Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz

INSTITUTO OSWALDO CRUZ
Pós-Graduação em Biologia Parasitária

DANIELE PEREIRA DE CASTRO

Fatores que interferem no desenvolvimento de tripanosomatídeos em *Rhodnius prolixus*: I-Efeito de fisalinas sobre o sistema imune; II-*Serratia marcescens* isolada da microbiota intestinal.

Tese apresentada ao Instituto Oswaldo Cruz como parte dos requisitos para obtenção do título de Doutor em Ciências

Orientadores: Prof. Dra. Patrícia de Azambuja Penna
Prof. Dr. Eloi de Souza Garcia

RIO DE JANEIRO
2009

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ORIENTADORES: Prof. Dra. Patrícia de Azambuja Penna
Prof. Dr. Eloi de Souza Garcia

Aprovada em: 17 / 03 / 2009

EXAMINADORES:

Prof. Dr. Alexandre Afrânio Peixoto
Prof. Dra. Leila de Mendonça Lima
Prof. Dr. Wanderley de Souza

Rio de Janeiro, 17 de março de 2009

Ficha catalográfica elaborada pela
Biblioteca de Ciências Biomédicas/ ICICT / FIOCRUZ - RJ

C355

Castro, Daniele Pereira de.

Fatores que interferem no desenvolvimento de tripanosomatídeos em *Rhodnius prolixus* : I- Efeito de fisalinas sobre o sistema imune; II- *Serratia marcescens* isolada da microbiota intestinal / Daniele Pereira de Castro. – Rio de Janeiro, 2009.

viii, 74 f. : il. ; 30 cm.

Tese (doutorado) – Instituto Oswaldo Cruz, Pós-Graduação em Biologia Parasitária, 2009.
Bibliografia: f. 57-74

1. Rhodnius prolixus. 2. Physalis angulata. 3. Fisalina. 4. Atividade imunosupressora. 5. Serratia marcescens. 6. Tripanosomatídeos. 7. Atividade tripanolítica. I. Título.

CDD 616 9363

Dedico ao meu marido Paulo, minha querida filha Letícia e aos meus pais, irmãos e sobrinha.

FOLHA DE AGRADECIMENTO

A minha orientadora Dra. Patrícia de Azambuja que me fez crescer como profissional cobrando meus deveres e ao mesmo tempo me deixando livre para criar. Agradeço por saber me escutar e sempre acreditar que eu era capaz.

Ao também orientador Dr. Eloi de Souza Garcia pelos ensinamentos tanto referentes à tese quanto sobre questões éticas e políticas do meio científico. Pela paixão à pesquisa e por mostrar o lado prático e simples de um ensaio.

Ao Dr. Reginaldo Brazil pelos amigáveis conselhos, por me escutar com tanta atenção, pelo carinho e apoio.

Ao Zildo pela criação dos insetos e à Aline aos ensinamentos das questões burocráticas, além da amizade.

Aos colegas do Laboratório de Bioquímica e Fisiologia de Insetos, em especial as grandes amigas Caroline, Andressa, Marcela, Rozana e Naty pelas colaborações, idéias, apoio, diversão, carinho e conversas. Novamente agradeço à Carol que com muito carinho, atenção e paciência, me recebeu no laboratório e me ensinou tantas coisas. Obrigada por ser tão prestativa!

Ao Dr. Sérgio Seabra pela orientação em relação á microscopia eletrônica de varredura e transmissão além dos ensaios de adesão e microscopia de vídeo.

Ao Dr. Wanderley de Souza pelos ensaios realizados no laboratório de Ultraestrutura Celular Hertha Meyer, Instituto de Biofísica Carlos Chagas Filho – UFRJ e pelas grandes idéias.

A Dra. Terezinha Tomassini e Ivone Ribeiro pela purificação das fisalinas e pela atenção dedicada durante os experimentos.

A Dra**.** Cécile Wandersman pelas variantes de *Serratia marcescens* utilizadas em nosso trabalho.

Ao Dr. Fernando Genta que participou do final de minha tese com grandes idéias.

Ao Dr. Marcelo Gonzalez pela grande amizade.

Ao Instituto Oswaldo Cruz/Fiocruz instituição aonde foi realizada minha tese e ao curso de pós-graduação em Biologia Parasitária, especialmente à Luciane da secretaria do curso.

Aos órgãos financiadores CAPES e FAPERJ.

Ao Paulo e Letícia por fazerem do dia-a-dia meu motivo de alegria e por me amarem tanto.

A minha família e a família do Paulo por estarem sempre ao meu lado com apoio e amor.

A todos aqueles que, embora não nomeados, me apoiaram em distintos momentos e por suas presenças afetivas, o meu reconhecido e carinhoso muito obrigada!

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RESUMO – TESE DE DOUTORADO

Daniele Pereira de Castro

Nesta tese, foram investigados fatores que interferem no desenvolvimento dos parasitas, *Trypanosoma cruzi* e *T. rangeli*, no inseto-vetor, *Rhodnius prolixus*, com a administração de substâncias extraídas de *Physalis angulata*, fisalinas, bem como fatores relacionados a microbiota bacteriana intestinal do inseto, como *Serratia marcescens*. As fisalinas B, D, F e G administradas junto ao sangue alimentar não alteraram a fisiologia de ninfas de 5° estádio de *R. prolixus*, entretanto quando inoculados com *T. rangeli* ou *Enterobacter cloacae* β12 causaram alta mortalidade. Nos insetos tratados com fisalina B e infectados com parasita observou-se diminuição na formação de microagregados de hemócitos e produção de óxido nítrico. As fisalinas B e F reduziram a atividade de lisozima e a fisalina D reduziu a atividade antibacteriana, ambas de insetos inoculados com bactéria. Insetos tratados com as fisalinas B, D, F e G exibiram reduções significativas na atividade fagocitária. O tratamento dos insetos com as fisalinas B, F e G e inoculação de bactéria ocasionou menor contagem na formação de microagregados de hemócitos e o tratamento com as fisalinas B e F menor número de hemócitos circulantes na hemolinfa. As atividades de fagocitose e microagregação de hemócitos inibidas pelo tratamento oral com fisalina B foram revertidas pela inoculação de ácido araquidônico (10 μg/inseto) e fator de agregação plaquetária (PAF) (1 μg/inseto) na hemocele dos insetos tratados. O tratamento dos insetos com fisalina B não alterou a atividade da PLA_2, porém aumentou a atividade de PAF-AH. Noutro enfoque da interação inseto-parasita, foram estudados os efeitos citotóxicos da *S. marcescens*. Ensaios *in vitro*, de incubação da bactéria com *T. cruzi* ou *T. rangeli* juntamente com o carboidrato D-manose (0,2M) resultaram na proteção dos parasitas da adesão e da lise de *S. marcescens* (variantes SM365 e RPH) numa relação dose dependente. Entretanto, a D-manose não interferiu na atividade hemolítica das variantes SM365 e RPH de *S. marcescens*. Enquanto com estas variantes a adesão das bactérias ao parasita ocorria em apenas alguns segundos, a variante DB11 não aderiu e não lisou o parasita. A adesão bacteriana é densa e compacta e ocorre por finos filamentos que com o tempo se desenvolve formando biofilme em volta da superfície do parasita lisado. Portanto, concluímos que dentre os fatores que interferem na interação entre inseto e *T. cruzi e T. rangeli* estudados, podemos citar as fisalinas e *S. marcescens* por suas atividades imunosupressoras e lítica contra os parasitas, respectivamente. Enquanto a fisalina B atua diminuindo os níveis de análogo de PAF (iPAF) na hemolinfa e elevando os níveis de PAF-AH, com ação inibitória sobre o sistema imune de *R. prolixus*, a bactéria *S. marcescens* lisa parasitas resultando em formação de biofilme, os quais regulam, direta ou indiretamente, o desenvolvimento do parasita no insetos vetor.

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ABSTRACT - TESE DE DOUTORADO

Daniele Pereira de Castro

Factors influencing the relationship between the parasites, *Trypanosoma cruzi* and *T. rangeli*, with the insect-vector, *Rhodnius prolixus*, were investigated by administration of compounds extracted from *Physalis angulata,* physalins, and by bacterial microbiota, *Serratia marcescens* of the insect's intestine. *R. prolixus* treatment with physalins B, D, F or G did not present alteration on its physiology; however with *T. rangeli* or *Enterobacter cloacae* β12 hemocelic infection we observed high insect mortality. Insects fed with blood containing physalin B and infected with *T. rangeli* presented low hemocyte microaggregate formation and low nitric oxide production. Insects treated with physalin B and F and inoculated with bacteria showed differences on lysozyme activity and reduced antibacterial activity upon administration of physalin D. Insects treated with physalins B, D, F and G exhibited drastic reductions in phagocyte activity. Insects fed with physalins B, F and G and inoculated with bacteria showed reduction in microaggregate formation and with physalins B and F lower number of total hemocyte count in the hemolymph. Hemocyte phagocytic activity and formation of hemocyte microaggregates were strongly blocked by oral treatment of this insect with physalin B. These inhibitions, induced by physalin B, were rescued by exogenous arachidonic acid (10 μg/insect) or PAF (1 μg/insect) applied by hemocelic injection. Following the treatment with physalin B there are no significant alteration in PLA_2 activities, but a significant enhancement of PAF-AH was observed. In another view of parasite-insect interaction *S. marcescens* cytotoxic effects were investigated. *In vitro* assays of *T. cruzi* and *T. rangeli* with *S. marcescens* (SM365 e RPH) and addition of D-mannose (0,2M) protected the parasites, markedly diminishing the lysis caused by the bacteria. This car bohydrate is unable to interfere with the hemolysis induced by SM365 and RPH variants. However D-mannose protects the parasites from adhesion and lyses in a dose dependent manner. This bacterium rapidly adhered to the protozoan surface through $_D$-mannose recognizing fimbriae and rapidly induced its complete lysis. In contrast, the DB11 variant of the same bacterial species did not adhere and did not induce protozoan lysis. Bacteria-protozoan attachment is dense and tight and is constituted by long filamentous structures. Bacteria adhesion develops into biofilm over the parasite surface after longer periods of interaction. We conclude that parasite-insect interactions are influenced by factors including physalins and *S. marcenscens* through immune depression and parasite lytic activities, respectively. While physalin B acts by depressing insect PAF analogous (iPAF) levels in hemolymph, and increasing PAF-AH levels, which plays a role in the cellular immune reactions of *R. prolixus,* the bacteria *S. marcescens* causes parasite lysis resulting in biofilm formation which are responsible for parasite development in insect vectors.

INTRODUÇÃO

Rhodnius prolixus Stal, 1859, é um inseto de grande importância médica na América Latina por ser uma das espécies transmissoras do *Trypanosoma cruzi,* agente etiológico da Doença de Chagas**,** sendo apontado como principal vetor na Colômbia e Venezuela. No Continente Americano, a doença de Chagas tem sido responsabilizada por aproximadamente 18000 mortes por ano. Há registros que mostram que cerca de nove milhões de pessoas estão infectadas e por volta de 40 milhões de pessoas vivendo em áreas de risco (Schofield et al., 2006; Moncayo e Ortiz Yanine, 2006).

Plantas que contenham substâncias bioativas capazes de afetar a transmissão dos parasitas infecciosos pelos insetos vetores têm sido pesquisadas como forma de controle do inseto e/ou da doença. A azadirachtina é um composto extraído da semente da árvore *Azadirachita indica* a qual, na Índia, tem sido amplamente utilizada no controle de insetos há centenas de anos. Atua sobre o desenvolvimento fisiológico e imunológico de uma grande variedade de insetos impedindo danos agrícolas e médicos (Schumutterer, 1990, 1995; Mordue e Blackwell 1993; Mulla e Su. 1999; Boeke et al. 2004; Garcia e Azambuja 2004).

Dentre a busca por novas substâncias bioativas isoladas de plantas, as fisalinas despertam grande interesse por possuírem atividade imunomoduladora, atividade tripanossomicida, bactericida, moluscidas, antiinflamatória e antitumoral (Lin *et al*., 1992; Chiang *et al*., 1992; Soares *et al*., 2003; Santos *et al*., 2003; Hwang *et al*., 2004). Recentemente tem sido estudada em mamíferos como droga antiinflamatória atuando similarmente a glucocorticóides, como dexametasona (Soares et al., 2003, 2006; Vieira et al., 2005).

Nesse trabalho foi investigada a ação de diferentes fisalinas sobre a fisiologia, comportamento e imunologia de *R. prolixus* desafiados com parasitas ou bactérias. Como foram observados maiores efeitos das fisalinas no sistema imunológico dos insetos assim foi aprofundada as investigações no controle do sistema imune celular e humoral, especialmente nas vias de eicosanóides e via de fator de ativação de plaquetas (PAF). Na presente tese, a discussão destas vias foi revistada à luz da via Toll/Imd.

O sistema imunológico dos insetos é extremamente importante para os parasitas que precisam superar às diferentes condições fisiológicas adversas durante o estabelecimento da infecção em seu hospedeiro invertebrado. Nesse contexto, o parasita

também enfrenta outros obstáculos no inseto como a interação com outros microorganismos situados no trato digestório, tais como as bactérias constituintes da microbiota.

Todos os vertebrados e invertebrados possuem em seu sistema uma grande variedade de bactérias patogênicas ou não. Muitas convivem em simbiose com o hospedeiro, como no caso da maioria das bactérias que habitam o trato digestivo. Essas bactérias podem ajudar na digestão e evitar a proliferação de micróbios patogênicos no hospedeiro enquanto que esse fornece habitat adequado e nutriente para as bactérias. Quando o hospedeiro possui apenas uma fonte de alimentação, como no caso de insetos hematófagos, a microbiota se torna essencial, pois além de ajudar na digestão, fornece vitaminas, nutrientes e enzimas deficientes ao inseto (Terra, 1990; Beard et al., 2002; Genta et al., 2006).

O *R. prolixus* possui diversas bactérias colonizando seu trato digestivo. Azambuja et al. (2004) isolaram a bactéria *Serratia marcescens* do tubo digestivo e demonstraram sua ação hemolítica e tripanolítica em ensaios *in vitro*. Dando prosseguimento a estes achados, foi investigada a ação tripanolítica de diferentes variantes de *S. marcescens* sobre os parasitas *T. cruzi* e *T. rangeli*. Além disso, possíveis inibidores (carboidratos) da atividade tripanolítica e da adesão bacteriana foram pesquisados bem como as estruturas da bactéria envolvidas nesse processo (fimbrias e biofilmes).

Para melhor compreensão, esse trabalho será apresentado em duas partes. A primeira em que será discutida a ação de diferentes fisalinas na resposta imune de *R. prolixus* e a segunda parte os efeitos citotóxicos da *S. marcescens* sobre os parasitas transmitidos por este inseto-vetor.

Fatores que interferem no desenvolvimento de tripanosomatídeos em *Rhodnius prolixus*:

I-Efeito de fisalinas sobre o sistema imune.

1. REVISÃO DE LITERATURA

1.1 *Rhodnius prolixus*

Inseto da ordem Hemiptera, família Reduviidae e subfamília Triatominae. Seu ciclo de vida compreende as fases de ovo, cinco estádios de ninfa e adulto cujo desenvolvimento ocorre através de metamorfose incompleta. Entre os estádios de ninfa e da fase jovem para adulto o inseto sofre um processo conhecido como muda ou ecdise que consiste na troca do exoesqueleto antigo por um novo (Carcavallo *et al*, 1997; Wigglesworth, 1972).

A transmissão do agente etiológico da Doença de Chagas, *Trypanosoma cruzi*, está relacionada com o hábito de vida hematófago dos vetores. *R. prolixus* é uma das espécies de insetos vetores mais importantes do *T. cruzi*. Alimenta-se exclusivamente de sangue em todas as fases de seu ciclo evolutivo, bastando apenas um repasto sanguíneo saturante para ocorrer a ecdise ou ovipostura (Garcia *et al*., 1975). No ambiente domiciliar, *R. prolixus* alimenta-se principalmente de sangue humano, embora utilize também, como fonte alimentar, cães e gatos. No ambiente silvestre faz uso de marsupiais e roedores, como fonte de alimento. Vivem em média entre um a dois anos, com grande capacidade de reprodução e, dependendo da espécie, com intensa resistência ao jejum. Para seu adequado desenvolvimento necessita de temperatura em torno de 27 a 30ºC e umidade relativa do ar de 75% (Azambuja *et al.,* 1991). Em laboratório, com condições controladas de temperatura e umidade e uso de aparatos artificiais de alimentação, é de fácil manutenção em colônias (Garcia *et al*. 1984).

1.2 Tripanossomatídeos

Os tripanosomatídeos, *T. cruzi* e *T. rangeli*, pertencem a ordem Kinetoplastida e família Tripanossomatidae. O *T. cruzi* é o agente etiológico da Doença de Chagas, o qual se encontra amplamente distribuído nas áreas endêmicas pela prevalência e distribuição de insetos vetores infectados com o parasita (Silveira *et al*, 1984). Ao contrário do *T. cruzi*, o *T. rangeli* não é considerado patogênico para os hospedeiros vertebrados (Tobie, 1970; Watkins, 1971). Entretanto esse protozoário deve ser estudado no contexto epidemiológico da Doença de Chagas por apresentar características biológicas e distribuição geográfica coincidentes com a do *T. cruzi* (Guhl e Vallejo, 2003).

Para completar o seu ciclo de vida os parasitas se alternam entre hospedeiro vertebrado e invertebrado, assumindo as formas replicativas (epimastigota no inseto e amastigota no hospedeiro vertebrado) e infectivas (tripomastigotas metacíclico no inseto e tripomastigota sanguíneos no hospedeiro vertebrado) (Garcia e Azambuja, 1991).

Os parasitas, *T. cruzi* e *T. rangeli*, possuem ciclo de vida diferente no hospedeiro invertebrado. O *T. cruzi* é um tripanosomatídeo do grupo stercoraria cujo ciclo de desenvolvimento no vetor se completa na região posterior do trato digestório sendo transmitido pelas fezes. Já o *T. rangeli* é do grupo salivaria que compreende as espécies cujo ciclo de desenvolvimento no vetor se completa na região anterior, no meio salivar, sendo transmitido através da inoculação pelas picadas dos vetores em seus hospedeiros. Assim o *T. cruzi* completa seu ciclo de vida no inseto apenas no sistema digestório enquanto o *T. rangeli* invade a hemocele através da parede epitelial do intestino médio. Livre na hemolinfa, ou associado a células, esses parasitas se multiplicam e invadem as glândulas salivares aonde se diferenciam para forma infectiva. O parasita em seguida é transmitido para o hospedeiro vertebrado por via inoculativa, no momento do repasto sanguíneo dos insetos infectados (Guhl e Vallejo, 2003).

Uma vez na hemolinfa do inseto, o *T. rangeli* pode ser reconhecido ativando o sistema de defesa do inseto. Diversos aspectos das respostas imune humoral e celular, envolvendo a interação de *T. rangeli* com os triatomíneos já foram investigados (Azambuja e Garcia, 2005). Alguns triatomíneos, particularmente do gênero *Rhodnius*, são susceptíveis à infecção por diferentes cepas desse parasita (Cuba Cuba *et al.*, 1972, Cuba Cuba 1998, D´Alessandro 1976, D´Alessandro-Bacigalupo e Gore-Saravia 1992, 1999; Machado *et al*., 2001).

1.3 Sistema imunológico dos insetos

Os insetos possuem um eficiente sistema de defesa responsável por evitar e eliminar possíveis infecções por partículas estranhas em sua circulação. Esse sistema inclui as barreiras físicas e químicas além das respostas relacionadas ao sistema imunológico. Apesar dos insetos aparentemente não possuírem imunidade adquirida, possuem um sistema imune inato bem desenvolvido (Lavine e Strand, 2002).

O sistema imune dos insetos compreende a ativação do sistema profenoloxidase, indução de fatores antimicrobianos, formação de microagregados, fagocitose, encapsulação e formação de reativos de oxigênio e nitrogênio. Este sistema pode ser subdividido em resposta imune celular, referente às defesas mediadas por hemócitos e

humoral, relacionadas ao plasma, porém ambos atuam de maneira integrada tornando o sistema mais eficiente no sentido de minimizar ou conter a multiplicação de patógenos (Azambuja e Garcia, 1987; Sörderhall *et al*.,1996; Gillespie e Kanost, 1997; Azambuja *et al*., 1997; Feder *et al*., 1997; Moreira-Ferro *et al*., 1998; Whitten e Ratcliffe 1999; Vizioli 2001; Whitten *et al*., 2001).

A imunidade humoral é constituída por um sistema complexo de transdução e sinalização, conhecido como via do Toll e Imd, descobertos em *Drosophila*, que direcionam a produção de peptídeos antimicrobianos e de proteínas específicas dependendo do microorganismo invasor (Hoffmann, 2003, Lemaitre e Hoffmann, 2007). Em *Drosophila* já foram identificados sete peptídeos antimicrobianos distintos que possuem efeitos direcionados a microorganismos diferentes. Em infecções causadas por fungos os peptídeos antimicrobianos atuantes na resposta imune são as drosomicinas e mechnikowina. Para as bactérias Gram-negativas são as atacinas, cecropinas, drosocinas e diptericinas e para Gram-positivas as defensinas e lisozimas (Hoffman, 2003).

