite-resistant snail strain. Photomicrograph of a transversal section of antennae tissue from *B. tenagophila* Taim 15 days after *S. mansoni* infection (20 miracidium/snail), showing a focal cellular reaction (arrow) with a granuloma-like appearance (200x). Diffuse and focal cellular infiltration is observed in cephalopodal tissue of parasite infected resistant snails and has been associated with the parasite penetration and destruction. Hematoxylin-Eosin stain

hemolymph from *B. tenagophila* Taim was able to destroy a small, but statistically significant, percentage of *S. mansoni* sporocysts even in absence of hemocytes (Pereira, 2005). The importance of *B. tenagophila* Taim cell-free hemolymph in *S. mansoni* control was also confirmed *in vivo*, since susceptible snails treated with *B. tenagophila* Taim cell-free hemolymph had lower percentage of infectivity (Pereira, 2005; Coelho and Bezerra, 2006), and the snails that got infection produced lower number of sporocysts and cercariae (Pereira, 2005).

The main components of *S. mansoni* sporocysts tegument is glicoproteins and glicolipides (Zelck and Becker, 1990; Uchikawa and Loker, 1991). Johnston and Yoshino (1996) showed that lectins from *B. glabrata* cell-free hemolymph bind to glicoproteins extracted from the parasite tegument. More recently (Adema *et al.*, 1997a,b) a group of proteins, homologous to fibrogen and that has been associated with recognition, was identified in *B. glabrata* hemolymph, being its expression enhanced after infection of the mollusc with *Echinostoma paraensei,* another digenetic trematode. These results suggested that lectins would serve as cell surface receptors for carbohydrate structures from trematode parasites. Moreover, soluble lectins would also participate in the recognition mechanism by binding to carbohydrate structures from both hemocytes and parasite tegument (van der Knapp *et al.*, 1990).

Besides participating in the recognition of *S. mansoni*, lectins can also activate hemocytes. Hemocytes of susceptible and resistant *B. glabrata* strains were stimulated with bovine albumin associated with one of the six carbohydrates: mannose, galactose, fucose, N-acetyl-glucosamine, N-acetyl-galactosamine and lactose, that are present in the tegument of *S. mansoni* sporocysts. Hemocytes stimulated with BSA-galactose, BSA-mannose, and BSA-fructose were able to produce reactive oxygen-species (ROS) (Hahn *et al.*, 2000).

Our results also confirmed the participation of soluble lectins in *S. mansoni*-sporocysts recognition mechanisms by *B. tenagophila* species. Circulating hemocytes recovered from *B. tenagophila* - both Taim and Cabo Frio strains - were intensively labelled by FITC-conjugated lectins, such as PNA, SBA, and WGA. Moreover, *S. mansoni* infection in resistant snail strain (Taim) resulted in initial reduction in number of labelled-hemocytes in circulation (Martins-Souza *et al.*, 2006). The reduction of circulating hemocytes during the first few hours after *S. mansoni* infection has been associated with the cell recruitment to the infection site (Bezerra *et al.*, 1997; Martins-Souza, 2003).

Other proteins similar to pro-inflammatory cytokines of vertebrates have been identified in hemolymph of molluscs and can participate in activation of hemocytes during the destruction process of parasites. Specifically in *B. glabrata* was identified a protein with immunological and functional similarity to interleukin-1-like (IL-1 like). In this snail, IL1-like protein is induced by *S. mansoni* infection, and the level was significantly higher in resistant snail strains (Granath *et al.*, 1994). The inoculation of human recombinant IL-1α in susceptible strain of *B. glabrata* resulted in increased production of ROS by circulating hemocytes and reduced number of *S. mansoni* cercariae upon parasite infection (Connors *et al.*, 1995). These authors confirmed, *in vitro*, that cell-free hemolymph recovered from rhIL-1α-treated snail, but not only rhIL-1α, was capable of destroy *S. mansoni* sporocysts, suggesting that IL-1 would activate *B. glabrata*-hemocytes to produce and secrete soluble cytotoxic mediators (Connors *et al.*, 1998).

The effector mechanisms by which activated hemocytes are able to kill trematode larvae are not fully understood yet. Dikheboom *et al.* (1988a,b) showed for the first time that gastropod hemocytes do produce reactive oxygen species (ROS) in response to trematode infection. The initial reduction of O_2 to superoxide anion (O_2) is catalyzed by NADPH-oxidase and O_2 can be converted to other ROS, including hydrogen peroxide (H_20_2) and hypochlorous acid (HOCl) (Hampton *et al.*, 1998; Bayne *et al.*, 2001). NADPH oxidase-like activity has been identified in gastropod hemocytes (Adema *et al.*, 1993) and ROS production

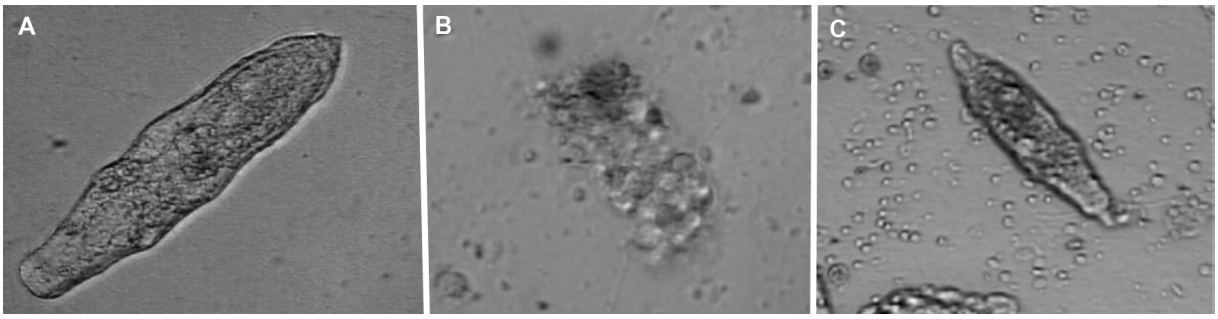

Fig. 4 Interaction of *Schistosoma mansoni* sporocyst and hemocytes from *Biomphalaria tenagophila* Taim (**B**) or *Biomphalaria glabrata* BH (**C**). (**A**) *S. mansoni* sporocyst in Chernin's Balanced Salt Solution, showing an intact parasite larva (200x). (**B**) *S. mansoni* sporocyst completely encapsulated by hemocytes from *B. tenagophila* Taim, a snail strain totally resistant to parasite infection (100x). (**C**) *S. mansoni* sporocyst incubated with hemocytes from *B. glabrata* BH, snail highly susceptible to parasite infection, showing an intact parasite with very few cell attached, x100

by *B. glabrata* hemocytes has been associated with *S. mansoni* resistance (Adema *et al.*, 1994). Additionally, molluscan hemocytes also generate nitric oxide (NO) from molecular oxygen and arginine (Conte and Ottaviani, 1995; Arumugan *et al.*, 2000). Experimental evidences of ROS and/or NOS participation in killing of *S. mansoni* sporocysts by *B. glabrata* hemocytes were obtained using specific oxidant scavengers or enzyme inhibitors during the *in vitro* association. The results showed that inhibition of H_2O_2 and NO production do favor sporocysts survival, indicating that this reactive species would be toxic to trematode larvae (Hahn *et al.*, 2001 a,b). However, attempts to associate H_2O_2 or NO production with snail strain susceptibility to *S. mansoni* infection have not been conclusive (Hahn *et al.*, 2000; Bayne *et al.*, 2001). Similarly, we were able to estimate NO production by hemocytes isolated from *B. glabrata* or *B. tenagophila* at different time after *S. mansoni* infection and re-stimulated *in vitro* with *S. mansoni* egg antigen (SEA). At each experimental point, as well as for each snail strain, analyses were performed in triplicate with total hemolymph collected and gathered together from 3 snails. After centrifugation, the hemocyte pellet was ressuspended in Chernin´s Balanced Salt Solution (5 x 10^5/ml of CBSS) containing 100 μg/ml of SEA, plated in 96-well tissue culture plates and incubated at 26 °C and 5 % CO_2. After 18 h of incubation, NO presence was assayed directly in the cell supernatant by the Greiss reaction (Green *et al.*, 1982) that quantifies the nitrite contents of the supernatants, as detailed by Perreira *et al.* (2006). The NO level increased in hemocytes recovered after the first few days of *S. mansoni* infection, however there were no detectable differences in NO level between the snails, although each species or strain shows remarkable difference in parasite susceptibility (Fig. 5). However, it is important to confirm if supernatant level do reflect the local production in hemocyte-sporocyts interaction. Besides producing reactive species of oxygen and nitrogen, microscopy analyses indicate that hemocytes from parasite-resistant snails would phagocvte portions of sporocyst tegument leading to mechanical lesion that may be also lethal to the parasite (van der Knaap and Loker, 1990).

Finally, in order to evaluate the efficiency of *Biomphalaria spp* defense system in the destruction of *S. mansoni* larvae, one must consider that there are many evidences indicating that the parasite is able to develop strategies to allow its evasion. Thus, it has been described that the primary sporocyst of *S. mansoni* acquires quickly the antigens present in the host´s hemolymph (Bayne *et al.*, 1986), as well as express in the tegument antigens similar to those expressed by the host´s cells (Yoshino and Bayne, 1983) hindering the recognition process of the parasite by hemocytes. It has been also reported that components of the excreted/secreted material by the miracidium during the transformation process may reduce the motility of hemocytes, as well as their phagocytic capacity (Connors and Yoshino, 1990; Lodes and Yoshino, 1990), thus justifying the cellular reaction almost inexistent observed around *S. mansoni* sporocysts present in the tissue of *B. glabrata* susceptible strains.

In addition to avoid the hemocytes´ approach, it has been also reported that sporocysts incubated *in vitro* with hemocytes of susceptible strains may be encapsulated, but not destroyed (Boehmler *et al.*, 1996; Hahn *et al.*, 2001a), suggesting the existence of anti-oxidant mechanisms.

Perspectives

The most recent studies related to the internal defense system of invertebrates have shown new aspects of the invertebrate-pathogen relationship. These aspects afforded us a better understanding of the recognition and cellular activation mechanisms, which are also present in the innate defense response of vertebrates. *Biomphalaria-Schistosoma mansoni* interaction, besides being an important model in human health, constitutes an experimental approach that may add important information to the knowledge of the defense mechanism utilized by invertebrates, as well as of phylogenetic evolution of these mechanisms.

In this context, our group has carried out researches dealing with the phenotypic and functional

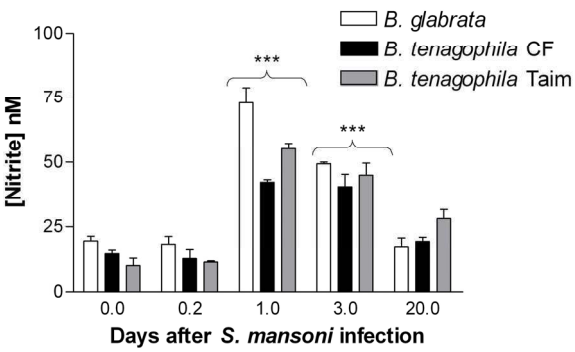

Fig. 5 Nitric oxide (NO) level in supernatant of circulanting hemocytes recovered from *Biomphalaria glabrata*, *B. tenagophila* Cabo Frio e *B. tenagophila* Taim during the *S. mansoni* infection. NO levels was indirectly estimated by quantification of nitrite, using the Greiss reaction, in hemocyte supernatant recovered after 18 h of *in vitro* re-stimulation with soluble egg antigen (SEA 100 μg/ml). *** for $P < 0.001$ when comparing with nitrite level obtained after stimulation of non-infected hemocytes from the same snail strain. At each time point, there was no statistically significant difference in the nitrite level between the snail strains

characterization of the hemocytes and hemolymph from different strains of *B. glabrata* and *B. tenagophila.* Our goal is to identify possible mechanism of trematode recognition and hemocyte activation in *B. tenagophila* Taim that would be responsible to the fast parasite destruction and consequent resistance against *S. mansoni* infection observed in this snail strain. In parallel, we are investigating the heritage of the resistance character from *B. tenagophila* Taim to the offspring resulted of crossbreeding with the susceptible snail strains. A more comprehensive identification of these mechanisms would expand the theorical base that gives support to mass introduction of *B. tenagophila* resistant strain from Taim in areas where transmission is maintained by this species (Coelho *et al.*, 2004).

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14

Biomphalaria e *Schistosoma mansoni*: papel do sistema interno de defesa do molusco na susceptibilidade ao parasito

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Esporocisto primário de *Schistosoma mansoni* incubado com hemolinfa total de *Biomphalaria tenagophila* da linhagem Taim. Observar a deposição de hemócitos e deformação do tegumento do esporocisto. Fonte: Ary Corrêa Jr. e Deborah Negrão-Corrêa.

Apesar da grande variedade e complexidade observada no desenvolvimento de trematódeos digenéicos, a maioria utiliza moluscos gastrópodas como primeiro hospedeiro invertebrado. Uma característica que marca a interação molusco-Digenea é o elevado grau de especificidade; tem sido freqüentemente relatado que um número restrito de espécies, ou mesmo linhagens geográficas, de *Biomphalaria* são susceptíveis a uma determinada cepa de *Schistosoma mansoni*. Um dos fatores que influencia na susceptibilidade de *Biomphalaria* à infecção por *S. mansoni* é a capacidade que o sistema interno de defesa deste hospedeiro tem de destruir o parasito. O sistema interno de defesa de *Biomphalaria sp* ainda não é completamente conhecido; sabe-se que é composto por elementos celulares, denominados hemócitos, e componentes solúveis, os quais poderiam diretamente afetar as larvas ou atuar no reconhecimento do parasito e ativação dos hemócitos. Desta maneira, acredita-se que, logo após a penetração do miracídio, hemócitos circulantes são atraídos para o sítio da infecção e podem destruir o miracídio ou encapsular o esporocisto primário. As lectinas solúveis ou expressas na membrana dos hemócitos provavelmente participam no mecanismo de reconhecimento do esporocisto, sendo possível que a rapidez e intensidade da reação antiparasitária variem em diferentes linhagens de hospedeiro. Alguns esporocistos encapsulados podem ser destruídos por lesões tegumentares e/ou pela citotoxicidade decorrentes da ativação dos hemócitos. O nível de susceptibilidade do molusco tem sido atribuído à capacidade do hemócito de envolver e destruir o parasito, o que será foco deste capítulo.

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Participation of cell-free haemolymph of *Biomphalaria tenagophila* in the defence mechanism against *Schistosoma mansoni* sporocysts

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SUMMARY

Biomphalaria tenagophila *of Taim strain is able to completely destroy* Schistosoma mansoni *sporocyst few hours after parasite penetration, although the mechanism is still not well known. In this experimental work we show that passive transference of cell-free haemolymph, especially from* B. tenagophila *Taim, resulted in higher resistance of* B. tenagophila *Cabo Frio to* S. mansoni *infection. This effect was demonstrated* in vivo*, by the reduction in the infection rate, and the significantly lower production of sporocysts and cercariae of the parasite in snails treated with Taim cell-free haemolymph compared to CBSS-inoculated snails. The protective effect of Taim cell-free haemolymph was also observed during the* in vitro *interaction between haemocytes and sporocysts. In this system, addition of* B. tenagophila *cell-free haemolymph, especially from Taim strain, was responsible for significant increase in sporocyst mortality compared to* B. glabrata *cell-free haemolymph or culture medium. Moreover, the combination of Taim cell-free haemolymph and Cabo Frio haemocytes increased significantly the mortality of sporocysts. The results show that Taim cell-free haemolymph would act direct and indirectly on destruction of* S. mansoni *sporocysts. The results also suggest that cell-free haemolymph indirectly increases parasite recognition by the circulating granulocytes and it is species specific.*

Keywords Biomphalaria tenagophila*, cell-free haemolymph factors, haemocyte activation, internal defence system of mollusks,* Schistosoma mansoni

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INTRODUCTION

Invertebrates, which constitute the great majority of animal species are exposed to a broad variety of infectious agents. Although invertebrates lack an adaptive immune system, with specificity and memory as observed in vertebrates, their internal defence system (IDS) is capable of keeping the invertebrate self-integrity (1,2). Recent work showed evidences that internal defence system response has many similarities with the innate immune response of vertebrates (3,4). The similarities include the capabilities of internal defence system to recognize nonself molecules and activate phagocytic cells to produce toxic oxygen and nitrogen metabolites (2,5).

Most experimental work has shown that *Biomphalaria* circulating haemocytes participate of the parasite control during a trematode infection. Histopathological analysis of *S. mansoni*-infected *Biomphalaria* snail showed that haemocyte infiltration around the parasite larvae was faster and stronger in snail strains that are more resistant to parasite infection (6,7). Direct evidence of the haemocyte participation on the *S. mansoni* infection control was provided by experiments that transferred the Amoebocyte-producing organ (APO) from resistant to susceptible snail strains. In *B. glabrata*, the transference of APO from miracidia-exposed resistant strain (13–16-R1 snails) to susceptible NIH snail resulted in significantly higher mortality of sporocysts than the observed during *S. mansoni* infection in NIH snail controls (8). Recently, Barbosa *et al*. (9) described a marked increase in the resistance of *B. tenagophila* Cabo Frio strain that received the APO from *B. tenagophila* Taim, a snail strain completely resistant to *S. mansoni*.

Even though haemocytes have been identified as the main effectors element during *S. mansoni* control inside the snail, there are some experimental evidences indicating that soluble elements of the haemolymph participate in the protective mechanism. Granath and Yoshino (10) reported that susceptible strain of *B. glabrata* (PR) previously inoculated with cell-free haemolymph from resistant snails (10-R2) had lower infective rate after exposure to *S. mansoni* miracidia. A

reduction in *S. mansoni* infective rate was also reported in *B. tenagophila* Cabo Frio inoculated with cell-free haemolymph recovered from *B. tenagophila* Taim, the parasite resistant snail strain (11). Moreover, Vasquez and Sullivan (12) showed that cell-free haemolymph collected from susceptible *B. glabrata* previously implanted with the APO from resistant snail strain donors were able to lower the infective rate of *S. mansoni* in the recipients, suggesting that haemocytes from APO implant were able to secrete soluble factors that participate in the parasite control mechanism.

The participation of soluble elements of *B. glabrata* haemolymph, such as lectins, in parasite recognition mechanism was initially suggested by Fryer and Bayne (13), who demonstrated that particles of polystyrene coated with *B. glabrata* soluble haemolymph are more efficiently phagocyted by haemocytes than the untreated particles. More recently, Saap and Loker (14) showed that cell-free haemolymph from non-compatible snail is able to rapidly kill trematode larvae, suggesting also a direct haemolymph effect.

Most of the *S. mansoni*-snail interaction studies were done using different strains of *B. glabrata* as experimental model. Although other snail species, such as *B. tenagophila*, have a growing importance in Schistosomiasis transmission, very few studies have been done with this snail species. This experimental work intent to evaluate the participation of cell-free haemolymph from *B. tenagophila* Taim, a snail strain that is able to completely destroy *S. mansoni* mother sporocysts, in parasite control mechanism.

MATERIALS AND METHODS

Parasites

The LE strain of *S. mansoni*, originally isolated from a patient from Belo Horizonte, Brazil, was used in all the experiments. This parasite strain has been maintained in successive passages through *B. glabrata* and hamsters – *Mesocricetus auratus* (15), in the Laboratory of Schistosomiasis, Department of Parasitology, Institute of Biological Science, Federal University of Minas Gerais (ICB, UFMG, Brazil), since its isolation in 1968.

Snails

Biomphalaria glabrata and *B. tenagophila* used in this study have been bred and maintained according to the procedures previously described by Pellegrino and Katz (15) at the mollusk room of the Laboratory of Schistosomiasis (ICB, UFMG, Brazil). *Biomphalaria glabrata* used in the experiments was collected at the Pampulha Lake, Belo Horizonte, Brazil. This snail strain, designated as BH, is highly susceptible to *S. mansoni* infection (16). Two strains of *B. tenagophila* were also selected for the study. The Taim strain was collected at the Ecological Station of Taim, Rio Grande do Sul, Brazil, and the Cabo Frio strain collected at Cabo Frio, Rio de Janeiro, Brazil. *Biomphalaria tenagophila* Taim is totally resistant to *S. mansoni* infection, LE (parasite strain originally isolated from an infected patient) or SJ strain (parasite strain originally isolated form naturally infected *B. tenagophila*), while Cabo Frio strain is highly susceptible to *S. mansoni* SJ and is partially resistant to *S. mansoni* LE (17,18).

Haemolymph collection

Snail haemolymph was collected through cardiac puncture (18,19). Briefly, each snail shell was cleaned with 70% alcohol solution and the cardiac region was identified and perforated with disposable 1-mL syringe (Syringe Plastipak 21 G needle). The recovered haemolymph was transferred to a Polypropylene tube and kept on ice until use in different procedures.

Transference of cell-free haemolymph

Biomphalaria tenagophila of Cabo Frio strain, measuring 12–14 mm in diameter, were used as recipient snails for testing the *in vivo* effect of cell-free haemolymph transference in the *S. mansoni* susceptibility. To obtain cell-free haemolymph used in the experiment, whole haemolymph was collected from a pool of noninfected *B. tenagophila* Taim or *B. tenagophila* Cabo Frio snails and centrifuged at 80 ***g*** for 5 min at 4°C. The supernatant, designated as cell-free haemolymph, was transferred to new polypropylene tubes and immediately injected into the recipient snails. The recipient snails were anaesthetized by keeping them into chlorine-free water containing sodium pentobarbital solution (0·4 mg/mL) for 8 h, as previously described elsewhere (20). Anaesthetized snails were randomly divided in four experimental groups; the first group was injected, into cephalopodal region of the snail, with 30 μL of cell-free haemolymph from noninfected *B. tenagophila* Taim (11·2 mg of total protein/mL of haemolymph), the second group was injected with 30 μL of cell-free haemolymph from noninfected *B. tenagophila* Cabo Frio (10·6 mg of total protein/mL of haemolymph), the third group was injected with 30 μL of Chernin's Balanced Salt Solution (CBSS buffer, 48 mM NaCl, 2·0 mM KCl, 0·5 mM Na_2HPO_4, 1·8 mM $MgSO_4 \cdot 7H_2O$, 3·6 mM $CaCl_2 \cdot 2H_2O$, 0·6 mM $NaHCO_3$, 5·5 mM glucose and 3 mM threalose, pH 7·4) and the last group was not inoculated. After the inoculations, twenty snails of each experimental group were kept noninfected and the remaining snails of all the experimental groups were individually infected with 20 miracidia of *S. mansoni*. The designation of each experimental group is detailed in Table 1.

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Table 1 Description of the treatment and the designation of each experimental group used in the cell-free haemolymph transference experiments

Experimental groups	
Designation	Treatment
CF	*B. tenagophila* Cabo Frio not inoculated and noninfected
CF-CBSS	*B. tenagophila* Cabo Frio injected with 30 μL of CBSS
CF-Taim	*B. tenagophila* Cabo Frio injected with 30 μL of cell-free haemolymph from *B. tenagophila* Taim
CF-CF	*B. tenagophila* Cabo Frio injected with 30 μL of cell-free haemolymph from noninfected *B. tenagophila* Cabo Frio
CF infected	*B. tenagophila* Cabo Frio infected with *S. mansoni*
CF-CBSS infected	*B. tenagophila* Cabo Frio injected with 30 μL of CBSS and infected with *S. mansoni*
CF-Taim infected	*B. tenagophila* Cabo Frio injected with 30 μL of cell-free haemolymph from *B. tenagophila* Taim and infected with *S. mansoni*
CF-CF infected	*B. tenagophila* Cabo Frio injected with 30 μL of cell-free haemolymph from *B. tenagophila* Cabo Frio and infected with *S. mansoni*

Parasitic infection

Schistosoma mansoni miracidia obtained from livers of experimentally infected hamsters were counted and used to infect *B. tenagophila* following the procedure described by Pellegrino and Katz (15). For the infection 20 miracidia were added to each flask containing one snail in chlorine-free water and kept for at least 5 h, under artificial light stimulation. Snails with 12–14 mm diameter in size, at the time of infection, were used in all the experiments.

Cell counts

For obtaining circulating haemocyte kinetics during *S. mansoni* infection, whole haemolymph was collected from each snail and 5 snails per group were examined at different time points. Total viable haemocytes were counted in Neubauer's chamber after dilution of whole haemolymph in CBSS medium containing 0·4% Trypan Blue (Sigma, St. Louis, MO) and differential haemocyte counts (granulocytes and hyalinocytes) were done after dilution in CBSS buffer containing 0·05% of Neutral Red (Sigma), as previously described (18).

Evaluation of infection

Schistosoma mansoni infection level of *B. tenagophila* Cabo Frio from each experimental group was evaluated by counting the production of *S. mansoni* daughter (secondary) sporocysts and cercariae from each snail. Daughter sporocysts were recovered and quantified from cephalopodal region of each *B. tenagophila* (5 snails/treatment) between the 15th and 30th day of *S. mansoni* infection, according to Pereira *et al.* (21) and modified by Martins-Souza *et al.* (18). *Schistosoma mansoni* cercaria emergence was also quantified after 4 h of stimulation with artificial light in each survived snail from 30 to 50 days after infection, as previously described by Pellegrino and Macedo (22). During this period, mortality and infective rates for each experimental group were recorded.

In vitro assay

To confirm the effect of the cell-free haemolymph on *S. mansoni* sporocysts and to evaluate its possible mechanism of action, an *in vitro* assay was performed based on Bayne *et al.* (23). Initially, *S. mansoni* miracidia were aseptically obtained from liver of infected hamsters, washed with ice-cooled CBSS buffer, and incubated in RPMI medium supplemented with 5% foetal bovine serum for 24 h at 28°C and 5% CO_2 for sporocyst transformation (24). Transformed sporocysts were washed in CBSS and re-suspended in CBSS supplemented with 2% of bovine serum albumin, 25 mM HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin (Sigma) and amino acids solution (Atlanta Biologicals, Norcross GA). Approximately 100 sporocysts were plated per well in 96-well tissue culture plates (Corning-Costar) and the exact number of sporocysts in each well was counted under microscopy to obtain the precise number of parasites in the beginning of the culture.

Total haemolymph was collected from a pool of noninfected *B. glabrata*, *B. tenagophila* Taim and *B. tenagophila* Cabo Frio and kept in separated tubes. Haemocytes from each snail strain were separated from the haemolymph by centrifugation in polypropylene-tube at 80 ***g*** for 5 min at 4°C. The cell-free haemolymph was transferred to new polypropylene tubes and the cell pellet was re-suspended in supplemented CBSS, counted and 100 μL/well containing 2×10^5 cells were transferred to each well of the assay plate.