As lisozimas foram os primeiros fatores antimicrobianos a serem purificados da hemolinfa dos insetos. Atuam na hidrólise da ligação glicosídica entre o ácido N-acetilmurâmico e a N-acetilglicosamina, que constituem a camada de peptideoglicano da parede celular de bactérias Gram-positivas (Powning e Davidson, 1976; Moreira-Ferro et al., 1998). Em 1987, Azambuja e Garcia demonstraram que a atividade de lisozima aumenta mediante inoculação de bactérias em *R. prolixus*. Ratcliffe e Rowley (1987) também observaram em *R*. prolixus que durante o processo de coagulação da hemolinfa, no local da ferida, ocorre liberação de lisozimas podendo atuar como prevenção à invasão microbiana da hemocele.

Os fatores antimicrobianos atuam em sinergismo, principalmente as lisozimas com cecropinas e atacinas. O mecanismo de ação das cecropinas consiste na alteração da estrutura das membranas bacterianas através da formação de poros causando a ruptura e morte da célula (Cocianchi et al., 1994). Porém já foi sugerida sua ação na síntese de prolina em bactérias levando a perda de potássio e ATP pela célula (Okada e Satori, 1985). Já as atacinas possuem maior especificidade atuando somente em bactérias Gram-negativas, tais como *Escherichia coli* (Hultmark et al., 1983), alterando a permeabilidade da membrana e permitindo a entrada de lisozimas e cecropinas (Engstrom et al., 1984). Outro grupo de peptídeos antimicrobianos induzidos são as defensinas que atuam somente em bactérias Gram-positivas. Sua biossíntese ocorre

principalmente no corpo gorduroso sendo ativada em resposta a injúrias no corpo do inseto (Hoffmann e Hoffmann, 1990).

Injúrias mecânicas também ativam outras respostas imune de insetos como o sistema profenoloxidase (proPO) que é uma reação em cadeia envolvendo eventos proteolíticos, similares a coagulação do sangue em mamíferos (Nappi, 1973), que resulta na deposição de melanina ao redor do tecido danificado ou do microorganismo invasor. Esse sistema se inicia com a ativação da enzima profenoloxidase (PPO) através de atividade proteolítica mediada por serino proteinases específicas formando a fenoloxidase (PO) (Jiang e Kanost, 2000). A atividade dessas proteinases é controlada por uma série de vias de sinalização após reconhecimento de partículas estranhas. O reconhecimento ocorre provavelmente por vias de ativação similares ao sistema complemento de vertebrados através de imunolectinas (Yu e Kanost, 2004). As enzimas do tipo profenoloxidade (PPO) recém sintetizadas ficam confinadas em hemócitos específicos como oenocitóides e são liberadas para o plasma, aonde são ativadas, através do rompimento da célula (Jiang et al., 1997; Ashida e Brey, 1998).

As enzimas ativadas, do tipo fenoloxidases (PO), catalisam a oxidação de monofenóis e difenóis, promovendo a conversão de catecolaminas a quinonas (Sugumaran, 1996). As quinonas são subseqüentemente convertidas em melaninas ou reagem com proteínas formando complexos de proteína-catecol, os quais são importantes reativos bioquímicos especialmente no reconhecimento e melanização de partículas estranhas durante respostas imunológicas (Cerenius e Soderhall, 2004). A atividade dessa enzima oxidativa parece ser controlada para evitar sua própria injúria ou danos ao inseto (Andersen, 1985). A sua atividade tem sido relacionada ao sistema de defesa contra microorganismos invasores pela presença de intermediários metabólicos citotóxicos e também pelo depósito de melanina em torno do objeto estranho, o qual dificulta a passagem de oxigênio, resultando na asfixia e morte do microorganismo invasor (Söderhall, 1982).

A ativação do sistema proPO resulta também na produção de espécies reativas de oxigênio (ROS) (ânion superóxido - O_2^-, radical hidróxi - OH e peróxido de hidrogênio - H_2O_2), que podem desempenhar um papel importante no combate às infecções (Nappi e Vass, 1993, Nappi et al., 1995). Em *Drosophila*, a liberação de ROS no trato digestivo é muito importante para o controle de infecções bacterianas, porém a produção desses reativos deve ser balanceada para evitar toxicidade ao próprio inseto (Lemaitre e Hoffmann, 2007).

O ânion superóxido (O_2^-) pode ainda reagir com o radical livre óxido nítrico (NO) produzindo peroxinitrito e outras moléculas conhecidas como reativos intermediários de nitrogênio (RNIs). O óxido nítrico, produzido por ação da enzima óxido nítrico sintase (NOS), é um radical extremamente importante, pois reage rapidamente tendo vasta participação em funções biológicas. Ambos os reativos intermediários de oxigênio e nitrogênio são altamente tóxicos para as células, funcionando como resposta imunológica no controle de infecções, porém devem ser produzidos de forma equilibrada, pois podem afetar as próprias células dos insetos.

Existem várias evidências da atividade de óxido nítrico (NO) em invertebrados, porém poucos trabalhos relatam sua função na resposta imunológica. Sua ação vasodilatadora tem sido associada a insetos vetores que se alimentam de sangue de vertebrados. NO é encontrado em glândulas salivares de inúmeras espécies hematófagas (Garcia *et al*., 1984; Yuda *et al*., 1996; Walker, 2005). Em relação à resposta imune alguns trabalhos têm demonstrado aumento na produção de NO em insetos inoculados com partículas estranhas (Azambuja e Garcia, 2005). Recentemente, Whitten *et al*. (2007) descreveram a importância de NO na resposta imune de *R. prolixus* infectados por *T. rangeli*, *T. cruzi* e componentes da parede celular de bactérias (LPS). Os autores relataram que a produção de NO ocorre em locais diferentes de acordo com cada patógeno sendo produzida no trato digestivo pela infecção por parasitas e no corpo gorduroso e hemócitos pela inoculação de LPS.

Atuando de modo integrado ao sistema humoral, as respostas do tipo celular são mediadas pelos hemócitos, células especializadas que circulam na hemocele dos insetos. Azambuja *et al*., (1991), estudando os hemócitos de diferentes espécies de triatomíneos, observaram que alguns tipos celulares são instáveis, lisam facilmente em condições adversas e tem sua morfologia alterada de acordo com o diluente utilizado. Os mesmos autores identificaram sete tipos de hemócitos: prohemócito, plasmatócito, granulócitos, cistócitos, oenócitos, adipohemócitos e células gigantes.

As respostas de defesa celular de insetos ou reações mediadas por hemócitos são caracterizadas pela interação direta entre hemócitos do hospedeiro e partículas invasoras. As reações de defesa celulares incluem fagocitose, microagregação, nodulação e, em caso de partículas grandes, encapsulação (Lavine e Strand, 2002; Stanley e Miller, 2006 Ratcliffe, 1982; Götz e Boman, 1985; Lackie, 1988). Em uma primeira etapa, ocorre o reconhecimento de componentes "non-self". Em seguida, toma parte a degranulação, migração de hemócitos e ativação do sistema profenoloxidase

(proPO) (Soderhall, 1999). Posteriormente, ocorre a formação dos nódulos, bem como o início da atividade fagocítica pelos plasmatócitos (Ratcliffe e Rowley, 1979).

A formação de nódulos é um evento que ocorre muito antes da indução de fatores antimicrobianos, indicando que esta é inicialmente a linha de defesa mais importante contra invasão de bactérias. Os nódulos são microagregados multicelulares, formados por hemócitos, que podem aprisionar, em sua matriz extracelular, uma grande quantidade de agentes invasores evitando assim a dispersão do patógeno (Ratcliffe e Gagen, 1977; Walters e Ratcliffe, 1983). A formação de nódulos é uma reação rápida que demanda uma mudança nos hemócitos circulantes, que passam de células não adesivas para células adesivas aptas a se ligar ao alvo (Lavine e Strand, 2002).

Em *Galleria mellonella*, células granulares após ligarem a partículas estranhas degranulam, formando um pequeno coágulo ao redor da partícula invasora (Ratcliffe e Rowley, 1979; Ratcliffe e Gagen, 1976). Logo depois da degranulação dos hemócitos granulares a cascata de profenoloxidase é ativada, o que induz o depósito de melanina em volta da bactéria com cinco a trinta minutos de exposição (Söderhall e Smith, 1986). Após o reconhecimento inicial, vários hemócitos, principalmente plasmatócitos, ligam-se ao coagulo formando um nódulo multicelular (Ratcliffe e Rowley, 1979; Mandato et al 1997). Alguns agregados podem eventualmente aderir a tecidos e serem encapsulados posteriormente (Salt, 1967; Ratcliffe e Gagen, 1977; Dunn, 1986).

A formação de microagregados de hemócitos (nódulos) pode ser induzida por LPS, zimosan, laminarina e algumas glicoproteínas presentes na parte externa da parede celular de bactérias e outros microrganismos (Lackie, 1988, Brookman *et al*., 1989). Em *R. prolixus*, foi observado que a formação de nódulos em infecções dos insetos com *T. cruzi* ocorre mais rapidamente do que em infecções por *T. rangeli*, sugerindo que este protozoário está mais apto a sobreviver e multiplicar na hemolinfa (Mello *et al*., 1995).

A fagocitose é um processo de defesa celular altamente conservado, constituindo a primeira resposta dos hemócitos a invasão do organismo por partículas estranhas em pequenas concentrações (Lavine e Strand, 2002; Stuart e Ezekowitz, 2008). Um dos primeiros cientistas a estudar a fagocitose em insetos foi Metalnikov no início do século XX, quando observou a dinâmica da resposta, através do estudo de hemócitos e sua participação no processo fagocítico, através da injeção de bactérias em duas espécies de Lepidoptera: *G. mellonella* e *Pyrausta nubilalis*. Após eliminar as bactérias da hemolinfa, os fagócitos se aglomeram, formando plasmodes, e migram da circulação

para órgãos internos, principalmente a porção posterior da aorta dorsal. O autor ressalta que a fagocitose não é somente um tipo de reação isolada, mas um fenômeno extremamente complexo capaz de manifestar vários graus de intensidade dependendo da infecção (Metalnikov, 1924 citado por Brey e Hultmark, 1998).

A fagocitose de uma partícula estranha envolve diferentes passos, tais como: adesão, reconhecimento, transdução de sinal, formação de pseudópodos, ingestão, fusão de lisossomos e eliminação da partícula fagocitada (Gillipsie *et al*., 1997). Essas partículas são primeiramente reconhecidas pelos hemócitos através de lectinas presentes na superfície de células fagocíticas (Stuart e Ezekowitz, 2008). Após reconhecimento e adesão ocorre uma série de eventos de sinalização em cascata que induz a formação de fagossomos, vacúolos grandes, que se fundem a lisossomos responsáveis pela ingestão e digestão da partícula estranha (Aderem e Underhill, 1999; Blander e Medzhitov, 2004; Shrestha e Kim, 2007a). A bactéria fagocitada é subseqüentemente morta e seus restos são digeridos (Götz & Boman, 1985). De uma maneira geral, dependendo da espécie de insetos, plasmatócitos e/ou células granulares são os primeiros hemócitos requeridos para iniciar o processo de reconhecimento e fagocitose (Kurucz e Vilmos, 1998).

A encapsulação ocorre nos insetos em resposta à invasão por parasitas e/ou microorganismos grandes, os quais devido ao tamanho não podem ser fagocitados por hemócitos (Ratcliffe, 1982). O processo de encapsulação resulta no envolvimento de granulócitos e mais externamente plasmatócitos, que ficam dispostos em camadas multicelulares ao redor do agente estranho, variando o grau de achatamento celular de fora para dentro da cápsula. Em várias espécies de insetos, a melanina é depositada na região interna, perto da superfície do objeto estranho (Dunn, 1986).

1.4 Sistema de sinalização

Para ambos os sistemas imune, celular e humoral, a via dos eicosanóides, principalmente pela produção de prostaglandinas, é de extrema importância para a sinalização celular (Stanley, 2006b). Ensaios com diferentes espécies de bactérias, fungos, parasitóides, protozoários e vírus indicam que eicosanóides atuam na resposta imune contra uma grande variedade filogenética de organismos invasores (Stanley, 2005, 2006b; Stanley e Miller, 2006; Büyükgüzel et al., 2007; Durmus et al., 2008; Stanley e Shapiro, 2007). Inúmeros grupos de pesquisa indicam que eicosanóides são mediadores cruciais para fagocitose, microagregação, migração de células e reações de

nodulação (revisão de Stanley e Miller, 2006). A principal enzima que inicia a via dos eicosanóides é a fosfolipase A_2 responsável pela liberação do ácido araquidônico que pode sofrer oxidação, por três vias distintas, formando os eicosanóides. Recentemente, alguns trabalhos têm demonstrado a importância da fosfolipase A_2 para resposta imune dos insetos (Tunaz et al., 2003; Park et al., 2005; Stanley, 2006a; Shresta e Kim, 2007a e 2008). Como essa enzima também participa da via de formação de plaquetas, alguns autores sugerem a participação de fatores de agregação de plaquetas (PAF) na sinalização celular de insetos (Machado et al., 2006; Figueiredo et al., 2008a; 2008b). Aminas biogênicas, citocinas ou fatores citocinas-"like" também podem estar envolvidas na sinalização celular do sistema imunológico de insetos (Gillespie et al., 1997).

Os peptídeos antimicrobianos da resposta imune humoral de insetos são sintetizados de acordo com o patógeno invasor pelas vias de sinalização Toll/Imd (referentes aos genes *Toll* e *imd* "immune deficiency"). No caso de infecção por fungos e bactérias Gram-positivas a via de sinalização é Toll e na infecção por bactérias Gram-negativas é a via de imuno deficiência (IMD) (Ferrandon et al., 2007).

Diversos trabalhos demonstram que existe uma correlação entre essas diferentes vias de sinalização. A fosfolipase A2 é a enzima que inicia tanto a via de eicosanóides como PAF pela liberação de ácido araquidônico e liso-PAF, respectivamente (Stanley, 2006a, Figueiredo et al., 2008a; 2008b). Alguns trabalhos também relatam a importância da fosfolipase A2 para a ativação da via Imd através de fatores nucleares-kappaB (NF-kB) (Choi et al., 2007; Yajima et al., 2003). PAF exerce suas diversas funções biológicas (início de inflamação, aumento da resistência contra infecções microbianas) através da ativação de NF-kB (Im et al., 1997; Han et al., 1999; Han et al., 2002; Ko et al., 2002; Choi et al., 2007).

1.4.1 Fosfolipase A2

As fosfolipases A_2, (PLA_2), também conhecidas por fosfatidil-acil-hidrolases, são enzimas lipolíticas que agem especificamente na catálise da hidrólise da ligação acil-éster na posição sn-2 de fosfoglicerídeos. Esta reação libera quantidades equimolares de ácidos graxos livres e lisofosfolipídeos, que são considerados substratos igualmente adequados para os processos hidrolíticos catalisados pelas demais fosfolipases (Verheij et al, 1981; Mukherjee et al., 1994; Lambeau e Gelb, 2008). Em mamíferos está relacionada a processos de sinalização de células que compõem a resposta inflamatória.

A hidrólise do fosfolipídeo (1-alquil-2-araquidonil-sn-glicero-3-fosforilcolina) pela enzima fosfolipase A_2 (PLA_2) inicia a biossíntese tanto de fator de ativação de plaquetas (PAF) como eicosanóides. No caso dos eicosanóides a PLA_2 libera o ácido araquidônico desse fosfolipídeo o qual pode ser oxidado tanto pela lipoxigenase como pela cicloxigenase formando os leucotrienos e prostaglandinas, respectivamente (Fig 1). A porção restante do fosfolipídeo liberada pela ação da PLA_2 é o lisofosfolipídeo, Liso-PAF, que em seguida é convertido em PAF pela adição de um grupo acetato pela liso-PAF acetiltransferase (Prescott et al, 2000) (Fig 2). Em ambos os casos, tanto pela formação de prostaglandinas como PAF temos a sinalização da resposta fagocítica (Stanley, 2006b; Figueiredo et al., 2008).

A fosfolipase A_2 tem sido relatada como atuante sobre a digestão de insetos, resposta imune, reprodução e metabolismo (para uma revisão ver Stanley 2006a). A caracterização da fosfolipase A_2 citosólica ou celular no corpo gorduroso (Uscian e Stanley-Samuelson, 1993) e em hemócitos circulantes (Schleusener e Stanley-Samuelson, 1996), que são os principais tecidos responsáveis pelo sistema de defesa de insetos, levou às investigações da ação dessa enzima na resposta imune. Alguns trabalhos também revelaram que a fosfolipase A_2 citosólica intracelular associadas a esses tecidos são independentes de cálcio (Stanley et al., 2002). Sendo que em mamíferos a fosfolipase A2 que regula a via dos eicosanóides também é independente de cálcio como encontrado nos insetos (Dennis, 1997; Balsinde et al., 1999 citado por Stanley et al., 2002).

1.4.2 Eicosanóides

Eicosanóides são metabólitos oxigenados contendo em sua estrutura de ácido graxo 20 carbonos poliinsaturados. Existem três grandes grupos de eicosanóides incluindo as prostaglandinas, ácidos epoxieicosatrienóicos e os vários produtos da lipoxigenase (Stanley et al., 2002). Cada grupo de eicosanóides é formado por vias específicas conhecidas como cicloxigenase, lipoxigenase e epoxigenase. Primeiramente, ocorre a liberação do ácido araquidônico através da atividade catalítica da fosfolipase A_2 (PLA_2) sobre fosfolipídeos. Em seguida o ácido araquidônico é oxidado por vias diferentes formando os eicosanóides (Fig 1) (Stanley 2000, 2006b; Shrestha e Kim, 2008).

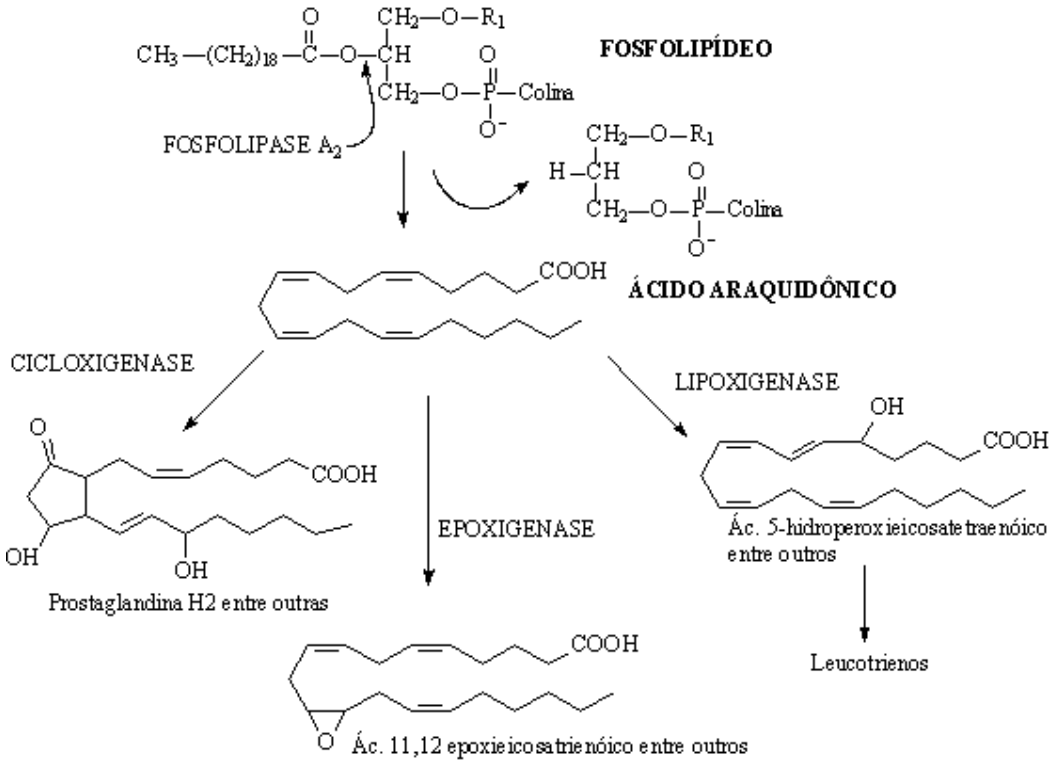

Figura 1: Via dos eicosanóides

Os eicosanóides são fundamentais para a sinalização da resposta imune de insetos (Büyükgüzel et al., 2007). Em relação à nodulação, vários autores já demonstraram que os eicosanóides são essenciais tanto na fase de migração de células como na formação de microagregados (Miller *et al.*, 1994; Miller e Stanley, 2001; Stanley-Samuelson *et al*., 1997). Em 1997, Mandato *et al.* utilizando *G. mellonella* como modelo de estudo, observaram que o tratamento com inibidores de eicosanóides interfere na migração de hemócitos, bem como na ativação do sistema profenoloxidase e no processo fagocítico sendo revertido pela adição de ácido araquidônico. Miller e Stanley (2001) verificaram diminuição no processo de microagregação de hemócitos quando larvas de *Manduca sexta* desafiadas com *Serratia marcescens* eram tratadas com dexametasona e que a administração de ácido araquidônico revertia tal efeito.

Recentemente, Park *et al.* (2003) demonstraram que a bactéria *Xenorhabdus nematophilus*, um patógeno natural para insetos, reduz a reação de nodulação em *M. sexta*, pela inibição da biossíntese de eicosanóides. Phelps *et al.*, (2003), também com experimentos *in vitro*, confirmaram esses resultados, acrescentando que as prostaglandinas (PGH_2), produto da ciclooxigenase, seriam os mediadores primários da nodulação em *M. sexta* inoculados com *S. marcescens.* Garcia *et al*., (2004a)

demonstraram que dexametasona, um inibidor de fosfolipase A_2 (PLA_2), indometacina, um inibidor de ciclooxigenase e ácido nordihidroguaiarético (NDGA), um inibidor não específico de lipoxigenase, inibem a formação de nódulos em insetos desafiados com *T. rangeli*. Os mesmos autores observaram que o ácido araquidônico reverte o efeito de dexametasona nos insetos impedindo a infecção de *T. rangeli* (Garcia *et al*., 2004a).