Cell-free haemolymph was further centrifuged at 1200 ***g*** for 30 min at 4°C and 50 μL of supernatant (20% of the total volume) was also added to each well of the assay plate.

For each experiment, *S. mansoni* sporocysts were plated and incubated in supplemented CBSS medium alone as a negative control of mortality under the incubation conditions. The mortality rate of *S. mansoni* sporocysts was evaluated after incubation with homologous combination of haemocytes (2×10^5 cell/treatment) plus cell-free haemolymph (20% total volume) from *B. glabrata*, *B. tenagophila* Cabo Frio or *B. tenagophila* Taim. Heterologous combinations, in which sporocysts were incubated with haemocytes from *B. glabrata* or from *B. tenagophila* Cabo Frio and cell-free haemolymph from *B. tenagophila* Taim, were also tested. Finally, the mortality rate of sporocysts was also measured after incubation with cell-free haemolymph from each snail strain tested without haemocytes. The assay was set up in triplicate for each treatment. The assay plates were kept at 26°C and 5% CO_2 for 18 h. At the end of the incubation time, 0·4% Trypan Blue dye solution was added to each well and the total number of sporocysts was counted under microscopy. The mortality was calculated by adding the number of sporocysts stained by Trypan Blue dye to the number of missing sporocysts based on the initial count.

Statistical analysis

Means of normally distributed data were compared using one-way ANOVA and *P*-values were assigned using Tukey *post hoc* analysis. For the data with distributions other than normal (*S. mansoni* cercariae and sporocysts) the nonparametric method of Kruskal-Wallis was used. Kaplan–Meier Survival Curves and the Log-Rank Test was used to analyse snail survival after haemolymph- or CBSS-inoculation.

RESULTS

Effect of *in vivo* inoculation of *Biomphalaria tenagophila* Taim cell-free haemolymph on haemocyte response

Schistosoma mansoni infection, but not the inoculation of CBSS-buffer or cell-free haemolymph, increased significantly *B. tenagophila* mortality during the first 5 days after treatment compared to normal snails (Figure 1a). Moreover, experimental groups that received parasite infection combined with cell-free haemolymph treatment had significantly higher snail mortality compared to other experimental groups (Figure 1b).

Inoculation of CBSS or cell-free haemolymph did not alter significantly the circulating haemocyte profile compared to nontreated snails. In contrast, *S. mansoni* infection resulted in significant alteration in the number and cell type

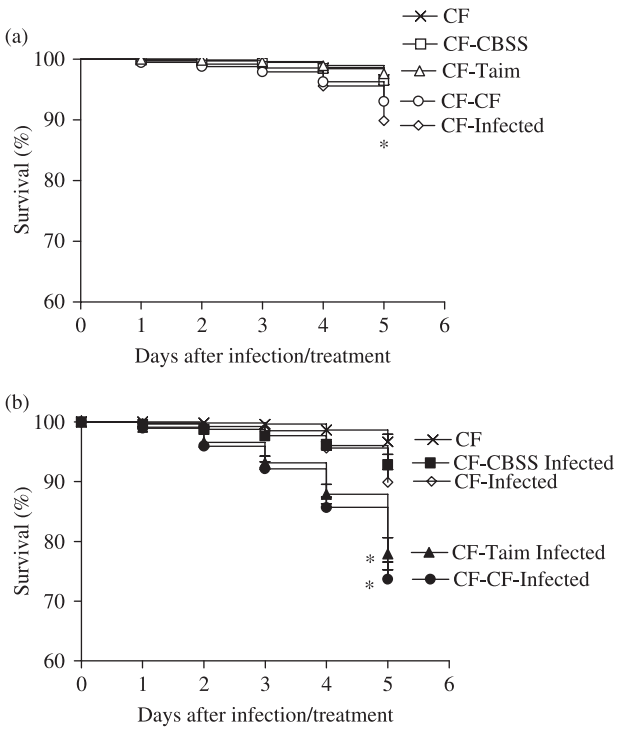

Figure 1 Effect of the haemolymph treatment and/or *Schistosoma mansoni* infection on the survival curve of *B. tenagophila*.
(a) Survival curve of snails during the first 5 days after cell-free haemolymph transference or CBSS-inoculation. CF, non infected and nontreated snails of Cabo Frio strain; CF-CBSS, CBSS-inoculated and noninfected snails; CF-Taim, snails treated with cell-free haemolymph from Taim; CF-CF, snails treated with cell-free haemolymph from Cabo Frio; CF-infected, *S. mansoni* infected snails from Cabo Frio strain. *Represent survival curve of CF-infected was statistically different from survival curve of CF.
(b) Survival curve of snails during the first 5 days after cell-free haemolymph transference or CBSS-inoculation plus *Schistosoma mansoni* infection. CF, non infected and nontreated snails of Cabo Frio strain; CF-CBSS infected, CBSS-inoculated and *S. mansoni* infected snails; CF-Taim infected, snails treated with cell-free haemolymph from Taim and infected with *S. mansoni*; CF-CF, snails treated with cell-free haemolymph from Cabo Frio and infected with *S. mansoni*; CF-infected, *S. mansoni* infected snails from Cabo Frio strain. *Represent survival curve of CF-Taim infected and CF-CF infected were statistically different from survival curve of CF.

of circulating haemocyte from *B. tenagophila* Cabo Frio. Snails that were inoculated with CBSS-buffer and infected showed a significant increase in total number of circulating haemocytes at 1 and 5 days post-infection compared to the number of circulating haemocytes observed in noninfected snails. The increased number of circulating haemocytes in these infected snails was mainly due to higher number of phagocytic cells (granulocytes), although in the first day after infection the number of hyalinocytes was also

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Table 2 Total and differential cell count in circulating haemolymph recovered from noninfected (CF) and *S. mansoni* infected *B. tenagophila* Cabo Frio that were previously inoculated with CBSS-buffer (CF-CBSS), with cell-free haemolymph from *B. tenagophila* Cabo Frio (CF-CF) or with cell-free haemolymph from *B. tenagophila* Taim (CF-Taim)

Circulating haemocytes population ($\times 10^4$ per mL of haemolymph)					
Time (days post-infection	Groups	Granulocytes	Hyalinocytes	Dead cells	Total cells
0	CF	70 ± 2·3	9 ± 3	9 ± 1·6	86 ± 5·3
1	CF-CBSS infected	**194 ± 16**	**55 ± 5**	2·5 ± 0·8	**236 ± 19**
	CF-CF infected	132 ± 19	30 ± 12	3·5 ± 2	160 ± 30
	CF-Taim infected	69 ± 2·4[a]	**160 ± 21**[a,b]	**60 ± 21**[a,b]	**255 ± 16**[b]
3	CF-CBSS infected	84 ± 13	33 ± 11	2 ± 0·9	112 ± 22
	CF-CF infected	109 ± 14	21 ± 4	8 ± 2	130 ± 08
	CF-Taim infected	116 ± 25	28 ± 4	19 ± 3	**181 ± 17**
5	CF-CBSS infected	**258 ± 59**	30 ± 6	12 ± 1	**317 ± 36**
	CF-CF infected	**204 ± 12**	8 ± 3	20 ± 3	**215 ± 10**
	CF-Taim infected	120 ± 19[a]	**52 ± 6**[b]	16 ± 2	**175 ± 09**[a]

Bold numbers represents number of cells statistically ($P \leq 0{\cdot}05$) different from the number in noninfected snails for the same cell type; [a]number of cells of CF-Taim statistically ($P \leq 0{\cdot}05$) different from the number in CF-CBSS for the same cell type and period of infection; [b]number of cells of CF-Taim statistically ($P \leq 0{\cdot}05$) different from the number in CF-CF for the same cell type and period of infection.

increased (Table 2). An increased number of circulating haemocytes was also detected in snails that were previously inoculated with cell-free haemolymph from Taim after 1, 3 and 5 days of *S. mansoni* infection. However, in Taim-treated and infected snails there was no increment in the number of circulating granulocytes and the higher number of total circulating haemocytes was due to significant increment of dead cells and hyalinocytes (Table 2). A significant alteration in hemocyte cell type was detected only after 5 dpi in snails that were treated with cell-free haemolymph from Cabo Frio and infected (Table 2).

Effect of *in vivo* inoculation of *Biomphalaria tenagophila* Taim cell-free haemolymph on *Schistosoma mansoni* snail susceptibility

The transference of cell-free haemolymph, especially from Taim snails, resulted in increased resistance of *B. tenagophila* Cabo Frio to *S. mansoni* infection measured by the number of migrating sporocysts (Figure 2), infective rate and cercaria shedding (Figure 3). Change in snail susceptibility to *S. mansoni* infection induced by the treatment with cell-free haemolymph from Taim was already detected during sporocysts migration through the snail. In 20% of CBSS-inoculated and *S. mansoni*-infected snails, migrating sporocysts was already detected at 15 days post-infection. The infective rate increased up to 70% on 20 dpi, reaching 100% of the examined snails on 25 dpi, and after this period it was not possible to recover migrating sporocysts in the examined snails. In contrast, the maximum infective rate observed in *B. tenagophila* Cabo Frio snails previously treated

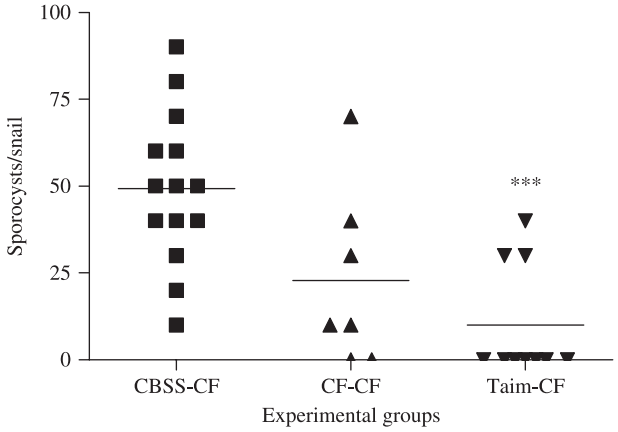

Figure 2 Number of migrating sporocysts from cell-free haemolymph treated and nontreated *B. tenagophila* of Cabo Frio strain at 25 days after *Schistosoma mansoni* infection. Snails were injected with 30 μL of CBSS (CBSS-CF), 30 μL of Cabo Frio cell-free haemolymph (CF-CF) or 30 μL of Taim cell-free haemolymph (Taim-CF) just before the infection with *Schistosoma mansoni* (20 miracidia/snail). Each point represents the number of sporocysts recovered from one infected snail. ***Represents number of sporocysts statistically different compared the number recovered in CBSS-CF group ($P \leq 0{\cdot}001$).

with Cabo Frio cell-free haemolymph was 70%, and migrating sporocysts were observed in only 30% of the infected snails that received Taim cell-free haemolymph. There was a delay in the beginning of the migration period of daughter sporocysts from the cephalopodal region to the hepatopancreas site, with migrating sporocysts being recovered only after 20 dpi in the snails that received cell-free haemolymph. Moreover,

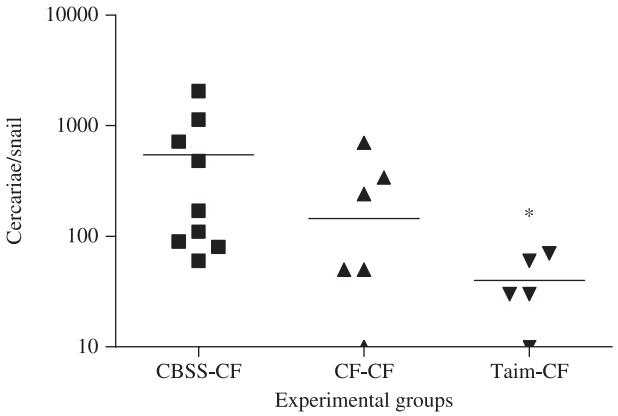

Figure 3 Number of cercariae recovered from cell-free haemolymph treated and nontreated *B. tenagophila* of Cabo Frio strain at 45 days after *Schistosoma mansoni* infection. Snails were injected with 30 μL of CBSS (CBSS-CF), 30 μL of Cabo Frio cell-free haemolymph (CF-CF) or 30 μL of Taim cell-free haemolymph (Taim-CF) just before the infection with *Schistosoma mansoni* (20 miracidia/snail). Each point represents the number of cercariae recovered from one infected snail. *Represents number of cercariae statistically different compared the number recovered in CBSS-CF group ($P \leq 0{\cdot}05$).

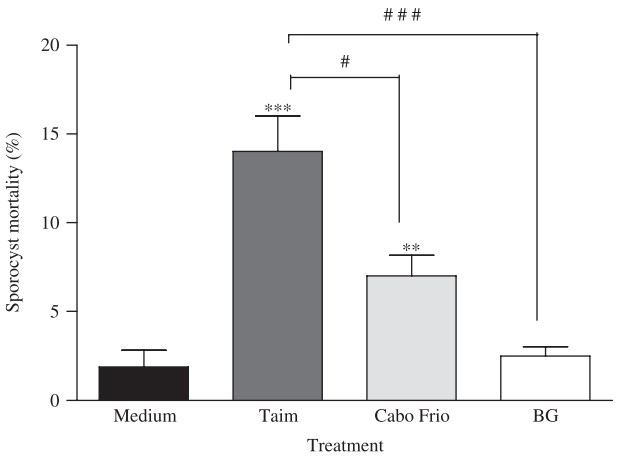

Figure 4 Effect of cell-free haemolymph from noninfected *B. glabrata* (BG), *B. tenagophila* Taim (Taim) or *B. tenagophila* Cabo Frio (Cabo Frio) on mortality rate of axenically transformed *S. mansoni* sporocysts. One hundred axenically transformed sporocysts of *S. mansoni* were incubated, in triplicate, with supplemented CBSS medium or with supplemented CBSS medium containing cell-free haemolymph of BH, Cabo Frio or Taim snail for 18 h. The percentage of dead sporocysts was calculated for each treatment and results showed the mean ± SD of three independent experiments. Statistically significant differences comparing the sporocysts mortality in cell-free haemolymph treatment vs. nontreated are indicated by ***$P \leq 0{\cdot}001$, **$P \leq 0{\cdot}01$; and between each treatment are indicated by #$P \leq 0{\cdot}05$ or ###$P \leq 0{\cdot}001$.

the number of migrating sporocysts recovered from snails previously treated with Taim cell-free haemolymph was statistically lower than the number recovered from the CBSS-inoculated group (Figure 2).

In all experimental groups the emergence of cercaria started at 40 dpi, however, the infection rate of snails that received Taim cell-free haemolymph was lower than the infected controls. At 45 dpi, 35% and 25% of the infected snails were shedding cercariae in CBSS-inoculated and cell-free Cabo-Frio haemolymph-treated snails, respectively, but cercariae were found in only 11% of the infected snails that received Taim cell-free haemolymph. Moreover, Taim cell-free haemolymph-treated snails showed a statistically lower number of cercariae than the CBSS-inoculated snails (Figure 3).

In vitro interaction

The culture conditions used in our experiments resulted in very low *S. mansoni*-sporocyst mortality (< 1%) and the addition of cell-free haemolymph from noninfected *B. glabrata* BH was not able to significantly alter the parasite mortality. In contrast, addition of *B. tenagophila* cell-free haemolymph, Taim or Cabo Frio, increased significantly the sporocyst mortality compared to the control or BH cell-free haemolymph groups. Moreover, the effect of Taim cell-free haemolymph on sporocyst mortality was twofold stronger than the effect of Cabo Frio cell-free haemolymph ($P \leq 0{\cdot}001$). The results indicated a direct effect of soluble factor of *B. tenagophila* haemolymph, especially from the Taim strain, on the sporocyst damage (Figure 4).

It is important to note that the sporocyst destruction induced by haemolymph from different snail strains *in vitro* reflected the level of snail susceptibility reported *in vivo*, that is, haemocytes plus cell-free haemolymph from Taim strain, the parasite resistant snail, induced the highest sporocysts mortality *in vitro*, while no significant sporocyst destruction was observed with addition of haemocytes plus cell-free haemolymph from *B. glabrata* BH, the high susceptible snail strain (Figure 5). Under this experimental conditions, it was detected a sporocyst mortality rate around 20% in the presence of haemocytes and cell-free haemolymph from Cabo Frio snail strain, and 40% with haemocytes and cell-free haemolymph from Taim strain, mortality being statistically higher ($P > 0{\cdot}001$) compared with treatments using larvae that were only incubated with supplemented culture medium alone or with haemolymph elements from *B. glabrata* BH. Moreover, the addition of *B. tenagophila* Cabo Frio haemocytes and Taim cell-free haemolymph to the sporocyst culture resulted in a significant increase in the number of dead parasites, reaching levels similar to the sporocyst

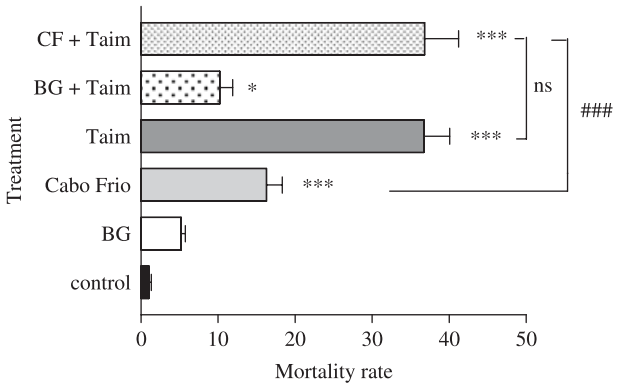

Figure 5 Mortality rate of *S. mansoni* sporocysts cultured *in vitro* in supplemented CBSS medium or in the presence of circulating haemocytes plus cell-free haemolymph recovered from different snail strain. Treatments: Control represents sporocyst cultured in supplemented CBSS medium; BG, sporocysts cultured in CBSS medium containing haemocytes plus cell-free haemolymph recovered from noninfected *B. glabrata* BH, Cabo Frio, sporocysts cultured in CBSS medium containing haemocytes plus cell-free haemolymph recovered from noninfected *B. tenagophila* Cabo Frio, Taim, sporocysts cultured in CBSS medium containing haemocytes plus cell-free haemolymph recovered from noninfected *B. tenagophila* Taim; BG + Taim, sporocysts cultured in CBSS medium containing haemocytes from noninfected *B. glabrata* plus cell-free haemolymph recovered from noninfected *B. tenagophila* Taim; CF + Taim, sporocysts cultured in CBSS medium containing haemocytes from noninfected *B. tenagophila* Cabo Frio plus cell-free haemolymph recovered from noninfected *B. tenagophila* Taim. The percentage of dead sporocysts was calculated for each treatment, done in triplicate and results showed the mean ± SD of three independent experiments. Statistically significant differences comparing the sporocysts mortality in treatment vs. control are indicated by $***P \leq 0{\cdot}001$, $**P \leq 0{\cdot}01$; and between each treatment are indicated by $\#P \leq 0{\cdot}05$ or $\#\#\#P \leq 0{\cdot}001$. ns – Not significant.

destruction observed in culture containing haemocytes plus cell-free haemolymph from *B. tenagophila* Taim, a parasite resistant snail strain (Figure 5).

The sporocyst mortality obtained when haemocytes from *B. glabrata* and Taim cell-free haemolymph were added to the culture medium was similar to the mortality rate obtained by addition of Taim cell-free haemolymph only (Figures 4 and 5).

DISCUSSION

The initial goal of this study was to evaluate the role of cell-free haemolymph from completely resistant *B. tenagophila* (Taim strain) in the mechanism of *S. mansoni* larvae destruction. Our data demonstrated that cell-free haemolymph from *B. tenagophila* of Taim strain was capable to significantly increase, *in vivo*, the resistance level to *S. mansoni* infection in susceptible snail strain, such as *B. tenagophila* of Cabo Frio strain. Moreover, Taim cell-free haemolymph by itself or in combination with haemocytes from susceptible snail strain increased *S. mansoni* sporocysts mortality.

The importance of the soluble factors of haemolymph for the protection mechanism against *S. mansoni* infection was previously demonstrated by Granath and Yoshino (10) and by Coelho and Bezerra (11). The authors reported a decrease in *S. mansoni* infection rate of *B. glabrata* PR and *B. tenagophila* Cabo Frio that were simultaneously inoculated with cell-free haemolymph from resistant snail strain. In *B. glabrata* (10), only the cell-free haemolymph from infected snail strain diminished the infection rate of the recipient snails; moreover, the number of cercariae produced by treated and infected snails was similar to the number of cercariae yielded by untreated infected snails (10). In our study, it was demonstrated that the transference of Taim cell-free haemolymph recovered from noninfected snails is sufficient to increase resistance during *B. tenagophila–S. mansoni* interaction. Moreover, we demonstrated that besides a reduction in the number of infected snails, the transference of Taim cell-free haemolymph to susceptible snails also resulted in a significantly reduction of parasite burden early in the parasite infection, suggesting the possibility of different mechanisms mediated by haemolymph from *B. glabrata* and *B. tenagophila.* The increased resistance of cell-free haemolymph treated snails was accompanied initially by high snail mortality. Haemolymph composition is complex; therefore the initial mortality effect would be completely independent of the protective effect on parasite control. Moreover, the groups that received haemolymph inoculation, especially from Taim snail, showed statistically higher number of dead circulating haemocytes (Table 2). Therefore, higher snail mortality could be a consequence of stronger cellular activation after cell-free haemolymph transference, leading to higher parasite destruction and tissue lesion.

In *B. tenagophila*, the increased snail resistance detected in cell-free haemolymph-treated snails was accomplished by modification in circulating haemocyte profile, i.e. haemolymph-treated snails showed higher hialinocyte counts and increased number of dead cells during early infection but no increase in circulating granulocytes was reported. One possible explanation for that fact is that cell-free haemolymph may activate granulocyte migration to the site of parasite penetration and/or improve the parasite recognition, promoting an increase in the destruction of sporocysts. In agreement with this hypothesis, histological analysis showed an association between strong cellular reaction around the parasite larvae and higher resistance to *S. mansoni* infection (6,7,25). Previous work (18) also showed a decrease in the number of circulating haemocytes during the first hours after parasite penetration into the snail. More

recently (26), we demonstrated that, in *B. tenagophila* of Taim strain, the haemocytes that leave the circulation just after *S. mansoni* infection are mainly large cells that were intensively labelled by FITC-conjugated lectins (WGA and PNA), suggesting that the activated cells would migrate to the infection site.

The participation of cell-free haemolymph in the mechanism of *S. mansoni* larvae destruction by the snail defence system is not understood. Studies (27–29) identified differences in the protein composition of haemolymph from resistant and susceptible *B. glabrata* strains, but no correlation between such differences and resistance has been reported. Bayne *et al*. (30) evaluated *in vitro* the effect of *B. glabrata* haemolymph on the axenically transformed primary sporocysts of *S. mansoni*. In those studies, the authors demonstrated that the cell-free haemolymph, obtained from susceptible or resistant *B. glabrata* are unable to alter the morphology of the sporocyst *in vitro*, the same occurs with haemolymph containing haemocytes from susceptible strain. Similar results were obtained in our *in vitro* system, in which there was no significant increase in sporocyst mortality with the addition of cell-free haemolymph or cell-free haemolymph plus haemocytes from *B. glabrata* BH to the culture medium (Figures 4 and 5). Nevertheless, as far as *B. tenagophila* snails are concerned, the possibility of a direct effect of cell-free haemolymph on *S. mansoni* larvae must be considered, since the cell-free haemolymph is able to kill 8% to 16% of *S. mansoni* sporocysts *in vitro*, even in the absence of haemocytes (Figure 4). Some mechanisms of direct lesion of pathogens, such as production of antimicrobial peptides (31) and activation of melanization process (32) have been described in invertebrates, but there is no experimental confirmation regarding the participation of these mechanisms in the destruction of *S. mansoni* in *Biomphalaria* spp. Bayne *et al*. (5,33) reported the presence of high levels of reactive oxygen or nitrogen species in cell-free haemolymph from *B. glabrata* and the possible effect of such compounds in the destruction of *S. mansoni* larvae. However, a possible involvement of these strongly reactive elements in parasite control observed during *in vivo* transference of Taim cell-free haemolymph or *in vitro* culture assays is still undefined.

In addition to the direct effect of some soluble factors of snail defence system, elements of cell-free haemolymph could act indirectly, facilitating parasite recognition and/or activating the haemocytes. Experimental evidences of a possible indirect effect of cell-free haemolymph have been demonstrated in *B. glabrata–S. mansoni* interaction, in which haemocytes from susceptible snail strains associated with soluble factors of haemolymph from resistant *B. glabrata* strains acquire the capacity of killing *S. mansoni* sporocysts (13,29,34). In our system, the effect of Taim cell-free haemolymph on haemocytes observed *in vivo* was confirmed *in vitro* by the high mortality rate of sporocysts obtained in culture system containing haemocytes/haemolymph from *B. tenagophila* Taim and by the increased mortality rate obtained by addition of cell-free haemolymph from Taim snails to haemocytes from *B. tenagophila* Cabo Frio (Figure 5). It is important to point out that, in our experimental model, the high level of sporocyst mortality *in vitro* was associated with higher number of haemocytes bound to the parasite observed when Taim cell-free haemolymph was added to culture medium, suggesting that some components in Taim cell-free haemolymph may increase parasite recognition by haemocytes. Many studies have demonstrated that carbohydrates associated with the tegument of *S. mansoni* sporocyst can bind to lectins expressed by haemocytes and/or soluble in *B. glabrata* haemolymph, therefore lectins would mediate parasite recognition (1,35–38). The involvement of lectins in *S. mansoni* sporocysts recognition by *B. tenagophila* of Taim strain is under investigation in our laboratory.

The cooperative effect of haemocytes and cell-free haemolymph was not observed between different snail species, since there was no significant change in sporocysts mortality by addition of Taim cell-free haemolymph plus *B. glabrata* BH haemocytes to the cell culture medium compared to the mortality rate observed with Taim cell-free haemolymph only. More recently, Martins-Souza *et al*. (26), using the same snail and parasite strains demonstrated that *B. glabrata* haemocytes showed different staining pattern with fluorescent lectins, when compared with *B. tenagophila* (Taim or Cabo Frio strains), thus suggesting differences in the expression of glycoproteins in the haemocyte membrane, both in intensity and quality, would justify the absence of indirect effect of cell-free haemolymph from *B. tenagophila* on *B. glabrata* haemocytes.

Even though the defence mechanisms employed by *B. tenagophila* to control trematode infections are far from being fully understood, the participation of cell-free haemolymph from resistant snail strains was demonstrated in our experiments. Our data also suggested that soluble components of the haemolymph can directly damage *S. mansoni* larvae and also can mediate parasite recognition by circulating haemocytes. Further studies should be undertaken to elucidate possible components responsible for such events.