Miller e Stanley (2004) confirmaram a importância de algumas prostaglandinas como mediadoras das reações de microagregação de hemócitos de *M. sexta* desafiados com LPS. Miller (2005) relatou que os eicosanóides mediam a migração de hemócitos na lagarta do tabaco, *M. sexta*. Recentemente, Merchant et al., (2008) demonstraram que os hemócitos são capazes de detectar e migrar em direção ao principal peptídeo N-formil-Met-Leu-Phe (fMLP) de *E. coli*. Além disso, demonstraram que a migração de hemócitos é inibida pelo tratamento com indometacina e dexametasona e que esse efeito é revertido pela adição de ácido araquidônico, concluindo que a migração de hemócitos é mediada pela via dos eicosanóides.

A importância dos eicosanóides na sinalização da atividade fagocítica em insetos, tem sido demonstrada em trabalhos recentes (Mandato et al., 1997). Shrestha e Kim, 2007a demonstraram a importância dos eicosanóides para fagocitose em *S. exigua* através da atividade patogênica de *Xenorhabdus nematophila*, a qual possui efeito de inibição da PLA_2 (Park e Kim, 2000, 2003). Como esperado *X. nematophila* inibiu a atividade fagocítica de hemócitos assim como dexametasona, inibidor específico da PLA_2, tendo sido esse efeito inibitório revertido com administração de ácido araquidônico.

Em insetos, dois grupos de eicosanóides parecem influenciar a fagocitose por hemócitos como demonstrado em *G. mellonella* (Mandato et al., 1997). Nesta espécie, tanto indometacina (inibidor da cicloxigenase) e esculetina (inibidor da lipoxigenase) significativamente inibiram a fagocitose (Mandato et al., 1997). Sabe-se que as vias da cicloxigenase e da lipoxigenase regulam a polimerização de actina necessária para a formação de pseudópodes em hemócitos, etapa inicial do processo de fagocitose (Miller, 2005).

Alguns trabalhos também relatam a sinalização de eicosanóides na resposta imune humoral. Em 1997, Morishima et al. relataram que a biossíntese de proteínas antimicrobianas depende da via dos eicosanóides em *Bombyx mori*. Yajima et al. (2003) demonstraram que a via de eicosanóides está ligada à via de imuno deficiência (Imd – "immune deficiency") responsável pela resposta humoral em *Drosophila*. A PLA_2

participa da ativação da via Imd dependente de LPS através da liberação de ácido araquidônico (Yajima et al., 2003).

Eicosanóides também estimulam a atividade fenoloxidase em vários sistemas imune de insetos incluindo *Spodoptera exígua* (Park e Kim, 2003; Shrestha e Kim, 2007b). Nesta espécie inibidores específicos da biossíntese de eicosanóides foram capazes de inibir a atividade de fenoloxidase (Park e Kim, 2003; Shrestha e Kim, 2007b). Recentemente, Shrestha e Kim (2008) demonstraram que os hemócitos do tipo oenocitóides são extremamente importantes para a cascata fenoloxidásica liberando a forma inativa da enzima, profenoloxidase, na hemocele. A ruptura dos oenocitóides é mediada por eicosanóides, prostaglandinas, já que sua produção foi inibida pelo tratamento com dexametasona bem como por bromofenacil bromídio, um inibidor específico de fosfolipase A_2 secretada e tendo sida esta ação revertida com tratamento por ácido araquidônico (Shrestha e Kim, 2008).

1.4.3 Fator de ativação de plaquetas

Paralelamente aos eicosanóides, existe o fator de ativação de plaquetas (PAF), 1-0-alquil-2-acetil-sn-glicero-3-fosforilcolina, composto naturalmente encontrado na membrana fosfolipídica e responsável pela sinalização de resposta imune em mamíferos (Prescott et al., 2000).

A síntese enzimática de PAF é altamente regulada e envolve duas etapas. A primeira etapa é a hidrólise de longas cadeias de ácidos graxos, como alquil-colina fosfoglicerídeo, a um intermediário 1-o-alquil-sn-glicero-3-fosfocolina (Liso-PAF) e um ácido graxo pela ação da fosfolipase A_2 (PLA_2). A segunda etapa consiste na conversão de liso-PAF em PAF pela adição de acetato através da enzima liso-PAF acetiltransferase (Snyder et al. 1985) (Fig 2).

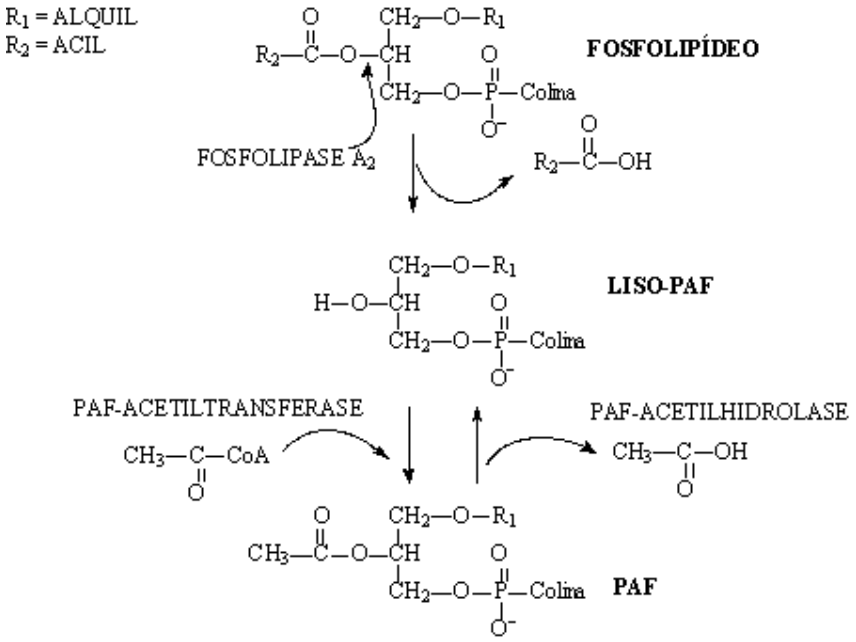

Figura 2: Via de PAF

Em mamíferos, PAF induz expressivo repertório de respostas fisiológicas incluindo fagocitose, agregação, adesão, degranulação, assim como diversas modificações morfológicas (Stafforini et al., 2003; Kordan et al., 2003; Faria Neto et al., 2005). Em relação à fagocitose, existem vários trabalhos mostrando a importância da via PAF para essa resposta celular. Os autores Showell e Williams (1989) e Au et al. (2001) demonstraram que o pré-tratamento de neutrófilos com o antagonista do receptor de PAF, UK-74505, inibe significativamente a fagocitose. Também foi observado que o tratamento com PAF interfere na atividade fagocítica de monócitos humanos (Bussolino et al., 1989), macrófagos (Ichinose et al., 1994) e leucócitos (Ishii e Shimizu, 2000; Au et al., 2001).

Apesar de PAF ser um importante lipídio distribuído extensamente em invertebrados (Sigiura et al., 1992) é raramente estudado em insetos. A atuação de PAF sobre o sistema imunológico de insetos foi recentemente investigada por Machado et al., (2006) e Figueiredo et al., (2008) que demonstraram que a via de sinalização do PAF é mediadora tanto da formação de microagregados de hemócitos como da fagocitose em *R. prolixus*. Além disto, o tratamento dos insetos com WEB2086, droga antagonista de PAF, inibe as respostas imune celular de microagregação e fagocitose sendo ambas revertidas quando os insetos tratados são inoculados com PAF na hemolinfa.

Outro trabalho de Figueiredo et al. (2008b) demonstra que cultura de *T. rangeli* inibe a fagocitose de hemócitos de *R. prolixus* através da inibição da atividade da enzima PLA_2, presente nas vias de sinalização de eicosanóides e PAF, bem como aumenta a atividade de PAF-acetilhidrolase (PAF-AH). Essa enzima é responsável pela eliminação do grupamento acetila de PAF formando liso-PAF (Derewenda e Ho, 1999). Dessa forma a enzima PAF-AH impede a formação de plaquetas pelo organismo inativando PAF. Os autores sugerem que o balanço entre a inibição de PLA_2 e o aumento de PAF-AH pode ser o fator determinante no bloqueio da fagocitose pela via do PAF durante a infecção por *T. rangeli*. Essa enzima é intensamente distribuída pelos tecidos e sangue de mamíferos e classificada em dois tipos: intracelular (citosólica) e extracelular (plasma) (Arai, 2002; Arai et al., 2002).

No genoma de *D. melanogaster* foram observados homólogos das subunidades α e β de PAF-acetilhidrolase de mamíferos sendo o homólogo da subunidade β altamente conservado (Sheffield et al, 2000).

Em insetos hematófagos têm sido identificados na glândula salivar vários inibidores de PAF e a enzima PAF-acetilhidrolase atuando na diminuição da ação inflamatória do hospedeiro no local da picada do inseto vetor e assim aumentando o sucesso da parasitemia. Esses inibidores interferem na agregação de plaquetas de animais facilitando a alimentação sanguínea pelo inseto vetor (Ribeiro, 1987; Basanova et al., 2002; Morita et al., 2006). Em *R. prolixus* foi descrito recentemente o inibidor de agregação (RPAI-1) que se liga a ADP e diminui a agregação de plaquetas (Francischetti et al., 2002). A atividade de PAF-acetilhidrolase, bem como fosfolipase A_2, tem sido descrita em glândulas salivares de insetos como *M. sexta* e espécies de pulga, bem como em carrapatos (Bowman et al., 1997; Cheeseman et al., 2001; Tunaz e Stanley, 2004).

1.4.4 Vias Toll/Imd

Para a produção de peptídeos antimicrobianos, são necessárias duas vias de sinalização intracelular que foram originalmente observadas em Drosophila através de mutantes nos genes *Toll* e *imd* (immune deficiency) (Lemaitre et al., 1995). Na via Toll, a sinalização do receptor de membrana codificado pelo gene *Toll* ativa dois fatores de transcrição, o Dif (Dorsal-related immune factor) e o Dorsal. Em *Drosophila* o fator Dif está relacionado a respostas imunológicas enquanto o Dorsal determina a polaridade dorso-ventral no embrião. Normalmente Dif e Dorsal formam um complexo inativo

com Cactus, um membro da família de inibidores de NF-kB (IkB). A sinalização de Toll resulta na fosforilação de Cactus, seguida de sua degradação. Dif e também Dorsal são, então, transportados para o núcleo, onde participam na ativação da transcrição de genes que codificam agentes antimicrobianos e sinais secundários (revisado em Hultmark, 2003).

Na via Imd existe o fator tipo Rel/NF-kB denominado Relish que é uma proteína composta tanto de domínios NF-kB-"like" quanto de domínios inibitórios IkB-"like" (Stoven et al., 2000). A ativação da via Imd ativa a clivagem endoproteolítica de Relish que libera domínios homólogos de Rel do domínio inibitório e permite a sua translocação para o núcleo (Stöven et al., 2003).

A via Toll media respostas imune a bactérias Gram-positivas e fungos enquanto a via Imd ativa defesas contra bactérias Gram-negativas e alguns bacilos Gram-positivos (Ferrandon et al., 2007). *Drosophila* utiliza proteínas de reconhecimento de peptideoglicano (PGRPs) e proteínas de ligação Gram-negativas (GNBPs) para discriminar entre as bactérias Gram-negativas e Gram-positivas. No caso de fungos a GNBP3 detecta componentes da parede celular, principalmente pelo reconhecimento de glucanos (Ferrandon et al., 2007).

1.5 Inibidores da sinalização

Em insetos, tem sido utilizado tratamento com compostos que atuam inibindo e revertendo diferentes etapas das vias de sinalização da resposta imune no intuito de melhor compreender os mecanismos que regulam o sistema imunológico. Em relação à via dos eicosanóides, agem como inibidores da via de ciclooxigenase, a indometacina e da via lipoxigenase, a esculetina e o NDGA (inibidor não específico), sendo descritas a sua reversão com os eicosanóides, prostaglandinas e ácidos epoxieicosatrienóicos respectivamente para as duas vias (Mandato et al., 1997; Phelps *et al.*, 2003; Garcia et al., 2004a).

Tanto para a via dos eicosanóides como a via do PAF, a fosfolipase A_2 é uma enzima essencial responsável pela hidrólise do fosfolipídeo de membrana. Sua ação resulta na liberação de ácido graxo e lipídeos essenciais para a formação de eicosanóides e PAF. No caso da via de ativação de plaquetas, tem sido utilizados antagonistas de PAF, como WEB2086 (Machado et al., 2006; Figueiredo et al., 2008) e antagonista do receptor de PAF, UK-74505 (Showell e Williams 1989; Au et al. 2001).

Como inibidores de fosfolipase A_2, alguns autores têm utilizado a bactéria *X. nematophila* (Park e Kim, 2000, 2003), bromofenacil bromídio, inibidor específico de fosfolipase A_2 secretada (Shrestha e Kim, 2008), metilaraquidonil fluorofosfato, inibidor específico de fosfolipase A_2 citosólica (Park et al., 2005) e dexametasona, inibidor da fosfolipase A_2. Recentemente foi demonstrado que o protozoário *T. rangeli* administrado junto ao sangue alimentar de *R. prolixus* foi capaz de inibir a atividade da fosfolipase A_2 (Garcia et al., 2004b, Figueiredo et al 2008b). Nesta espécie, os autores, Garcia et al. (2004b), observaram que o tratamento com ácido araquidônico dos insetos previamente infectados com o parasita reverte o efeito inibidor causado pelo parasita em respostas imune celulares como microagregação. Recentemente Figueiredo et al (2008b) demonstraram que a inibição da atividade fagocítica dos hemócitos de insetos tratados com *T. rangeli* é revertida tanto com adição de ácido araquidônico como PAF. Além disso, observaram inibição da atividade de PLA_2 secretada e celular e elevação dos níveis de PAF-AH na hemolinfa de insetos tratados com o parasita. Os autores sugerem que fatores bioquímicos presentes em *T. rangeli* estão envolvidos na modulação de respostas imune atuando nas vias de sinalização de eicosanóides e PAF pela inibição de PLA_2 (Figueiredo et al 2008b).

1.5.1 Glucocorticóides

Os glucocorticóides são antiinflamatórios potentes e drogas imunossupressoras que agem em quase todos os tipos de células e formam complexos que interagem com a expressão e função de múltiplos mediadores. São compostos do grupo dos esteróides, encontrados naturalmente como hormônios, como é o caso do cortisol, responsável pelo metabolismo de glicose no sangue.

Os glucocorticóides se ligam aos receptores de glucocorticóides citoplasmáticos. Após se ligarem ao receptor correspondente, podem permanecer como monômeros e interagir inibindo fatores de transcrição de genes de citocina (transrepressão) ou podem formar dímeros e interagir com elementos de resposta de glucocorticóides (GRE) induzindo a transcrição de genes (transativação). Na transativação, o complexo receptor ligante recém formado se transloca para o núcleo da célula, aonde se liga a vários GREs na região promotora dos genes alvo. O receptor de hormônio ativado interage com fatores de transcrição específicos regulando, portanto a transcrição de genes alvo (Gupta et al 1984; Beato & Klug, 2000; Lowenberg et al., 2008).

Glucocorticóides são capazes de impedir a transcrição de muitos genes relacionados ao sistema imune. Eles induzem a síntese de lipocortina-1 (anexina-1), a qual se liga à membrana celular, impedindo que a PLA_2 entre em contato com seu substrato, fosfolipídeo (Nakano et al., 1990; Lowenberg et al., 2008). Isso leva a queda da produção de eicosanóides. A expressão da ciclooxigenase é também inibida, potencializando seu efeito. Em outras palavras, os dois produtos principais da inflamação, prostaglandinas e leucotrienos, são inibidos pela ação de glucocorticóides (Danon e Assouline, 1980; Hirata et al., 1980).

Um glucocorticóide sintetizado bastante conhecido é a dexametasona, que vem sendo utilizada como droga anti-inflamatória em mamíferos e em estudos relacionadas às vias de sinalização de resposta imunológica (Fig 3). Esta droga tem efeito mediador da liberação da proteína inibidora da $PLA_{2,}$ ao se ligar com receptores de glucocorticóides (Nakano et al., 1990).

Os glucocorticóides também possuem efeito sobre os fatores nucleares-kB impedindo sua translocação para o núcleo. Sua ação ocorre através do estímulo de transcrição das proteínas inibitórias IkBα (Auphan et al., 1995; Scheinman et al., 1995) e por se associar aos receptores de NF-kB (Heck et al., 1997; Wissink et al., 1997; De Bosscher et al., 2003; Lowenberg et al., 2008).

O glucocorticóide dexametasona bloqueia as ações de PAF o qual é capaz de induzir a rápida degradação das proteínas IkBα influenciando na ativação de NF-kB induzida pro LPS (Han et al., 1999).

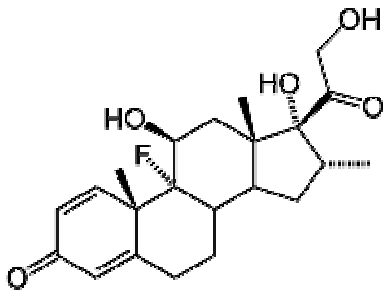

Figura 3: Estrutura da dexametasona

1.5.2 Imuno depressores naturais

A utilização de variadas classes de produtos do metabolismo secundário de plantas para o controle de pragas agrícolas e vetores de doenças parasitárias tem sido amplamente explorada no campo da investigação científica. Uma substância natural bastante conhecida e de grande sucesso como regulador do desenvolvimento, ou droga antihormonal, é a azadiractina, extraída de plantas da família Meliaceae como *Azadirachta indica* e *Melia azedarach* (Brahmachari, 2004). Azadiractina é um triterpeno, mais especificamente um limonóide, que além da ação fagoinibidora, causa distúrbios fisiológicos, alterando o desenvolvimento, reprodução e fisiologia hormonal, o que conseqüentemente abala a resposta imunológica dos insetos (Schumutterer, 1990, 1995; Mordue e Blackwell 1993; Mulla e Su. 1999; Boeke et al. 2004; Garcia e Azambuja 2004).

Alguns trabalhos relatam o efeito inibidor da azadiractina sobre a resposta imunológica de *R. prolixus*. Azambuja *et al.* (1991), observaram diminuição na indução da atividade antibacteriana e de lisozima, bem como no processo de microagregação de hemócitos de *R. prolixus* desafiados com *Enterobacter cloacae*. Recentemente, Fiqueiredo *et al.* (2006) demonstraram que a azadiractina, atuando como antagonista da ecdisona, exerce, indiretamente, um papel fundamental na inibição da fagocitose em *R. prolixus*. Os autores reportaram que o tratamento oral simultâneo de insetos com a azadirachtina e ecdisona, resultou em índice normal de atividade fagocítica semelhante ao grupo de insetos não tratados (controle).

1.5.2.1 Fisalinas

Algumas substâncias isoladas de plantas apresentam bioatividade sobre a imunidade de mamíferos e, em alguns casos, atuam no sistema imune de insetos. As fisalinas, além de possuírem atividade imunomoduladora, possuem atividade tripanossomicida, bactericida, moluscida, antiinflamatória e antitumoral (Lin *et al*., 1992; Chiang *et al*., 1992; Soares *et al*., 2003; Santos *et al*., 2003; Hwang *et al*., 2004). Assim como os corticosteróides, usados hoje para controlar o sistema imune de humanos especialmente no controle da rejeição em transplantes de órgãos, as fisalinas atraíram a atenção dos cientistas por pertencerem também ao grupo dos esteróides.

As fisalinas são compostos isolados do metabolismo secundário de plantas do gênero *Physalis* da família Solanácea. A espécie *P. angulata* L. é muito utilizada no

Norte e Nordeste no tratamento de inúmeras enfermidades, de dermatite a reumatismo. É amplamente distribuída em regiões tropicais e subtropicais, incluindo a Amazônia, e conhecida vulgarmente como mullaca, camapu, bate-testa, entre outros nomes (Tomassini *et al*., 2000).

As fisalinas extraídas do tronco, galhos e folhas desta planta foram isoladas e caracterizadas quimicamente como seco-esteróides. Possuem estruturas bastante complexas, caracterizando-se como derivados esteroidais do tipo 13, 14-seco-16,24 cicloergostanos, carbonilados em C-15 (Tomassini *et al*., 2000) (Fig 4). Seco-esteróides são moléculas com estruturas similares aos esteróides, quatro anéis esteroidais, porém o anel B encontra-se aberto.

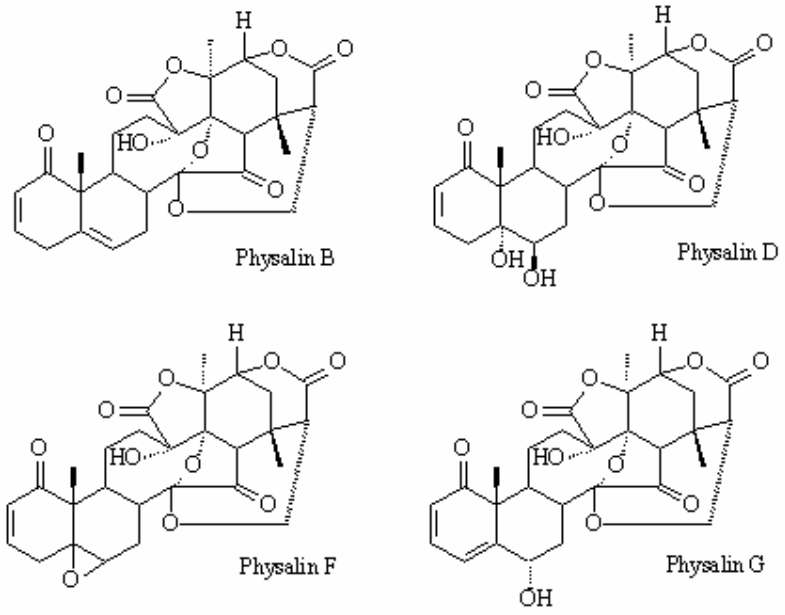

Figura 4: Estruturas das fisalinas B, D, F e G

Extratos de diclorometano da *P. angulata* apresentaram alta atividade parasiticida contra *T. brucei rhodesiense* (agente causador da doença do sono) com valores de IC_{50} de 0,1µg/ml (Freibughauss et al., 1996). Recentemente Nagafuji *et al.*, (2004), observaram atividade tripanossomicida e citotóxica tanto das fisalinas como vitanolídeos extraídos da planta *P. angulata,* sendo, ambos os produtos com atividade mais intensa para as formas tripomastigotas do que nas epimastigotas de *T. cruzi*. Dentro do grupo de esteróides, os seco-esteróides apresentam atividade inseticida, fagoinibidora (fagodeterrente) e antagonista de ecdisteróides tendo sido também relacionados a mecanismos de defesa química. Estudos foram realizados em larvas da

Musca domestica, os quais demonstraram alto efeito fagoinibidor de compostos vitanolídeos isolados da planta *Salpichroa origanifolia* (Mareggiani *et al.*, 2000).

A atividade antibacteriana foi demonstrada por Tomassini *et al.* (1997) através dos extratos etanólicos e aquosos frente às cepas de *Staphylococcus aureus* e *Escherichia coli*. Hwang *et al.*, (2004) observaram atividade contra *Streptococcus mutans* utilizando 50 mg/ml de extrato metanólico de *P. angulata* e constataram rápida mortalidade bacteriana após dois minutos. Em 2002, Januário *et al.* demonstraram que a fisalina D possui atividade sobre *Mycobacterium tuberculosis* tendo sido estabelecida a sua concentração mínima inibitória em 32 μg/mL. Constatou-se também atividade molusquicida utilizando fisalinas isoladas de homogeneizado de folhas da plantas. Em concentração de 100 mg/ml da cultura de *Biomphalaria tenagophila* houve mortalidade acima de 90% (Santos *et al.* 2003).

Soares *et al*. (2003) confirmaram a atividade imunomodulatória das fisalinas B, F e G em camundongos. Estes foram tratados com as diferentes fisalinas e constatou-se uma potente inibição da produção de óxido nítrico em macrófagos ativados por lipopolissacarídeos e interferon gama. Houve uma redução de 90 a 100% da produção de óxido nítrico, enquanto que a dexametasona reduz em apenas 30% a produção deste mediador químico. Estes autores observaram que as fisalinas inibem as respostas de macrófagos por mecanismo distinto do ocorrido com dexametasona.

Por outro lado, Vieira et al. (2005) demonstraram que as fisalinas B e F atuam similarmente à dexametasona, diminuindo a ação inflamatória em tratamentos de isquemia intestinal e lesões de reperfusão em camundongos. A atividade tanto das fisalinas como dexametasona foi revertida após tratamento com um antagonista de receptor de esteróide, RU486, confirmando a ação sobre receptores de glucocorticóides.

Recentemente, Soares et al. (2006) observaram que o tratamento de camundongos com as fisalinas B, F e G previne a rejeição de transplante de coração. Entretanto, o tratamento com o antagonista de receptor glucocorticóide (RU486) não reverteu a ação das fisalinas sobre linfócitos Con A-estimulados. Portanto ou as fisalinas atuam sobre receptores de glucocorticóide por um mecanismo diferente ou ativando um receptor diferente.

2. OBJETIVOS

Geral

Avaliar o efeito de diferentes fisalinas sobre o sistema imunológico do inseto desafiado com *Trypanosoma rangeli* e/ou *Enterobacter cloacae* bem como seu mecanismo de ação sobre a imunidade do inseto.

Específicos

I. Avaliar efeito das fisalinas sobre a fisiologia dos insetos (ecdise, mortalidade) inoculados ou não com *T. rangeli* e/ou *E. cloacae;*
II. Investigar o sistema humoral (profenoloxidase, produção de óxido nítrico, atividade lisozima e atividade antibacteriana) de insetos tratados oralmente com fisalina e inoculados com *T. rangeli* e/ou *E. cloacae*;
III. Investigar a imunidade celular (número de hemócitos livres circulantes na hemolinfa, formação de microagregados de hemócitos, fagocitose) de insetos tratados oralmente com fisalina e inoculados com *T. rangeli* e/ou *E. cloacae*;
IV. Investigar se as fisalinas agem sobre a imunidade do inseto pela via dos eicosanóides e/ou PAF

3. RESULTADOS

ARTIGOS:

Anexo 1 - *Trypanosoma rangeli*: Effects of physalin B on the immune reactions of the infected larvae of *Rhodnius prolixus*. Garcia ES, Castro DP, Ribeiro IM, Tomassini TC, Azambuja P. Experimental Parasitology 2006 Jan;112(1):37-43.

Anexo 2 - Cellular immune response in *Rhodnius prolixus*: role of ecdysone in hemocyte phagocytosis. Figueiredo MB, Castro DP, S Nogueira NF, Garcia ES, Azambuja P. Journal of Insect Physiology 2006 Jul;52(7):711-6.

Anexo 3 - Immune depression in *Rhodnius prolixus* by seco-steroids, physalins. Castro, DP, Figueiredo MB, Ribeiro IM, Tomassini TCB, Azambuja P, Garcia ES. Journal of Insect Physiology 2008 Mar; 54(3):555-62.

Anexo 4 – Physalin B inhibits *Rhodnius prolixus* hemocyte phagocytosis and microaggregation by activation of endogenous PAF-acetylhydrolase activities. Castro, DP, Figueiredo MB, Genta FA, Ribeiro IM, Tomassini TCB, Azambuja P, Garcia ES. Journal of Insect Physiology 2009; 55:532-537.

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Experimental Parasitology
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Trypanosoma rangeli: Effects of physalin B on the immune reactions of the infected larvae of *Rhodnius prolixus*

Eloi S. Garcia [a], Daniele P. Castro [a], Ivone M. Ribeiro [b], Therezinha C.B. Tomassini [b], Patrícia Azambuja [a,*]

[a] *Departamento de Bioquímica e Biologia Molecular, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz (Fiocruz), Av. Brazil 4365, Rio de Janeiro 21045-900, RJ, Brazil*
[b] *Far Manguinhos, Fiocruz, Rio de Janeiro, RJ, Brazil*

Received 17 June 2005; received in revised form 12 September 2005; accepted 19 September 2005
Available online 3 November 2005

Abstract

Physalins are seco-steroids obtained from plants of the family Solanaceae. Herein, we tested *Physalis angulata* L purified physalin B as an immunomodulatory compound in 5th-instar larvae of *Rhodnius prolixus*, which were systemically infected with the H14 *Trypanosoma rangeli* strain protozoan. In uninfected insects, the effective concentration of physalin B, which inhibited 50% of the blood ingested (ED_{50}) volume, was 15.2 ± 1.6 μg/ml of the meal. Ecdysis processes and mortality in uninfected larvae, treated orally with physalin B in concentrations ranging from 1 to 10 μg/ml, was similar to that observed in insects not treated with physalin B. However, *R. prolixus* larvae previously fed on blood containing 1.0, 0.1, and 0.01 μg of physalin B/ml exhibited mortality rates of 78.1, 54.3, and 12.7%, respectively, 6 days after inoculation of *T. rangeli* (1×10^3 parasites/insect), whereas only 7.2% mortality was observed in the control group, injected with sterile culture medium. The insects treated with physalin B (0.1 μg/ml) and inoculated with *T. rangeli* did not modify the phenoloxidase (PO) activity and total hemocyte count in the hemolymph. However, physalin B treatment caused a reduction in hemocyte microaggregation and nitric oxide production and enhanced the parasitemia in the hemolymph. These results demonstrate that physalin B from *P. angulata* is a potent immunomodulatory substance for the bloodsucking insect, *R. prolixus*.

Index Descriptors and Abbreviations: Seco-steroids; *Rhodnius prolixus*; *Physalis angulata* L; Physalin; *Trypanosoma rangeli*; Hemocytes; Microaggregation; Nitric Oxide; Prophenoloxidase

1. Introduction

Trypanosoma rangeli is a hemoflagellate protozoan parasite of triatomines and mammals. Unlike *Trypanosoma cruzi* which develops only in the gut of *Rhodnius prolixus*, *T. rangeli* also develops in the gut, but commonly invades the hemolymph. Development is completed in the salivary glands, in a key step prior to transmission by direct inoculation feeding on vertebrate host (Garcia et al., 1994; Hecker et al., 1990; Hoare, 1972; Takle, 1988; Tobie, 1970). Apparently, the parasite is harmless for humans and a variety of wild and domestic animals, however it can be pathogenic to the insect vector (Watkins, 1971).

Once in the hemocoel, *T. rangeli* must overcome its insect vector's defense system which depends on many aspects of the humoral and cellular responses, including lysozyme and trypanolytic activities (Mello et al., 1995), prophenoloxidase (proPO) activation (Gomes et al., 2003), phagocytosis and hemocyte microaggregate formation (Garcia et al., 2004; Mello et al., 1995; Takle, 1988), agglutination (Mello et al., 1995; Ratcliffe et al., 1996), and superoxide and nitric oxide production (Whitten et al., 2001).

Physalis angulata belongs to the Solanaceae family and is widely distributed throughout tropical and subtropical

* Corresponding author. Fax: +55 21 2590 3495.
E-mail address: azambuja@ioc.fiocruz.br (P. Azambuja).

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doi:10.1016/j.exppara.2005.09.003

regions of the world. Extracts from this plant have been used in different countries in traditional medicines against a variety of diseases such as malaria, asthma, hepatitis, dermatitis, and rheumatism (Chiang et al., 1992; Lin et al., 1992). Several compounds from *P. angulata* have been isolated and chemically characterized. A group of seco-steroids, known as physalins, are found in *P. angulata* stems and leaves (Lin et al., 1992). Recently, Soares et al. (2003) reported that physalins inhibited macrophage activation and nitric oxide production as well as causing lipopolysaccharide-induced death in a murine model of endotoxic shock.

Since many aspects of physiological processes in mammals are mediated by physalins, we hypothesized that physalin B can alter the normal immunological responses of *R. prolixus* infected with *T. rangeli*. This is a suitable and interesting biological model for investigation since the hemocoelic infection by *T. rangeli* is similar to that occurring in nature. Herein, we provide evidence that insects previously fed on blood containing physalin B become more susceptible to systemic *T. rangeli* infection and that physalin B is a potential immunomodulatory compound for the triatomine *R. prolixus*.

2. Materials and methods

2.1. Insects

Fifth-instar *R. prolixus* larvae were used throughout these studies. After molting, insects starved for 15–20 days were randomly chosen. All insects were raised and maintained as previously described (Garcia et al., 1984) feeding on citrated rabbit blood through a membrane feeding apparatus (Azambuja and Garcia, 1997).

2.2. Parasites

Trypanosoma rangeli strain H14 (CT-IOC 038, supplied by Dr. M.A. Sousa, Fiocruz, Brazil) was grown in a Liver Infusion Tryptose (LIT, Difco) culture medium supplemented with 20% heat-inactivated fetal calf serum at 28 °C according to Garcia and Azambuja (1997). The short epimastigotes (99% purity) were obtained from the log-growth phase of the parasites (until day 7 of cultivation), washed and resuspended in sterile culture medium (Garcia et al., 2004).

2.3. Physalin purification

Physalins were purified as described by Soares et al. (2003). Basically, *P. angulata* L stem ethanolic extracts, obtained from dried plant material collected in Belém do Pará (Brazil), were dissolved in methanol and mixed with a lead acetate solution. Activated charcoal (Merck Darmstadt, Germany) was added to the mixture and stirred up. The solution was filtered, poured into a separatory funnel and then extracted with chloroform. The chloroform layer was evaporated under reduced pressure, producing a pool of physalins. This pool was chromatographed using a liquid medium pressure chromatographic column (MPLC). The elution was carried out with a gradient solvent system (hexane–ethyl acetate 7:3; through 100% ethyl acetate). The collected fractions, after evaporation, were assayed on thin layer chromatography (TLC) plates, against standard physalins. After recrystallization in ethyl acetate, the procedure yielded physalin B of 96% purity and other types of physalins. The chemical structure of physalin B is represented in Fig. 1.

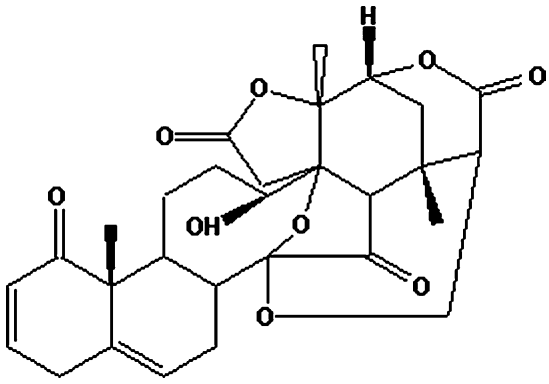

Fig. 1. Structure of the physalin B.

2.4. Effects of physalin B on blood ingestion rate and development

Before initiating the experiments of *T. rangeli* inoculation in *R. prolixus* (infected groups), we conducted a series of general control experiments and assessments to ensure that oral treatment with physalin B did not affect feeding, ecdysis processes, and death rate. First, to determine the antifeedant effect, physalin B was dissolved in ethanol–0.15 M NaCl (1:1) at a concentration of 4 mg/ml and added to the blood meal to achieve final concentrations varying from 1 to 30 μg/ml. Groups of 5th-instar larvae were allowed to feed through a membrane feeding apparatus (Azambuja and Garcia, 1997) for 30 min. Blood intake was determined by body weight difference just before (32.5 ± 3.4 mg) and after feeding. The effective antifeedant concentration (ED_{50}) was determined by using the linear regression, method of least squares (Snedecor, 1964), correlating the blood meal intake and the physalin B concentration. Secondly, to investigate possible physalin B toxicity and ecdysis affects we fed 5th-instar insect larvae on blood containing physalin B at concentrations varying from 1 to 10 μg/ml. A control group (not-treated with physalin) fed on blood with the solvent (ethanol/saline). Finally, we observed the mortality and molting during 30 days following feeding.

2.5. Insect infection with T. rangeli

Groups of 5th-instar *R. prolixus* larvae were allowed to feed for 30 min on a blood meal. For the treated groups the

blood was supplemented with physalin B (0.01, 0.1, and 1 μg/ml) previously diluted in solvent. For the control insects the meal consisted only of blood containing ethanol/saline. Only fully gorged insects were used (180.5 ± 22.1 mg); partially fed insects were discarded. For hemocoelic infection at 5 days after feeding, 1 μl of protozoa suspension in culture medium (1×10^3 parasites/insect) was inoculated laterally into the thorax using a 10-μl Hamilton syringe connected to a fine-needle (infected groups). Sterile culture medium was inoculated under the same conditions for non-infected control insects.

2.6. Mortality in infected insects

To evaluate mortality caused by *T. rangeli* infection in insects that had been previously fed with blood containing physalin B at different concentrations (0.01, 0.1, and 1 μg/ml, treated insects), we inoculated groups of insects with protozoa (infected groups) at day 5 after feeding and noted the mortality on days 1, 3, and 6 post-infection. A control group untreated with physalin B received inoculation of sterile culture medium. To simplify, only cumulative values at day 6 post-infection are reported. After this period the mortality was negligible.

2.7. Hemolymph collection, hemocyte and hemocyte microaggregation counts

To determine possible physalin B interference with total hemocyte counts and hemocyte microaggregate formation, hemolymph samples were collected carefully from a cut leg with 5-μl Pyrex calibrated micropipettes (Sigma Chemical, USA). Samples were immediately diluted in anticoagulant solution (0.01 M ethylenediamine tetraacetic acid, 0.1 M glucose, and 0.062 M sodium chloride, 0.03 M trisodium citrate, 0.026 M citric acid, pH 4.6) as described by Azambuja et al. (1991). At different intervals after inoculation the insects with flagellates, the number of hemocytes microaggregates (operationally defined as a cluster of five or more cells—larger than 100 μm in diameter) formed and the total hemocytes numbers were determined in each sample by direct observation of every field in a hemocytometer through phase-contrast microscopy.

2.8. Prophenoloxidase-activating assay

For observation of the Prophenoloxidase (proPO)-activating system, hemolymph samples were collected at different intervals after infection as already described and immediately diluted to 10% in hypotonic calcium-cacodylate buffer (0.01 M sodium cacodylate, 0.01 M $CaCl_2$, pH 7.4). Aliquots of 10 μl of the diluted samples were added to 35 μl with the same buffer, and reactions were initiated by the addition of 15 μl of L-DOPA saturated solution (Sigma Chemical Company, USA) (4 mg/ml). The formation of DOPA-chrome at room temperature was measured spectrophotometrically at 492 nm in an ELISA plate reader (Model Anthos Labtec HT2 from Dely Instruments, England). Assays were run at 0, 15, 30, and 60 min after incubation to assess the kinetics of PO activity. For simplicity, only values for 60 min were considered. The enzyme unit was expressed as $A_{492} \times 100$/mg protein.

2.9. Nitrite and nitrate determinations

Pooled hemolymph samples (80 μl) were diluted in a proportion 1:1 with anticoagulant solution and processed as described by Whitten et al. (2001). A 50 μl mixture was processed using a Griess Reagent System Assay Kit (Promega, WI, USA) for nitrate and nitrite content, which can be indicative of reactive nitrogen intermediate (RNI) metabolism (reviewed by Moncada et al., 1991). Samples were handled following the manufacturer's instruction and absorbance of the product was measured at 550 nm. Nitrite content was quantified per milligram of protein using a range of sodium nitrate standards.

2.10. Protein determination

Proteins of hemolymph samples were quantified with a protein assay kit (BCA* Protein Assay Reagent, Pierce, USA) using bovine serum albumin (BSA) as the standard.

2.11. Statistical analyses

The results were analyzed using ANOVA and Student–Newman–Keuls (SNK) multiple range test of comparison of means according to Stats Direct Statistical Software, version 2.2.7 for Windows 98. For mortality studies, a X^2 test was applied. Data are reported as means ± standard (SD) deviation of isolated experiments. Differences among groups were considered not statistically significant when $p > 0.05$. Probability levels are specified in the text.

3. Results

3.1. Control experiments

When the drug was added to blood at concentrations ranging from 1 to 30 μg/ml, the ED_{50} (effective concentration which inhibits 50% of blood meal ingestion) was 15.2 ± 1.6 μg/ml of blood meal (mean of five experiments with 35–50 insects for each experiment), as determined by computing the linear regression line of the correlation between blood meal intake and the concentrations of physalin B. Concentrations below 5 μg/ml had no anti-feeding effect.

Experiments to investigate the effects of physalin B on ecdysis processes demonstrated that 5th-instar larvae treated orally at concentrations ranging from 1 to 10 μg/ml as well as the control group (non-treated insects) were able to molt between 18 and 22 days after feeding. Even though the treated group, which fed on blood containing 10 μg/ml of physalin ingested a mere 65% of the amount consumed

by non-treated control insect, 100% of ecdysis resulted. When comparing the mortality at day 30 after blood feeding, we observed that the death rate was less than 10% for all doses of physalin B-treated and non-treated groups.

3.2. Physalin B and mortality of infected insects

We then evaluated the mortality of insects that had been previously fed with blood containing different concentrations of physalin B and inoculated with a suspension of *T. rangeli* H14 strain or insects fed on blood containing solvent and inoculated with sterile culture medium (non-treated control group) (Table 1). The mortality in the treated groups was significantly higher (78.1% at the concentrations of 1 μg/ml, 54.3% at 0.1 μg/ml, at day 6 post-infection) than that for non-treated control insects (7.2%) ($p < 0.0001$). However, the mortality induced by treatment with physalin B at the concentration of 0.01 μg/ml (12.7%) was not significantly different when compared with the non-treated control insects ($p > 0.05$).

3.3. Physalin B and prophenoloxidase-activating system

Fig. 2 demonstrates that at 24 and 48 h post-injection the parasite-induced proPO activity was significantly elevated in the hemolymph of *T. rangeli* infected insects in both, groups that had been fed with blood containing physalin B and controls as compared to that of control group of insects fed on blood containing solvent and inoculated with sterile culture medium ($p < 0.001$ and $p < 0.05$, respectively at 24 and 48 h). However, parasite inoculation failed to elicit significantly enhanced proPO activity during the entire experiment, when we compared infected insects that had fed on blood containing parasites and infected control insects fed on blood with solvent ($p > 0.05$). Inoculation of sterile culture medium failed to enhance proPO activity during the entire experiment in insects that had fed with blood containing solvent. At 12 and 72 h there was no difference among the three groups of insects ($p > 0.1$).

Table 1
Effects of physalin B on the mortality of 5th-instar larvae of *Rhodnius prolixus* previously fed on blood containing the compound and inoculated with *Trypanosoma rangeli* strain H14[a]

Treatment (μg/ml)	Inoculation	Mortality (%)	
		Mean[b]	Range
Non-treated control	Culture medium	7.2	5.9–9.9
1	*T. rangeli*	78.1	68.4–86.7
0.1	*T. rangeli*	54.3	47.2–62.1
0.01	*T. rangeli*	12.7	8.1–14.5

Data represent cumulative mortality at day 6 after infection.