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Potential Role of the Chemokine Macrophage Inflammatory Protein 1α in Human and Experimental Schistosomiasis

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In human schistosomiasis, the concentrations of the chemokine macrophage inflammatory protein 1α (MIP-1α/CCL3) is greater in the plasma of patients with clinical hepatosplenic disease. The objective of the present study was to confirm the ability of CCL3 to detect severe disease in patients classified by ultrasonography (US) and to evaluate the potential role of CCL3 in *Schistosoma mansoni*-infected mice. CCL3 was measured by enzyme-linked immunosorbent assay in the plasma of *S. mansoni*-infected patients. CCL3-deficient mice were infected with 25 cercariae, and various inflammatory and infectious indices were evaluated. The concentration of CCL3 was higher in the plasma of *S. mansoni*-infected than noninfected patients. Moreover, CCL3 was greater in those with US-defined hepatosplenic than with the intestinal form of the disease. In CCL3-deficient mice, the size of the granuloma and the liver eosinophil peroxidase activity and collagen content were diminished compared to wild-type mice. In CCL3-deficient mice, the worm burden after 14 weeks of infection, but not after 9 weeks, was consistently smaller. The in vitro response of mesenteric lymph node cells to antigen stimulation was characterized by lower levels of interleukin-4 (IL-4) and IL-10. CCL3 is a marker of disease severity in infected humans, and experimental studies in mice suggest that CCL3 may be a causative factor in the development of severe schistosomiasis.

Schistosomiasis is one of the most prevalent helminth diseases in the world and is caused by blood flukes of the *Schistosoma* genus (36). In infected individuals, the granulomatous inflammation in response to egg deposition in the liver in the case of *Schistosoma mansoni* is the major pathological finding and accounts for most of the clinical symptoms. Worm oviposition at 5 to 6 weeks poses a strong Th2 bias in the immune response against infection (6) while also inhibiting the Th1 component (12, 42). Nonetheless, the granulomatous response that is maximal during the first few weeks after initial oviposition is also subjected to immunomodulation. Apart from interleukin-10 (IL-10) (29), other factors may also be involved in this process. For example, recent literature suggests that, in animal models, the granulomatous response that occurs at chronic time points is dependent on the soluble form of the IL-13 receptor α2 (27). In patients, failure in modulating the response might lead to the development severe schistosomiasis later in life (15). Indeed, a direct consequence of the persistence of an exacerbated immune response appears to be the development of large granulomatous reactions associated with intense collagen deposition (20) and the development of hepatosplenic schistosomiasis. Therefore, there is much scientific interest in the understanding of the mechanisms and inflammatory mediators underlying egg-induced granulomatous response, with the ultimate goal of proposing strategies to modulate fibrosis. It is known, however, that prevention of granuloma formation may be dangerous, since lethality has been reported in mice that fail to form granulomas (21).

A few studies have evaluated the role of chemokines in *S. mansoni* granuloma formation. Most of them have been carried out in mice by intravenous administration of egg antigen-conjugated beads (9). Overall, there is good evidence to suggest that chemokines play an important role in defining the leukocyte infiltrate and subsequent immune response that occurs around beads (9). For instance, the chemokines (32), macrophage inflammatory protein 1α (MIP-1α/CCL3) and regulated on activation and normal T-cell expressed and secreted (RANTES/CCL5), are released during the granulomatous response and blocking of their action alters the size and other granuloma characteristics in a model of pulmonary embolization (10, 25, 26, 38). Studies with chemokine receptor-deficient mice also suggested a role for chemokines in the development of granulomas (9, 19, 41, 52).

Fewer studies have evaluated the role of chemokines for human schistosomiasis. A recent study from our group has shown that concentrations of CCL5, eotaxin/CCL11, and CCL3 are elevated in the plasma in patients with chronic schistosomiasis. Importantly, patients with high concentrations of CCL3 were more likely to develop hepatosplenomegaly

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(18). However, this assumption may have been biased, since organomegaly was not confirmed by ultrasonography (US). Moreover, blockade of receptors for CCL3 was associated with a decrease in size of in vitro granuloma around antigen-conjugated beads (18). In the present study, we wanted to confirm and extend our previous data, but now using patients classified by US from a different area of schistosomiasis endemicity. We also investigated likely roles of CCL3 in a murine model of infection that could account for differences in disease morbidity in patients. To this end, the course of *S. mansoni* infection was monitored in CCL3-deficient and wild-type mice.

MATERIALS AND METHODS

Blood collecting and outpatient clinical and US examination. Plasma samples were obtained from patients living in Boa União and Nova União, two adjacent localities, in Minas Gerais, Brazil. Study groups were composed of 32 intestinal disease patients, 13 hepatosplenic patients, and 12 controls. The diameter of the portal and mesenteric veins, the sizes of the liver and spleen, and wall thickening of central and peripheral branches of the portal vein were evaluated according to World Health Organization guidelines (1, 14, 22, 27). The criteria for inclusion in the hepatosplenic group was that patients had to present thickening of the portal branches of >0.3 cm and hepatic and splenic organomegaly as shown by US performed with a portable Hitachi EUB-200 apparatus. This is an important aspect of the present study since sole clinical organomegaly is a bad predictor of disease outcome (1). In our patient population, only grade I fibrosis (periportal thickening of >0.3 and <0.5) was found, confirming previous observations that more severe presentations of the disease seem to be less prevalent probably due to the treatment of infection (2, 3). Patients were treated for infection regardless of their adherence to the study. Those younger than 5 years and those exhibiting liver damage suggestive of other diseases, such as steatosis, in the absence of pipe stem fibrosis, were excluded. The control group was composed of noninfected individuals living in the area of endemicity and healthy volunteers from an area where the disease is not endemic. Negative results for schistosome infection were attested by three consecutive parasitological examinations by the Kato-Katz method (24). All patients signed an informed consent for the use of blood samples.

Animals and Infection. Male and female C57BL/6 and CCL3-deficient mice (13) were obtained from the animal facility of the Centro de Pesquisas Ren, Rachou. The animals were 8 weeks old at the beginning of the experiment and kept at the animal facility of the Grupo Interdisciplinar de Estudos em Esquistossomose. Rodent chow and water were given ad libitum. LE strain *S. mansoni* cercariae (50) released from *Biomphalaria glabrata* snails were suspended in saline 0.9% and subcutaneously injected (25 cercariae per animal) by an experienced technician. Within the same experiment, all groups of mice, wild type or knockout, were infected with the same cercaria suspension. To assess parasite burden, mice had to be subjected to mesenteric perfusion for collecting of adult worms. The latter procedure induces a degree of tissue damage and thus prevented the use of the tissues and blood from these animals for the measurement of other parameters. Thus, separate groups of animals were used to assess either parasitological or immunological parameters. Female mice were used for the parasitological studies (egg and worm burdens), and male mice were used for all other parameters described here. Infection-induced morbidity and mortality were observed in CCL3-deficient and wild-type mice throughout the experiment, which lasted 9 or 14 weeks, and so represented the acute and chronic phases of the infection. All experiments were repeated and, since the means of a given variable were similar in these experiments, the results were pooled for presentation.

Determination of egg content in the feces. Animals were kept in individual gridded-floor cages the night prior to sacrifice. In each cage a piece of wet absorbent paper kept feces humid. On the following morning, feces were collected, weighed, homogenized in 10% buffered formaldehyde, and stored. Two aliquots of 100 μl each were counted by light microscopy, and the results are expressed as number of eggs per gram of feces.

Recovering of worms from the portal system. Animals were sacrificed by cervical displacement, and viscera were exposed. A cut was made at the portal vein, and a needle conjugated to a perfusion pump was introduced into the abdominal aorta (45). Saline (0.9%) was perfused and collected in a Becker apparatus. Decantation was allowed for 30 min, and the supernatant was discarded. Washing steps were performed until the supernatant was clear. Male and female worms were counted in a laboratory magnifier.

Determination of egg content in the tissues. The livers, lungs, intestines, and spleens of infected mice were weighted and solubilized in 5% KOH solution until no sign of intact tissue was present. Tissue suspensions were centrifuged at 200 × *g* for 1 min at room temperature, washed five times, and stored at 4°C. Eggs in aliquots of 100 μl were counted under a light microscope, and values were extrapolated to the total organ content. The tissue egg retention index, a measure of worm fecundity, was calculated as the sum of the absolute number of eggs present in tissues divided by the number or female worms, regardless of whether they were found mated or not (17).

Differential blood cell count and tissue histology. Animals were sacrificed under ether anesthesia, followed by cervical displacement, and 20 μl of blood was collected from each brachial plexus. The blood was diluted in Turk's solution for total cell count. A blood smear was prepared and stained with Giemsa and May-Grunwald stains. Differential blood count of mononuclear cells, neutrophils, and eosinophils was performed from a total of 300 cells. Fragments of liver were removed, fixed in 10% buffered formaldehyde, and stained with either hematoxylin and eosin or Picrus Sirius (30). About 500 μl of blood were collected for the quantification of cytokines.

Cell culture. After sacrifice, animals not subjected to hepatic perfusion had the abdominal wall opened in sterile conditions. Mesenteric lymph nodes (MLN) were removed, and cells were isolated. The resultant cell suspension was diluted to 10^7 cells/ml in HEPES-modified RPMI supplemented with potassium-penicillin at 10 IU/ml and 1 μg of gentamicin-containing 5% fetal calf serum/ml. Cells were plated in the presence of concanavalin A (1 μg/ml), a nonspecific T-cell stimulator (11), and schistosome egg antigen (SEA) (5) or soluble adult worm antigenic preparation (SWAP), both at the concentration of 50 μg/ml. The supernatant was collected 48 h later for cytokine quantification.

Cytokine determination by ELISA. The concentration of cytokines in plasma and culture supernatant was performed by sandwich enzyme-linked immunosorbent assay (ELISA) as described elsewhere (31). The ELISA kits for mouse IL-4, IL-10, IL-13, gamma interferon (IFN-γ), and human CCL3 were obtained from R&D Systems. The antibody pairs and standards used in the assay for mouse IL-1 were kindly provided by Steve Poole. The specific recommendations of the manufacturers were followed. Human plasma samples were subjected to acid-salt precipitation to avoid cross-reactivity in ELISA (18).

Determination of the activity of eosinophil peroxidase (EPO). The assay was performed as described previously (47). Briefly, 100 mg of the liver and intestine of the animals were weighted and homogenized in 1.9 ml of phosphate-buffered saline and centrifuged at 12,000 × *g* for 10 min. The supernatant was discarded, and the erythrocytes were lysed. The samples were then centrifuged, the supernatant was discarded, and the pellet was suspended in 1.9 ml of 0.5% hexadecyltrimethyl ammonium bromide in phosphate-buffered saline, frozen three times in liquid nitrogen, and centrifuged at 4°C at 12,000 × *g* for 10 min. The supernatant was used in the enzymatic assay by the addition of an equal amount substrate (1.5 mM *o*-phenylenediamine–6.6 mM H_2O_2 in 0.075 mM Tris-HCl [pH 8]). The reaction was stopped with 50 μl of 1 M H_2SO_4, and the absorbance was read at 492 nm.

Hydroxyproline determination. Fragments (100 mg) of liver and intestine were removed for hydroxyproline determination as an indirect measure of collagen content (39). Briefly, tissues were homogenized in 0.9% saline, frozen, and lyophilized. The assay was performed with 20 mg of the lyophilized material subjected to alkaline hydrolysis in 300 μl of H_2O plus 75 μl of 10 M NaOH at 120°C for 20 min. An aliquot of 50 μl of the hydrolyzed tissue was added to 450 μl of Chloramine T oxidizing reagent (0.056 M Chloramine T–*n*-propanol 10% in acetate-citrate buffer [pH 6.5]) and allowed to react for 20 min. A hydroxyproline standard curve was prepared likewise. Color was developed by the addition of 500 μl of 1 M *p*-dimethylaminebenzaldehyde diluted in *n*-propanol–perchloric acid (2:1 [vol/vol]). The absorbance was read at 550 nm.

Morphometric analysis. Slides with sections of the liver were analyzed under a light microscope for the characterization of the inflammatory infiltrate and the composition of granulomas. For the quantitative analysis of granuloma area, images were captured with a digital camera (Optronics DEI-470) connected to a microscope (Olympus IX70) and analyzed with Image ProPlus. For each infected animal, the first nine granulomas found in either of three liver sections meeting the criteria of being isolated and of presenting a central viable egg were photographed and analyzed. Granulomas in which more than one or no egg was visible were excluded from the analysis. The area of each of the nine granulomas was measured three times and averaged.

Histological egg densitometry. Images covering 1,500 μm^2 from liver sections of male wild-type and $CCL3^{-/-}$ mice were captured with a digital camera (Optronics DEI-470) connected to a microscope (Olympus IX70) with a magnification of × 100. The number of images taken was about 30 to 50 for each animal and was equal to the highest number of whole snapshots that could be taken without including serial slices in a given fragment of stained tissue. The schistosome eggs per image were counted, and the average value was entered as

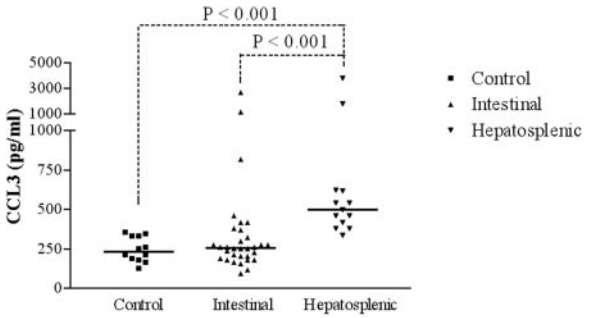

FIG. 1. Chemokine levels in the plasma of schistosome-infected patients and control individuals. Individual concentrations are shown with the median of each group expressed as a horizontal line. Patients were classified as intestinal ($n = 32$) or hepatosplenic ($n = 13$) according to clinical and US examinations. A control group was composed of 12 individuals. Plasma samples were processed for elimination of cross-reactivity and used for CCL3 determination by ELISA. The data were analyzed by the Kruskal-Wallis test, followed by Dunn's comparison test.

an individual value for each animal. The final value is referred to here as the "egg densitometric index" and was used to compare to egg counts obtained from whole-tissue digestions performed in female mice. As hydroxyproline content was only measured in livers and intestines of male mice, the egg densitometric index was useful to calculate a ratio describing the collagen deposition per tissue egg content.

Statistical analysis. Results for mice are expressed as means ± the standard error of the means (SEM) and were analyzed by analysis of variance, followed by the Newman-Keuls multiple comparison test. MLN cultures were analyzed by using an unpaired *t* test when detectable levels of cytokines were found only in infected groups. Human data were analyzed by Kruskal-Wallis test, followed by Dunn's multicomparison test. A cutoff value of MIP-1α that differentiated patients with or without hepatosplenomegaly was selected empirically. Relative risk was calculated by using the Fisher exact test in a 2×2 contingency table.

RESULTS

Concentration of CCL3 in plasma of patients. To confirm and validate our previous finding that CCL3 marked a group of patients with severe disease (18), we evaluated the concentrations of CCL3 by sandwich ELISA in plasma samples of US-assessed schistosome-infected individuals. In agreement with previous findings, infected patients had greater concentration of CCL3 in plasma than noninfected individuals ($P < 0.05$). The concentrations of CCL3 were significantly higher in plasma of patients with organomegaly than in those without organomegaly ($P < 0.001$, Fig. 1). The value of 400 pg/ml was found to be best to separate patients with or without organomegaly. Indeed, infected patients with a concentration of CCL3 of >400 pg/ml had a 14-fold greater chance of having organomegaly than those with lower concentrations of this chemokine ($P < 0.001$). However, there was no tendency for patients with high levels of CCL3 within the intestinal group to exhibit higher levels of fibrosis (not shown).

Infection indices in CCL3-deficient and wild-type mice. Initial experiments were designed to assess whether CCL3 deficiency interfered with worm burden and/or fecundity. No statistically significant difference in the lethality rates of infected mice was found, although percentages tended to be smaller in the CCL-3-deficient (28.5%) than in the wild-type group (37.5%) at 14 weeks postinfection. Of the approximately 25 injected cercariae, ca. 10 adult worms were recovered after

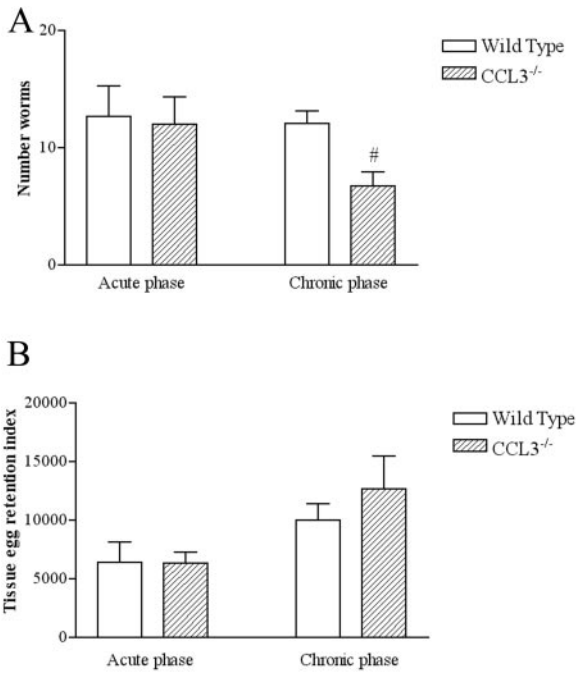

FIG. 2. Total number of worms in the portal system and fecundity of *S. mansoni* harvested from wild-type or CCL3-deficient mice. Values are the means ± the SEM of worms from perfusates of female mice. (A) The male and female adult mated and unmated worms present in the portal system of infected wild-type (□; acute phase, $n = 6$; chronic phase, $n = 12$) or CCL3-deficient (▨; acute phase, $n = 6$; chronic phase, $n = 11$) mice were counted after hepatic perfusion at 9 (acute phase) or 14 (chronic phase) weeks after infection. (B) The ratio between the total number or eggs present in all analyzed tissues, i.e., lungs, liver, intestine, and spleen divided by the number of female worms gives the tissue egg retention index for each group. The numbers of female worms are equal to those used to calculate the averages shown in panel A. #, $P < 0.01$ as determined by the Newman-Keuls test for CCL3-deficient mice compared to wild-type ones at the same phase of infection.

perfusion from either wild-type or CCL3-deficient mice at 9 weeks postinfection (Fig. 2A). At 14 weeks, there was a similar number of worms in the wild-type group, but a we observed a significant ($P < 0.01$) reduction in CCL3-deficient mice (Fig. 2A). Despite changes in worm numbers, the tissue egg retention index, as calculated by the total number of eggs trapped in tissues divided by the total number of female worms, was equal in CCL3-deficient and wild-type mice during both the acute and the chronic phases (Fig. 2B).

These results indicate that CCL3 deficiency does not alter worm fecundity. However, tissue- and phase-specific analysis reveals important differences in egg burdens calculated in female mice through the digestion of whole tissues with 5% KOH solution. During both the acute and chronic phases, a smaller number of eggs per gram of feces was found in CCL3-deficient mice (Fig. 3A). At the chronic phase other tissue specific differences were found. A reduction in the absolute number of eggs deposited in the liver (Fig. 3B, $P < 0.05$) and spleen (Fig. 3C, $P < 0.05$) was observed in the CCL3-deficient group. There was no difference in the number of eggs in the intestines (Fig. 3D) or lungs (not shown). Confirming data on egg counts, the evaluation of egg number by histology demonstrated a smaller number of

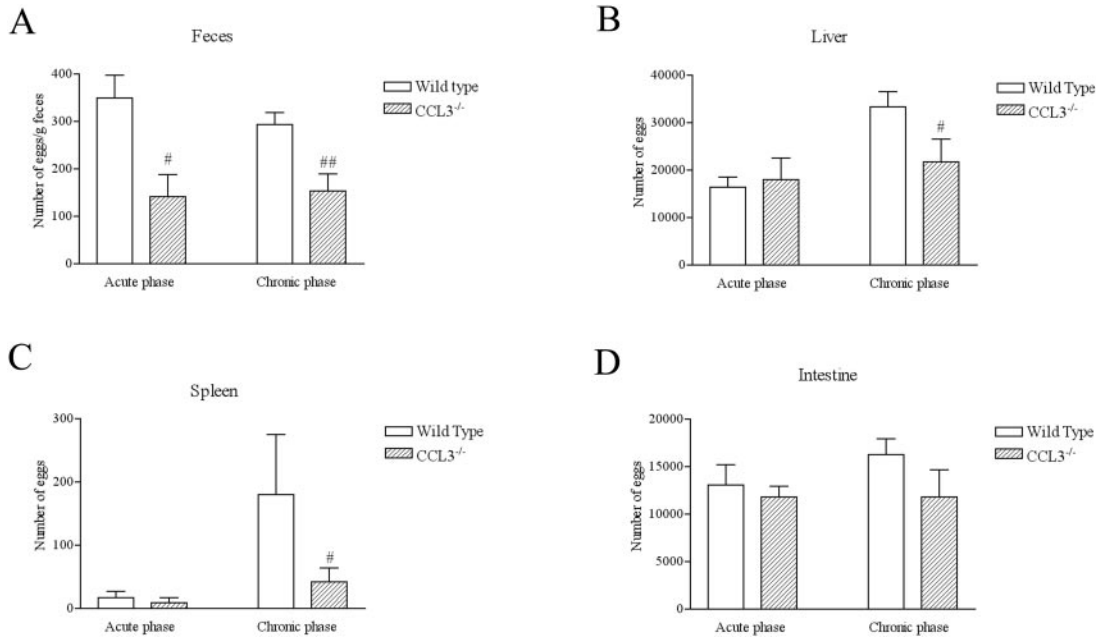

FIG. 3. *S. mansoni* egg burdens in different tissues from wild-type or CCL3-deficient mice. Values are the means ± the SEM of eggs counted from tissues or feces from female mice. At the day prior to sacrifice, animals were kept in special cages for the collection of their feces. Eggs in the feces (A) of wild-type (□; acute phase, $n = 6$; chronic phase, $n = 12$) or CCL3-deficient (▨; acute phase, $n = 6$; chronic phase, $n = 11$) mice are expressed per grams. After sacrifice, mice had their livers (B), spleens (C), and intestines (D) excised, weighed, and dissolved in 5% KOH. After the counting of aliquots in a light microscope, the total number of eggs in the tissues was calculated. # and ##, $P < 0.05$ or $P < 0.01$, respectively, as determined by the Newman-Keuls test for MIP-1a/CCL3-deficient mice compared to wild-type mice at the same phase of infection.

eggs in the liver of CCL3-deficient compared to wild-type mice during the chronic phase of infection (3.7 ± 0.2 versus 2.6 ± 0.3, respectively; $P < 0.05$ [unpaired t test]). The difference in egg density was not accompanied by changes in organ mass between the two groups (results not shown).

Hepatic and intestinal inflammation in CCL3-deficient and wild-type mice. Eosinophils represent the major cellular component of Th2 granulomas (4). The eosinophil relative content in tissues was deduced by EPO activity. There was minimal EPO activity in the liver of noninfected animals both groups (Fig. 4A). *S. mansoni* infection induced an increase in the EPO activity in the liver during the acute phase of infection, and this was persistent at 14 weeks. Comparisons within the same phase of infection demonstrated that the levels of EPO in the liver of CCL3-deficient mice were similar at 9 weeks. There was a significant 15% reduction of liver EPO ($P < 0.05$) at 14 weeks compared to wild-type mice (Fig. 4A).

Just as no statistical difference was found in the egg number in the intestines of wild-type animals compared to CCL3-deficient animals, the EPO activity was also similar between these groups in this organ (Fig. 4B). Blood eosinophilia is found in infected animals, but no difference was observed between the two groups (Fig. 4C).

Next, we evaluated whether CCL3 deficiency interfered with the size of liver granulomas. Comparison between the acute and chronic phases shows an approximate 50% reduction in granuloma size both in wild-type and CCL3-deficient mice (Fig. 5A), indicating extensive immunomodulation. The area of granulomas formed in the liver of CCL3-deficient animals was similar to those of wild-type mice in the acute phase of the infection but significantly smaller ($P < 0.01$) at 14 weeks with a 28% reduction (Fig. 5A). Despite the clear reduction in granuloma size, there was no apparent tendency of livers of CCL3-deficient mice to present larger stretches of hepatic degeneration or fibrosis. Since no major differences have been observed between the two animal groups at 9 weeks, the following results will focus on the chronic phase of the infection.

As liver fibrosis is a major alteration associated with morbidity, we evaluated the hydroxyproline content of livers from wild-type and CCL3-deficient mice. As shown in Fig. 5B, the amount of hydroxyproline present in the liver, an indirect measure of collagen content, is increased in infected animals compared to noninfected ones. The hydroxyproline content in the liver of wild-type animals was higher than that of CCL3-deficient ones (Fig. 5B). No statistically significant differences were found for the hydroxyproline concentrations in the intestine in any group of mice (results not shown).

To dissect whether collagen deposition depended directly on the action of CCL3 or was resultant from the decreased egg retention in the liver of CCL3-deficient mice, we calculated in the same animal the ratio between the hydroxyproline content and the number of eggs in tissue, as assessed histologically. Thus, the ratio between a marker of fibrosis and a marker of egg burden was similar in wild-type and CCL3-deficient mice (0.62 ± 0.11 versus 0.67 ± 0.13, respectively).