[a] Insects were fed on blood meal containing physalin B (treated groups) or ethanol/saline as solvent (non-treated control insects). Five days after feeding, insects were inoculated with 1 μl of *T. rangeli* strain H14 (1×10^3 cells/insect) (infected groups) or 1 μl of sterile culture medium (non-treated control insects).

[b] Mean of five experiments ($N = 15$–20 insects for each treatment).

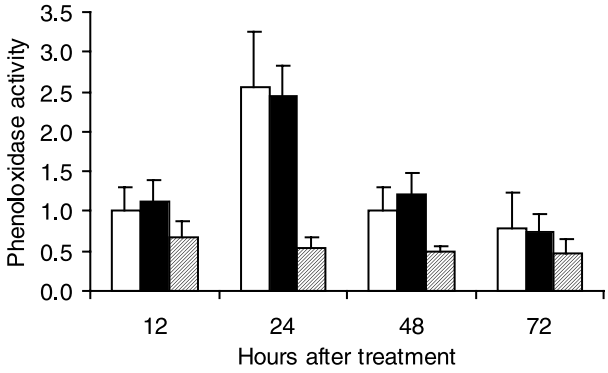

Fig. 2. Effects of physalin B on the phenoloxidase (PO) activity of 5th-instar *R. prolixus* larvae inoculated with *T. rangeli* 5 days after oral treatment. Insects fed on blood meal containing solvent and inoculated with parasites (white column); insects fed on blood meal containing 0.1 μg/ml physalin B and inoculated with parasites (black column); insects fed on blood meal containing solvent and inoculated with sterile culture medium (shaded column). Values represent means ± SD of PO activity ($A_{492} \times 100$/mg protein) of 18–24 insects per each point.

3.4. Physalin B, hemocyte, and hemocyte microaggregation counts

Inoculation with *T. rangeli* in *R. prolixus* larvae previously fed on blood with solvent or blood containing physalin B (0.1 μg/ml) demonstrated that, despite minor fluctuations, there were no significant changes in the total number of hemocyte counts, THC, $p > 0.1$ (approximately from 1.8×10^6 to 2.2×10^6 cells/ml) (Fig. 3A). A similar scenario resulted with insects fed on blood containing solvent whether or not they were inoculated with sterile culture medium (not shown).

As shown in Fig. 3B, a significant hemocyte microaggregation number was observed 2 days after inoculation in control insects fed on blood containing vehicle alone and injected with parasites in contrast to that displayed by infected insects that had fed with blood containing physalin B ($p < 0.01$). Afterwards a large number of hemocyte microaggregates (approximately 3.5×10^5 and 2.5×10^5 microaggregates/ml, respectively, at days 4 and 6 after infection) was detected in control insects fed on blood containing solvent and injected with *T. rangeli* as opposed to that of physalin B-treated insects infected with flagellates ($p < 0.0001$, Fig. 3B). Negligible numbers of hemocyte microaggregates were formed in the hemolymphs during the entire experiment when physalin B-treated insects or insects that had been fed with blood containing solvent were injected with sterile culture medium (not shown).

Fig. 3C demonstrates that insects that had previously been fed with blood containing physalin B and inoculated with *T. rangeli* at day 5 after feeding, exhibited a significantly higher number of parasites at day 2, 4, and 6 post-injection (between 2.7 and 3.9×10^5 parasites/ml of hemolymph) in contrast to that observed in the control insects that had fed on blood containing solvent and injected with parasites (less than 0.7×10^5 parasites/ml during the whole experiment) ($p < 0.001$).

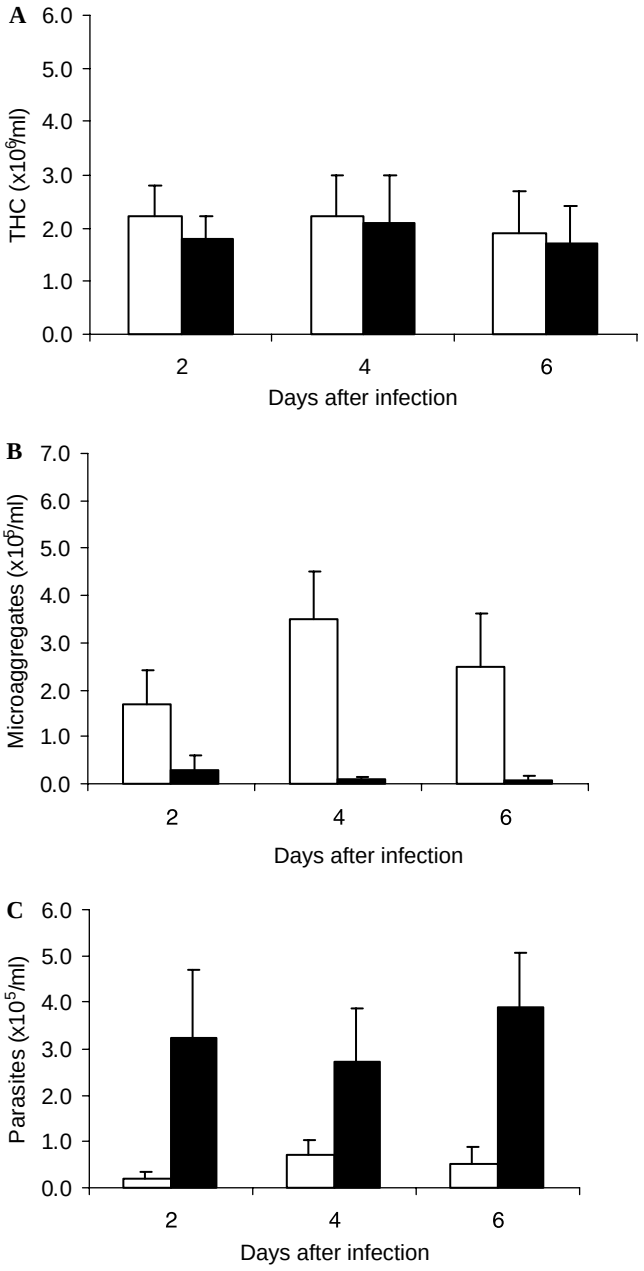

Fig. 3. Effects of physalin B on total hemocyte counts (A), hemocyte microaggregation (B), and parasitemia (C) of 5th-instar *R. prolixus* larvae injected with *T. rangeli* 5 days after oral treatment. Insects fed on blood meal containing solvent and inoculated with parasites (white column); insects fed on blood meal containing 0.1 μg/ml physalin B and inoculated with parasites (black column). Values represent means ± SD of the number of hemocytes, hemocyte microaggregation and parasites per milliliter of hemolymph of 14–20 insects per each point.

3.5. Physalin B and nitrite and nitrate production

Combined nitrate and nitrite levels, which represent metabolic products of nitric oxide reactions, were greatly enhanced at 24 and 48 h after injection in pooled hemolymph samples from insects fed on blood with solvent and inoculated with *T. rangeli* (21.6 and 19.1 μM/mg protein, respectively) in contrast to those of the infected insects that had been fed with blood containing physalin B (9.2 and 7 μM/mg protein, respectively) ($p < 0.01$) (Fig. 4). There was no significant difference observed between sterile culture medium inoculated control insects that had fed on blood containing solvent (6.5 and 7.2 μM/mg protein, respectively) and flagellate inoculated insects treated with physalin B ($p > 0.05$).

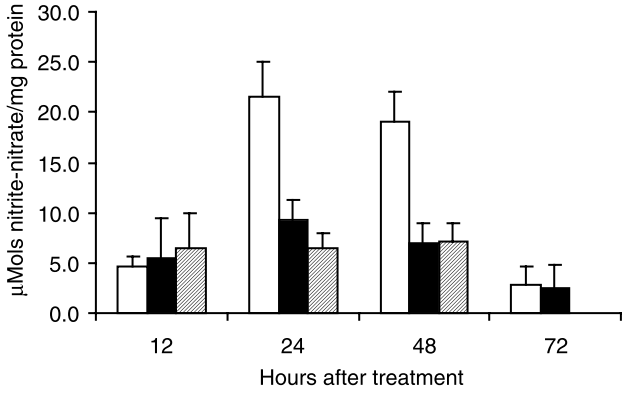

Fig. 4. Effects of physalin B on the total nitrite/nitrate content in the hemolymph of 5th-instar *R. prolixus* larvae injected with *T. rangeli* five days after oral treatment. Insects fed on blood meal containing solvent and inoculated with parasites (white column); insects fed on blood meal containing 0.1 μg/ml physalin B and inoculated with parasites (black column); insects fed on blood meal containing solvent and inoculated with sterile culture medium (shaded column). Values represent means ± SD of the total nitrite/nitrate (μM nitrite/nitrate per milligram of protein) of 6–8 pools of hemolymph taken from 14–20 insects for each pool.

4. Discussion

A variety of compounds found in plants, often called secondary chemicals, interfere with specific physiological functions of insects, including feeding and development. For example, 6,7-dimethoxy-2,2-dimethyl-3-chromene, designated precocene II, azadirachtins, and some lignoids presented powerful anti-feeding and anti-ecdysis effects in *Rhodnius* (Azambuja et al., 1982, 1981; Garcia and Azambuja, 2004; Garcia and Rembold, 1984; Garcia et al., 1986).

Blood feeding is essential for complete *R. prolixus* development. Using a suitable feeding bioassay (Azambuja et al., 1982), we demonstrated that the ingestion of blood containing different concentrations of physalin B gave an ED_{50} (effective concentration which inhibits 50% of blood meal ingestion) of 15.2 ± 1.6 μg/ml of blood meal. Thus, the amount of blood ingestion was not altered by the addition of physalin B at concentrations of 1 μg/ml or less. Similarly, exposition of the insects to physalin B, (concentrations of 1 μg/ml or less) influenced neither the development and ecdysis processes nor the mortality of uninfected control insects. The data from control experiments reported here indicate that physalin B does not evoke any physiological manifestations that might interfere in *Rhodnius* physiology and biology.

Studies carried out with azadirachtin and the lignoids, nordihydroguaiaretic acid (NDGA), and burchellin,

demonstrated that they affect the establishment of *Trypanosoma cruzi* infection in the gut of *R. prolixus* (Cabral et al., 1999; Garcia et al., 1989; Gonzalez et al., 1999). On the other hand, a systemic injection of *T. rangeli* short epimastigotes into insects previously fed on blood containing eicosanoid biosynthesis inhibitors as a phospholipase A2 inhibitor, dexamethasone, a specific inhibitor of the cyclooxygenase pathway, indomethacin and a non-selective lipoxygenase inhibitor, NDGA, reduced hemocyte microaggregation and enhanced parasitemia and mortality induced by the parasite challenge (Garcia et al., 2004). Application of arachidonic acid counteracted the hemocyte microaggregation inhibition as induced by dexamethasone (Garcia et al., 2004). It seems that *Rhodnius* cellular immune responses to a parasite infection are modulated by a physiological system that includes eicosanoid biosynthesis pathways. However, nothing yet has been revealed about on the effects of physalin B on eicosanoid pathways in insects.

To assess whether or not nitric oxide generative activities could operate during *T. rangeli* infection in *R. prolixus* treated with physalin B, we measured nitrite/nitrate and RNI breakdown products in inoculated hemolymphs. The injection of H14 short epimastigotes provoked the highest levels of nitrite/nitrate metabolic products and RNI metabolism in *R. prolixus* that did not receive physalin B at 24 and 48 h after infection. This experimental group also had the lowest parasitemia, and a large percentage of the H14 parasites were observed attached to hemocytes soon after injection, possibly triggering hemocyte microaggregation and the pathway of signalling events that culminated in activation of nitric oxide production. In insects inoculated with epimastigotes of *T. rangeli* that were fed with blood containing physalin B, the RNI released and nitrite/nitrate concentration were significantly lower than that stimulated by the systemic parasite infection in untreated control insects.

There is an implication that nitric oxide is involved in *Drosophila* immunity and that it mediates an early step of the signal transduction pathway, inducing response to natural infection with gram-negative bacteria in the insect (Nappi et al., 2000). Although in this paper we did not attempt to distinguish the mechanism of nitric oxide production utilized by the insects against trypanosomes, there is evidence, for example, that the mosquito restricts malaria parasite development with inducible synthesis of nitric oxide. Dietary provision of the NOS substrate L-arginine reduces *Plasmodium* infections in *Anopheles stephensi*. In contrast, dietary provision of the NOS inhibitor, *N*-nitro-L-arginine methylester, significantly increases the parasite number in infected mosquitoes (Luckhart et al., 1998). On the other hand, the mode of action of this gas on the parasites is well known in mammals (e.g., Gobert et al., 1998; Rottenberg et al., 1996).

Whitten et al. (2001) studied the role of superoxide, RNI and nitrite/nitrate production in *R. prolixus–T. rangeli* interactions and concluded that nitric oxide production is important in terms of anti-trypanosome defenses in this insect. Nitric oxide synthase expression and nitrite/nitrate levels are also modulated in the stomach, midgut and fat body following challenge with *T. cruzi* and *T. rangeli* (Whitten et al., personnel communication). Takle (1988) and Mello et al. (1995) demonstrated that hemocyte aggregation follows injection of *T. rangeli* in *R. prolixus*. However, the number of hemocyte microaggregates was significantly lower in infected insects receiving treatment with physalin B than that stimulated by *T. rangeli* infection in untreated control insects. These arguments reinforce our data that nitric oxide production together with hemocyte microaggregate formation is important in terms of anti-trypanosome defenses in *R. prolixus*.

Soares et al. (2003) tested the immunomodulatory activities of physalins in mice. They demonstrated that (i) physalins caused a reduction of nitrite/nitrate production by macrophages stimulated with lipopolysaccharide and interferon; (ii) macrophages, in the presence of physalin B, stimulated with lipopolysaccharide alone or in combination with interferon, produced lower levels of tumor necrosis factor (TNF), interleukin-6 and interleukin-12; and (iii) the inhibitory activity of physalin B, unlike that of dexamethasone, was not reversed by an antiglucocorticoid, and treated mice had lower levels of TNF than control mice after lipopolysaccharide challenge. They also concluded that physalins are immunomodulatory substances and act through a mechanism distinct from that of dexamethasone (Soares et al., 2003).

The data presented in this paper support the hypothesis that physalin B is an immunomodulator to *T. rangeli* challenge in *R. prolixus*. Four main points four support this hypothesis. First, the mortality in response to our standard parasite challenge was expressed in a concentration-dependent way in insects treated with concentrations ranging from 0.01 to 1 μg of physalin B/ml of blood meal. Second, the death rate was significantly enhanced in insects that received concentrations of 0.1 and 1 μg of physalin B and were infected with flagellates. Third, the hemocyte microaggregation response and nitrite/nitrate concentration, which represent metabolic products of nitric oxide reactions and RNI metabolism against *T. rangeli* infection, was significantly reduced in the hemolymph of insects treated with physalin B (0.1 μg/ml) when compared with infected untreated controls. Fourth, the number of parasites in the hemolymph of treated-insects was significantly higher than that observed in insects feeding on blood without physalin B. Based on these results we infer that physalin B is a mediator of microaggregation and nitric oxide reactions to parasite challenge in 5th-instar *R. prolixus* larvae. This study is the first to demonstrate that physalin B may alter the normal immunological responses during protozoan infection in an insect vector of disease.

Acknowledgments

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico

(CNPq) to E.S.G. and P.A., Fundação Oswaldo Cruz (Papes project to P.A.). E.S.G. and P.A. are CNPq Senior Research Fellows. D.P.C. is a post-graduate student with CAPES scholarship. The paper was revised by Mitchell Raymond Leshen, Chicago, Illinois-UCLA.

References

Azambuja, P., Garcia, E.S., 1997. Care and maintenance of triatomine colonies. In: Crampton, J.M., Beard, C.B., Louis, C. (Eds.), Molecular Biology of Insect Disease Vectors: A Method Manual. Chapman & Hall, London, pp. 56–64.

Azambuja, P., Bowers, W.S., Ribeiro, J.M.C., Garcia, E.S., 1982. Antifeedant activity of precocenes and analogs on *Rhodnius prolixus*. Experientia 38, 1054–1055.

Azambuja, P., Garcia, E.S., Ratcliffe, N.A., Warthen Jr., J.D., 1991. Immune-depression in *Rhodnius prolixus* induced by the growth inhibitor, Azadirachtin. J. Insect Physiol. 37, 771–777.

Azambuja, P., Ribeiro, J.M., Garcia, E.S., 1981. Effects of ecdysone on the metamorphosis and ecdysis prevention induced by precocene II in *Rhodnius prolixus*. Gen. Comp. Endocrinol. 45, 100–104.

Cabral, M.M.O., Azambuja, P., Gottlieb, O.R., Garcia, E.S., 1999. Neolignans inhibit *Trypanosoma cruzi* infection of its triatomine vector, *Rhodnius prolixus*. Parasitol. Res. 85, 184–187.

Chiang, H.C., Jaw, S.M., Cehn, P.M., 1992. Inhibitory effects of physalin B and physalin F on various human leukemia cells in vitro. Anticancer Res. 23, 1155–1162.

Garcia, E.S., Azambuja, P., 1997. Infection of triatomines with *Trypanosoma cruzi*. In: Crampton, J.M., Beard, C.B., Louis, C. (Eds.), Molecular Biology of Insect Disease Vector: A Method Manual. Chapman & Hall, London, pp. 146–155.

Garcia, E.S., Azambuja, P., 2004. Lignoids in insects: chemical probes for the of ecdysis, excretion, and *Trypanosoma cruzi* triatomine interactions. Toxicon 44, 431–440.

Garcia, E.S., Azambuja, P., Contreras, V.T., 1984. Large-scale rearing of *Rhodnius prolixus* and production of metacyclic trypomastigotes of *Trypanosoma cruzi*. In: Morel, C.M. (Ed.), Genes and Antigens of Parasites, A Laboratory Manual, UNDP/World Bank/WHO and Fundação Oswaldo Cruz, Rio de Janeiro, pp. 43-46.

Garcia, E.S., Machado, E.M.M., Azambuja, P., 2004. Effects of eicosanoid biosynthesis inhibitors on the prophenoloxidase-activating system and microaggregation reactions in the hemolymph of *Rhodnius prolixus* infected with *Trypanosoma rangeli*. J. Insect Physiol. 50, 157–165.

Garcia, E.S., Gonzales, M.S., Rembold, H., Azambuja, P., 1989. Chagas'disease and its insect vector. Effect of azadirachtin A on the interaction of a triatomine host (*Rhodnius prolixus*) and its parasite (*Trypanosoma cruzi*). Z. Naturforsch. 44c, 317–322.

Garcia, E.S., Mello, C.B., Azambuja, P., Ribeiro, J.M.C., 1994. *Rhodnius prolixus*: salivary antihemostatic components decrease with *Trypanosoma rangeli* infection. Exp. Parasitol. 78, 287–293.

Garcia, E.S., Rembold, H., 1984. Effects of azadirachtin on ecdysis of *Rhodnius prolixus*. J. Insect Physiol. 30, 939–941.

Garcia, E.S., Uhl, M., Rembold, H., 1986. Azadirachtin, a chemical probe for the study of moulting processes in *Rhodnius prolixus*. Z. Naturforsch. 41c, 771–775.

Gobert, A.P., Semballa, S., Daulouede, S., Lesthelle, S., Taxile, M., Veyret, B., Vincendeau, P., 1998. Murine macrophages use oxygen- and nitric-dependent mechanisms to synthesize *S*-nitroso-albumin and to kill extracellular trypanosomes. Infect. Immun. 66, 4068–4070.

Gomes, S.A.O., Feder, D., Garcia, E.S., Azambuja, P., 2003. Suppression of the prophenoloxidase system in *Rhodnius prolixus* orally infected with *Trypanosoma rangeli*. J. Insect Physiol. 49, 829–837.

Gonzalez, M.S., Nogueira, N.F.S., Mello, C.B., de Souza, W., Schaub, G.A, Azambuja, P., Garcia, E.S., 1999. Influence of brain on midgut organization and *Trypanosoma cruzi* development in the vector, *Rhodnius prolixus*. Exp. Parasitol. 92, 100–108.

Hecker, H., Scwarzenbach, M., Rudin, W., 1990. Development and interactions of *Trypanosoma rangeli* in and with the reduviid bug, *Rhodnius prolixus*. Parasitol. Res. 76, 311–318.

Hoare, C.A., 1972. The Trypanosomes of Mammals. A Zoological Monograph. Blackwell, Oxford-Edinburgh.

Lin, Y.S., Chiang, H.C., Kan, W.S., Hone, E., Won, M.H., 1992. Immunomodulatory activity of various fractions derived from *Physalis angulata* L. extract. Am. J. Chem. Med. 20, 233–243.

Luckhart, S., Vodovotz, Y., Cui, L., Rosenberg, R., 1998. The mosquito *Anopheles stephensi* limits malaria parasite development with inducible synthesis of nitric oxide. Proc. Natl. Acad. Sci. USA 95, 5700–5705.

Mello, C.B., Garcia, E.S., Ratcliffe, N.A., Azambuja, P., 1995. *Trypanosoma cruzi* and *Trypanosoma rangeli*: interplay with hemolymph components of *Rhodnius prolixus*. J. Invertebr. Pathol. 65, 261–268.

Moncada, S., Palmer, R.M.J., Higgs, E.A., 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol. Rev. 43 (2), 109–142.