Figure 6A and B shows the normal architecture of the liver of noninfected wild-type and CCL3-deficient animals which were apparently similar. In Fig. 6C, we noticed a strong egg-induced granulomatous response in the liver of a wild-type mouse at 14 weeks postinfection. There was an intense eosin-

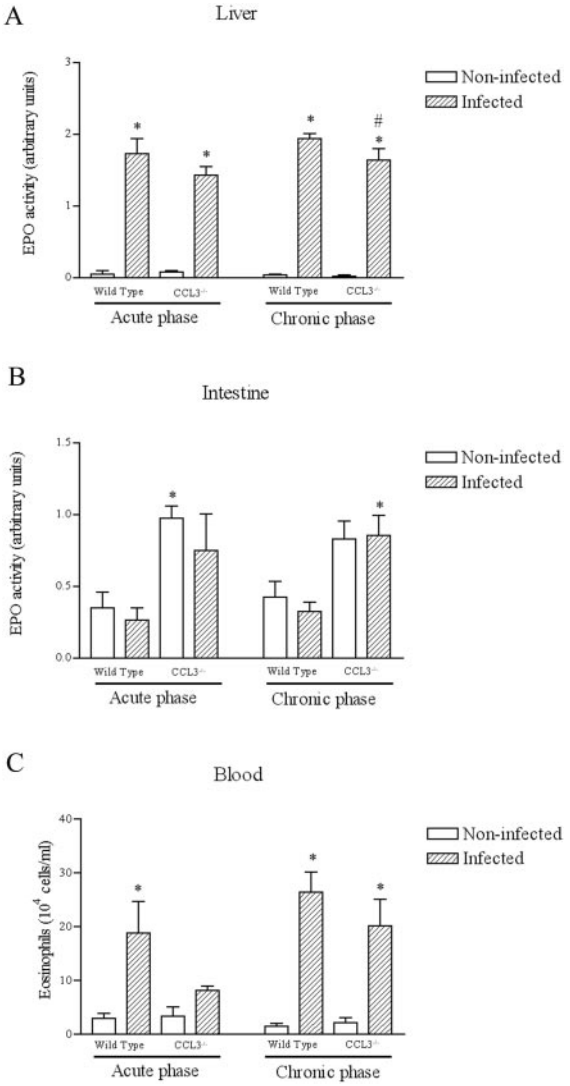

FIG. 4. Blood eosinophils and EPO activity in the livers and intestines of wild-type and CCL3-deficient mice. Values are the means ± the SEM from male mice. At 9 (acute phase) or 14 (chronic phase) weeks after infection, a fragments of the livers (A) and intestines (B) of noninfected (□) or infected (▨) wild-type (acute phase noninfected, $n = 4$; infected, $n = 6$; chronic phase noninfected, $n = 8$; infected, $n = 12$) and CCL3-deficient (acute phase noninfected, $n = 5$; infected, $n = 4$; chronic phase noninfected, $n = 8$; infected, $n = 12$) mice were weighed and processed for quantification of EPO activity. Blood smears were also prepared, fixed, stained with hematoxylin and eosin, and then subjected to differential cell counting. The percentages of eosinophils are shown in panel C. ✱, $P < 0.001$ (in panel A), $P < 0.05$ (in panel B), or $P < 0.01$ (in panel C) as determined by the Newman-Keuls test for infected versus noninfected mice of the same phenotype at the same phase of infection. #, $P < 0.05$ for infected CCL3-deficient compared to infected wild-type mice at the same phase of infection.

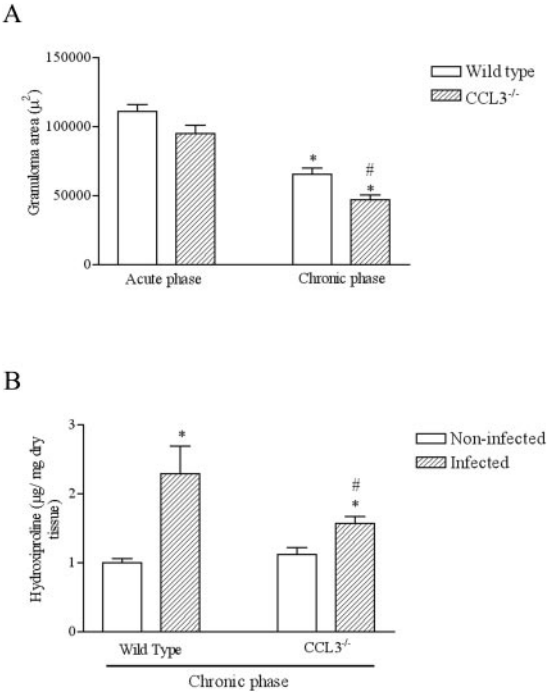

FIG. 5. Area of the granulomas formed around eggs and hydroxyproline content in the liver of wild-type and CCL3-deficient mice at the acute and chronic phases of infection. Hydroxyproline content was used as a measure of collagen deposition. Values are the means ± the SEM of liver samples from male mice at the chronic phase of the infection. (A) At 9 (acute phase) or 14 (chronic phase) weeks after infection, paraffin-fixed liver slices of wild-type (□ acute phase, $n = 8$; chronic phase, $n = 12$) or CCL3-deficient (▨; acute phase, $n = 8$; chronic phase, $n = 12$) mice were stained with eosin and hematoxylin. Isolated granuloma images were captured and analyzed in the software KS300 2.0 for the measurement of the area. ✱, $P < 0.01$ as determined by the Newman-Keuls test for infected mice at the acute phase compared to infected mice of the same phenotype at the chronic phase; #, $P < 0.01$ for CCL3-deficient mice compared to wild-type mice at the same phase of infection. (B) At 14 weeks (chronic phase) after infection, a fragment of the liver of noninfected (□) or infected (▨) wild-type (noninfected, $n = 4$; infected, $n = 6$) and CCL3-deficient (noninfected, $n = 4$; infected, $n = 7$) animals was used for the quantification of hydroxyproline. ✱, $P < 0.05$ for infected mice compared to noninfected ones of the same phenotype; #, $P < 0,05$ for infected MIP-1α/CCL3-deficient mice compared to wild-type mice.

ophilic infiltrate and extensive collagen deposition, as shown by Picrus Sirius staining. However, Fig. 6D illustrates the result shown in Fig. 5A that granulomas around eggs in the CCL3-deficient group were smaller than that of wild-type mice.

Cytokine production by MLN cells during the chronic phase of infection. The in vitro cytokine production from SEA-stimulated MLN cells from mice is shown in Fig. 7. As expected, noninfected mice of both groups produced little or no cytokine against SEA stimulation. In contrast, MLN cells from infected animals produced cytokines of both the Th1 (IFN-γ) and the Th2 (IL-4, IL-10, and IL-13) type. As observed in Fig. 7A and B, the cytokines IL-4 ($P < 0.01$) and IL-10 ($P < 0.001$) were produced at lower levels by cells from CCL3-deficient mice. However, the production of TNF-α (Fig. 7C), IFN-γ (Fig. 7D), and IL-13 (Fig. 7E) were similar in the two mouse strains.

Table 1 shows the negative and positive control groups for the data given in Fig. 7 and also shows the responses to SWAP stimulation. Unstimulated MLN cells of both wild-type and CCL3-deficient mice produced very little or no IL-4, IL-10, IL-13, IFN-γ, and TNF-α, regardless of the infection status. Nonetheless, concanavalin A-stimulated MLN cells from infected animals of both groups produced higher amounts of IL-4 ($P < 0.001$ and < 0.01 for wild-type and CCL3-deficient mice, respectively) and IL-10 ($P < 0.05$ and < 0.01 for wild-

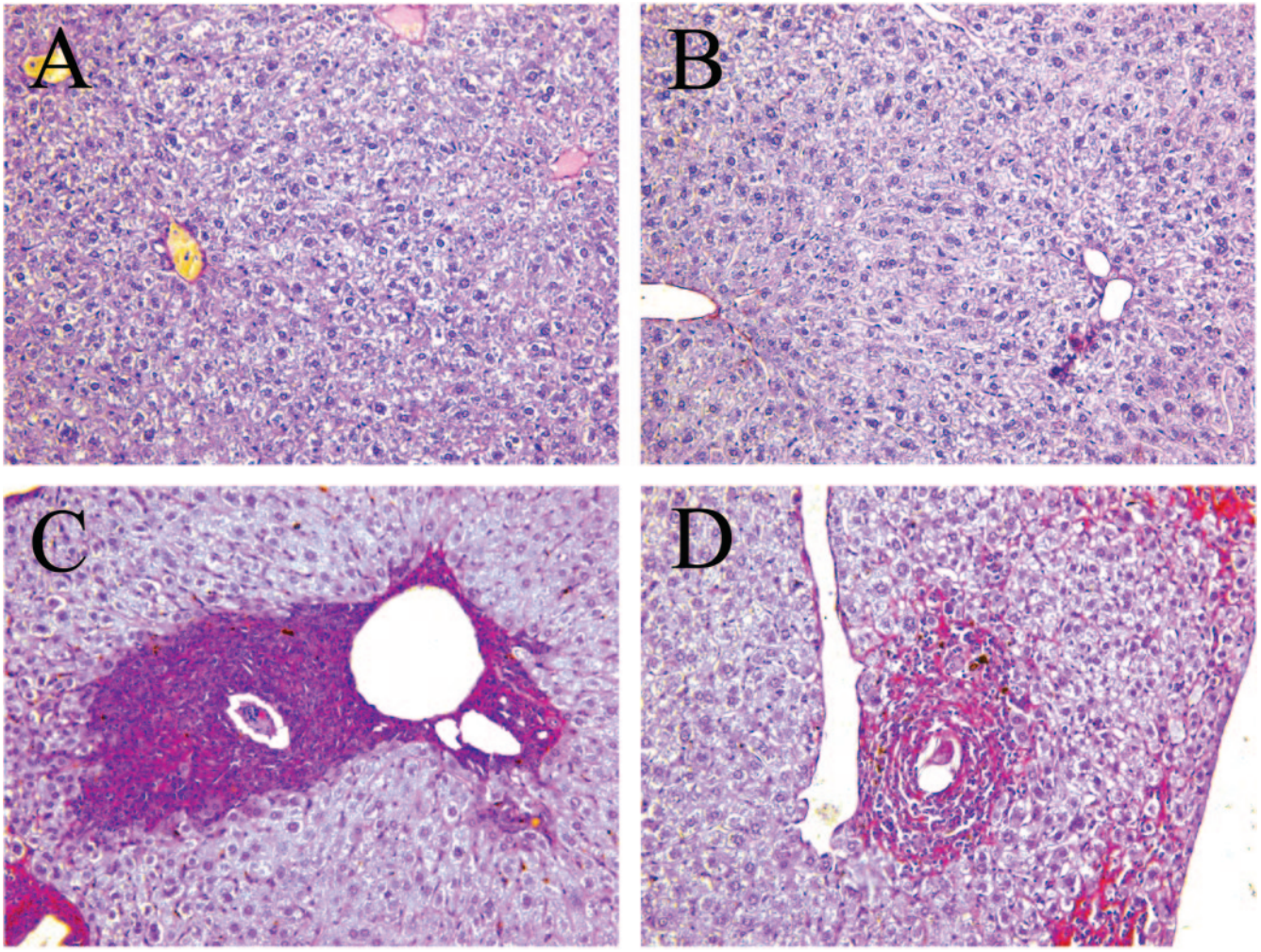

FIG. 6. Histological sections of the liver of noninfected or infected male wild-type and CCL3-deficient animals at the chronic phase of infection. Images are from Picrus Sirius-stained histological sections representative of the group. (A) Normal architecture of the liver of a noninfected wild-type animal. (B) Normal architecture of the liver of a noninfected CCL3-deficient animal. (C) Appearance of the liver of a wild-type infected mouse at the chronic phase of the infection. Notice the strong granulomatous response formed around the eggs. (D) Appearance of the liver of a CCL3-deficient infected mouse at the chronic phase of the infection. Many of the alterations described in panel C are also visible.

type and CCL3-deficient mice, respectively),but no difference was found in IFN-γ, IL-13, or TNF-α production. Similar results were observed for SWAP-stimulated cells, where noninfected animals did not respond stimulation, producing no detectable levels of cytokines. There was always cytokine production in infected animals.

DISCUSSION

We have recently demonstrated a correlation between elevated concentrations of CCL3 and the likelihood of presentation of severe schistosomiasis in humans (18). The present study had two main purposes: (i) to confirm the latter findings by using US to identify patients with severe disease and (ii) to investigate the existence of a causal relationship between CCL3 and morbidity in an animal model of *S. mansoni* infection. Using plasma of patients categorized according to US findings, we were able to confirm previous findings (18) that levels of CCL3 in plasma were a good marker of hepatosplenic schistosomiasis. Indeed, patients with hepatosplenic disease had greater concentrations of this chemokine in plasma than those with intestinal disease or controls. Moreover, infected patients with CCL3 concentrations in plasma of >400 pg/ml had a 14-fold-greater chance of presenting with hepatosplenomegaly than those with lower concentrations. Thus, the two studies, which comprise two distinct populations, strongly suggest that CCL3 concentrations could be good markers of severity in *S. mansoni* infection.

Important differences were found in *S. mansoni* infection in mice in the absence of CCL3. (i) The numbers of eggs in the liver and adult worms were decreased in CCL3-deficient mice at the chronic phase of the infection. (ii) Chronic-phase granulomas were smaller in CCL3-deficient mice than in wild-type mice. (iii) The livers of infected CCL3-deficient mice had a lower hydroxyproline content. Of note and in contrast to the findings at the chronic phase, there were no dramatic changes in infection or inflammatory indices at 9 weeks.

We have shown here that the parasitic load of chronically

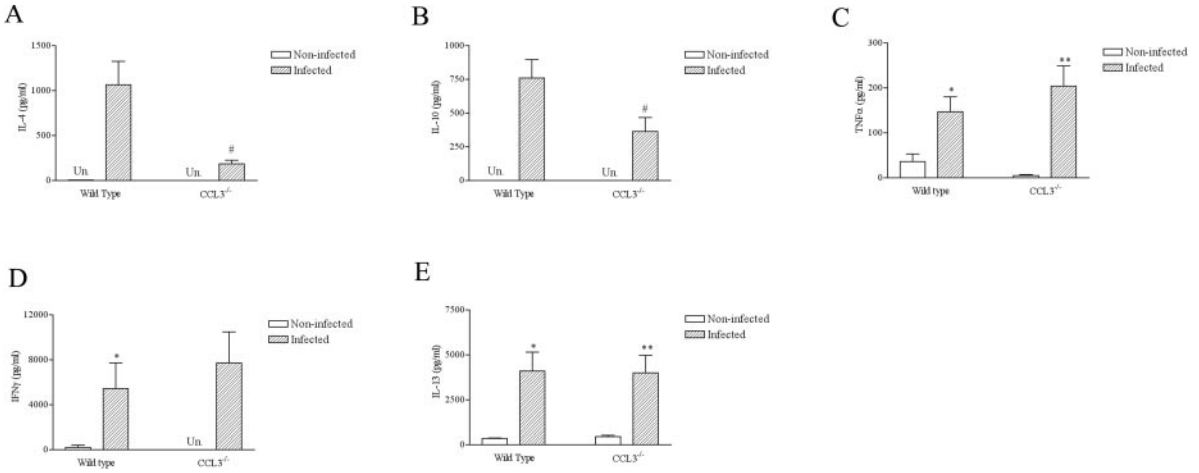

FIG. 7. Cytokine production by MLN cells from male infected and noninfected wild-type and CCL3-deficient mice in response to SEA stimulation at the chronic phase of the infection. Values are the means ± the SEM of supernatants from four mice. At 14 weeks postinfection, lymph node cells were obtained and stimulated with SEA. The supernatant was collected 48 h later for the quantification of IL-4 (A), IL-10 (B), TNF-α (C), and IFN-γ (D). ✻ and ✻✻, $P < 0.05$ or $P < 0.01$, respectively, for infected mice compared to noninfected ones of the same phenotype as determined by the Newman-Keuls test; #, $P < 0.05$ for infected CCL3-deficient compared to infected wild-type mice as determined by unpaired *t* test. Un., undetectable.

infected CCL3-deficient animals is reduced compared to wild-type animals. There are two possible explanations for this. First, CCL3 deficiency may interfere with the immune response to adult worms, making it more efficient and so capable of causing their clearance. Second, the absence of CCL3 may directly or indirectly alter worm survival. The testing of these hypotheses is beyond the scope of the present study and is currently under way in our laboratory. In any case, further studies are needed to address this question.

It is worth emphasizing that all groups of animals were infected with the same cercaria suspension by the same person in a random order and with no delay between groups, so that differences in cercarial virulence or viability could not account for the latter findings. Besides, worm burdens were investigated in groups of female wild-type and CCL3-deficient mice, excluding the possibility that sex-specific susceptibilities to infection could have biased the results (16, 33).

It is not clear in the literature whether mice can mount an effective immune response against adult *S. mansoni* worms. Our results, however, do indicate that the difference in worm burdens appeared in CCL3-deficient mice only after 9 weeks of infection and was responsible for the slight tendency, although not statistically significant, of wild-type mice to have higher mortality rates than CCL3-deficient mice. If a change in immune response was the reason for adult worm killing, then a difference in MLN responses to SWAP stimulation between wild-type and CCL3-deficient mice could have been noticeable. However, among cytokines that have been shown to have a role in worm killing in other models of parasitic infection, we have shown that CCL3-deficient mice responded to SWAP stimulation, producing less IL-4, IFN-γ, and TNF-α than wild-type animals. This is more suggestive of a less intense response to egg antigens, secondary to decreased parasitic load, rather than an enhanced immune response to adult worms in this group. On the other hand, CCL3 may be an important factor for the production of Th2 cytokines in vitro. Moreover, other studies investigated the role of CCL3 in models of Th2 inflammation (18, 34, 35). These have usually shown an exacerbation of the response against SEA in the presence of high CCL3 concentrations or an inhibition when CCL3 was blocked with antibodies. These results also corroborate the data shown herein, so that a contribution of CCL3 in some Th2 cytokine amplification loops should not be excluded.

CCL3 binds to the chemokine receptors CCR1 and CCR5 (29) and is chemotactic for monocytes (51, 54), lymphocytes (48), and eosinophils (40), among other cell types, which are important for egg-induced granuloma formation (4). Indeed, similar to the inhibition observed in our model of infection, one study based on the pulmonary embolization model demonstrated a 20 to 40% inhibition in granuloma size in anti-CCL3-treated animals (26). It is noteworthy that the inhibition observed (28%) may be underestimated as the egg itself accounts for some of the granuloma area that, if excluded, would revert in a larger percent inhibition. The slight fall in EPO activity found in the present study is in agreement with previous data in that eosinophils should be less recruited in the absence of CCL3 (49). A reduction in the number of eosinophils may have contributed to the smaller size of granulomas present in the liver of CCL3-deficient mice. Reduction of other cell types is also a possibility, considering the lack of selectivity of the chemotactic activity of CCL3 toward any specific cell type present in the schistosome egg granuloma. Regardless of the cellular composition, the smaller granuloma area found in CCL3-deficient mice is in agreement with the lower granuloma index previously reported for peripheral blood mononuclear cells treated with met-RANTES, a CCR1 and CCR5 antagonist (37), in a model of in vitro granuloma (18). Thus, a reduced egg content in tissues associated with smaller granulomas could reflect diminished morbidity in CCL3-deficient mice.

We have shown that the liver collagen content of infected CCL3-deficient mice was smaller than that of wild-type mice. There is indeed evidence in the literature to suggest that CCL3 is involved in fibrosis both in humans and in mice (44). CCL3 is detectable in the bronchoalveolar lavage fluid of patients

TABLE 1. In vitro cytokine production from MLN cells (chronic phase)

Cytokine	Infection status[a]	Mean production[b] ± SEM (pg/ml) in:		
		Cell medium	Concanavalin A	SWAP
IL-4				
Wild type	NI	ND	6 ± 2	ND
	I	ND	97 ± 22‡	150 ± 58
$CCL3^{-/-}$	NI	ND	4 ± 3	ND
	I	ND	98 ± 9†	29 ± 15
IL-10				
Wild type	NI	ND	61 ± 7	ND
	I	ND	231 ± 37*	244 ± 81
$CCL3^{-/-}$	NI	ND	14 ± 2	ND
	I	ND	306 ± 85†	121 ± 63
IL-13				
Wild type	NI	300 ± 7	353 ± 37	411 ± 31
	I	30 ± 8	2,886 ± 558	1,208 ± 301
$CCL3^{-/-}$	NI	390 ± 51	401 ± 59	514 ± 75
	I	179 ± 121	4,044 ± 1,286*	1,291 ± 524
IFN-γ				
Wild type	NI	123 ± 72	7,049 ± 5,799	ND
	I	168 ± 13	6,989 ± 1,800	1,458 ± 430
$CCL3^{-/-}$	NI	11 ± 8	1,277 ± 586	ND
	I	226 ± 49	4,885 ± 1,969	253 ± 75
TNF-α				
Wild type	NI	9 ± 4	109 ± 36	7 ± 2
	I	7 ± 3	111 ± 9	30 ± 8*
$CCL3^{-/-}$	NI	4 ± 1	62 ± 8	3 ± 1
	I	12 ± 10	76 ± 12	14 ± 5

[a] NI, not infected; I, infected.

[b] ND, not detectable. *, $P < 0.05$; †, $P < 0.01$; ‡, $P < 0.001$. P values were determined by the Newman-Keuls test for male infected mice compared to noninfected mice of the same phenotype.

with sarcoidosis and idiopathic pulmonary fibrosis (43). Moreover, anti-CCL3 antibody treatment in mice inhibits bleomycin-induced inflammatory infiltrate and fibrosis (41). Besides, several reports have shown the role of IL-4 in granulomatous inflammation and its interference on various aspects of the response, including collagen synthesis (7, 8, 28). So, it is possible that the inhibition of IL-4 production observed in CCL3-deficient mice could account for at least some of the observed inhibition of collagen deposition. From these evidences, it could have been possible that CCL3 also affected the amount of collagen that is produced in the liver. However, unlike the pipe stem fibrosis found in humans, the granulomas contain most of the disease-induced collagen deposition in mice. Accordingly, by calculating the ratio between the hydroxyproline content and the liver egg content, we could observe that the difference in fibrosis deposition was eliminated if the effect of egg burdens was excluded. These data indicate that the reduced collagen content of liver of CCL3-deficient mice was resulted mainly from the decreased number of eggs present in these animals. A direct effect of CCL3 on fibrogenesis in our model could not be demonstrated but remains a possibility that deserves further investigation.

In summary, we have shown that CCL3 was elevated in the plasma of patients with US-defined hepatosplenomegaly and marked a group under greater risk of developing severe disease. We also showed that CCL3 deficiency is associated with decreased morbidity in a murine model of infection. This may derive from distinct actions of this chemokine, i.e., one that results in reduced worm burdens and their associated consequences and whose mechanism is unknown. One such associated consequence, whose importance is undisputed in schistosomiasis, is a reduction in collagen content that was demonstrated in CCL3-deficient mice. Second, CCL3 deficiency was associated with decreased granulomas, possibly reflecting a direct action of the chemokine on the various cell types that compose the granulomatous inflammation (46). Altogether, these results indicate that schistosomiasis is milder in the absence of CCL3 and suggest that CCL3 may be a causative factor in the development of severe schistosomiasis in humans.

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Differential lectin labelling of circulating hemocytes from *Biomphalaria glabrata* and *Biomphalaria tenagophila* resistant or susceptible to *Schistosoma mansoni* infection

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Lectins/carbohydrate binding can be involved in the Schistosoma mansoni *recognition and activation of the* Biomphalaria *hemocytes. Therefore, expression of lectin ligands on* Biomphalaria *hemocytes would be associated with snail resistance against* S. mansoni *infection. To test this hypothesis, circulating hemocytes were isolated from* B. glabrata *BH (snail strain highy susceptible to* S. mansoni*),* B. tenagophila *Cabo Frio (moderate susceptibility), and* B. tenagophila *Taim (completely resistant strains), labelled with FITC conjugated lectins (ConA, PNA, SBA, and WGA) and analyzed under fluorescence microscopy. The results demonstrated that although lectin-labelled hemocytes were detected in hemolymph of all snail species tested, circulating hemocytes from both strains of* B. tenagophila *showed a larger number of lectin-labelled cells than* B. glabrata*. Moreover, most of circulating hemocytes of* B. tenagophila *were intensively labelled by lectins PNA-FITC and WGA-FITC, while in* B. glabrata *small hemocytes were labeled mainly by ConA. Upon* S. mansoni *infection, lectin-labelled hemocytes almost disappeared from the hemolymph of Taim and accumulated in* B. glabrata *BH. The role of lectins/carbohydrate binding in resistance of* B. tengophila *infection to* S. mansoni *is still not fully understood, but the data suggest that there may be a correlation to its presence with susceptibility or resistance to the parasite.*

Key words: *Schistosoma mansoni* - *Biomphalaria tenagophila* - snail susceptibility to trematode - lectin labelling of hemocytes - host-parasite interaction

Some snail species of the genus *Biomphalaria* are obligatory hosts for the development of the larval stages of *Schistosoma mansoni* life cycle. The capacity of the inner defense system (IDS) of that host to destroy the parasite is one of the factors that may determinate *Biomphalaria* susceptibility to *S. mansoni* infection (Souza et al. 1997, Yoshino et al. 2001, Martins-Souza et al. 2003). The IDS of *Biomphalaria* remains not completely understood, but it is well known that it is composed of cellular elements named hemocytes, and soluble components that could directly affect the larvae and/or act on the recognition of the parasite and activation of hemocytes.

Hemocytes are the main mediators of the defense system of molluscs, taking part in phagocytosis of particles (e.g. bacteria and protozoans), as well as in encapsulation of larger parasites, as for example helminthic larvae (van der Knaap & Loker 1990). Nevertheless, participation of the soluble fraction of *B. glabrata* hemolymph (Granath & Yoshino 1984) or of *B. tenagophila* (Pereira 2005) in the mechanism of destruction of *S. mansoni* sporocysts is also important. This was confirmed in vivo by the increase of resistance to the parasite in susceptible snails treated with hemolymph of resistant ones.

The presence of substances in *B. glabrata* hemolymph that promote agglutination of hemocytes surrounding *S. mansoni* sporocysts was reported by Loker et al. (1984). Afterwards, Fryer and Bayne (1996) demonstrated that particles of polystyrene treated with soluble factors of hemolymph from resistant strains of *B. glabrata* are more significantly phagocytized by hemocytes from susceptible strains than the untreated particles, thus suggesting that soluble factors of hemolymph participate in the recognition mechanism and opsonization of particles by hemocytes.

In the search for possible mediators related to the parasite's recognition mechanism, it was demonstrated that purified lectins of *Conavalia ensiformis* (ConA), *Erythrina corallodendrom* (ECA), *Glycine max* (SBA), *Tetragonolobus purpurea* (TPA), and *Triticum vulgaris*, among others, were able to bind to proteins present on the surface of *S. mansoni* sporocysts (Yoshino 1976, Uchikawa & Loker 1991, Johnston & Yoshino, 1996). Johnston and Yoshino (1996) showed that proteins from the soluble fraction of *B. glabrata* hemolymph were also capable of binding to the majority of sporocyst proteins recognized by purified lectins. Binding of these hemolymph proteins was strongly inhibited when sporocyst proteins were treated with periodate, suggesting participation of carbohydrates in this interaction. Thus, lectins could functionally intermediate binding of hemocytes to the larval tegument of trematodes (van der Knaap & Loker 1990). Moreover, the binding lectin-carbohydrate possibly leads to a structural change of the complex, that could induce hemocyte activation (Bayne 1990), resulting in an

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increase of the phagocitary activity (Fryer et al. 1989) and/or of production of reactive oxygen derivatives (ROI) (Hahn et al. 2000). For this reason, the resistance or susceptibility of species or strains of *Biomphalaria* to *S. mansoni* infection could be related to qualitative or quantitative differences in the production of some lectins (Zelck & Becker 1990). Nevertheless, various experiments showed no difference in lectin composition of hemolymph from susceptible and resistant strains of *Biomphalaria* (Johnston & Yoshino 1996).