Nappi, A.J., Vass, E., Frey, F., Carton, Y., 2000. Nitric oxide involvement in *Drosophila* immunity. Nitric Oxide 4, 423–430.

Ratcliffe, N.A., Nigam, Y., Mello, C.B., Garcia, E.S., Azambuja, P., 1996. *Trypanosoma cruzi* and erythrocyte agglutinins: a comparative study of occurrence and properties in the gut and hemolymph of *Rhodnius prolixus*. Exp. Parasitol. 93, 83–93.

Rottenberg, M.E., Castaños-Velez, E., De Mesquita, R., Laguardia, O.G., Biberfeld, P., Õrn, A., 1996. Intracellular localization of *Trypanosoma cruzi* and inducible nitric oxide synthase (iNOS): evidence for dual pathway of iNOS induction. Eur. J. Immunol. 6, 3203–3213.

Snedecor, G.W., 1964. Statistical Methods Applied in Experiments in Agriculture and Biology. Iowa State University Press.

Soares, M.B., Bellintani, M.C., Ribeiro, I.M., Tomassini, T.C., Ribeiros dos Santos, R., 2003. Inhibition of macrophage activation and lipopolysaccharide-induced death by seco-steroids purified from *Physalis angulata* L. Eur. J. Pharmacol. 459, 107–112.

Takle, G.B., 1988. Studies on the cellular immune response of insects toward the insect pathogen *Trypanosoma rangeli*. J. Invertebr. Pathol. 51, 6472.

Tobie, E.J., 1970. Observation on the development of *Trypanosoma rangeli* in the haemocoel of *Rhodnius prolixus*. J. Invertebr. Pathol. 16, 118–125.

Watkins, R., 1971. Histology of *Rhodnius prolixus* infected with *Trypanosoma rangeli*. J. Invertebr. Pathol. 17, 59–66.

Whitten, M.M.A., Mello, C.B., Gomes, S.A.O., Nigan, Y., Azambuja, P., Garcia, E.S., Ratcliffe, N.A., 2001. Role of superoxide and reactive nitrogen intermediates in *Rhodnius prolixus* (Reduviidae)/*Trypanosoma rangeli* interactions. Exp. Parasitol. 98, 22–57.

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Journal of Insect Physiology 52 (2006) 711–716
Journal of Insect Physiology
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Cellular immune response in *Rhodnius prolixus*: Role of ecdysone in hemocyte phagocytosis

Marcela B. Figueiredo[a], Daniele P. Castro[a], Nadir F. S. Nogueira[b], Eloi S. Garcia[a], Patrícia Azambuja[a,*]

[a]*Departamento de Bioquímica e Biologia Molecular, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Avenida Brasil 4365, Manguinhos, CEP 21040-900 Rio de Janeiro, Brazil*
[b]*Laboratório de Biologia Celular e Tecidual, CBB, Universidade Estadual do Norte Fluminense, Campos de Goytacazes, RJ, Brazil*

Received 31 January 2006; received in revised form 23 March 2006; accepted 27 March 2006

Abstract

In this paper we investigate in vivo and in vitro effects of orally administered azadirachtin and ecdysone on the phagocytic responses of *Rhodnius prolixus* 5th-instar larval hemocytes to the yeast *Saccharomyces cerevisiae*. Groups of insects fed non-treated blood (control) and insects that received azadirachtin plus ecdysone in the blood meal were inoculated with yeast cells in the hemocele. The injected yeast cells disappeared rapidly from the hemolymph, being removed completely by 90 min after inoculation. In the insects treated only with azadirachtin the clearance of free yeast circulating particles was significantly delayed compared to the two previously mentioned groups. It was demonstrated that the binding of yeast cells to hemocytes was reduced in the insects treated only with azadirachtin in comparison to both non-treated control and azadirachtin plus ecdysone-treated groups. Phagocytosis occurred when yeast cells were added to hemocyte monolayers prepared with hemolymph from blood fed insects, treated or not with azadirachtin plus ecdysone, so that yeast cells were rapidly bound to hemocytes and internalized in high numbers. By contrast, insects treated with azadirachtin exhibited a drastic reduction in the quantity of yeast cell–hemocyte binding and subsequent internalization. In all groups, the hemocytes attached to the glass slides were predominantly plasmatocytes. The magnitude and speed of the cellular response suggests that hemocyte phagocytosis is one of the main driving forces for the clearance of free circulating yeast cells from the hemolymph. We propose that ecdysone modulates phagocytosis in *R. prolixus* larvae, and that this effect is antagonized by azadirachtin.

Keywords: Phagocytosis; Ecdysone; Azadirachtin; Yeast; *Rhodnius prolixus*; Hemocytes

1. Introduction

There is an implicit assumption that azadirachtin, a tetranortriterpenoid from the neem tree (*Azadirachta indica* A. Juss), is a natural growth inhibitor which strongly influences insect hormones through interference with the neuroendocrine system (Rembold, 1987). Garcia et al. (1986) demonstrated that ecdysteroid levels in the hemolymph of *Rhodnius prolixus* larvae treated with azadirachtin are too low for induction of ecdysis. The synthesis and release of prothoracicotropic hormone (PTTH) by the brain, which in turn induces ecdysone synthesis by the prothoracic glands, was deficient in the azadirachtin-treated insects (Garcia et al., 1990). Thus, in *R. prolixus* larvae, azadirachtin has a powerful anti-ecdysis effect, which can be completely reversed by administration of ecdysone (Garcia and Rembold, 1984). Furthermore, it has been demonstrated that therapy with azadirachtin interferes in several parameters of the cellular and humoral immune responses in *R. prolixus* larvae (Azambuja et al., 1991a; Feder et al., 1997).

When microorganisms invade the hemolymph, cellular components of the insect's immune system may be rapidly mobilized. An essential component of hemocyte-mediated defense reactions is phagocytosis, a process by which the cell engulfs particles from the extracellular environment into cytoplasmic vesicles. In order to internalize a

*Corresponding author. Tel: +55 21 3865 8134; fax: +55 21 2590 3495.
E-mail address: azambuja@ioc.fiocruz.br (P. Azambuja).

0022-1910/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jinsphys.2006.03.011

phagocytic target from the extracellular environment, binding to the cell surface must first occur (Vogel et al., 1980; Gerson et al., 1982). The phagocytic process is influenced by several factors such as (i) different inert particles (Brehélin and Hoffmann, 1980), (ii) biological inducers and inhibitors (Costa et al., 2005), (iii) quantity and types of hemocytes and (iv) their potential role in self and non-self recognition (Ratcliffe, 1993; Gupta, 1991). So far, with regard to insect physiology, there is little information on the mechanisms that underlie hemocyte phagocytosis in insects. One plausible possibility is that the neuroendocrine system in insects is involved in modulating this physiological event.

We have considered whether or not ecdysone can interfere in insect cellular immunity by using in vivo inoculation of insects and hemocyte monolayers as a tool to study phagocytosis in the interaction between yeast cells (*Saccharomyces cerevisiae*) and *R. prolixus* larvae. In this paper, we provide evidence, through in vivo experiments, that insects treated with azadirachtin become less competent to clear free circulating yeast cell from the hemolymph and demonstrate that ecdysone is required for this process. Analyses of hemocyte monolayer experiments confirm that treatment with azadirachtin of *R. prolixus* larvae reduces binding of yeast cells to hemocytes as well as the subsequent phagocytic responses, and that ecdysone treatment can counteract these inhibitory effects of azadirachtin. We hypothesize that ecdysone modulates phagocytosis of foreign particles by *R. prolixus* hemocytes in vivo.

2. Materials and methods

2.1. Insects

All experiments were undertaken with *R. prolixus* 5th-instar larvae reared and maintained as previously described (Azambuja and Garcia, 1997). After molting, insects were randomly chosen, starved for 15–20 days, and then fed citrated human blood through a membrane feeding system (Azambuja and Garcia, 1997). In some experiments azadirachtin (Sigma) or α-ecdysone (Sigma), both dissolved in 1:4 ethanol–saline, were added to the blood meal in final concentrations of 1 and 2.5 μg/ml, respectively.

2.2. Preparation of yeast cells

S. cerevisiae, dried baker's yeast cells kindly donated by Dr. Norman Ratcliffe, were diluted at a concentration of 5% in phosphate buffer-saline PBS, autoclaved at 120 °C for 15 min and kept at 4 °C for a maximum period of 30 days prior to usage. For experiments the heat-killed yeast cell suspension samples were washed three times in sterile PBS by centrifugation (5000 *g* for 10 min) and then resuspended at concentrations from 10^6 to 10^9 yeast cells/ml in insect saline (18 g L^{-1} *D*-glucose, 12.2 g L^{-1} KCl, 0.36 g L^{-1} $NaHCO_3$, 380 mOsm, pH 7.8) as described in Whitten et al.(2001).

2.3. Insect hemocelic inoculation: in vivo experiments

R. prolixus 5th-instar larvae weighting 34.6 ± 2.4 mg were allowed to feed on blood containing either azadirachtin or azadirachtin plus ecdysone. A control group was fed with blood containing 1 μl ethanol–saline per ml of blood. All experiments were performed only with fully gorged insects weighting 192.8 ± 14.3 mg.After a period of 5-8 days after feeding, the insects were inoculated laterally into the thorax with 1 μl of yeast suspension at a concentration 5×10^8 yeast cells/ml in sterile insect saline using a 10-μl Hamilton syringe. For control, one group of insects was fed with blood containing ecdysone alone and inoculated with the same number of yeast cells.

2.4. Hemolymph collection and cell counting: in vivo experiments

R. prolixus hemolymph samples were collected carefully, from a severed leg with 5-μl calibrated micropipettes (Sigma). The product was immediately diluted in anticoagulant solution (0.01 M ethylenediamine tetraacetic acid, 0.1 M glucose, 0.062 M sodium chloride, 0.026 M citric acid, pH 4.6) as described by Azambuja et al. (1991b). At different times after inoculation, total hemocyte count, free circulating yeast particles and yeast cell associated hemocytes were determined by direct observation in a hemocytometer chamber by phase-contrast optics microscopy. Differential hemocyte counts were performed prior to inoculation according to Azambuja et al. (1991b).

2.5. Preparation of FITC-labelled yeast

For *S. cerevisiae* cell labeling, the method described by Rohloff et al. (1994) was modified. Basically, yeast autoclaved cells suspended in PBS were washed 3-times with the same sterile solution, and the pellet was finally resuspendend in carbonate buffer (0.2 M Na_2CO_3, 0.2 M $NaHCO_3$, pH 9.4). The sample suspensions were adjusted to give a concentration of 5.0×10^8 yeast cells/ml in carbonate buffer containing fluorescein isothiocyanate (FITC) 0.1 mg/ml and incubated for 30 min in the dark at room temperature on a rotary mixer. The solution was then washed 3 times with insect saline to remove all traces of free FITC, and then the conjugated FITC-yeast resuspended in the sterile Grace insect medium (GIM) (Gibco-BRL) at a concentration of 1×10^8 cells/ml was stored at −20 °C until application.

2.6. Hemocyte monolayers and phagocytosis assay (in vitro experiments)

Hemolymph was gathered in a glass micropipette, and then pooled in an Eppendorf tube containing sterile insect

saline with traces of phenylthiourea (PTU) in a proportion of 20 µl of hemolymph to the same volume of insect saline–PTU solution. Immediately upon collection, hemolymph samples were applied onto sterile 12 mm diameter round glass cover slips located at the bottoms of plate composed of 24-well (Corning) and incubated at room temperature for 60 min.The monolayer was gently rinsed three times in 250 µl of sterile insect saline to remove non-attached cells. Immediately after washing, 250 µl of FITC-labeled yeast suspension in GIM solution (5×10^8 cells/ml) was added to each well and incubated for 1 h at room temperature. Non-adherent yeast cells were rinsed from the cover slips three times using insect saline. Non-internalized yeast cells were quenched with insect saline containing 1.4 mg/ml trypan blue for 15 min according to the method described by Asgari and Schmidt (2003). After washing three times with insect saline, cell monolayers were then fixed with 2.0% glutaraldehyde in insect saline for 60 min at room temperature. The cover slips were washed in PBS and kept at 4 °C overnight in a dark, humid chamber before being inverted onto glass slides and studied in the microscope. To quantify phagocytic activity for each cover slip, five different fields containing 100 hemocytes were examined. In all treatments the numbers of associated and internalized yeast cells were determined through hemocyte images from a Nikon Eclipse E200 microscope. In each field phase-contrast was alternated with visualization of internalized yeast cells under indirect UV illumination.

2.7. Hemocyte viability

Hemocyte viability was assessed by trypan blue exclusion method (Boyes et al., 1964). Basically, 0.1% trypan blue in insect saline was added directly onto the cover slips prepared with washed hemocyte monolayers of different treated groups of insects and evaluated under phase contrast microscopy after incubation for 10 min at room temperature.

2.8. Statistical analyses

For all experiments conducted, the results were analyzed using ANOVA and the Student–Newman–Keuls (SNK) multiple range test of comparison of means according to Stats Direct Statistical Software, version 2.2.7 for Windows 98. Differences between samplings were considered not statistically significant at a probability more than 5% ($p > 0.05$). Probability levels are specified in the text.

3. Results

3.1. In vivo experiments

In order to determine whether or not the treatment with azadirachtin and ecdysone would affect cellular immune reactions we compared the total number of hemocytes and measured the percentage of plasmatocytes, granular cells and other types of hemocytes circulating in the hemolymph prior yeast cell inoculation. No significant differences were encountered between the total hemocyte numbers obtained from blood fed insects (control) and the groups fed with blood containing azadirachtin or azadirachtin plus ecdysone (Fig. 1A) ($p > 0.1$). We also observed that percentages of plasmatocytes and granular cells varied from 33% to 49% with no significant difference among groups (Fig. 1B) ($p > 0.05$).

In order to verify whether or not the treatments with azadirachtin and ecdysone would affect hemocyte activities, in background experiments we measured their viability applying the trypan blue exclusion method. The viability indexes of control, azadirachtin and azadirachtin plus ecdysone treated insects, surpassed 83% in all cases.

In order to investigate the effects of azadirachtin and ecdysone on hemocyte phagocytosis, groups of treated larvae and controls were challenged with 1 µl of a suspension of 5×10^8 yeast cells/ml on days 5–8 after blood meal ingestion. Controls and azadirachtin plus

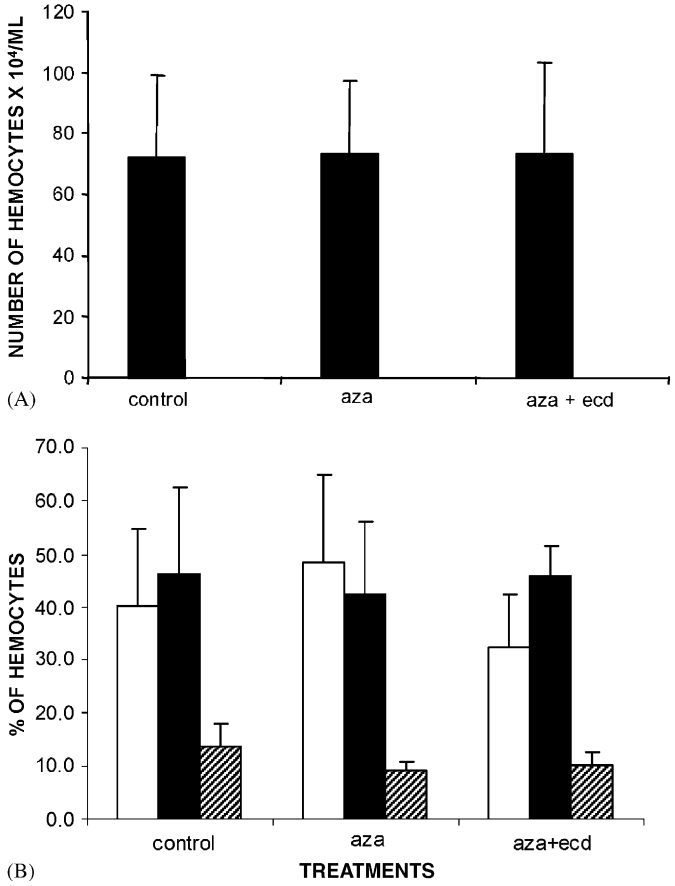

Fig. 1. Effects of azadirachtin and ecdysone on total hemocyte counts (THC) and percentage of plasmatocytes and granular cells in the hemolymph of *R. prolixus* 5th-instar larvae. Insects were previously fed a blood meal containing azadirachtin (1 µg/ml), azadirachtin plus ecdysone (2.5 µg/ml) or solvent (control), and hemolymph was extracted 5–8 days after the treatments. (A) Total hemocyte counts; (B) percentage of plasmatocytes (□), granular cells (■), other types of hemocytes (▨). Values represent mean ± SD; $n = 15$ insects.

ecdysone treatment at 30 and 90 min after yeast inoculation exhibited a faster decrease in the percentage of insects with yeast cells circulating free in hemolymph than did azadirachtin treated insects (Table 1). In addition, insects treated with azadirachtin presented a number of yeast cells in hemolymph ranging from 0 to 21×10^4 cells/ml at 90 min, while in azadirachtin plus ecdysone and control groups no cells were detected by this time (Table 1).

The number of yeast cells associated with hemocytes in the group treated with azadirachtin was lower than in untreated control insects and in the azadirachtin plus ecdysone group ($p < 0.0001$) (Fig. 2). However, the numbers of hemocytes not associated with yeast cells were not significantly different among the three groups ($p > 0.05$). The group of insects fed on blood containing ecdysone presented similar results to the control group fed on blood (not shown).

Table 1
Effects of azadirachtin and ecdysone on percentage of insects with yeast cells circulating in the hemolymph of *R. prolixus* 5th-instar larvae inoculated with *Saccharomyces cerevisiae*

Treatments	% insects with yeast cells/(range $\times 10^4$ cells/ml)	
	30 min[a]	90 min[b]
Azadirachtin	100/(0–39)	50/(0–21)
Azadirachtin + Ecdysone	67/(0–10)	0/(0–0)[b]
Control	67/(0–2)	0/(0–0)[b]

Insects fed on blood containing azadirachtin (1 μg/ml), blood with azadirachtin plus ecdysone (2.5 μg/ml) or blood containing only solvent (control). Five-eight days after treatments, insects were inoculated with yeast suspension (5×10^8 cells/ml).
Values represent mean ± SD; $n =$ 5–8 insects. The experiment was repeated three times yielding similar results.
[a]Minutes after inoculation.
[b]Below hemocytometer chamber counting limit.

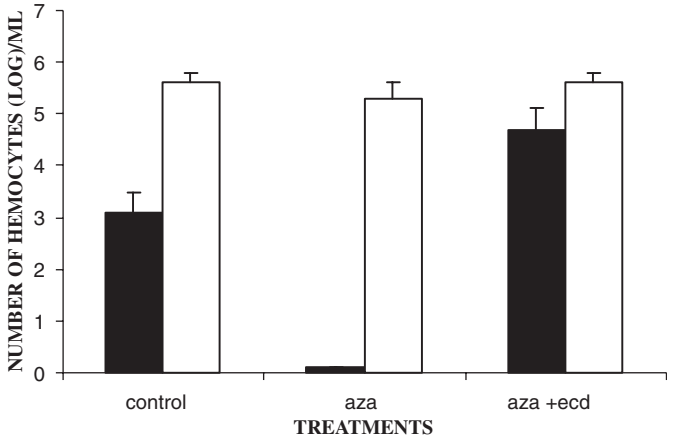

Fig. 2. Effects of azadirachtin and ecdysone on the numbers of hemocytes binding yeast cells (■) hemocytes with no yeast cells bound (□) in the hemolymph of *R. prolixus* 5th-instar larvae 30 min after inoculation with *Saccharomyces cerevisiae*. Insects previously fed on blood containing azadirachtin (1 μg/ml), blood with azadirachtin plus ecdysone (2.5 μg/ml) or blood containing only solvent (control). Five-eight days after treatments, insects were inoculated with yeast suspension (5×10^8 cells/ml). Values represent mean ± SD; $n = 5$–8 insects. The experiment was repeated three times yielding similar results.

3.2. *In vitro experiments*

3.2.1. *Effects of azadirachtin and ecdysone on the number of yeast cells associated with and internalized by hemocytes*

Background experiments for hemocyte monolayers using hemolymph of *R. prolixus* 5th-instar larvae demonstrated that plasmatocytes were the predominant cell type (> 95%) attached to the glass slides for all insect groups (not shown). The number of yeast cells associated with hemocytes was proportionally related to the number of *S. cerevisiae* cells in the incubation medium in the case of hemocytes taken from insects fed on blood plus solvent (Fig. 3).

Under the same experimental conditions, we observed a significant difference in the percentages of hemocytes associated with at least one yeast cell when the hemocytes were obtained from insects previously fed with blood containing azadirachtin (28%) whilst in controls as well as in insects treated with azadirachtin plus ecdysone, almost 80% of the hemocytes were bound to at least one yeast cell. Consequently, the percentage of yeast-free hemocytes was significantly higher in the groups of insects treated with azadirachtin than in both controls and insects which received azadirachtin plus ecdysone added to the blood meal.