In molluscs, lectins are synthesized by hemocytes and released in hemolymph, or are expressed on the surface of circulating hemocytes, where they are able to act as cytophylic receptors (Richards & Renwrantz 1991). Thus, another possibility that has been poorly explored in the literature is that differences in the recognition of the parasite could be related to differences in the expression of lectin ligands in hemocytes of different snail strains. Therefore, the objective of this experimental study was to verify lectin binding on circulating hemocytes from *B. glabrata* BH, a snail strain that is highly susceptible to *S. mansoni* infection (Paraense & Corrêa 1963), as well as in hemocytes from *B. tenagophila* Cabo Frio, snail strain with moderate susceptility to *S. mansoni*, and *B. tenagophila* Taim that is completely resistant to the parasite infection (Martins-Souza et al. 2003). Moreover, we intent to verify if the resistance of *B. tenagophila* Taim to *S. mansoni* would be associated with the variation of expression of lectin-hapten on the circulating hemocytes.

MATERIALS AND METHODS

Parasite - The snails above mentioned were infected with *S. mansoni* of LE strain (isolated from a patient at Belo Horizonte, MG, Brazil). This strain has been maintained at the laboratory of the Schistosomiasis Research Unit, Federal University of Minas Gerais, Brazil, for 35 years.

Snails - Snail species of *B. glabrata* and *B. tenagophila* used in this study has been bred and maintained according to the procedures previously described by Pellegrino and Katz (1968) at the mollusc room of the Schistosomiasis Research Unit, Institute of Biological Sciences, Federal University of Minas Gerais, Brazil, for at least 25 years. The lineage of *B. glabrata* utilized and named BH strain proceeds from the Pampulha lake, Belo Horizonte, MG, Brazil. This snail strain is highly susceptible to the infection to *S. mansoni* (Paraense & Corrêa 1963). Two strains of *B. tenagophila* were selected for the study. The Taim strain collected at the Ecological Station of Taim, Rio Grande do Sul, Brazil, and the Cabo Frio strain collected at Cabo Frio, Rio de Janeiro, Brazil. *B. tenagophila* Taim is totally resistant to *S. mansoni* infection, LE or SJ strain (Santos et al. 1979, Martins-Souza et al. 2003), while Cabo Frio strain is highly susceptible to *S. mansoni* SJ and is partially resistant to *S. mansoni* LE (Corrêa et al. 1979, Martins-Souza et al. 2003).

Infection of snails - Hamsters (*Mesocricetus auratus)* infected with the LE strain of *S. mansoni* were sacrificed 45-50 days after infection, for obtention of miracidia. The snails were individually infected with 20 miracidia, according to the technique described by Pellegrino and Katz (1968). Snails with 12-14 mm diameter in size were used in all experiments, at the moment of infection.

Labelling of molecules expressed on the surface of circulating hemocytes - Labelling on the surface of circulating hemocytes from different strains of *Biomphalaria* was performed with lectins isolated from Concanavalin A – ConA, *Glycine max* – SBA, *Triticum vulgaris* – WGA and *Arachis hypogaea* – PNA conjugated with fluorescein isothyocynate – FITC (EY Laboratories, San Mateo). The cells presenting fluorescence after treatment were considered as labelled.

Circulating hemocytes were isolated from the total hemolymph collected, by means of cardiac puncture, from *B. glabrata* or *B. tenagophila* (Cabo Frio and Taim), uninfected, and after 5, 24, 72, and 120 h post-infection with 20 miracidia of *S. mansoni,* according to the technique described by Martins-Souza et al. (2003).

At each experimental point, as well as for each snail strain, analyses were performed with hemocytes, collected and gathered together, from the total hemolymph of 15 snails. Hemocytes present in each group were separated from the hemolymph by centrifugation (200 × g for 5 min, at room temperature), washed with Chernin's balanced salt solution – CBSS (Chernin 1970), glycose and trealose-free (incomplete CBSS). After that, the sediment of hemocytes was resuspended into 1 ml incomplete CBSS, containing 2% bovine albumin. A sample of each group was diluted into 1/10 CBSS containing 0.4% Trypan Blue. Total counting and cellular viability of each sample were estimated in Neubauer's chamber. It was observed that the hemocytes used showed more than 90% viability. Throughout countings, the hemocytes were morphologically classified as small, medium, and large.

Each treatment was carried out in triplicate using 1×10^5 hemocytes for each replica, the hemocytes being distributed into 1.5-ml polypropilene tubes. Simultaneously, a control was performed with 1 x 10^5 hemocytes, in triplicate, for each labelling in relation to the lectin-hemocyte specificity. After distribution of hemocytes, the tubes were centrifuged (200 × g for 3 min), the cells resuspended in 200 µl incomplete CBSS containing 2% bovine albumin and 10 µg/ml lectin conjugated with FITC (Con A-FITC, WGA-FITC, SBA-FITC or PNA-FITC), and incubated at room temperature, in the dark, for 1 h. The labelled hemocytes in the control tubes were centrifuged and resuspended in incomplete CBSS containing 0.2M of specific carbohydrates for each lectin. Mannose was the carbohydrate used for treatments with Con A-FITC, whereas N-acetyl-glycosamine was utilized for treatments with WGA-FITC and D-galactose in the competition trials with the lectins PNA-FITC and SBA-FITC.

After incubation with labelled lectins and addition of carbohydrates related to the control treatments, the hemocytes were washed and resuspended in 100 µl incomplete CBSS for analysis under epifluorescence microscope using the appropriate filters (Olympus IX70). A representative number of digital images of hemocytes were obtained from each treatment by means of a digital camera Optronics model DEI-470 and the processing image

software Image Pro-Plus 5.0. For each treatment, digital images were used to analyze at least 100 hemocytes, defining cell type (small, medium or large) and presence of label.

Statistical analysis - The results obtained were statistically analyzed, using analysis of variance or Student's t test for the parametric data, as well as Krushal-Wallis test for non-parametric data.

RESULTS

Circulating hemocytes isolated from *B. glabrata* (BH strain) and *B. tenagophila* (Cabo Frio and Taim strain) expressed ligands that were able to bind to tested lectins Con A, WGA, SBA, and PNA. However, the proportion of labelled hemocytes in relation to each tested lectin was statistically different concerning the species and strains of *Biomphalaria* (Fig. 1). Specifically, about 50% hemocytes from uninfected *B. glabrata* and *B. tenagophila* Cabo Frio were labelled by ConA, however a statistically lower rate (30%) of circulating hemocytes from *B. tenagophila* Taim presented carbohydrates recognized by ConA. In contrast, the proportion of circulating hemocytes labelled with PNA-FITC was statistically higher in hemocytes isolated from *B. tenagophila* Taim (64% of the total) compared with the result obtained in hemocytes from *B. tenagophila* Cabo Frio and *B. glabrata* (24 and 10% of total circulating hemocytes, respectively), and similar pattern was also observed in circulating hemocytes labeled by SBA. Finally, the proportion of circulating hemocytes labelled by WGA-FITC was high, mainly in cells isolated from *B. tenagophila* (70-75% of cells).

Specificity of lectin binding to hemocytes was confirmed by addition of concentrated carbohydrate solution: Mannose for Con A, N-acetylglycosamine for WGA, and Galactosamine for PNA or SBA. In all cases, treatment with the corresponding carbohydrate presented as a result elimination or significant reduction of the fluoresce labelling of hemocytes.

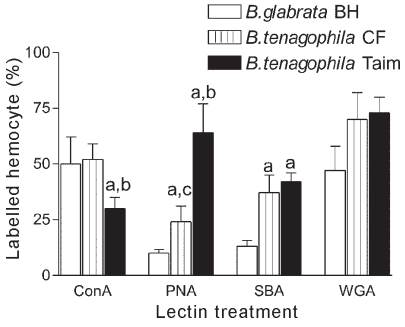

Fig. 1: percentage of total circulating hemocytes isolated from non-infected *Biomphalaria glabrata* BH strain, *B. tenagophila* Cabo Frio strain, and *B. tenagophila* Taim strain, labeled by FITC conjugated ConA, PNA, SBA or WGA purified lectins. a: represents a value significantly different from the value obtained with hemocytes from *B. glabrata* BH; b: represents a value significantly different ($p < 0.05$) from the value obtained with hemocytes from *B. tenagophila* Cabo Frio, and c: represents a value significantly different from the value obtained with hemocytes from *B. tenagophila* Taim.

Besides the difference in the proportion of hemocytes labelled by different lectins, it was possible to observe that there were differences in the labelling pattern and in the morphological type of labelled hemocytes between the different strains or species of tested *Biomphalaria*. In hemocytes isolated from uninfected *B. glabrata*, a diffuse and weak labelling of the cells could be observed, being lectins WGA-FITC (Fig. 2a, b) and Con A-FITC (Fig. 2c, d) bound to carbohydrates on the surface of small and medium hemocytes, whereas only few small cells were labelled by PNA-FITC (Fig. 2e, f). In contrast, hemocytes isolated from uninfected *B. tenagophila* (Taim and Cabo Frio) showed a more intense label than in *B. glabrata,* with large cells presenting a strong labelling on the membrane by WGA (Figs 3b, 4b), while small circulating hemocytes were diffused labeled by ConA (Fig. 3d). Another marked difference was the high number of large and medium hemocytes labelled by PNA-FITC, especially in *B. tenagophila* Taim (Fig. 4c).

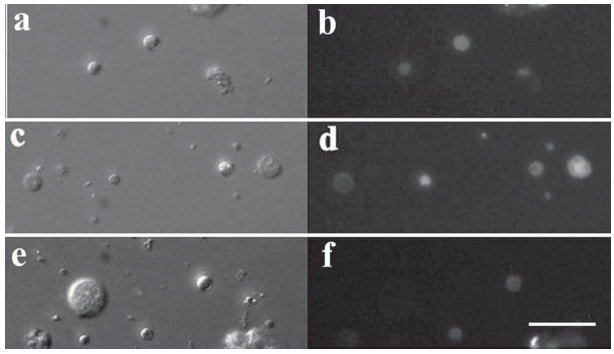

Fig. 2: labelling pattern of circulating hemocytes isolated from non-infected *Biomphalaria glabrata* BH strain, after incubation with FITC conjugated purified lectins. a and b: hemocytes labeled with WGA-FITC; c and d: hemocytes labeled with PNA-FITC; e and f: hemocytes labeled with ConA-FITC; a, c, and e show the cells on Nomarski microscopy while b, d, and f represents the same field on fluorescence mycroscopy (Bar = 10 μm).

Attempting to verify variations on the surface of circulating hemocytes that could alter the binding of lectins during *S. mansoni* infection, hemocytes were isolated from different species and strains of *Biomphalaria*, after the first 120 h post-infection, and incubated with lectin conjugated with FITC. *S. mansoni* infection induced an increase in the proportion of hemocytes from *B. glabrata* labelled by Con A. The increase occurred between 5 and 24 hpi, as a consequence of the higher number of small and medium labelled hemocytes. After 72 hpi, the proportion of hemocytes labelled by ConA decreased to a similar level of that observed in uninfected snails. However, only small hemocytes showed labelling, while medium and large ones labelled by lectin were nearly not observed. Reduction in the proportion of circulating hemocytes from *B. glabrata* labelled by Con A was more intense after 120 hpi (Fig. 5a). *S. mansoni* infection also induced a little increase in the proportion of *B. glabrata* hemocytes la-

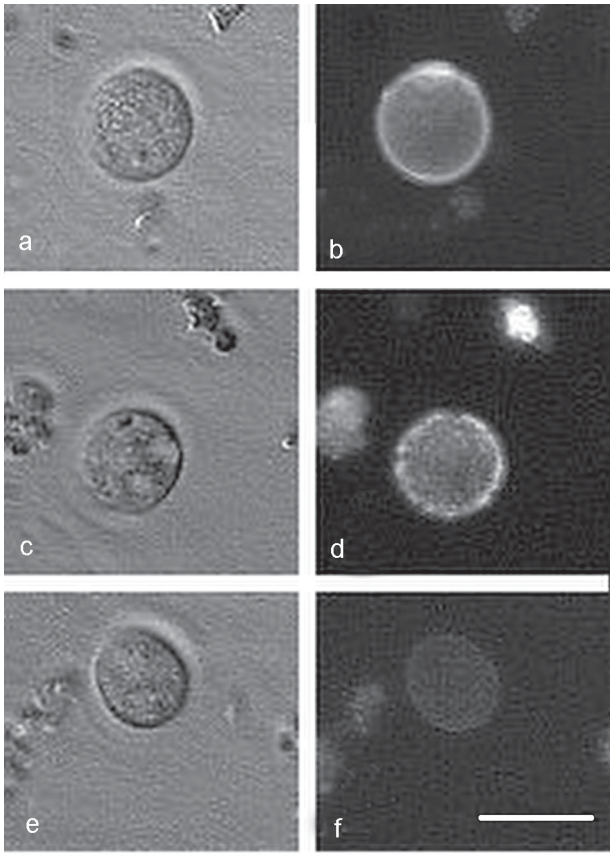

Fig. 3: labelling pattern of circulating hemocytes isolated from non-infected *Biomphalaria tenagophila* Cabo Frio strain, after incubation with FITC conjugated purified lectins. a and b: hemocytes labeled with WGA-FITC; c and d: hemocytes labeled with PNA-FITC; e and f: hemocytes labeled with ConA-FITC; a, c, and e show the cells on Nomarski microscopy while b, d, and f represents the same field on fluorescence mycroscopy (Bar = 10 μm).

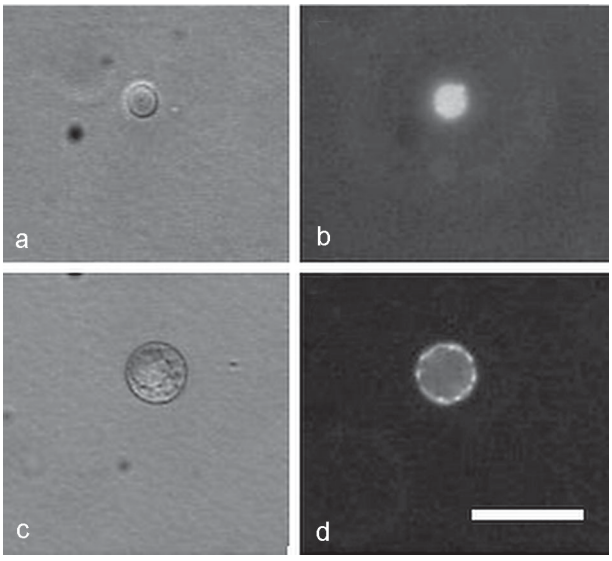

Fig. 4: labelling pattern of circulating hemocytes isolated from non-infected *Biomphalaria tenagophila* Taim strain, after incubation with FITC conjugated purified lectins. a and b: hemocytes labeled with WGA-FITC; c and d: hemocytes labeled with PNA-FITC; a and c show the cells on Nomarski microscopy while panels b and d represents the same field on fluorescence mycroscopy (Bar = 10 μm).

belled by PNA-FITC and SBA-FITC, being this labelling almost exclusively due to small hemocytes, and that increase was maintained high throughout the observation period (Fig. 5b, c). Analyzing the labelling pattern of *B. glabrata* hemocytes by WGA-FITC, it was observed a high number of labelled cells, being most of them represented by small hemocytes. Nevertheless, the number of medium and large hemocytes that were labelled by WGA was considerably higher than the number of hemocytes labelled by other lectins. During infection, it was detected a decrease in the number of medium hemocytes labelled after 5 h post-infection, as well as an increase in the number of large hemocytes labelled after 72 h (Fig. 5d).

S. mansoni infection did not increase the proportion of circulating hemocytes isolated from *B. tenagophila* (Cabo Frio strain) and labelled by ConA-FITC (Fig. 6a) or by WGA-FITC (Fig. 6d). However, it was observed that there was an increase in the proportion of hemocytes labelled by PNA-FITC and SBA-FITC, as a result of the increase in small hemocytes that expressed ligands to these lectins (Fig. 6b, c). Moreover, it is interesting to note that large hemocytes labelled by PNA and WGA nearly disappeared from circulation after 5 h post-infection. Conversely, the proportion of circulating hemocytes labelled by ConA-FITC, PNA-FITC, SBA-FITC, and WGA-FITC decreased after 5 h post-infection with *S. mansoni* in *B. tenagophila* Taim (Fig. 7). Only in labelling by ConA it could be observed an increase in the proportion of hemocytes labelled after 24 and 72 hpi, in relation to non-infected snails (Fig. 7a).

DISCUSSION

Various authors demonstrated that circulating hemocytes in hemolymph of *Biomphalaria* is a heterogeneous cell population. Heterogenity of these cells was confirmed morphologically (Sminia 1981, Joky et al. 1983, Barraco et al. 1993, Bezerra et al. 1997, Matricon-Gondran & Letorcart 1999, Johnston & Yoshino 2001), and functionally (Granath & Yoshino 1983, Matricon-Gondran & Letorcart 1999b, Martins-Souza et al. 2003). Our results confirmed that circulating hemocytes express ligands (carbohydrates) for lectin binding in a very heterogeneous manner. It is interesting to note that the results demonstrated a great variety detected in the pattern and in the type of binding of lectins in hemocytes from *B. glabrata* when compared to *B. tenagophila*. A high proportion of hemocytes isolated from *B. tenagophila* showed a marked labelling by different types of lectin, specially PNA and WGA, whereas in circulating hemocytes of *B. glabrata* only incubation with ConA presented as a result a high proportion of labelled cells. Some differences were also detected in the labelling pattern of circulating hemocytes isolated from Cabo Frio and Taim strains. Binding between lectins and the surface of hemocytes showed to be specific, since labelling

was inhibited by a high concentration of the corresponding carbohydrate.

Histological studies carried out with different strains of infected *B. glabrata* or *B. tenagophila* has documented that in highly susceptible molluscs there are sporocysts and cercariae in abundance in the host´s tissue, and an early absolute lack of cellular reaction around the parasite. At the other extreme, there are resistant snails, shedding fewer cercariae, but showing an intense hemocyte infiltration in the host´s tissue, frequently found around parasitary structures under disintegration (Guaraldo et al. 1981, Sullivan & Richards 1981, Souza et al. 1995, 1997).

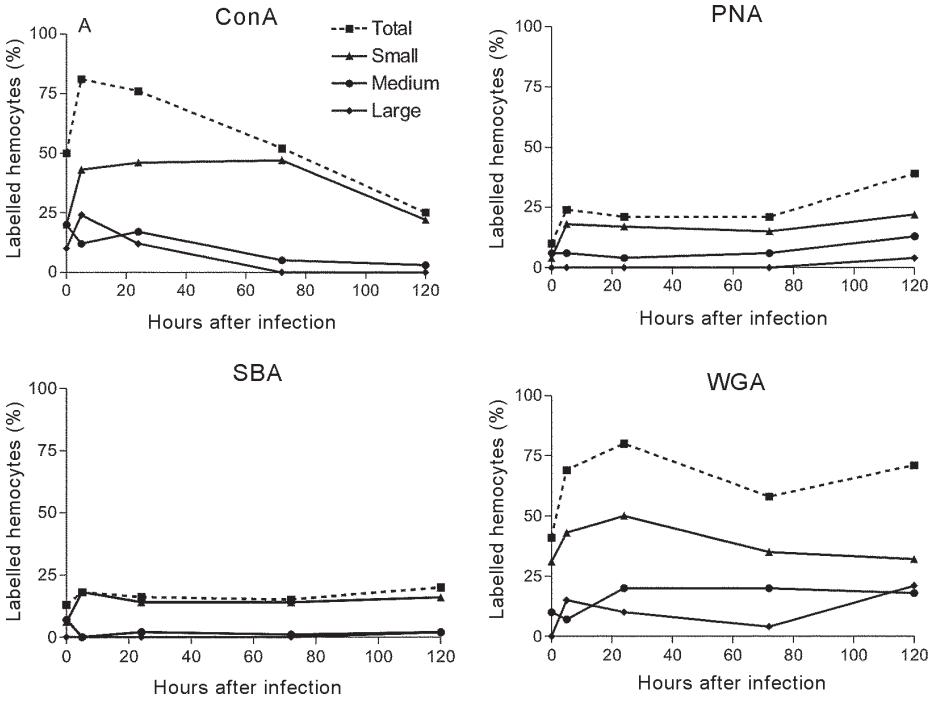

Fig. 5: kinectics of labelled hemocytes of *Biomphalaria glabrata* BH strain, after incubation with FITC conjugated purified lectins, during the first 120 h of *Schistosoma mansoni* infection.

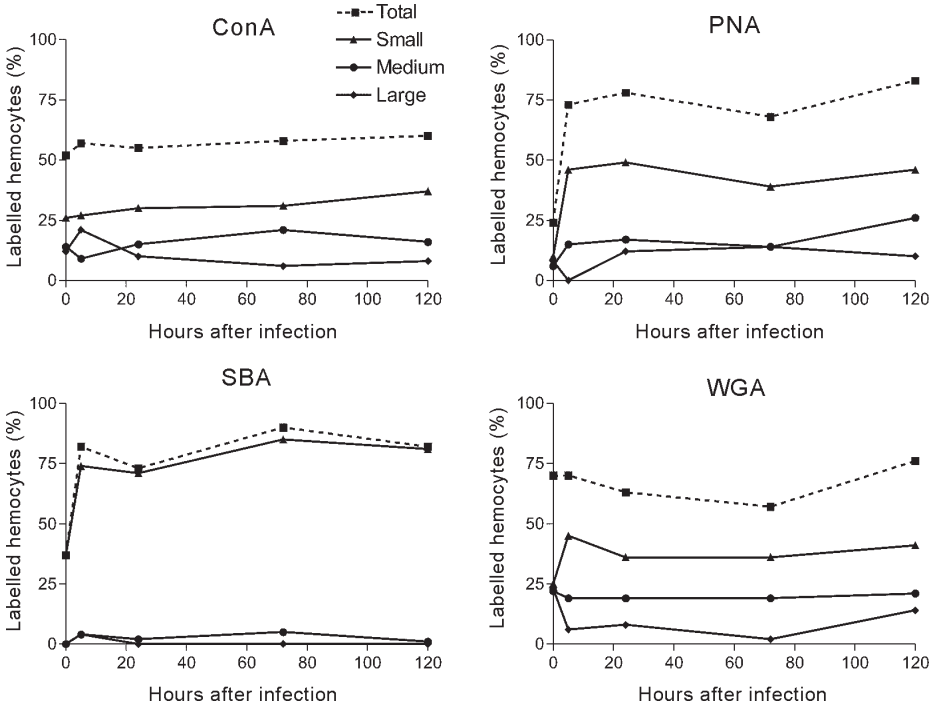

Fig. 6: kinectics of labelled hemocytes of *Biomphalaria tenagophila* Cabo Frio strain, after incubation with FITC conjugated purified lectins, during the first 120 h of *Schistosoma mansoni* infection.

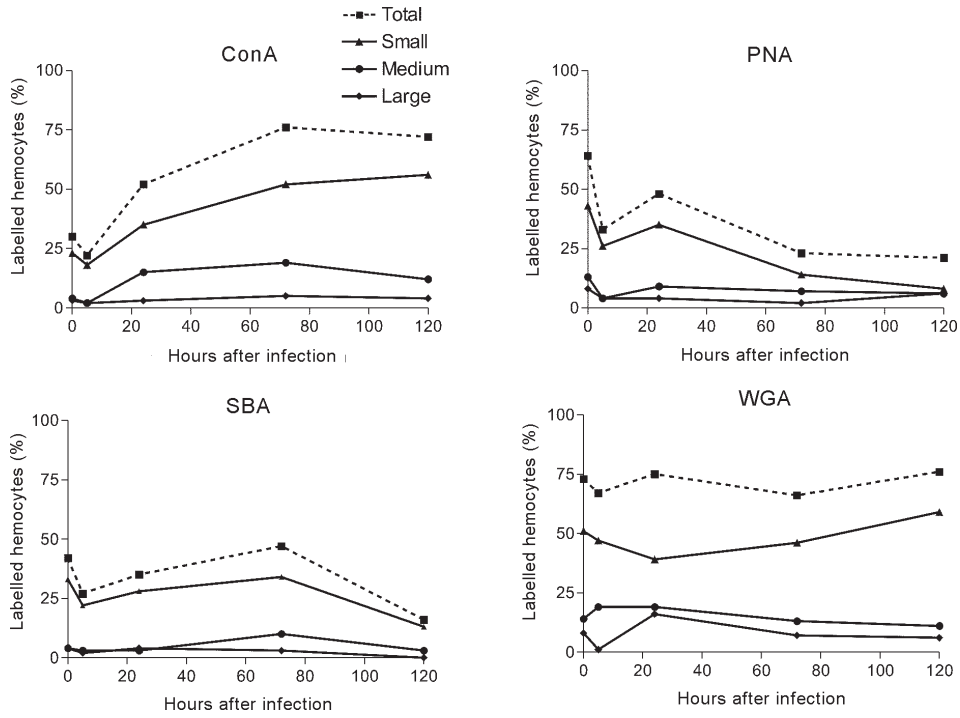

Fig. 7: kinectics of labelled hemocytes of *Biomphalaria tenagophila* Taim strain, after incubation with FITC conjugated purified lectins, during the first 120 h of *Schistosoma mansoni* infection.

Therefore, the significant variation on labelling pattern by lectin observed among different species and strains of *Biomphalaria* used in this study could help to explain hemocyte binding around the parasite observed in some snail strain and, therefore it would be an important factor for determination of differences in the susceptibility level to *S. mansoni* infection reported in the snail species (Corrêa et al. 1979, Martins-Souza et al. 2003, Coelho et al. 2004, Pereira 2005, Rosa et al. 2005).

Lectin/carbohydrate binding has been associated with recognition of trematode larvae, and could exert influence upon the hemocyte capacity of encapsulating trematode larvae (van der Knaap & Lokert 1990, Yoshino et al. 2001, Guillou et al. 2004). In this sense, various authors demonstrated that the tegument, at the larval stages of trematodes, is coated with a high level of glycosidic compounds, and the majority of the tested lectins were specifically able to bind either to axenically transformed larvae (Uchikawa & Loker 1991) or to proteins isolated from its tegument (Johnston & Yoshino 1996). On the other hand, the presence of lectins has been detected in hemolymph of *Biomphalaria*. However, it was not possible to associate the presence of lectins in snail hemolymph with the susceptibility level to *S. mansoni* infection of the snail strain (Zelck et al. 1995, Johnston & Yoshino 1996). Our results show that there were differences in the expression of haptens (glycoproteins or glycolipids) on the hemocyte membrane of different species or strains of *Biomphalaria*. These differences could result in greater capacity of hemocytes, from different strains, of recognizing and adhering to the parasite.