When phagocytosis assays were performed in vitro with hemocyte monolayers, the numbers of yeast cells associated with hemocytes were different according to the previous treatment of the donor insects. Considering a quantity equal or higher than 15 yeast cells bound to hemocytes, there was a significant reduction in the group treated with azadirachtin when compared to the control group ($p < 0.001$) and the azadirachtin plus ecdysone treated insects ($p < 0.001$) (Fig. 4A). A similar significant result was

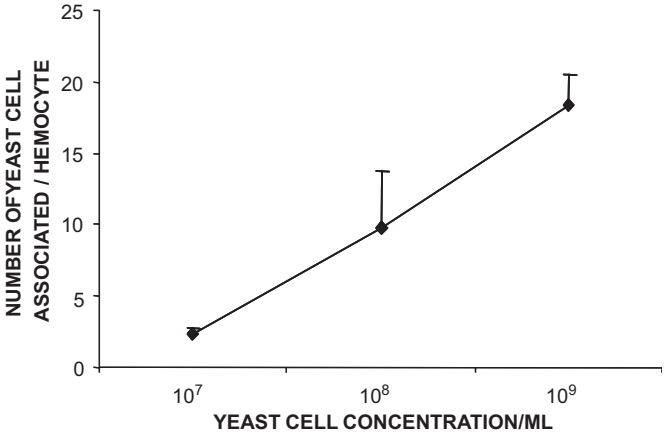

Fig. 3. Association effect of different concentrations of *Saccharomyces cerevisiae* incubated with *R. prolixus* 5th-instar larvae hemocyte monolayers. Hemolymph was obtained 5–8 days after feeding (see details in Section 2). Values represent mean ± SD; $n =$ 10 monolayers.

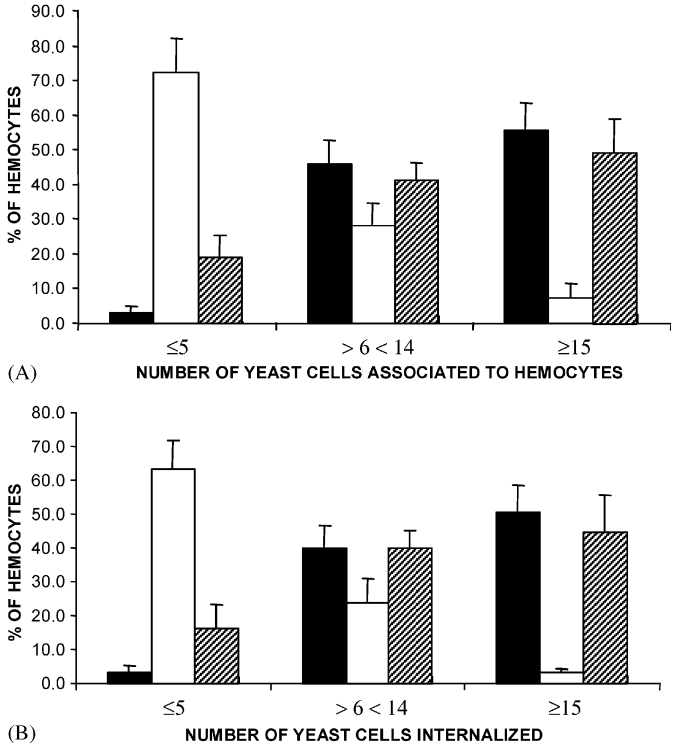

Fig. 4. Effects of azadirachtin and ecdysone on phagocytosis of *R. prolixus* 5th-instar larval hemocyte monolayers. (A) Percentage of hemocytes associated to yeast cells, (B) percentage of hemocytes with yeast cells internalized. Hemocyte monolayers were incubated with 5×10^7 FITC conjugated yeast cells/ml for 60 min (for details see Section 2). Insect groups were fed blood (control) (■); blood containing azadirachtin (1 μg/ml) (□); or azadirachtin plus ecdysone (2.5 μg/ml) (▨). Values represent mean ± SD; $n = 10$ monolayers.

obtained for internalization of yeast cells: more than 40% of hemocytes phagocytized 15 or more yeast particles in the control group and in the case of insects previously treated with azadirachtin plus ecdysone, whilst only 3% of hemocytes were able to internalize the same number of yeast cells in the group of insects which had received azadirachtin treatment ($p < 0.0001$) (Fig. 4B). Because of the consistently high percentage of hemocytes on a monolayer ingesting 15 or more yeast particles in the two former groups, in the insects treated with azadirachtin the number of hemocytes that bound only 5 or fewer yeast cells significantly predominated ($p < 0.001$) (Fig. 4A). Very similar results were displayed with respect to the number of yeast cells internalized by hemocytes (Fig. 4B). Considering the number of yeast cells bound to and/or internalized by hemocytes on a monolayer of 6–14 cells, there were no significant differences among all groups ($p > 0.05$).

4. Discussion

Our principal findings may be stated as follows: azadirachtin treatment did not influence the viability and the total number of hemocytes circulating in the hemolymph indicating that the responses were not due to a toxic effect on the *R. prolixus* immune cells. With regard to the cell types involved in yeast cell–hemocyte association and phagocytosis, in vitro experiments indicated that plasmatocytes were the main cell type implicated in these processes. In vivo experiments, performed with hemolymph from azadirachtin-treated *R. prolixus* inoculated with *S. cerevisiae*, revealed that a reduced number of hemocytes attached to yeast cells. Similar results were seen in the case of hemocyte monolayers obtained from insects treated with azadirachtin, i.e. the hemocytes were diminished in their ability to react against yeast cells. As a consequence, an extremely low phagocytic activity of the plasmatocytes appeared in these azadirachtin experimental groups. On the other hand, the monolayer assay indicated that the inhibition of binding and internalization of yeast cells by azadirachtin treatment was counteracted by simultaneous treatment with ecdysone. Since the attachment of yeast cells to the hemocyte surface is an essential prerequisite to trigger phagocytic responses, we suggest that the cellular activity or recognition of yeast cells by hemocyte receptors may be compromised in the hemocytes of azadirachtin-treated insects. This raises a relevant question regarding the mechanism of action of azadirachtin on *R. prolixus* hemocyte phagocytosis.

Most of the published articles on phagocytosis in insects have concentrated on microorganism/hemocyte interactions emphasizing the complexities and sophistication of the process that consists of two steps, binding and internalization (Rabinovitch, 1967). Besides the interaction between receptors and ligands triggering the phagocytic responses (Greenberg and Silverstein, 1993), several authors have demonstrated that plasmatocytes are the predominant cell type involved (Rohloff et al., 1994; Anggraeni and Ratcliffe, 1991; Ratcliffe and Rowley, 1979; Ratcliffe et al., 1984).

Few aspects of cell-mediated insect immunity control are well understood and, for an adequate interpretation of our results, the interrelation between the neuroendocrine system and immune system must be established. The first evidence of endocrine system involvement in *R. prolixus* hemocyte regulation comes from the work of Jones (1967), who used ligature experiments to demonstrate that ecdysteroids could regulate the number of hemocytes as well as the volume of hemolymph and induce hemocyte differentiation. Hoffmann, (1970) showed that in *Locusta migratoria* the prothoracic glands stimulate production and differentiation of hemocytes. It was confirmed in *Drosophila* larvae infected by eggs of parasitic wasps, that when ecdysone levels were low or ecdysone signaling was blocked, the hemocyte encapsulation response was drastically reduced (Sorrentino et al., 2002). Recently, Franssens et al. (2006) described that 20-hydroxyecdysone and juvenile hormone play an important regulatory role in the nodulation response in flesh fly larvae, *Neobellieria bullata*.

Previous work from our laboratory indicated that azadirachtin adversely affected the *R. prolixus* immune reaction when the insects were challenged with *Enterobacter*

cloacae by decreasing nodule formation, antibacterial activity and lysozyme in the hemolymph as well as increasing the population of microorganisms in the hemocele (Azambuja et al., 1991a). Since azadirachtin inhibits ecdysis by irreversible blockage of ecdysone production in the prothoracic glands (Sieber and Rembold, 1983; Garcia et al., 1986, 1990), it seems reasonable to assume that a decrease in the number of yeast cells bound to the hemocyte surface and the subsequent hemocyte phagocytic responses in the azadirachtin-treated insects may reflect cellular immunity being under ecdysteroid control. Additional support for this hypothesis is evidenced by our present demonstration that simultaneous ecdysone and azadirachtin treatment enhanced both the number of hemocytes binding to yeast cells and also the number of yeast cells internalized, opposing the effect of treatment with azadirachtin alone.

To our knowledge this is the first report of the modulation by ecdysone of phagocytosis in insects. The mechanism(s) responsible for this immune deficiency is unclear, but we have postulated that ecdysone may have a direct influence on the *R. prolixus* hemocyte phagocytosis. This hypothesis will be further explored in our future work.

Acknowledgments

This work was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (Faperj) and Fundação Oswaldo Cruz (Papes) to PA. ESG and PA are senior CNPq Research Fellows, MBF and DPC are post–graduate students with Faperj and Capes scholarships. This paper was reviewed and revised by Mitchell Raymond Lishon, native of Chicago, ILl, USA-UCLA 69.

References

Anggraeni, T., Ratcliffe, N.A., 1991. Studies on cell-cell cooperation during phagocytosis by purified haemocyte populations of the wax moth, *Galleria mellonella*. Journal of Insect Physiology 37, 453–460.

Asgari, S., Schmidt, O., 2003. Is cell surface calreticulin involved in phagocytosis by insect hemocytes? Journal of Insect Physiology 49, 545–550.

Azambuja, P., Garcia, E.S., 1997. Care and maintenance of triatomine colonies. In: Crampton, J.M., Beard, C.B., Louis, C. (Eds.), Molecular Biology of Insect Disease Vectors; A Mehtods Manual. Chapman & Hall, London, pp. 56–64.

Azambuja, P., Garcia, E.S., Ratcliffe, N.A., Warthen Jr., J.D., 1991a. Immune-depression in *Rhodnius prolixus* induced by the growth inhibitor, azadirachtin. Journal of Insect Physiology 37, 771–777.

Azambuja, P., Garcia, E.S., Ratcliffe, N.A., 1991b. Aspects of classification of hemíptera hemocytes from six triatomine species. Memórias do Instituto Oswaldo Cruz 86, 1–10.

Boyes, E.A., Old, L.J., Charoulikov, J., 1964. Cytotoxic test for demonstration of antibodies. Methods in Medical Research 10, 39–51.

Brehélin, M., Hoffmann, J.A., 1980. Phagocytosis of inert particles in *Locusta migratoria* and *Galleria mellonella* study of ultrastructure and clearance. Journal of Insect Physiology 26, 103–111.

Costa, C.P., Ribeiro, C., Girard, P.A., Zumbihl, R., Brehélin, M., 2005. Modes of phagocytosis of Gram-positive and Gram-negative bacteria by *Spodoptera littoralis* granular haemocytes. Journal of Insect Physiology 51, 39–46.

Feder, D., Mello, C.B., Garcia, E.S., Azambuja, P., 1997. Immune responses in *Rhodnius prolixus*: influence of nutrition and ecdysone. Journal of Insect Physiology 43, 513–519.

Franssens, V., Smagghe, G., Simonet, G, Claeys, I., Breugelmans, B., de Loof, A., Broeck, J.V., 2006. 20-Hydroxyecdysone and juvenile hormone regulate the laminarin-induced nodulation reaction in larvae of the flesh fly, *Neobellieria bullata*. Developmental and Comparative Immunology, in press.

Garcia, E.S., Rembold, H., 1984. Effects of azadirachtin on ecdysis of *Rhodnius prolixus*. Journal of Insect Physiology 30, 939–941.

Garcia, E.S., Uhl, M., Rembold, H., 1986. Azadirachtin, a chemical probe for the study of molting processes in *Rhodnius prolixus*. Zeitschrift fur Naturforschung 41, 711–715.

Garcia, E.S., Luz, N., Azambuja, P., Rembold, H., 1990. Azadirachtin depresses the release of prothoracicotropic hormone in *Rhodnius prolixus* larvae: evidence from head transplantations. Journal of Insect Physiology 36, 679–682.

Gerson, D.F., Capo, C., Benoliel, A.M., Bongrand, P., 1982. Adhesion, phagocytosis and cell surface energy. The binding of fixed human erythrocytes to rat macrophages and polymethylpentene. Biochimie and Biophysic Acta 692, 147–155.

Greenberg, S., Silverstein, S.C., 1993. Phagocytosis. In: Paul, W.E. (Ed.), Fundamental Immunology. Raven Press, New York, pp. 941–965.

Gupta, A.P., 1991. Insect immunocytes and other haemocytes: roles in cellular and humoral immunity. In: Gupta, A.P. (Ed.), Immunology of Insects and Other Arthropods. CRC Press, Boca Raton, FL, USA, pp. 19–118.

Hoffmann, J.A., 1970. Endocrine regulation of production and differentiation of hemocytes in an orthopteran insect *Locusta migratoria migratoroides*. General and Comparative Endocrinology 15, 198–219.

Jones, J.C., 1967. Effects of repeated haemolymph withdrawals and of ligaturing the head on differential haemocyte counts of *Rhodnius prolixus*. Journal of Insect Physiology 13, 1351–1360.

Rabinovitch, M., 1967. The dissociation of the attachment and ingestion phases of phagocytosis by macrophages. Experimental Cell Research 46, 19–32.

Ratcliffe, N.A., 1993. Cellular defense responses of insects, unresolved problems. In: Beckage, N.E., Thompson, S.N., Federici, B.A. (Eds.), Parasites and Pathogens of Insects, vol. 1. Parasites, Academic Press, San Diego, pp. 267–304.

Ratcliffe, N.A., Rowley, A.F., 1979. Role of haemocytes in defense against biological agents. In: Gupta, A.P. (Ed.), Insect Hemocytes. Cambridge University Press, Cambridge, UK, pp. 331–414.

Ratcliffe, N.A., Leonard, C., Rowley, A.F., 1984. Prophenoloxidase activation nonself recognition and cell cooperation in insect immunity. Science 226, 557–559.

Rembold, H., 1987. The azadirachtins—potent insect growth inhibitors. Memórias do Instituto Oswaldo Cruz 82 (Suppl. 3), 61–66.

Rohloff, L.H., Wiesner, A., Gotz, P., 1994. A fluorescence assay demonstrating stimulation of phagocytosis by haemolymph molecules of *Galleria mellonella*. Journal of Insect Physiology 40, 1045–1049.

Sieber, K.P., Rembold, H., 1983. The effects of azadirachtin on the endocrine control of moulting in *Locusta migratoria*. Journal of Insect Physiology 29, 523–527.

Sorrentino, R.P., Carton, Y., Govind, S., 2002. Cellular immune response to parasite infection in the *Drosophila* lymph gland is developmentally regulated. Developmental Biology 243, 65–80.

Vogel, G., Thilo, L., Schwarz, H., Steinhart, R., 1980. Mechanism of phagocytosis in *Dictyostelium discoideum*: phagocytosis is mediated by different recognition sites as disclosed by mutants with altered phagocytic properties. Journal of Cell Biology 86, 456–462.

Whitten, M.M., Mello, C.B., Gomes, S.A.O., Nigam, Y., Azambuja, P., Garcia, E.S., Ratcliffe, N.A., 2001. Role of superoxide and reactive nitrogen intermediates in *Rhodnius prolixus* (Reduviidae)/Trypanosoma rangeli interactions. Experimental Parasitology 98, 44–57.

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Journal of Insect Physiology 54 (2008) 555–562
Journal of Insect Physiology
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Immune depression in *Rhodnius prolixus* by seco-steroids, physalins

D.P. Castro[a], M.B. Figueiredo[a], I.M. Ribeiro[b], T.C.B. Tomassini[b], P. Azambuja[a,*], E.S. Garcia[a]

[a]*Laboratório de Bioquímica e Fisiologia de Insetos, Instituto Oswaldo Cruz , Fundação Oswaldo Cruz, Av. Brasil 4365, Rio de Janeiro, RJ 21045 900, Brazil*
[b]*Far Manguinhos, Fiocruz, Av. Brasil 4365, Rio de Janeiro, RJ 21045 900, Brazil*

Received 31 July 2007; received in revised form 4 December 2007; accepted 7 December 2007

Abstract

A comparative study of the effects of physalins, seco-steroidal substances of *Physalis angulata* (Solanaceae), on the immune reactions of *R. prolixus* was carried out. Ecdysis and mortality were not affected by treatment with physalins B, D, F or G (1–10 μg/ml of blood meal). *R. prolixus* larvae fed with blood containing physalins and inoculated with 1 μl of *Enterobacter cloacae* β12 (5×10^3/insect) exhibited mortality rates three times higher than controls. The insects treated with physalin B, and F (1 μg/ml) and inoculated with *E. cloacae* β12 showed significant differences on lysozyme activity in the hemolymph compared to untreated insects. Furthermore, physalin D (1 μg/ml) significantly reduced the antibacterial activity. Concerning cellular immune reactions, all insects treated with physalins (1 μg/ml), exhibited drastic reductions in the quantity of yeast cell–hemocyte binding and subsequent internalization. Insects inoculated with bacteria and treated with physalins B, F and G showed reductions of microaggregate formation but physalin D did not. Physalins B and F also reduced total hemocyte count in the hemolymph. These results suggest that, in different ways, probably due to their different chemical structures, physalin B, D, F and G are immunomodulatory substances for the bloodsucking insect, *R. prolixus*.

Keywords: Seco-steroids; *Rhodnius prolixus*; *Physalis angulata* L; Physalin; Immune response

1. Introduction

Some species of insect belonging to the subfamily Triatominae, family Reduviidae, are vectors of the protozoan parasite *Trypanosoma cruzi*, the causative agent of Chagas' disease, also called American trypanosomiasis, which is endemic in large areas of Latin America (for review see Garcia et al., 2007). Chagas' disease is responsible for the deaths of around 18,000 people a year and may affect about 9 million people, with about 40 million people living in what are considered to be high risk zones, and approximately 300,000 new cases occur every year (Schofield, 2006; Moncayo, 2006).

A group of substances with a seco-steroidal chemical structure, known as physalins, are found in *Physalis angulata* that belongs to the Solanaceae family. Physalin B has been proposed as a biogenetic precursor for all physalins isolated from this plant. Physalins D, F and G also used in our study, vary in the presence of a hydroxyl or epoxy group in ring B (C-5 and C-6) (Fig. 1) (Kuo et al., 2006). This plant is widely distributed throughout the tropical and subtropical regions of the world. Extracts from *P. angulata* have been used in different countries in popular medicine as a treatment for a variety of illnesses (Di Stasi et al., 1989). Physalins have been described as having potent antimycobacterial and antitumoral effects and potent cytotoxic activity against cancer cells (Chiang et al., 1992a, b; Lin et al., 1992; Januário et al., 2002; Kuo et al., 2006; Damu et al., 2007). A more recent study has demonstrated that physalins may also have anti-inflammatory activities in macrophages, as assessed by a marked inhibitory action on NO production and prevention of the lethality associated with lipopolysaccharide injection in mice (Soares et al., 2003). The *in vivo* anti-inflammatory actions of physalins are mostly due to activation of the

*Corresponding author. Tel.: +55 21 3865 8134; fax: +55 21 2590 3495.
E-mail address: azambuja@ioc.fiocruz.br (P. Azambuja).

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doi:10.1016/j.jinsphys.2007.12.004

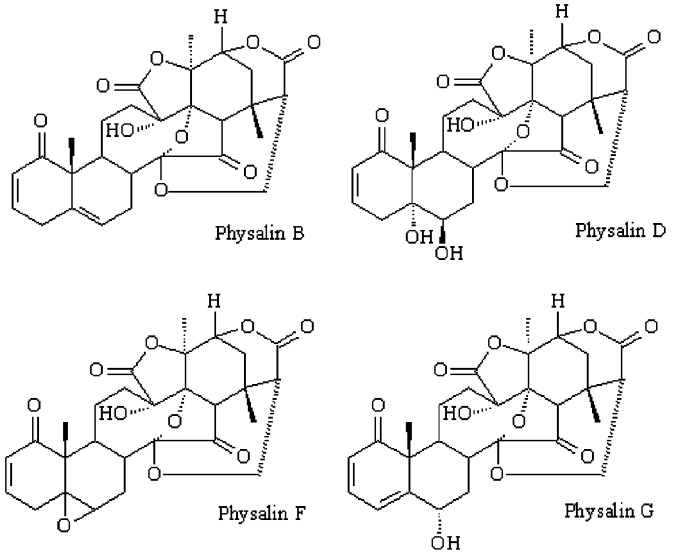

Fig. 1. Structures of physalins B, D, F and G.

glucocorticoid receptor (Vieira et al., 2005) and these compounds inhibit lymphocyte function and allogeneic transplant rejection (Soares et al., 2006).

Since many aspects of physiological processes in mammals are affected by physalins (Soares et al., 2003; Vieira et al., 2005), we hypothesized that physalins would also be able to modify the immune reactions in response to bacterial infection of *Rhodnius prolixus*. Also given the medical importance of this triatomine to the epidemiology of Chagas' disease we believe that these compounds may be used as a suitable and interesting biological model for investigating insect immunology. *R. prolixus* is capable of mounting both strong humoral and cellular immune responses against invading pathogens (Azambuja et al., 1991, 1999; Garcia et al., 2004a, b; Figueiredo et al., 2006). Though these defensive responses protect the insects effectively from many invaders, some microbes are highly infectious and able to overcome innate immune reactions. For example, we have shown that oral infection with *T. rangeli* reduces the phenoloxidase (PO) (Gomes et al., 2003) and hemocyte microaggregation reactions in *R. prolixus* larvae (Garcia et al., 2004a, b). On the other hand, previous results from our laboratory provide evidence that physalin B caused a reduction in hemocyte microaggregation and nitric oxide production in *R. prolixus* larvae with a systemic infection with *Trypanosoma rangeli* and we suggested that physalin B has a potential immunomodulatory activity for triatomines (Garcia et al., 2006).