Corroborating this hypothesis, our results also demonstrate that *S. mansoni* infection produced dissimilar effects on the labelling pattern of circulating hemocytes by lectins in different snail strains. In *B. glabrata,* it was observed that there was a significant increase on the proportion of circulating hemocytes labelled by lectins, up to 72 h post-infection. Conversely, 5 h after *S. mansoni* infection, the hemocytes labelled by lectins nearly disappeared from the circulation of *B. tenagophila* Taim, a strain able to destroy sporocysts completely. Previous works (Bezerra et al. 1997, Martins-Souza et al. 2003) report a decrease in the total number of circulating hemocytes in *B. glabrata* or *B. tenagophila* at the first hours after infection. However, the results here presented indicate that hemocytes which are out of circulation in *B. tenagophila* Taim are the ones that express haptens for lectins, suggesting that these hemocytes could be retained in the site of infection, thus justifying the intense cellular reaction observed in these snails that would result in early parasite destruction.

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In vitro evaluation of the effect of the nematophagous fungi *Duddingtonia flagrans*, *Monacrosporium sinense* and *Pochonia chlamydosporia* on *Schistosoma mansoni* eggs

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Abstract The in vitro effect of four isolates of the nematophagous fungi *Duddingtonia flagrans* (AC 001), *Monacrosporium sinense* (SF 53) and *Pochonia chlamydosporia* (VC 1 and VC 4) on eggs of *Schistosoma mansoni* was examined. One thousand *S. mansoni* eggs were plated on 2% water–agar with the grown isolates and control without fungus. After 7, 14 and 21 days, the eggs were removed and classified according to the following parameters: type 1, lytic effect without morphological damage to eggshell; type 2, lytic effect with morphological alteration of embryo and eggshell; and type 3, lytic effect with morphological alteration of embryo and eggshell, besides hyphal penetration and internal egg colonization. Significant differences ($P < 0.01$) were found among the studied fungal isolates for ovicidal activity, confirming type 3 effect for the isolates VC 1 and VC 4, which characterizes the ovicidal activity of a fungus. Type 3 effect was only found for *P. chlamydosporia* (VC 1 and VC 4) with 26.6 and 17.2%, 25.6 and 22.6%, 27.4 and 23.9% in the 7, 14 and 21 days respectively ($P < 0.01$). *P. chlamydosporia* can thus be a potential biological control agent for *S. mansoni* eggs.

Keywords Biological control · *Duddingtonia flagrans* · *Monacrosporium sinense* · Nematophagous fungi · *Pochonia chlamydosporia* · *Schistosoma mansoni*

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Introduction

Human schistosomiasis is caused by infection with digenetic trematodes of the genus *Schistosoma*, which are among the most abundant infection agents in humans (Disch et al. 2002). *Schistosoma mansoni* is however the only species endemic in Brazil, with estimates of 6.3 million infected people and about 26 million people under risk of infection. The states of Minas Gerais, Bahia, Sergipe, Pernambuco and Alagoas are the areas most prone to the disease (Katz and Peixoto 2000). The biological cycle of *S. mansoni* is characterized by a heteroxenic cycle, requiring a definitive host to complete its development and an intermediate host, which

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are some mollusk species of the genus *Biomphalaria*. Within the molluscs, *S. mansoni* larvae go through a series of asexual reproduction cycles, producing thousands of cercariae that are released into the aquatic environment over a long period of time. In the human, after migrating to the lung, the worms mature and reproduce in the hepatic portal system. In this way, part of the mature eggs of *S. mansoni* is retained in the host tissues, mainly liver and intestine. The remaining eggs are eliminated with the contaminated feces into the environment, hatching on contact with water to release the larvae. The miracidia hatch and swim actively around in search of an intermediate host for penetration (Coelho 1995). The schistosomiasis control programs, as the National Health Foundation has implemented in Brazil since 1975, are based on diagnosis and specific treatment for infected people living in endemic areas, which has resulted in a significant reduction in mortality and morbidity associated with schistosomiasis. However, this technique to control schistosomiasis has not proven efficient for the control of parasite transmission, and in some areas, reduction in disease prevalence after treatment has also not been found (Disch et al. 2002).

Alternative control measures for the intermediate host and phases of parasite life cycle, which are efficient and not harmful to the environment, are therefore of great importance for the control of schistosomiasis in Brazil (Coelho et al. 2004).

Biological control with nematophagous fungi has been suggested, among other alternative measures, as form of reducing this and other infections caused by gastrointestinal helminths (Araújo et al. 2006). These fungi are divided in three groups: endoparasites, predacious and opportunists (Mota et al. 2003). However no work demonstrating fungal action on eggs of helminths has previously been carried out. In the predacious group, the genera *Duddingtonia* and *Monacrosporium* stand out for their effectiveness in environmental control of nematodes (Dimander et al. 2003).

In the opportunist group, the chlamydospore-forming species *Verticillium chlamydosporium* stands out, being currently named *Pochonia chlamydosporia* due to examination and phylogenetic analysis of rDNA sub unities (Gams and Zare 2001).

The present study aimed at evaluating the in vitro action of the nematophagous fungi *Duddingtonia flagrans, Monacrosporium sinense* and *P. chlamydosporia* on *S. mansoni* eggs.

Materials and methods

Four isolates of nematophagous fungi, one from *D. flagrans* (AC 001), one from *M. sinense* (SF 53) and two from *P. chlamydosporia* (VC 1 and VC 4) were stored in test tubes containing 2% corn-meal-agar (2% CMA), in the darkness, at 4°C for 10 days.

Culture disks, 4 mm in diameter, were extracted from fungal isolates kept in the test tubes and plated into 9-cm diameter Petri dishes containing 20 ml of 2% potato-dextrose-agar (PDA 2%), and then stored in darkness at 25°C for 10 days. After isolate growth, new culture disks, 4 mm in diameter, were transferred to 9-cm diameter Petri dishes containing 20 ml of 2% water–agar (2% WA) for 10 days.

Eggs of *S. mansoni* were obtained in infected mice by using the Pellegrino technique (1957) with some modifications. Eggs were morphologically analysed under an optical microscope using a 10× objective lens and poured onto the surface of a plate containing only 2% WA medium, with isolates grown for 10 days and a control without fungus, with 25 replicates for each group. Each plate in the treatments contained one thousand *S. mansoni* eggs with only one fungal isolate. In the intervals 7, 14 and 21 days, the eggs were collected from each plate containing the isolate and from the control without fungus, as described

Table 1 Percentages for the ovicidal activity of the nematophagous fungi *Duddingtonia flagrans* (AC 001), *Monacrosporium sinense* (SF 53) and *Pochonia chlamydosporia* (VC 1 and VC 4) and the control group without fungi on eggs of *Schistosoma mansoni* at 7, 14 and 21 days

Isolates	Effect type 1[a]	Effect type 2[b]	Effect type 3[c]
Effect at 7 day			
AC 001	62.4^{A}	0^{B}	0^{C}
SF 53	70.2^{A}	0^{B}	0^{C}
VC 1	21.2^{B}	27.2^{A}	26.6^{A}
VC 4	27.0^{B}	27.8^{A}	17.2^{A}
Control	0^{C}	0^{B}	0^{C}
Effect at 14 day			
AC 001	62.2^{A}	0^{B}	0^{B}
SF 53	68.4^{A}	0^{B}	0^{B}
VC 1	21.8^{B}	30.2^{A}	25.6^{A}
VC 4	27.6^{B}	31.0^{A}	22.6^{A}
Control	0^{C}	0^{B}	0^{B}
Effect at 21 day			
AC 001	70.2^{A}	0^{B}	0^{C}
SF 53	74.8^{A}	0^{B}	0^{C}
VC 1	20.8^{B}	28.6^{A}	27.4^{A}
VC 4	27.6^{C}	31.4^{A}	23.9^{A}
Control	0^{D}	0^{B}	0^{C}

Percentages followed by same letter (A, B, C) in the same column are not significantly different ($P > 0.01$). Friedman test

[a] Lytic effect without morphological damage to eggshell, with hyphae adhered to the shell

[b] Lytic effect with morphological alteration of embryo and eggshell, without hyphal penetration through the eggshell

[c] Lytic effect with morphological alteration of embryo and eggshell, besides hyphal penetration and internal colonization

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by Araújo et al. (1995), placed in glass slides with a drop of 1% Amam blue and evaluated under a 40× lens according to parameters established by Lysek et al. (1982): type 1, lytic effect without morphological damage to eggshell, with hyphae adhered to the shell; type 2, lytic effect with morphological alteration of embryo and eggshell, without hyphal penetration through the eggshell, and type 3, lytic effect with morphological alteration of embryo and eggshell, besides hyphal penetration and internal colonization.

To identify the statistical differences among the independent groups, data of each group were examined by non-parametric Friedman test at 1% probability.

Results and discussion

Table 1 shows the ovicidal activity of the fungi *D. flagrans, M. sinense* and *P. chlamydosporia* at 7, 14 and 21 days respectively.

Examination by optical microscopy under a 40× lens showed hyphae colonizing egg surfaces in all the treatments with fungi. The groups treated with isolates VC 1 and VC 4 had, besides hyphae colonizing egg surfaces, showed hyphal presence also inside the eggs. Scanning electron microscopy showed the structures appressorium, hyphae and chlamydospores of *P. chlamydosporia* (Figs. 1 and 2).

Significant differences ($P < 0.01$) were found among the fungal isolates studied for ovicidal activity, confirming the type-3 effect for the isolates VC 1 and VC 4, which characterizes the ovicidal activity of a fungus. Mizobutsi et al. (2000) evaluated the action of 64 fungal isolates, *P. chlamydosporia* among them, on eggs of *Meloidogyne javanica*. They found a greater ovicidal activity of this fungus compared to the others, similarly to the results found in this work.

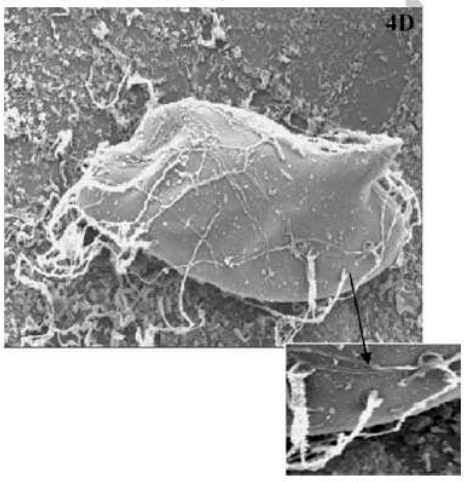

Fig. 1 Appressorium, hyphae (black arrow) of *Pochonia chlamydosporia*. Scanning Electron Microscopy—10 μm

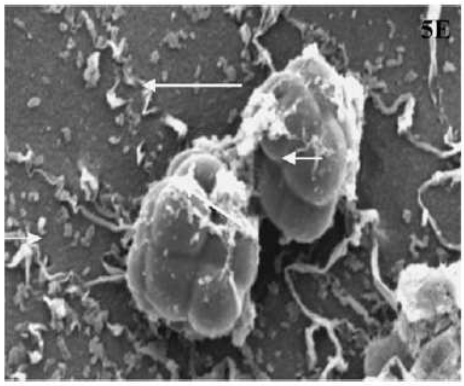

Fig. 2 Chlamydospores (white arrow) of *Pochonia chlamydosporia*. Scanning Electron Microscopy—10 μm

In this work, the presence of *D. flagrans* and *M. sinense* hyphae on the eggshell did not result in egg destruction. A more effective explanation of this fact would need the application of viability tests, in other words, to feed animals with eggs that have been previously colonized by fungi (Morgan-Jones et al. 1983).

This was the first study on the action of the fungal predators *D. flagrans*, *M. sinense* and *P. chlamydosporium* on eggs of *S. mansoni*, identifying *P. chlamydosporia* as a potential candidate for biological control of this helminth.

Acknowledgements The work has a financial support of CNPq.

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Flow cytometry analysis of the circulating haemocytes from *Biomphalaria glabrata* and *Biomphalaria tenagophila* following *Schistosoma mansoni* infection

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SUMMARY

Aiming to further characterize the haemocyte subsets in *Biomphalaria* snails, we have performed a detailed flow cytometric analysis of whole haemolymph cellular components using a multiparametric dual colour labelling procedure. Ethidium bromide/acridine orange fluorescence features were used to first select viable haemocytes followed by flow cytometric morphometric analysis based on the laser scatter properties (forward scatter-FSC and side scatter-SSC). Our findings demonstrated that *B. glabrata* (BG-BH, highly susceptible to *S. mansoni*) and 2 strains of *B. tenagophila* (BT-CF, moderately susceptible and BT-Taim, resistant to *S. mansoni*) have 3 major circulating haemocyte subsets, referred to as small, medium and large haemocytes. The frequency of small haemocytes was higher in BG-BH, while medium haemocytes were the most abundant cell-type in both *B. tenagophila* strains. *Schistosoma mansoni* infection resulted in early reduction of large and medium circulating haemocytes followed by an increase of small haemocytes. Although parasite infection induced haemocyte alterations in all *Biomphalaria* strains, the response was particularly intense in BT-Taim, the parasite-resistant snail. Interestingly, the trematode infection induces changes in haemocytes with less granular rather than in those with more granular profile. The results indicated that, in *B. tenagophila* of Taim strain, circulating haemocytes, especially the medium and high subset with less granular profile, are very reactive cells upon *S. mansoni* infection, suggesting that this cell subset would participate in the early parasite destruction observed in this snail strain.

Key words: *Biomphalaria*, *Schistosoma mansoni*, haemocyte, flow cytometry, defence system, molluscs.

INTRODUCTION

Biomphalaria glabrata and *B. tenagophila* are Brasilian fresh water Planorbids of great medical relevance as intermediate hosts of *Schistosoma mansoni*, a trematode parasite that causes human schistosomiasis, a disease that affects about 8 million people in Brazil (Paraense, 2001).

B. glabrata has an internal defence system (IDS) consisting of soluble components of haemolymph and circulating cells, termed haemocytes, which work in association during the snail responses against infectious agents (van der Knaap and Loker, 1990). In snails, circulating haemocytes, especially the phagocytic cell population, are the principal line of cellular defence involved in destruction of *S. mansoni* larvae inside the intermediate host (Bayne *et al*. 1980; Noda and Loker, 1989*a*; Zelck and Becker, 1992; Ottaviani, 1992; Adema *et al*. 1994; Sapp and Loker 2000; Negrão-Corrêa *et al*. 2007). Therefore, the characterization of circulating haemocytes is fundamental for understanding differences in the resistance of *Biomphalaria* species to *S. mansoni*. Most of the studies (Harris, 1975; Lo Verde *et al*. 1982; Lie *et al*. 1987; Barraco *et al*. 1993) have reported that *B. glabrata* circulating haemocytes are composed of at least 2 cell populations, based mainly on morphological and functional aspects: the hyalinocytes and the granulocytes. However, ultrastructural (Matricon-Gondran and Letorcart, 1999) and biochemical (Granath and Yoshino, 1983) analyses indicated that circulating granulocytes are very heterogeneous cells and could be involved in different processes during snail infection.

In agreement with the studies on *B. glabrata*, previous studies reported that circulating haemocytes from *B. tenagophila* are also composed of hyalinocytes and granulocytes (Martins-Souza *et al*. 2003). Further analysis showed that injection of silica into *B. tenagophila* resulted in temporary reduction of the granulocyte subset and increase in the snail

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susceptibility to *S. mansoni* infection (Martins-Souza *et al*. 2003), supporting the participation of these cells in the protective mechanism against *S. mansoni* infection. However, even with a high dose of silica only part of the granulocyte population was affected by the treatment, demonstrating that circulating granulocytes are an heterogeneous cell population.

Using flow cytometric analysis, we have now profiled, during *S. mansoni* infection, the circulating haemocyte populations from *B. glabrata* BH, a snail strain that is highly susceptible to *S. mansoni* infection (Paraense and Corrêa, 1963), haemocytes from *B. tenagophila* Cabo Frio, a snail that is moderately susceptible to *S. mansoni*, and *B. tenagophila* Taim that is completely resistant to the parasite infection (Bezerra *et al*. 1997: Martins-Souza *et al*. 2003; Coelho *et al*. 2004; Rosa *et al*. 2005).

MATERIALS AND METHODS

Parasite

LE strain of *S. mansoni* (isolated in 1968 by Pellegrino and Katz from a patient in Belo Horizonte, MG, Brazil) was used in all the experiments. The parasite has been maintained by successive passages through *B. glabrata* and hamsters (*Mesocricetus auratus*) in the laboratories of the Schistosomiasis Research Unit, Federal University of Minas Gerais, Brazil.

Snails

Two species of *Biomphalaria* were selected for the study, *B. glabrata* and *B. tenagophila*. The strain of *B. glabrata* was the BH strain (BG-BH), collected at Belo Horizonte, State of Minas Gerais, Brazil, which is highly susceptible to *S. mansoni* infection (Paraense and Corrêa 1963; Corrêa *et al*. 1979; Santos *et al*. 1979; Bezerra *et al*. 1997; Souza *et al*. 1997; Martins-Souza *et al*. 2003). Two strains of *B. tenagophila* were also used, the Cabo Frio strain collected at Cabo Frio (BT-CF), State of Rio de Janeiro, Brazil, moderately susceptible to the LE strain of the parasite (Martins-Souza *et al*. 2003), and the Taim strain collected at the Ecological Station of Taim (BT-Taim), State of Rio Grande do Sul, Brazil, that is totally resistant to *S. mansoni* infection (Corrêa *et al*. 1979; Santos *et al*. 1979; Bezerra *et al*. 1997: Martins-Souza *et al*. 2003; Coelho *et al*. 2004; Rosa *et al*. 2005). Both *B. glabrata* (BH) and *B. tenagophila* (Taim and Cabo Frio) were bred and maintained in the mollusc room of the Schistosomiasis Research Unit, Institute of Biological Sciences, Federal University of Minas Gerais, Brazil, for at least 25 years, according to the procedures previously described by Pellegrino and Katz (1968). The snails used in the experiments measured 12–14 mm in shell diameter at the time of miracidium exposure.

S. mansoni *infection*

The infection of *B. glabrata* and *B. tenagophila* with *S. mansoni* followed the procedure described by Pellegrino and Katz (1968). The eggs were obtained from homogenized livers of 45 to 50 day-infected hamsters. After several washes in cold saline, the miracidia were stimulated to hatch under artificial light. Samples of miracidia were collected, counted under a stereomicroscope, and 20 miracidia were added to each flask containing 1 snail to a final volume of 10 ml and incubated for at least 5 h under artificial light. *Schistosoma mansoni* cercaria emergence was examined after 4 h of stimulation with artificial light in each snail that survived after 40–45 days after infection, as previously described by Pellegrino and Macedo (1955).

Haemolymph collection and haemocyte count

Whole haemolymph was collected from BG-BH, BT-CF and BT-Taim at different times during *S. mansoni* infection. Each snail shell was cleaned with 70% alcohol, dried with absorbent tissue paper and the haemolymph was collected by cardiac puncture using a 21-G needle (Zelck and Becker, 1992; Bezerra *et al*. 1997). To avoid cellular agglutination, whole haemolymph was collected and diluted 1:1 in Chernin's balanced salt solution (CBSS) (Chernin, 1970) [47·7 mM of NaCl, 2·0 mM of KCl, 0·49 mM of $Na_2 HPO_4$ anhydrous, 1·8 mM of $MgSO_4$. 7 H_2O, 3·6 mM of $CaCl_2$. 2 H_2O, 0·59 mM of $NaHCO_3$, 5·5 mM glucose and 3 mM trehalose], containing citrate/EDTA [50 mM sodium citrate, 10 mM EDTA, and 25 mM sucrose] pH 7·2. After individual collection, the haemolymph from 3 snails of the same experimental group was pooled and 3 separated pools were prepared and tested for each experimental group in each point. The triplicates of pooled whole haemolymph were transferred to 3 separated 1·5 ml Eppendorff tubes. After sedimentation of small shell fragments for 2 min, the whole haemolymph was transferred to another 1·5 ml Eppendorff tube. Whole haemolymph was used for total haemocyte counts performed using 10 μl of whole pooled haemolymph diluted 1/10 in CBSS buffer containing 0·4% Trypan Blue. Viable haemocytes, i.e cells that did not stain with Trypan Blue, were counted immediately in a Neubauer's chamber. In parallel, each sample of whole haemolymph was also used for cytometric analysis described bellow.

Flow cytometry analysis

Flow cytometry analysis was performed after incubation of 200 μl of pooled whole haemolymph with an equal volume of ethidium bromide (Et-Br) and acridine orange (AO) solution (stock solution

Et-Br = 12·5 mg/ml, and AO = 4 mg/ml) diluted 1 : 1000 in CBSS citrate/EDTA (Parks *et al.* 1979). Haemocyte suspension was incubated for 1 h on ice, in the dark. After incubation, haemocyte suspension was immediately analysed using a FACScan flow cytometer (BD Bioscience, San Jose, CA, USA). Flow cytometry analysis of whole haemolymph cellular components was performed using instrument settings to capture the fluorescence signals from ethidium bromide and acridine orange, respectively at FL3 and FL1 detectors, using log amplification scales. In total, 20 000 events were analysed for each haemolymph pooled sample. CellQuestTM software package (BD Bioscience, San Jose, CA, USA) was used for data acquisition and analysis. Data analysis was initially performed using FL1 versus FL3 dot plot distribution graphs to differentiate live cells (AO positive/ Et-Br negative cells) from dead cells (AO negative/EtBr positive cells) from debris (AO and Et-Br negative events). After gating on live haemocytes, cells were selected on size (forward laser scatter – FSC) versus internal complexicity (side laser scatter – SSC) as illustrated in Fig. 1. Three major haemocyte supopulations were selected based on their laser forward scatter, referred to as small (R1 = FSC channels between 240–440), medium (R2 = FSC channels between 440–840) and large (R3 = FSC channels >840). Each of the three haemocyte subpopulations was further analysed based on the internal complexity properties, refereed as SSC^{Low} or less granular and SSC^{High} or more granular profiles (Fig. 2A). For this purpose, SSC versus AO/ FL1 dot plots were constructed for small, medium and large gated haemocytes and quadrant statistical analysis applied to quantify the less granular and more ganular haemocytes within each subset as illustrated in Fig. 2A. As the small, medium and large haemocytes presented distinct SSC properties, specific cut-off edges were used to categorize SSC^{High} subsets within small (SSC channels $\geqslant 100$), medium (SSC channels $\geqslant 200$) and large (SSC channels $\geqslant 300$) haemocyte subpopulations. Percentages of haemocyte subsets obtained from the flow cytometric analysis were further converted on absolute counts taking into account the total viable haemocyte counts (haemocytes not stained by Trypan Blue) performed on Neubauer's chamber obtained with the same haemolymph sample.

Statistical analysis

Data referring to the numbers of circulating haemocytes within each cell-subset are reported as mean ± standard deviation (S.D.), and analysed by using one-way analysis of variance (ANOVA). A two-way analysis of variance (ANOVA) was used to compare each haemocyte subtype between the 3 *Biomphalaria* strains during the infection with *S. mansoni*.

RESULTS

Haemolymph incubation with ethidium bromide and acridine orange solution allowed the separation of the viable circulating haemocytes from the dead cells and small fragments. As observed in Fig. 1A, viable circulating haemocytes from *Biomphalaria* snails could be separated into 3 major cell subpopulations based mainly on size (forward scatter – FSC) and granularity (side scatter – SSC) dot plot distribution. The haemocyte subpopulations have been denominated small haemocytes (R1 – FSC channels between 240–440), medium haemocytes (R2 – FSC channels between 440–840) and large haemocytes (R3 – FSC channels >840).

Analysis of absolute counts of haemocyte subpopulations demonstrated distinct profiles in *B. glabrata* (BG-BH), *B. tenagophila* Cabo Frio (BT-CF) and *B. tenagophila* Taim (BT-Taim) snails (Fig. 1B). In non-infected *B. glabrata*, the majority of circulating cells were small haemocytes (740 ± 150 cells/μl of haemolymph) while in non-infected *B. tenagophila* medium haemocytes represented the majority of circulating haemocytes (590 ± 140 cells/μl haemolymph in BT-CF and 670 ± 120 cells/μl of haemolymph in BT-Taim). Large haemocytes were detected in similar numbers in circulating haemolymph of *B. glabrata* and *B. tenagophila* (Fig. 1B). There was no detectable difference in circulating haemocyte profiles in non-infected *B. tenagophila* of Cabo Frio strain and *B. tenagophila* of Taim strain.

Fig. 2A shows analysis of 3 major circulating haemocyte subpopulations in *Biomphalaria* snails, based on their laser side scatter (SSC) versus acridine orange fluorescence intensity (FL1). Two subsets, referred to as less granular and more granular haemocytes, were identified in all 3 haemocyte subpopulations. The analysis of side-scatter properties of circulating haemocytes from the *Biomphalaria* spp., revealed that most of the small and medium haemocytes were confined within the less granular subset while the large haemocyte population shows a similar amount of cells with less granular and more granular properties (Fig. 2A). The most striking difference in haemocyte SSC properties between non-infected snails of different species was the fact that the higher number of small haemocytes observed in *B. glabrata* was due to the less granular cell subset compared to *B. tenagophila*. In contrast, both strains of *B. tenagophila* (BT-CF and BT-Taim) had a significantly higher frequency of more granular medium haemocytes than *B. glabrata*, as reflected in higher numbers of this cell type (Fig. 2B).

At 40 days after *S. mansoni* infection, cercariae were found in 58% of infected *B. glabrata* and 20% of *B. tenagophila* Cabo Frio strain. As reported previously (Martins-Souza *et al.* 2003), cercariae were not found in *S. mansoni*-infected *B. tenagophila* of Taim strain. *Schistosoma mansoni* infection in

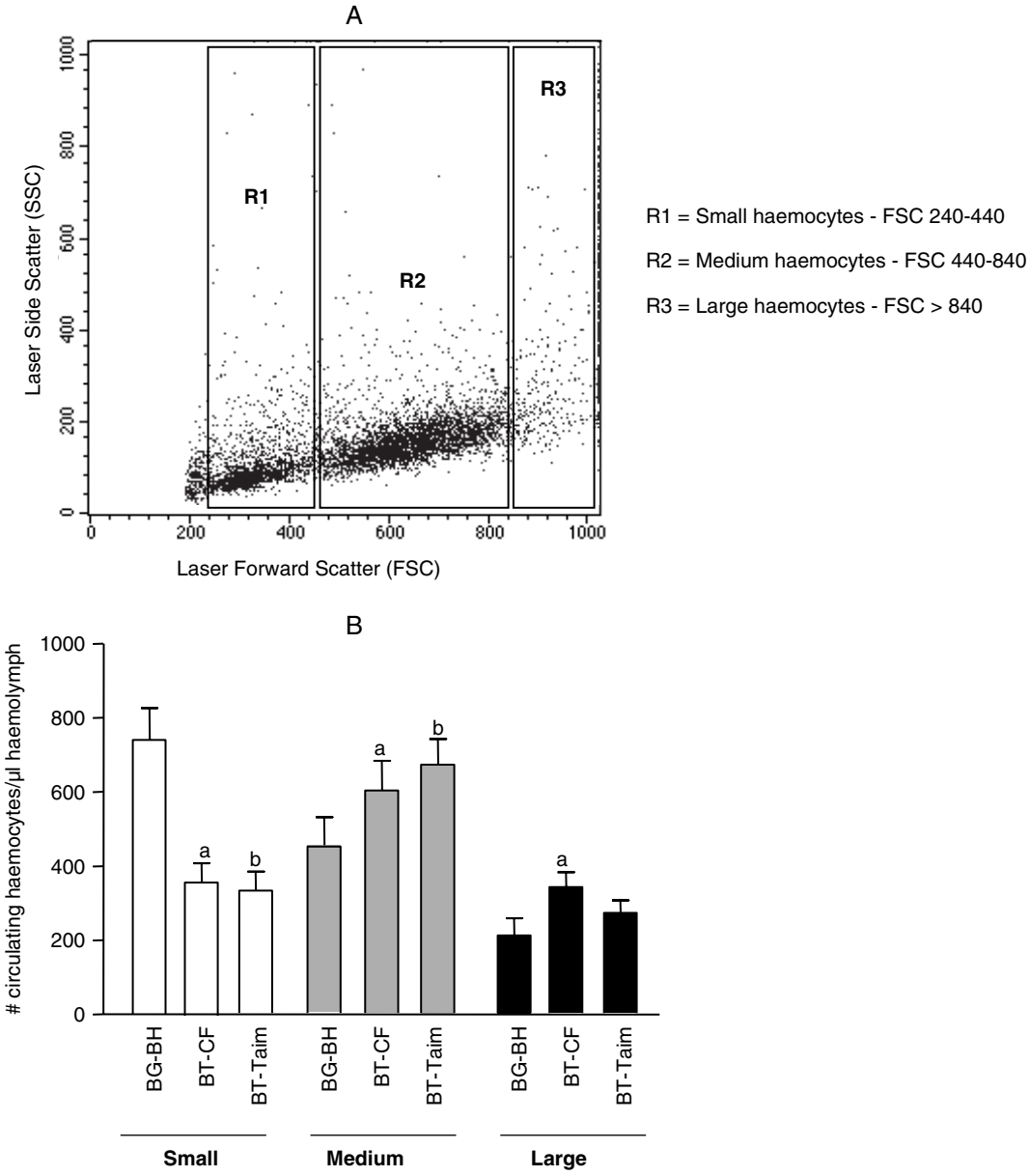

Fig. 1. (A) Profile of circulating haemocyte population in *Biomphalaria* snails. Three major haemocyte subpopulations (R1 = small – FSC between 240–440, R2 = medium – FSC between 440–840 and R3 = large – FSC > 840) can be identified by flow cytometric dot plot distributions based on their laser forward scatter (FSC) versus laser side scatter properties (SSC). (B) Absolute counts of small (R1 = □) medium (R2 = ■) and large (R3 = ■) haemocyte subpopulations in non-infected *B. glabrata* (BG-BH), *B. tenagophila* of Cabo Frio strain (BT-CF) and *B. tenagophila* of Taim strain (BT-Taim) snails. Data are presented as mean number ± standard deviation of circulating haemocyte subpopulations. (a) Represents significant differences ($P < 0{\cdot}05$) in the number of haemocytes of each subset (small, medium, and large) in BT-Cabo Frio compared to the number of haemocytes obtained in BG-BH. (b) Represents significant differences ($P < 0{\cdot}05$) in the number of haemocytes of each subset (small, medium, and large) in BT-Taim compared to the number of haemocytes obtained in BG-BH. There were no differences in the number of haemocytes of each subset between BT-Taim and BT-Cabo Frio (One – way. ANOVA, post-test Tukey).

Biomphalaria resulted in a significant reduction of large and medium circulating haemocytes as early as 5 h after the parasite infection (Fig. 3). However, in *B. glabrata* the cell reduction was transient, returning to non-infected levels briefly after parasite infection (Fig. 3A). In contrast, the parasite infection in *B. tenagophila* resulted in intense alteration of circulating haemocytes, especially in Taim strain. Infected *B. tenagophila* of Cabo Frio strain showed a significant decrease in number of medium and large circulating haemocytes only at 5 h after infection, followed by a gradual increase in medium-sized circulating cells (Fig. 3B). The parasite infection induced a more intense modification of the circulating haemocyte profile of *B. tenagophila* of Taim, the snail strain that is resistant to *S. mansoni* infection

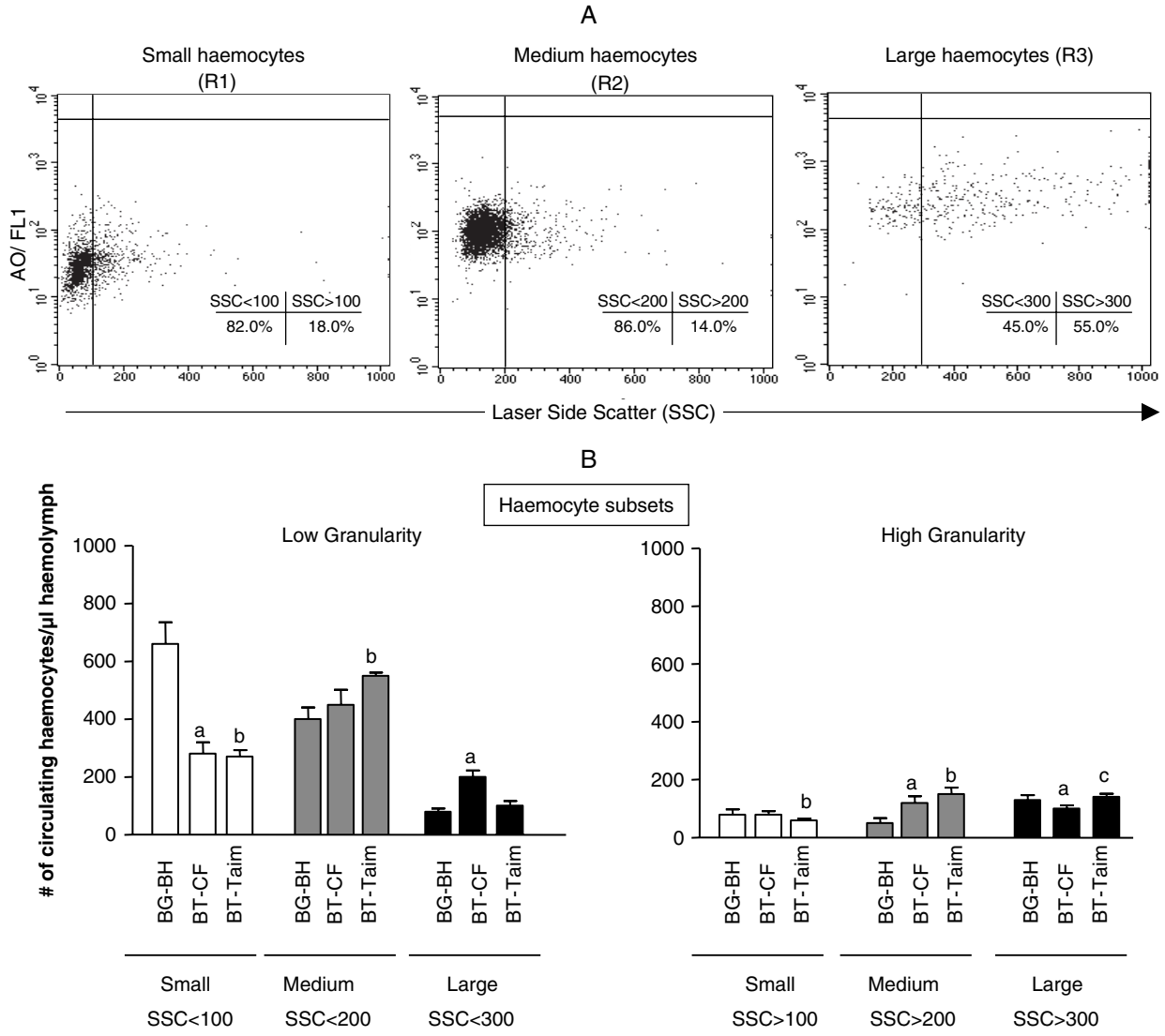

Fig. 2. Analysis of 3 major circulating haemocyte subpopulations in *Biomphalaria* snails, based on their laser side scatter properties (SSC) versus acidine orange fluorescence intensity (FL-1) profiles. (A) Two subsets referred to as SSC^{Low} and SSC^{High} haemocytes can be identified in all 3 haemocyte subpopulations. (B) Absolute counts of haemocyte subsets categorized as SSC^{Low} and SSC^{High} subsets in small (R1 = □) medium (R2 = ▩) and large (R3 = ■) haemocyte subpopulations of *B. glabrata* (BG-BH), *B. tenagophila* Cabo Frio (BT-CF) and *B. tenagophila* Taim (BT-Taim) snails. Data are presented as mean ± standard deviation of low and high granular haemocytes within the 3 major circulating haemocyte subsets. (a) Represents significant differences ($P < 0{\cdot}05$) in the number of haemocytes of each subset (small, medium, and large) in BT-Cabo Frio compared to the number of haemocytes obtained in BG-BH. (b) Represents significant differences ($P < 0{\cdot}05$) in the number of haemocytes of each subset (small, medium, and large) in BT-Taim compared to the number of haemocytes obtained in BG-BH. There were no differences in the number of haemocytes of each subset between BT-Taim and BT-Cabo Frio (One-way. ANOVA, post-test Tukey).

(Fig. 3C). In this strain, the number of large circulating haemocytes was significantly lower at 5 and 24 h after infection. Medium circulating haemocytes from infected Taim snails had an initial reduction, especially due to low numbers of the less granular cell subsets 5 h after parasite infection (Fig. 4), followed by a significant increase in less granular cells 24 h after infection, that reduced again at 72 h and 120 h after infection. The number of small circulating haemocytes in infected Taim snails gradually increased after *S. mansoni* infection, being significantly higher than the non-infected snails after 72 h post-infection (p.i.), when the number of small circulating haemocytes reached 720 cell/ μl of haemolymph compared to 340 detected before infection (Fig. 3C). The analysis of the SSC properties of circulating haemocytes during the *S. mansoni* infection revealed that most of the cellular alteration was due to less granular haemocyte subsets (Fig. 4). The number of circulating haemocytes with more granular profile, within the different haemocyte populations, (small, medium or large) was relatively constant after parasite infection in all the snail strains tested.

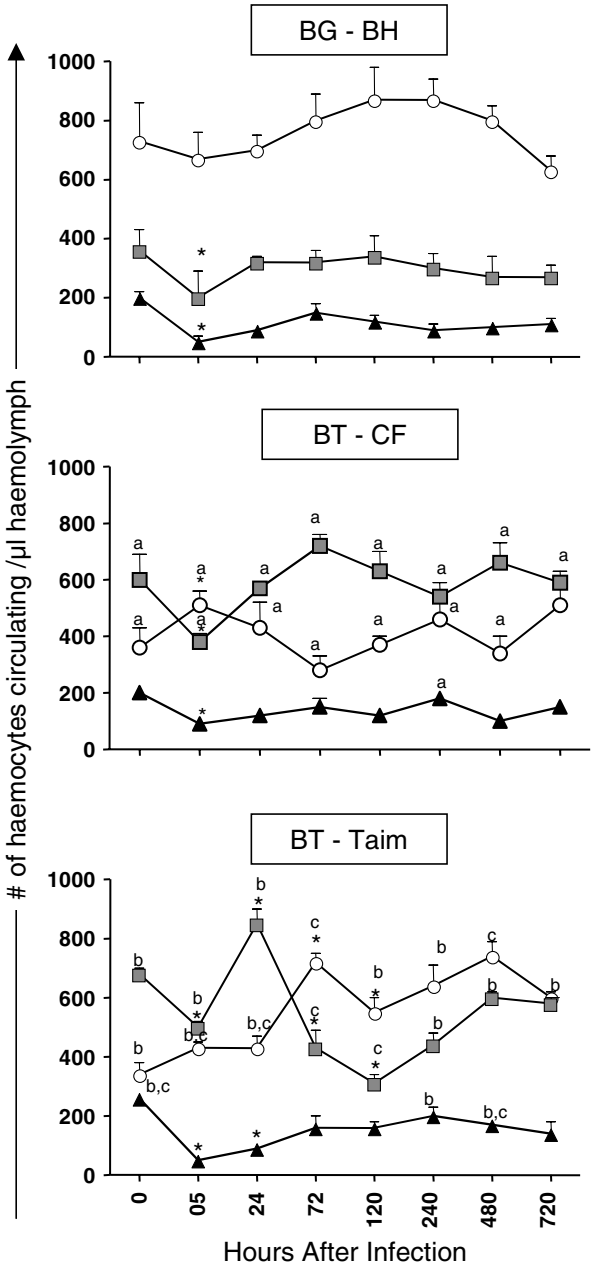

Fig. 3. Kinetic analysis of 3 major circulating haemocyte subpopulations in *Biomphalaria glabrata* (BG-BH), *B. tengophila* Cabo Frio (BT-CF) and *B. tenagophila* Taim (BT-Taim) snails following *S. mansoni* infection. Data are presented as mean number ± standard deviation of circulating haemocyte subpopulations, including small (o), medium (■) and large (▲) haemocytes during 30 days after infection. (a) Represents significant differences ($P<0{\cdot}05$) in the number of haemocytes in BT-Cabo Frio compared to the number of haemocytes obtained in BG-BH. (b) Represents significant differences ($P<0{\cdot}05$) in the number of haemocytes in BT-Taim compared to the the number of haemocytes obtained in BG-BH. (c) Represents differences in the number of haemocytes of each subset between BT-Taim and BT-Cabo Frio, during the infection (two–way ANOVA). * Represents significant differences ($P<0{\cdot}05$) in the number of haemocytes of each subset in each snail strain compared to non-infected (time 0) (One-way ANOVA, post-test. Tukey).

DISCUSSION

The characterization of circulating haemocytes in *Biomphalaria* has been described by various authors (Sminia 1981; Barraco *et al.* 1993; Bezerra *et al.* 1997; Matricon-Gondran and Letorcart, 1999 and Johnston and Yoshino, 2001), focusing on morphological and biochemical aspects of these cells. Using a flow cytometry-based methodology, Johnston and Yoshino (2001) described 2 major haemocyte subpopulations, referred as R1 and R2 type cells, in circulating haemocytes from *B. glabrata* snails free of infection. In the present experimental work, we also used flow cytometry analysis to characterize circulating haemocytes from *B. tenagophila*. The difference between this work and earlier reports is that the characterization of haemocytes was performed in living cells, and it was possible to examine alterations in the circulating haemocyte profile during *S. mansoni* infection. Moreover, we compared the effects of the parasite infection on numbers of circulating haemocytes in snails of different species (*B. glabrata* and *B. tenagophila*) and strains with different levels of susceptibility to *S. mansoni* infection.

Most of the earlier reports (Harris, 1975; Yoshino, 1976; Lo Verde *et al.* 1982; Lie *et al.* 1987; Barraco *et al.* 1993) differentiated 2 cell types in circulating haemocytes from *Biomphalaria* spp., designated as hyalinocytes (cells of smaller size and without granularity) and granulocytes (cells of greater size and high granularity). Using neutral red differential staining, previous studies from our laboratory (Bezerra *et al.* 1997; Martins-Souza *et al.* 2003) also identified 2 types of cells within circulating haemocyte populations from *Biomphalaria*: haemocytes that were not stained by neutral red (also called hyalinocytes), and red-stained granulocytes. However, red-stained haemocytes are a very heterogeneous cell population that includes both small and large cells (Negrão-Correa *et al.* 2007).

In the present work, cytometric analysis revealed that circulating haemocytes from *Biomphalaria* species consist of 3 major cell types, small, medium and large haemocytes. In fact, the analysis of the laser forward scatter versus laser side scatter dot plot distribution allowed us to identify a minor haemocyte subset, herein referred to as large haemocytes (P3) with FSC >840. The R3 haemocytes indicated here were also present in the cytometric profile of circulating haemocytes of *B. glabrata* reported by Johnston and Yoshino (2001), but those authors did not separate them from the R2 subset. The 3 circulating haemocyte subsets reported here are in agreement with the results of Matricon-Gondran and Letorcart (1999) who, based on their size and ultrastructural aspects, also identified 3 subpopulations of haemocytes in whole haemolymph of *B. glabrata* free of infection. The results also corroborated the earlier data of Martins-Souza *et al.*

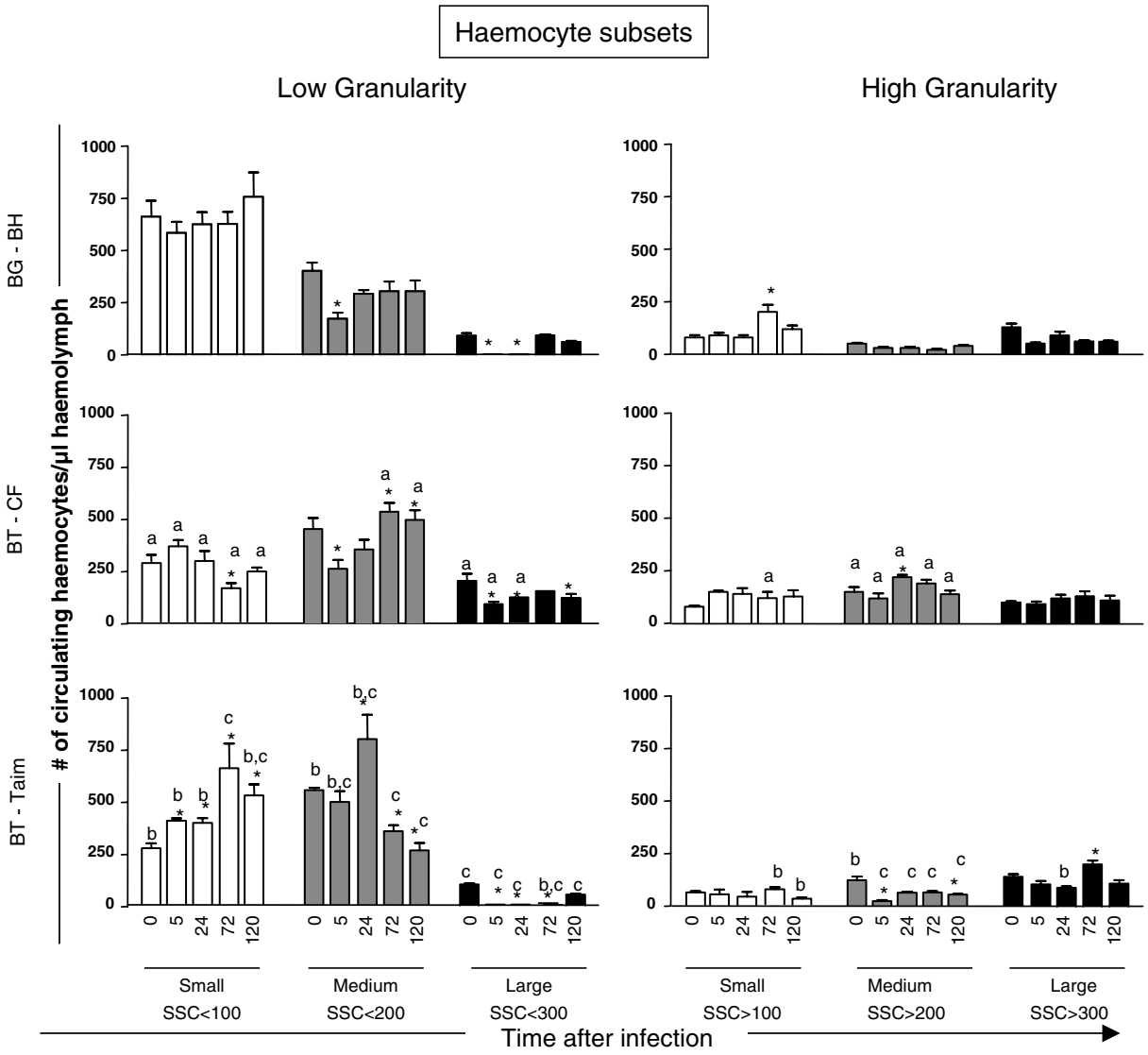

Fig. 4. Kinetic analysis of SSC^{Low} and SSC^{High} haemocyte subsets within the 3 major circulating haemocyte subpopulations in *Biomphalaria glabrata* (BG-BH), *B. tenagophila* Cabo Frio (BT-CF) and *B. tenagophila* Taim (BT-Taim) snails following *Schistosoma mansoni* infection. Data are presented as of SSC^{Low} and SSC^{High} haemocytes within the 3 major circulating haemocyte subpopulations, including small (□), medium (■) and large (■) haemocytes during 30 days after infection. (a) Represents significant differences ($P<0{\cdot}05$) in the number of haemocytes of each subset of SSC^{Low} and SSC^{High} in BT-Cabo Frio compared to the number of haemocytes obtained in BG-BH. (b) Represents significant differences ($P<0{\cdot}05$) in the number of haemocytes of each subset of SSC^{Low} and SSC^{High} in BT-Taim compared to the number of haemocytes obtained in BG-BH. (c) Represents differences in the number of haemocytes of each subset between BT-Taim and BT-Cabo Frio, during the infection (two-way ANOVA, post-test Tukey). * Represents significant differences ($P<0{\cdot}05$) in the number of haemocytes of each subset of SSC^{Low} and SSC^{High} in each snail strain compared to non-infected (time 0).

(2006) that identified 3 circulating haemocyte subsets in *Biomphalaria* using optical microscopy. Moreover, the authors showed that these cell subsets are differentially labelled by FITC-conjugated lectins and respond differently to *S. mansoni* infection, suggesting a functional role for them.

The analysis also showed that all the 3 subpopulations of haemocytes are heterogeneous with regard to their laser side scatter properties. Each haemocyte subset, in *B. glabrata* and *B. tenagophila*, is composed of 2 subpopulations of cells with different granularity profiles, referred to as less granular and more granular haemocytes. More importantly, the cytometric analysis allowed us to differentiate circulating haemocyte profiles of *B. glabrata* and *B. tenagophila*. In *B. glabrata* most of the circulating haemocytes were small cells with lower granularity profile while in *B. tenagophila* the majority of cells were medium, low granular haemocytes.

Another interesting finding of the present experimental work was the significant changes induced by *S. mansoni* infection in each snail strain or species. Bezerra *et al.* (1997) and Martins-Souza *et al.* (2003) showed that, 5 h after infection by *S. mansoni*, there

was a significant reduction in the number of cells circulating in all the snails studied, and the decrease was more intense in resistant strains. The authors also demonstrated that after the initial reduction of circulating haemocytes, there was an increase in total number of cells between 1–3 and 15 days after infection. *Biomphalaria glabrata* infected with the trematode *Echinostoma paraensei* showed an increased number of circulating haemocytes. The increased number of circulating cells was mainly due to the increase in the number of round small cells and the partially spread granulocytes (Noda and Loker, 1989*a*). However, phagocytic activity of circulating haemocytes was statistically lower in 8-day infected *B. glabrata* compared to non-infected haemocytes (Noda and Loker, 1989*b*), a result that would suggest that activated cells had migrated out of circulation. In this study we found that the reduction in circulating haemocytes induced by parasite infection was due to a significant decrease in the number of medium and large cells with more granular profile. Moreover, in *S. mansoni*-susceptible snails, such as *B. glabrata* (BG-BH) and *B. tenagophila* (BT-CF), the modified haemocyte profile was not intense and was transient. In contrast, in *B. tenagophila* of Taim strain, the resistant snail strain, the alteration in circulating haemocyte profile was very intense and prolonged until 120 h after infection. In these snails, large circulating haemocytes of less granular profile almost disappeared from the haemolymph, while small haemocytes gradually increased in number during the parasite infection. Moreover, medium haemocytes were very responsive during the first few days of *S. mansoni* infection in *B. tenagophila* of Taim, increasing in number, followed by a decrease that persisted throughout the infection. According to Sminia (1981), the small haemocytes, called hyalinocytes, have great mitotic activity and low phagocytic activity. He suggested that this cell type is a precursor for granulocytes – cells that have little mitotic activity, but high phagocytic activity. This hypothesis could explain the haemocyte response to parasite infection: mature cells, consisting of large and medium circulating haemocytes, migrate out of the haemolymph to the infection site. In parallel, immature small haemocytes proliferate and differentiate into the mature cells. Alternatively, it is possible that the infections may be inducing a degranulating effect on some cells or the formation of new granules in others. In previous work, Martins-Souza *et al*. (2006) had shown that medium and large circulating haemocytes, recovered from *B. tenagophila* of Taim strain, were intensively labelled by FITC-conjugated lectins, especially WGA and PNA. In addition, this cell population may be sequestred in the infection site. The participation of lectins in the haemocyte-sporocyst interaction has been well documented in the literature, most of them showing that lectins produced and secreted by haemocytes could facilitate binding of haemocytes to the larval tegument of trematodes (Van der Knaap and Loker 1990; Loker and Bayne, 2001; Yoshino *et al*. 2001 and Martins-Souza *et al*. 2006). Moreover, lectin-carbohydrate binding possibly leads to a structural change of the complex, that could induce haemocyte activation (Bayne, 1990), resulting in an increase of the phagocytic activity (Fryer *et al*. 1989) and/or of production of reactive oxygen species (ROS) (Hahn *et al*. 2000). Therefore, an intense circulating haemocyte response induced by *S. mansoni* infection would be associated with strong cellular infiltration around the parasite larvae and, consequently, with snail resistance against the infection.

In conclusion, our data clearly demonstrated that cytometric analysis is a useful tool for the characterization of circulating haemocytes in *Biomphalaria*, allowing us to quantify the changes in circulating haemocytes induced by parasite infection, such as *S. mansoni*. The comparison between haemocyte responses to *S. mansoni* infection in resistant and susceptible snail strains suggested that resistance observed in Taim strain is associated with intense haemocyte activation, and migration of medium and large haemocytes of less granular profile to the infection site.

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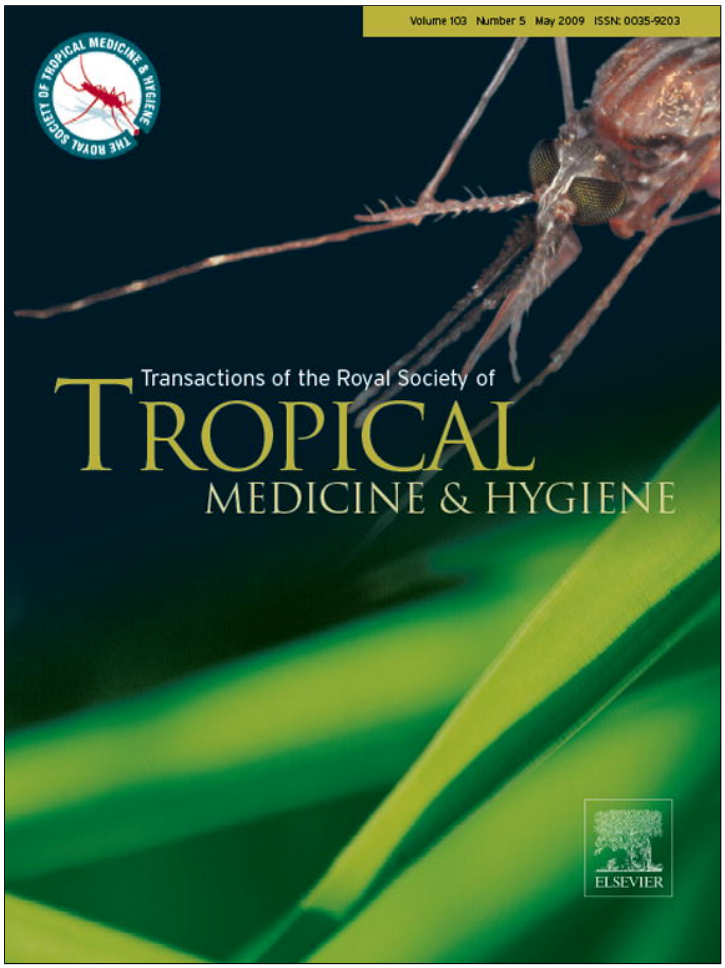

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Association between nutritional status, environmental and socio-economic factors and *Giardia lamblia* infections among children aged 6—71 months in Brazil

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Brazil

Summary A cross-sectional study was conducted on a randomised sample of 405 children aged 6—71 months in Brazil to investigate the association between nutritional status, environmental and socio-economic factors and *Giardia lamblia* infection. Data collection entailed an interview, anthropometric measurements and the collection of faeces and venous blood samples. The analysis was performed using multivariate logistic regression. The prevalence rate for *G. lamblia* was 26.3%. Nutritional status evaluation showed that 7.9% of the children had chronic malnutrition and 11.1% had acute malnutrition. The risk factors associated with infection by *G. lamblia* were an age of 2 years or older [odds ratio (OR) = 2.4], living in a two-bedroom house or smaller (OR = 2.3), living among a family of five or more people (OR = 2.4) and living in a house without access to a sewerage system (OR = 2.1). Non-participation in the social service programme was associated with a lower risk of infection (OR = 0.2). The model adjusted for age, including only biochemical and nutritional variables, showed weak associations with *G.lamblia* infection for two variables: inadequate animal protein intake according to the Dietary

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Reference Intake recommendation and low haemoglobin concentration. The sociodemographic and environmental risk factors classically described were associated with *G. lamblia* infection, but nutritional variables were only weakly associated with it.

1. Introduction

Parasite infections are a public health issue, especially in developing countries.[1,2] Among enteroparasites, *Giardia lamblia* is the protozoan most frequently found, with estimated prevalence rates of 20—30% in developing countries and 2—5% in developed countries.[3,4] Environmental and socio-economic factors, as well as hygiene habits, are important determinants of *G. lamblia* infection prevalence.[5,6]

Giardiasis is characterised as a wide-spectrum illness, although the majority (60—80%) of infected individuals is asymptomatic. The main clinical symptom is diarrhoea, but abdominal pain and weight loss are also reported.[7] In 2001, Katz and Taylor[8] suggested that giardiasis might influence the growth and development of infected children. Concomitantly, protein-energy malnutrition can increase susceptibility to and morbidity from *G. lamblia* and other parasite infections.[9] However, studies of the association between nutritional deficiency and giardiasis are contradictory.

In Brazil, some studies have shown that children infected by *G. lamblia* have a significantly lower weight per age (W/A) and height per age (H/A) than children who are not infected.[10,11] These results were corroborated by a study carried out in Ecuador[12] showing that children with giardiasis had a higher risk of growth deficits than did non-infected children. However, Sawaya et al.[13] did not find any association between infection by *Giardia* and nutritional status.

In this study, we investigated the association between nutritional status, environmental and socio-economic factors and *G. lamblia* infection among children aged 6—71 months who were living in Itinga, Minas Gerais, Brazil.

2. Materials and methods

2.1. Study design, area and population

A cross-sectional epidemiological study was conducted during July 2005 in 6- to 71-month-old children living in rural and urban areas of Itinga, Vale of Jequitinhonha, one of the poorest regions in the state of Minas Gerais in south-east Brazil. The human development index (HDI)[14] of Itinga is 0.624, placing it in 799th place of the state's 853 municipalities.

The sample size was determined on the basis of the following parameters: 1671 children aged 6—71 months were living in Itinga, according to the Brazilian Institute of Geography and Statistics (IBGE);[15] parasite infection prevalence was estimated to be between 18 and 78%;[6,16] the minimum difference tolerated between the estimates and the real values of such prevalence = 5%; alpha error = 0.05 and design effect = 1.5. We thus calculated that the study called for at least 393 children, and 405 were included.

The units sampled were households identified through the municipal health service records. The number of households selected in the rural and urban regions was proportional to the existing number of households in each region. The randomised sampling process was conducted in two stages: (1) a random sample of city blocks (urban area) and localities (rural areas) was selected proportionally to the existing blocks and localities; and (2) within each selected block and locality, random samples of households were drawn, proportional to the number of existing households. All children aged 6—71 months living in the selected households were invited to participate in the study.

2.2. Data collection

Upon arriving at the randomly selected households, teams of two trained interviewers obtained informed consent from a parent or other person responsible for the child. The interviewers were provided with instruction manuals and used a pre-coded questionnaire when conducting the interviews. Information was collected on the following groups of characteristics: demographic and social questions on child-related variables (e.g. sex, age, race, weight, height, reported morbidities, access to health service), socio-economic status of the parents (e.g. education level, income, schooling, employment, number of family members, number of children), household and environmental conditions (e.g. construction materials of walls, roof and floor, number of rooms, water quality, access to sanitation, public water, sewage availability, domestic refuse storage and disposal).

The interviews were complemented by evaluation of the children's food and dietary intake using a validated semi-quantitative food-frequency questionnaire, tailored for the dietary habits of the Vale of Jequitinhonha region. The person in charge of preparing the children's meals was interviewed. To standardise this information, the Food Photo Record Album[17] was used to help measure the meal portions and food preparations consumed by the infants.

To analyse the per capita/day consumption, the Brazilian Food Composition Tables were used. The translation of meals into weight or volume and calculation of macro- and micronutrient intake was carried out using the STATA software, version 9.0 (Stata Corp., College Station, TX, USA). To evaluate nutrient intake, the cut-off point method developed by Beaton was used.[18]

Estimates of daily consumption of each nutrient were compared with the Dietary Reference Intake (DRI) values used for the dietary evaluation of population groups. Inadequacy of diet was determined by calculating the prevalence of individuals with intake values lower than those recommended by the DRI (Estimated Average Requirement, Adequate Intake), taking into account sex and life stage.

Energy was calculated on the basis of the DRI's Estimated Energy Requirement.[18]

2.3. Anthropometric evaluation

Anthropometric evaluation was conducted using weight scales with a sensitivity of 50 g and a capacity of 150 kg. To measure height, a wooden anthropometer was used, with a ruler measuring up to 2000 mm. The following indicators were used: H/A and W/A *Z*-scores, according to the methodology used by the US Centers for Disease Control and Prevention (CDC).[19] It was preferred that the children not wear clothes while measurements were being taken.

2.4. Parasitological evaluation

For the collection of faecal material, a 50 ml flask with a tight lid was made available, containing 25 ml fixing solution (phosphate formula buffer with 10% formol). The collected samples were homogenised and stored under refrigeration until they were examined using the spontaneous sedimentation method of Hoffman, Pons and Janer.[20] These analyses were conducted at the Laboratory of Clinical Analysis of the Universidade Federal de Ouro Preto. Two slides were prepared for each sample, and they were examined by a laboratory technician and reviewed by the laboratory responsible.

2.5. Blood collection and haematocrit analysis

Blood was collected using 7.5 ml vacuum tubes, with and without EDTA. Serum and plasma were frozen at −70 °C. Before centrifugation of the blood collected in EDTA, two blood capillaries were prepared for each infant for haematocrit analysis. The capillaries were centrifuged at 1.000 rpm per 10 min in a microcentrifuge (Centimicro, model 211; Fanem, São Paulo, Brazil). Readings were carried out on graphs showing the haematocrit concentration counts. Children aged 6—59 months who presented with haematocrit values below 33% and children aged between 60 and 71 months with values below 34% were considered anaemic.[21]

2.6. Haemoglobin analysis

Haemoglobin concentration was measured using the HemoCue haemoglobin photometer. The HemoCue technique is based on an optical measuring cuvet of small volume (10 pl) in which its absorbance is measured at 565 and 880 nm.

2.7. Ferritin analysis

Ferritin levels were determined by immunoassay of enzymatic particles by Beckman Coulter (Access Immunoassay System), using Access Ferritin as a reagent. The concentration considered normal was above 10 ng/ml for infants up to 1 year old and above 12 ng/ml for children older than 1 year.[22,23]

2.8. Processing and analysis of data

The information from the interviews and the results of the laboratory tests were managed using EpiData 3.4 (The EpiData Association, Odense, Denmark). Statistical analysis of the data was conducted using the STATA 9.0 software (StataCorp, 2005).

Univariate analyses were carried out using the χ^2 test for proportions and Student's *t* and Wilcoxon tests for means and medians. To quantify the association between risk factors with *G. lamblia*, odds ratio (OR) and 95% CI were calculated. Multivariate analysis was performed using multivariate logistic regression. Variables with $P < 0.20$ in the univariate analysis and some variables considered important in the literature for *G. lamblia* infection were selected for multivariate logistic regression. Variables representing characteristics of low frequency or of collinearity were excluded from the final model. The multivariate logistic models were built using the backward process, and statistical significance was determined by the likelihood ratio test.[24]

3. Results

During field research, 328 households were visited and 405 infants and children were evaluated. The mean age was 39 ± 18.87 months, and the median age was 40 months (interquartile range 23—56 months); 52.8% were boys and 47.2% girls.

When socio-economic status was examined, 57.8% of the children were found to be in families with income below minimum wage, 31.8% with one to two times the minimum wage, 5.5% with two to four times the minimum wage, and 4.9% with more than four times the minimum wage (the Brazilian minimum wage is about US$260.00 per month). Among the mothers, 6.9% had no formal education, 24.0% had an incomplete elementary school education, 34.5% had completed elementary school, 30% had a high school education and 4.6% had a college education. As for family size and environment, 36.9% were in families with five members or more, 39.0% lived in houses with fewer than six rooms, 38.7% did not have running water and 70.6% had no access to a sewerage system.

Of 405 faecal exams analysed, 132 (32.6%) were from children who had parasite eggs or cysts in their faeces. The parasite most frequently observed was *G. lamblia*, with a prevalence rate of 26.3% (Table 1).

The symptoms presented by the children infected with *G. lamblia* ($n = 106$) in the 15 days before the study, as reported by a parent or guardian, were diarrhoea (14.5%), blood in the faeces (1.9%), vomiting (11.3%) and fever (28.3%). Only 3.1% of the infected children reported simultaneous diarrhoea, fever and vomiting.

Nutritional evaluation revealed that 11.1% of the children had acute malnutrition (W/A), and 7.9% (H/A) showed chronic malnutrition. However, the prevalence increased to 33.8% for acute malnutrition and 29.1% for chronic malnutrition when children who were at risk of malnutrition were added. Only 1.7% of the children assessed were overweight, and 5.7% were categorised as having an overweight risk.

Table 1 Prevalence of parasite infections in 405 children aged 6—71 months, Itinga, Minas Gerais, Brazil, 2005.

	n (%)
Protozoan infection	
Giardia lamblia	106 (26.3)
Entamoeba histolytica/E. dispar	17 (4.1)
Endolimax nana	5 (1.2)
Iodameba butschlii	3 (0.7)
Helminth infection	
Hymenolepis nana	8 (2.0)
Ancilostomideos	3 (0.7)
Enterobius vermicularis	3 (0.7)
Ascaris lumbricoides	2 (0.5)

Only *G. lamblia* infection was considered in the analysis of potential risk factors. First, the medians for age and the biochemical and nutritional parameters were compared between the infected and non-infected children (data not shown). Only the median values of age and ferritin serum concentration were higher in infected than non-infected children (47.5 vs. 37.0 months, $P = 0.002$; 36.8 vs. 28.5 ng/ml, $P = 0.007$).

Univariate analysis was carried out, taking into account all the variables, one at a time. However, Tables 2 and 3 show only the variables with $P < 0.20$ in univariate analyses and those that were known to have biological importance for *G. lamblia* infection.

The final multivariate model, with the crude and adjusted OR values and their respective 95% CI, is presented in Table 4. The variables found to be independently associated with *G. lamblia infection* were age 2 years or older (OR = 2.4), living in a two-bedroom house or smaller (OR = 2.3), living among a family consisting of five or more members (OR = 2.4), living in a household without access to a sewage system (OR = 2.1) and non-participation in the social service programme (the Children's Pastoral Program, a non-governmental organisation) was associated with a lower risk of *G. lamblia* infection (OR = 0.2).

A model with only biochemical and nutritional markers (animal protein intake, calorie intake, haematocrit, haemoglobin, ferritin) as predictors of infection by *G. lamblia* was also evaluated. In the final model, adjusted for age, only two variables showed weak associations with the infection: adequate animal protein intake, according to the recommendations of the DRI, was associated with lower risk (OR = 0.97, 95% CI 0.95—0.99, $P = 0.002$) and low haemoglobin serum concentration (10 mg/dl) was associated with higher risk (OR = 1.10, 95% CI 1.06—1.18, $P = 0.016$).

4. Discussion

The results of this study showed that *G. lamblia* was the most prevalent parasite infection (26.3%) among the children investigated. This finding corroborates the findings of more recent studies conducted in other regions of Brazil, which reported prevalence rates of *Giardia* infection ranging between 8.8 and 29.0%.[6,11,25,26]

The absence of infections with common helminths, such as *Ascaris lumbricoides* and *Ancilostomideos*, was an unexpected result given that Itinga is located in the Vale of Jequitinhonha, one of the poorest regions in the state of Minas Gerais, Southeast Brazil. One explanation for the absence of helminth infections is the fact that many of the children had previously been treated with anthelminthics. According to information collected during the interview, 20% of the children infected by *G. lamblia* and 23% of those not infected had been treated with anthelminthics just months before this study. In a prospective study in children, Northrop-Clewes et al.[27] showed that in the group treated with an anthelminthic (mebendazole), there was a reduction in the prevalence of *A. lumbricoides* and *T. trichiura* after a simple medication dose. However, infection by *Giardia* increased in the treated group (from 4 to 49%) after 4 months of observation. To gain a better understanding of the dynamics of intestinal parasite infections, a prospective study must be undertaken, with children monitored before and after treatment.

The prevalence of chronic malnutrition, 7.9%, based on height for age, was lower than the prevalence considered risky for public health. However, the prevalence of chronic malnutrition increased to 29.1% when children who were at risk of malnutrition were added. Data recently published by UNICEF[28] indicate that there is a global tendency towards decreased malnutrition prevalence and increased overweight. In Brazil, the prevalence of malnutrition was estimated to be 11% in 2007.[28]

In our study, univariate analysis showed that malnutrition risk, according to H/A, was associated with *G. lamblia* infection. However, in the final model, after adjustment for other variables, malnutrition was not associated with infection. Sadjjadi and Tanideh[5] found that H/A and W/A were significantly different between infected and non-infected children. In Brazil, Carvalho-Costa et al.[11] found an association between *G. lamblia* and the *Z*-scores for weight (WAZ) and height (WHZ) in a cross-sectional study conducted among children aged 6—84 months.

The main clinical symptom reported by the infected children was diarrhoea (14.5%), which is a characteristic of acute infection and is often short-lived and self-limiting. Other symptoms characteristic of acute infection, such as abdominal discomfort, vomiting and fever, were reported to have occurred simultaneously in only 3.1% of the infected children. It is worth mentioning that the interview may not have been sufficiently sensitive to detect the symptoms reported by the parents. Also, of the children infected by *G. lamblia* who had diarrhoea symptoms ($n = 15$), only two were chronically undernourished. A longitudinal study of children in Northeastern Brazil showed that children infected with symptomatic giardiasis, when compared with asymptomatic children, presented deficits in weight and height for age.[25]

Although infection with *Giardia* is classically influenced by environmental and socio-economic factors, the biochemical and nutritional markers showed that inadequate animal protein intake, according to the DRIs and low haemoglobin serum concentration (mg/dl) when adjusted for age, was significant for *Giardia* infection among the children analysed. However, these associations yielded ORs close to 1.0,

Table 2 Univariate analyses of parasite infection by *Giardia lamblia*, based on socio-economic, demographic and environmental factors in children aged 6—71 months, Itinga, Minas Gerais, Brazil, 2005.

Variable	*Giardia lamblia* Infection			
	Yes	No	Odds ratio (95% CI)	*P*-value
	n (%)	*n* (%)		
Family size (*n* = 405)				
≥5 members	58 (54.7)	120 (40.1)	1.8 (1.2—2.8)	0.011
<5 members	48 (45.3)	179 (59.9)	1	
House size (*n* = 405)				
≤6 rooms	74 (69.8)	180 (60.2)	1.5 (0.9—2.5)	0.080
>6 rooms	32 (30.2)	119 (39.8)	1	
No. bedrooms (*n* = 405)				
≤2 bedrooms	71 (66.9)	158 (52.8)	1.8 (1.1—3.0)	0.012
>2 bedrooms	35 (33.0)	141 (47.2)	1	
Family income (*n* = 396)				
<1 minimum wage	33 (31.1)	123 (42.4)	0.6 (0.4—0.8)	0.043
≥1 minimum wage	73 (68.9)	167 (57.6)	1	
Tap water (*n* = 405)				
No	57 (53.8)	108 (36.1)	2.1 (1.3—3.3)	0.001
Yes	49 (46.2)	191 (63.9)	1	
Sewerage system (*n* = 405)				
No	85 (80.2)	206 (68.9)	1.8 (1.1—3.1)	0.030
Yes	21 (19.8)	93 (31.1)	1	
Mother's age (*n* = 391)				
≤20 years	2 (2.0)	24 (8.2)	0.2 (0.5—0.9)	0.049
>20 years	97 (98.0)	268 (91.8)	1	
Mother's education (*n* = 392)				
None	11 (11.0)	17 (5.8)	2.0 (0.9—4.4)	0.088
More than 1 year of school	89 (89.0)	275 (94.2)	1	
No. brothers/sisters (*n* = 404)				
≥2	48 (45.7)	83 (27.8)	2.2 (1.4—3.5)	0.001
<2	57 (54.3)	216 (72.2)	1	
No. brothers and sisters <5 years (*n* = 334)				
Yes	67 (74.4)	128 (52.5)	2.7 (1.5—4.5)	0.000
No	23 (25.6)	116 (47.5)	1	
Social Service Program (The Children's Pastoral Program) (*n* = 405)				
Not registered	98 (92.5)	292 (97.7)	0.3 (0.1—0.8)	0.021
Registered	8 (7.5)	7 (2.3)	1	
Hospitalised during gestation (*n* = 385)				
Yes	3 (3.1)	26 (9.1)	0.3 (0.1—1.7)	0.064
No	95 (96.9)	261 (90.9)	1	

indicating weak associations between these factors and the infection.

The low prevalence of malnutrition, the low frequency of clinical symptoms and the weak association of biochemical and nutrition markers suggested that *G. lamblia* infection was acute in this population of children.

The most important risk factors associated with infection by *G. lamblia* identified in this study were children's age, family size, number of bedrooms, lack of access to a sewerage system, and participation in the Children's Pastoral Program.

Children aged 2 years or older had a higher risk of *G. lamblia* infection. In this study, 72% of the children were 2 years or older, but the higher proportion of children in this age group was reflective of the child population in Itinga (73% of the children are 2 years or older), according to the IBGE.[15] This association between age and *G. lamblia* infection may be explained by a longer and frequent exposure to infection sources. Okyay et al.[29] and Teixeira et al.[6] did not

Table 3 Univariate analysis of parasite infections by *Giardia lamblia*, based on individual characteristics of children aged 6 to 71 months, Itinga, Minas Gerais, Brazil, 2005.

Variable	*Giardia lamblia* infection			
	Yes	No	Odds ratio (95% CI)	*P*-value
	n (%)	*n* (%)		
Age group (*n* = 405)				
≥2 years	92 (86.8)	216 (72.2)	2.5 (1.4—4.7)	0.003
<2 years	14 (13.2)	83 (27.8)	1	
Feeling sick in the past 15 d (*n* = 405)				
Yes	37 (34.9)	70 (23.4)	1.8 (1.1—2.9)	0.022
No	69 (65.1)	229 (76.6)	1	
Hospitalised (*n* = 403)				
Yes	37 (34.9)	83 (28.0)	1.4 (0.9—2.2)	0.179
No	69 (65.1)	214 (72.0)	1	
Chronic malnutrition (*n* = 364)				
Yes	10 (10.7)	17 (6.3)	1.8 (0.8—4.0)	0.171
No	84 (89.3)	253 (93.7)	1	
Acute malnutrition (*n* = 364)				
Yes	37 (39.4)	80 (29.6)	1.5 (0.9—2.5)	0.083
No	57 (60.6)	190 (70.4)	1	
Animal protein deficiency (*n* = 405)				
Yes	72 (7.9)	157 (52.5)	1.9 (1.2—3.1)	0.006
No	34 (32.1)	142 (47.5)	1	
Serum hematocrit deficiency (*n* = 350)				
Yes	40 (44.9)	123 (47.1)	0.9 (0.5—1.5)	0.722
No	49 (55.1)	138 (52.9)	1	
Ferritin deficiency (*n* = 364)				
Yes	16 (17.4)	72 (26.5)	0.6 (0.3—1.1)	0.081
No	76 (82.6)	200 (73.5)	1	
Haemoglobin deficiency (*n* = 405)				
Yes	39 (36.8)	111 (37.1)	1.1 (0.7—1.6)	0.952
No	67 (63.2)	188 (62.9)	1	
Ferrous sulfate in the last 15 d (*n* = 402)				
No	104 (98.1)	270 (91.2)	5.0 (1.2—21.5)	0.030
Yes	2 (1.9)	26 (8.8)	1	

find any significant age-related associations with *G. lamblia* infection.

In this study, children whose families had five or more members had 2.4 times the risk of acquiring the infection than those in smaller families. This result supports the previously reported hypothesis of interpersonal transmission, particularly among children, who are the most vulnerable.[6,26]

Children who lived in houses with fewer than two bedrooms also had a greater chance of being infected (OR = 2.3).

Table 4 Risk factors for *Giardia lamblia* infection in children aged 6—71 months, Itinga, Minas Gerais, Brazil 2005.

Variable	Unadjusted odds ratio (95% CI)	Adjusted odds ratio (95% CI)
Age (≥2 years vs. <2 years)	2.5 (1.4—4.7)	2.4 (1.3—4.6)
No. bedrooms (≤2 vs. >2)	1.8 (1.1—3.0)	2.3 (1.4—3.8)
Family size (≥5 vs.<5)	1.8 (1.2—2.8)	2.4 (1.5—4.0)
Sewerage system (no vs. yes)	1.8 (1.1—3.1)	2.1 (1.3—3.5)
Social Service Program (no vs. yes)	0.3 (0.1—0.8)	0.2 (0.1—0.6)

Social Service Program: The Children's Pastoral Program.

This variable may be interpreted as an indirect measurement of socio-economic levels in the population (proxy variable); i.e. smaller homes reflect lower socio-economic levels.

The lack of a sewerage system in the households also made the Itinga children more susceptible to *Giardia* infection (OR = 2.1). This proxy variable may indicate water contamination, as lack of sanitation reflects possible contamination of different water sources.[30] Previous studies reported an association between households without indoor toilet and infection by *Giardia*.[6,26]

Participation of the children in the Children's Pastoral Program was inversely associated with infection. The likely explanation is that children involved in the social assistance programme are exposed to the worst health conditions, such as infections and malnutrition, and they demand the most monitoring.

Okyay et al.[29] found an association between the schooling level of the parent or person responsible and infection by *Giardia*. In this study, this variable was not found to be associated with infection. In Itinga, a high percentage of the mothers (30.9%) had less than an elementary school education, but only 6.9% of them were illiterate.

An important methodological limitation of cross-sectional studies is that they do not allow for causal inferences, because the temporal relationships between risk factors and infections are not evaluated. One of the limitations of this study was the loss of information due to interviewees' refusal to answer and during collection of the biological and anthropometrical data. However, there is no evidence that the missing data were related to *G. lamblia* infection; i.e. information bias is not likely. One important methodological advantage of this study was that the sampling process was conducted at different stages, which guaranteed external validity, as a representative sample of children from regions (urban and rural) and age distribution was selected from Itinga.

In conclusion, a high prevalence of *G. lamblia* infection was found in this study. The sociodemographic and environmental risk factors classically described in the literature were associated with infection. Although biochemical and nutrition markers presented weak associations with the infection, they must not be underestimated. Prospective studies must be conducted to investigate these associations in children infected by *G. lamblia*, with and without symptoms.

Authors' contributions: JAL and MC were the coordinators of the project and responsible for the study design; RRS and CAMS carried out the data collection and managed the data processing; CAJP and RLCN carried out the laboratory analysis; DNC helped in the interpretation of the data; RRS, DNC and MC drafted the manuscript. All authors read and approved the final manuscript. MC is guarantor of the paper.

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