Thus, the current study focuses on a comparative study of physalin B, D, F and G (Fig. 1) on the humoral and cellular immune reactions of *R. prolixus*. In particular, we investigated the effects of physalins on the immune reactions comprising lysozyme and antibacterial activities, hemocyte phagocytosis and microaggregate formation in response to foreign invaders in the hemolymph of *R. prolixus*.

2. Material and methods

2.1. Insects

Fifth-instar *R. prolixus* larvae were used throughout these studies. After molting, insects were starved for 15–20 days and were randomly chosen when required. All insects were raised and maintained as previously described (Garcia et al., 1984) feeding on defibrinated rabbit blood through a membrane feeding apparatus (Azambuja and Garcia, 1997).

2.2. Microorganisms

Enterobacter cloacae strain β12 and *Escherichia coli* strain D31 (gifts of Dr. H. Boman, Sweden) were maintained and aerobically grown at 37 °C in liquid brain-heart (BHI) medium. *E. cloacae* β12 used for inoculation of the insects was taken from 2-h-old BHI medium cultures. *E. coli* D31 was used to test antibacterial activity. *Saccharomyces cerevisiae*, dried baker's yeast cells kindly donated by Dr. Norman Ratcliffe, were diluted at a concentration of 5% in phosphate buffer-saline (PBS), autoclaved at 120 °C for 15 min and kept at 4 °C for a maximum period of 30 days prior to usage. For experiments heat-killed yeast cell suspensions were prepared as described by Figueiredo et al. (2006).

2.3. Physalin purification

Physalins B, D, F and G were purified from stems of dried *P. angulata* plants collected in Belém do Pará, Brazil. *P. angulata* was identified by Dr. Lucia Carvalho from the Rio de Janeiro Botanical Garden. A voucher specimen is held under number RFA 23907/8 at the Federal University of Rio de Janeiro, Brazil. Purification was carried out according to the previously described methodology (Soares et al., 2003). The purity of physalins was determined by the HPLC technique and the average range was between 96% and 98% for the *seco*-ergostane derivatives. Physalins are stable at room temperature (30–33 °C). They were kept in an amber flask and are soluble in ethyl acetate and methanol. Preparations of pure physalins were dissolved in ethanol and diluted in the blood meal.

2.4. Insect hemocoelic inoculation

R. prolixus fifth-instar larvae weighing 35.6 ± 3.4 mg were allowed to feed on blood containing physalin B, D, F and G. All experiments were performed only with fully gorged insects weighing 212.2 ± 12.3 mg. After a period of 5 days of feeding, the insects were inoculated laterally in the thorax with 1 µl of *E. cloacae* β12 suspension at a concentration 5×10^6 bacteria/ml in sterile culture medium using a 30 gauge hypodermic needle (BD PrecisionGlide) adapted to a 10 µl Hamilton syringe. The experiments were performed with three control groups: fed with blood

containing 1 μl ethanol per ml (control 1); fed with blood containing ethanol and inoculated with sterile culture medium (control 2); fed with blood containing ethanol and inoculated with *E. cloacae* β12 (control 3); fed with blood containing physalins and inoculated with sterile culture medium (control 4).

2.5. Mortality in infected insects

To evaluate mortality caused by *E. cloacae* β12 infection in insects that had been previously fed with blood containing physalin B, D, F and G at a dosage of 1 μg/ml of blood meal, we inoculated groups of insects with bacteria (infected groups) at day 3 after feeding and observed the mortality on days 1, 2 and 3 post-infection. A control group treated with ethanol received an injection of sterile culture medium (control 2). To simplify, only cumulative values at day 3 post-infection are reported. After this period, mortality was negligible.

2.6. Antibacterial assay

R. prolixus hemolymph samples were collected carefully from a severed leg with 5-μl calibrated micropipettes (Sigma). Individual samples of hemolymph were collected 3 and 6 days after inoculation with *E. cloacae* β12 and diluted in a proportion of 1:1 of calcium–cacodylate buffer (0.01 M sodium cacodylate, 0.01 M $CaCl_2$, pH 7.4). Plates were prepared using agarose 1% in liquid culture medium (BHI) containing streptomycin (100 μg/ml) and ampicillin (80 μg/ml), seeded with *E. coli* D31. Antibacterial activity in hemolymph samples (2 μl) was recorded as the percentage of insects that presented diameters of growth inhibition zones higher than 4 mm including the wells (2 mm) in the agarose plates and incubated at 37 °C for 24 h.

2.7. Lysozyme activity

Hemolymph was collected 3 and 6 days after inoculation with *E. cloacae* β12 and diluted in a proportion of 1:1 calcium–cacodylate buffer (0.01 M sodium cacodylate, 0.01 M $CaCl_2$, pH 7.4). Aliquots of 10 μl of hemolymph were incubated with 100 μl of *Micrococcus lysodeikticus* dried cells at 37 °C for 60 min. Turbidity was measured spectrophotometrically at 492 nm. The enzyme unit was expressed as a percentage of activity measured by A_{492}/mg protein considering the highest clearance among samples as 100% of activity.

2.8. Protein determination

Proteins of hemolymph samples were quantified with a protein assay kit (BCA Protein Assay Reagent, Pierce, USA) using bovine serum albumin (BSA) as the standard.

2.9. Total circulating hemocyte and microaggregation counts

Hemolymph, collected 3 and 6 days after inoculation, was immediately diluted in anticoagulant solution (0.01 M ethylenediamine tetraacetic acid, 0.1 M glucose, 0.062 M sodium chloride, 0.026 M citric acid, pH 4.6) as described by Azambuja et al. (1991). At different times after inoculation, total circulating hemocyte numbers and hemocyte microaggregates (operationally defined as a cluster of five or more cells—larger than 100 μm in diameter) were determined in each sample by direct observation in a hemocytometer chamber by phase-contrast optical microscopy.

2.10. Preparation of FITC-labeled yeast for phagocytosis assay

The method described by Rohloff et al. (1994), as modified by Figueiredo et al. (2006) was used. Basically, yeast autoclaved cells suspended in insect saline were washed three times with the same sterile solution and the pellet was finally resuspended in carbonate buffer (0.2 M Na_2CO_3, 0.2 M$NaHCO_3$, pH 9.4). The sample suspensions were adjusted to give a concentration of 5.0×10^8 yeast cells/ml in carbonate buffer containing fluorescein isothiocyanate (FITC) 0.1 mg/ml and incubated for 30 min in the dark at room temperature (range of 22–27 °C) in a rotary mixer. The solution was then washed three times with insect saline to remove all traces of free FITC and then the conjugated FITC-yeast resuspended in sterile Grace's insect medium (GIM) (Gibco-BRL) at a concentration of 1×10^8 cells/ml was stored at −20 °C until application.

2.11. Hemocyte monolayers and phagocytosis assay

Hemocyte monolayer were prepared as previously described by Figueiredo et al. (2006). Basically, hemolymph from physalin treated insects or ethanol treated insects (control 1) was collected in a glass micropipette and applied onto 12 mm diameter round glass cover slips, containing sterile insect saline with traces of phenylthiourea (PTU), located at the bottom of plates composed of 24-wells (Corning) and incubated at room temperature (range of 22–27 °C) for 60 min. Each cover slip contained 20 μl of hemolymph pool (2 insects) and the same volume of insect saline–PTU solution. The monolayer was gently rinsed three times in 250 μl of sterile insect saline to remove non-attached cells. Immediately after washing, 250 μl of FITC-labeled yeast suspension in GIM solution (1×10^8 cells/ml) was added to each well and incubated for 1 h at room temperature (range of 22–27 °C). Non-adherent yeast cells were rinsed from the cover slips three times using insect saline. Non-internalized yeast cells were quenched with insect saline containing 1.4 mg/ml trypan blue for 15 min according to the method described by Asgari and Schmidt (2003). After washing three times with

insect saline, cell monolayers were then fixed with 2.5% glutaraldehyde in insect saline for 60 min at room temperature (range of 22–27 °C). The cover slips were washed three times in insect saline and kept in a humid chamber before being inverted onto glass slides and studied microscopically. To quantify phagocytic activity for each treatment five cover slips (50 cells) were examined totaling 250 cells/treatment. The numbers of associated and internalized yeast cells were determined through hemocyte images from a Nikon Eclipse E200 microscope. Each field phase-contrast was alternated with visualization of internalized yeast cells under indirect UV illumination.

2.12. Statistical analyses

For all experiments conducted, the results were analyzed using ANOVA and the Student–Newman–Keuls (SNK) multiple range test for comparison of means according to SPSS version 12.0 for Windows. Differences between samplings were considered not statistically significant at a probability more than 5% ($p > 0.05$). Probability levels are specified in the text.

3. Results

3.1. Control experiments

Before initiating the experiments, we conducted a series of general control experiments and assessments to ensure that oral treatment with physalin B, D, F and G, at doses ranging from 1 to 10 μg/ml of blood meal, and, inoculation with sterile culture medium did not affect feeding, developmental processes related to ecdysis, death rate or hemocyte numbers. First, we observed by determining the body weight just before and after feeding that physalins did not have any anti-feeding effect on fifth-instar larvae (not shown). Also, when fifth-instar larvae were treated orally at all concentrations of physalins, 100% of the treated insects were able to molt between 14 and 22 days after feeding, as was also the case for insects fed on blood with ethanol control, and also non-inoculated insects (control 1). Comparing mortality at day 25 after blood feeding, we observed that the death rate was less than 10% for all physalin-treated and non-treated groups. Finally, treating insects with 1 μg/ml of all physalins did not interfere with morphology of freely circulating hemocytes, compared to any of the controls. However insects treated with physalins at a dose of 10 μg/ml displayed altered hemocyte morphology, and when inoculated with *E. cloacae* β12 presented mortality higher than 85% (not shown). Therefore, we standardized the dose of 1 μg/ml for all physalins tested in our comparative studies.

3.2. Physalin B, D, F and G and mortality of infected insects

We evaluated the mortality of insects that had been previously fed with blood containing 1 μg/ml of physalins in the blood meal and inoculated with a suspension of *E. cloacae* β12, comparing with insects fed with blood containing diluent and inoculated with sterile culture medium (control 2) or bacteria (control 3) (Table 1). The mortality in the treated groups was significantly higher (more than 60%) than the control groups treated with ethanol and infected with bacteria (control 3) (21.2% mortality) at day 3 post-feeding than that for the group treated with ethanol and inoculated with sterile culture medium (control 2) (19.9%).

3.3. Physalins and antibacterial activity

In this study, we inoculated live *E. cloacae* β12 into *R. prolixus* treated or not with different physalins and followed the percentage of insects with growth inhibition zone higher than 4 mm in individual hemolymph taken 3 and 6 days after inoculation. As expected, hemolymph taken from insects treated with ethanol and inoculated with *E. cloacae* β12 (control 3) presented a significantly higher percentage of insects with a positive antibacterial response 3 and 6 days after inoculation when compared to hemolymph from insects treated with ethanol but injected with sterile culture medium (control 2) ($p < 0.02$) (Fig. 2). The induction of antibacterial response obtained for the insects treated with different physalins was similar at day 3 ($p > 0.05$) to control 3, and was only significantly lower for physalin D at 6 days after inoculation of bacteria ($p < 0.02$) (Fig. 2).

3.4. Physalins and lysozyme activity

Experiments were performed with hemolymph taken from individual *R. prolixus* larvae treated with ethanol or physalins inoculated or not with *E. cloacae* β12. Injecting insects with bacteria induced a significant increase in lysozyme activity compared to control insects treated with ethanol and inoculated with sterile culture medium (control 2) at both 3 and 6 days after inoculation ($p < 0.001$ and $p < 0.001$, respectively) (Fig. 3). Insects treated with

Table 1
Effects of physalins (1 μg/ml) on the mortality of fifth-instar larvae of *Rhodnius prolixus* previously fed with blood containing the compounds and inoculated with *Enterobacter cloacae* β12

Treatment	Inoculation	Mortality (%)	
		Mean	Range
Ethanol (control 2)	Culture medium	19.9	15.0–24.8
Ethanol (control 3)	*E. cloacae* β12	21.2	10.9–31.5
Physalin B	*E. cloacae* β12	77.6	76.0–79.2
Physalin D	*E. cloacae* β12	66.7	54.0–79.5
Physalin F	*E. cloacae* β12	67.5	73.0–62.0
Physalin G	*E. cloacae* β12	60.7	52.9–68.5

Data represent cumulative mortality at day 3 after inoculation.
Physalins were previously diluted in ethanol.
Mean of 4 experiments ($N = 50$–80 insects for each compound).

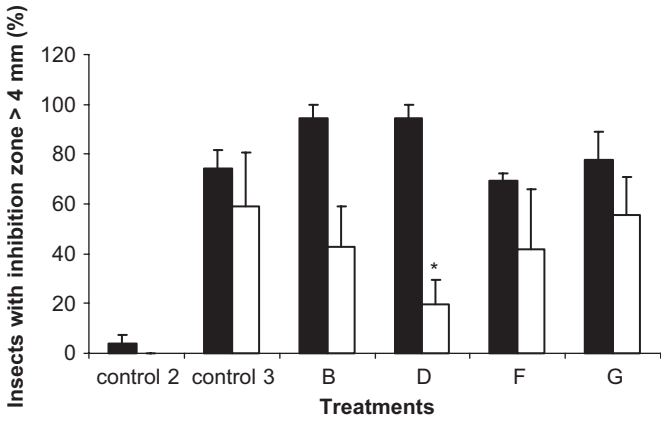

Fig. 2. Antibacterial activity of *Rhodnius prolixus* hemolymph from insects treated with ethanol (control) and treated with physalins B, D, F and G, and inoculated with *Enterobacter cloacae* β12. Antibacterial activity is represented as the percentage±SE of growth inhibition zone, defined as more than 4 mm, formed in each agarose plate cultured with *Escherichia coli* D31. Each agarose plate had at least 10 wells containing hemolymph from different individual insects of each treatment ($n = 10$). The experiments were repeated three times. Results observed from hemolymph taken after 3 days (black column) and 6 days (white column) after inoculation. Control 2—ethanol treated insects and inoculated with sterile culture medium. Control 3—ethanol treated insects inoculated with bacteria. B, D, F and G—insects treated with physalins and inoculated with bacteria. Significant results comparing groups treated with physalins and with control 3 in three and six days after inoculation are marked with an asterisk *($p<0.02$).

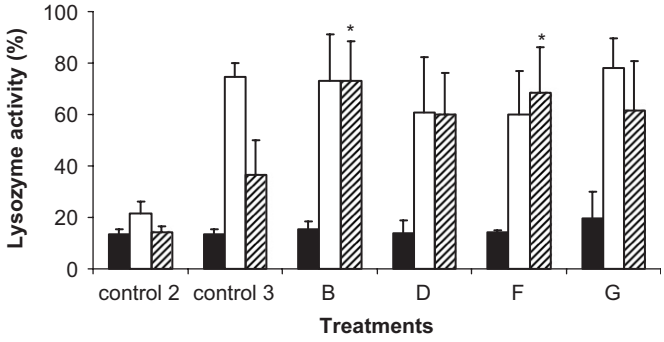

Fig. 3. Percentage of lysozyme activity of *Rhodnius prolixus* hemolymph taken at different days after inoculation with *Enterobacter cloacae* β12 or sterile culture medium. Results were observed from hemolymph collected at day zero (black column), day 3 (white column) and day 6 (shaded column) after inoculation. Control 2—ethanol treated insects inoculated with sterile culture medium; Control 3-ethanol treated insects inoculated with bacteria. B, D, F and G—insects treated with physalins and inoculated with bacteria. The enzyme unit was expressed as a percentage of activity measured by A_{492}/mg protein considering the highest clearance among samples as 100% of activity (15–20 insects per each point). Significant results comparing groups treated with physalins and with control 3 are marked with an asterisk * ($p<0.005$ for physalin B and $p< 0.05$ for physalin F at 6 days after inoculation).

physalins and inoculated with bacteria at 3 days after inoculation showed similar levels of lysozyme activity to controls given ethanol and then inoculated with bacteria (control 3) being values not significantly different among these treatments on day 3 ($p>0.1$) (Fig. 3). However, 6 days after inoculation hemolymph of insects treated with physalins B and F showed significant differences when comparing to hemolymph from untreated insects (control 3) ($p<0.005$, $p<0.05$, respectively) (Fig. 3).

3.5. Physalins and circulating hemocytes and formation of microaggregates

Feeding *R. prolixus* larvae with physalins B and F (1.0 μg/ml) prior to inoculation with *E. cloacae* β12 suspension in *R. prolixus* larvae caused a significant reduction in the number of circulating hemocytes (THC) at 3 days after inoculation ($p<0.01$ and $p<0.02$) when compared to ethanol treated insects inoculated with bacteria (control 3). Despite some fluctuations in THC following prior treatments with physalins D and G, there was no significant change in the total number of circulating hemocytes compared to controls at day 3 ($p>0.1$) (Fig. 4A). Insects previously fed with blood containing solvent or physalins B, D, F and G inoculated with bacteria demonstrated similar THC values in hemolymph collected 6 days after inoculation ($p>0.1$). A low THC value was observed at days 3 and 6 after inoculation with insects fed with blood containing solvent and injected with sterile culture medium (control 2) (Fig. 4A).

As shown in Fig. 4B a significant hemocyte microaggregation number was observed 3 days after inoculation in the control insects fed with blood containing solvent alone and injected with bacteria (control 3) in contrast to those displayed by infected insects that had fed with blood containing physalin B, F and G ($p<0.001$, $p<0.0001$ and $p<0.0001$, respectively). Physalin D had no effect on microaggregate formation at 3 days after inoculation ($p>0.1$) (Fig. 4B). The same results showing significant differences for physalins B, F and G ($p<0.0001$, $p<0.0001$ and $p<0.0001$, respectively) and no significant effect of physalin D ($p>0.05$) were observed at 6 days after bacteria inoculation compared with the group treated with ethanol and inoculated with bacteria (control 3) (Fig. 4B). Negligible numbers of hemocyte microaggregates were formed in the hemolymph during the entire experiment when physalin-treated insects (not shown) or insects that had been fed with blood containing solvent and inoculated with sterile culture medium (control 2) (Fig. 4B). Oral treatment with all physalins B, D, F and G (1 μg/ml), inoculated or not with sterile culture medium, did not interfere with hemocyte morphology.

3.6. Physalins and hemocyte phagocytosis

Phagocytosis assays performed with hemocyte monolayers demonstrated that the number of yeast cells associated with hemocytes was different according to the previous treatment of the hemolymph donor insects. Taking into account a quantity equal or higher than 15 yeast cells bound to hemocytes, there was a significant reduction in the groups treated with physalin B, D, F and G

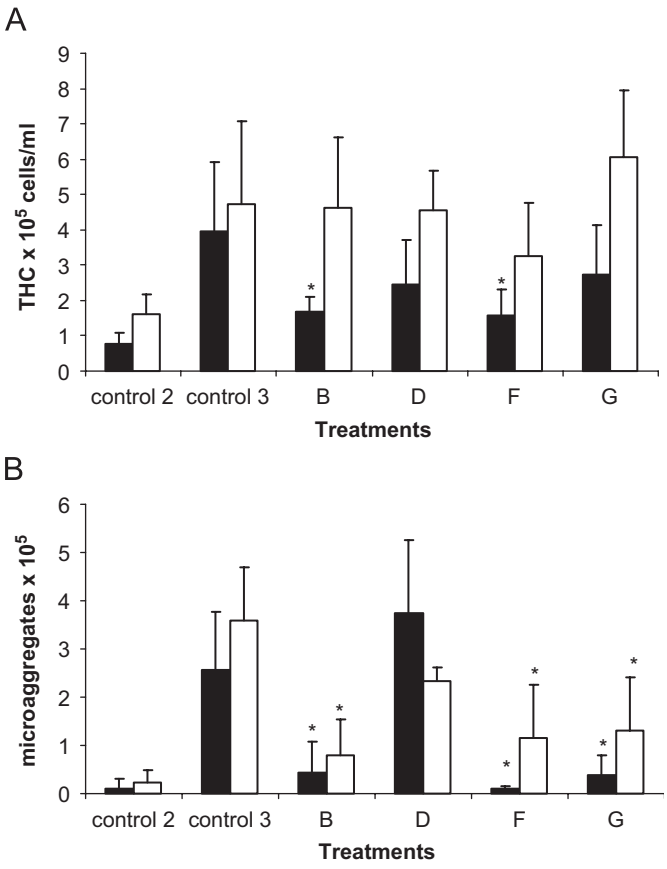

Fig. 4. Total circulating hemocyte counts (A) and microaggregates (B) of *Rhodnius prolixus* hemolymph from insects treated with ethanol and treated with physalins and inoculated with *Enterobacter cloacae* β12. Results observed from hemolymph taken after 3 days (black column) and 6 days (white column) of inoculation. Control 2—ethanol treated insects inoculated with sterile culture medium; Control 3—ethanol treated insects inoculated with bacteria. B, D, F and G—insects treated with physalins and inoculated with bacteria. Values represent means ± SD of the number of circulating hemocytes and hemocyte microaggregation, per milliliter of hemolymph of 10–15 insects per each point. (A) Significant differences between groups treated with physalins and with control 3 in three and six days after inoculation are marked with an asterisk* ($p<0.01$ for physalin B and $p<0.02$ for physalin F). (B) Significant differences between groups treated with physalins and with control 3 are marked with an asterisk * in day 3 ($p<0.001$ for physalin B and $p<0.0001$ for physalins F and G) and 6 after inoculation ($p<0.0001$ for physalins B, F and G).

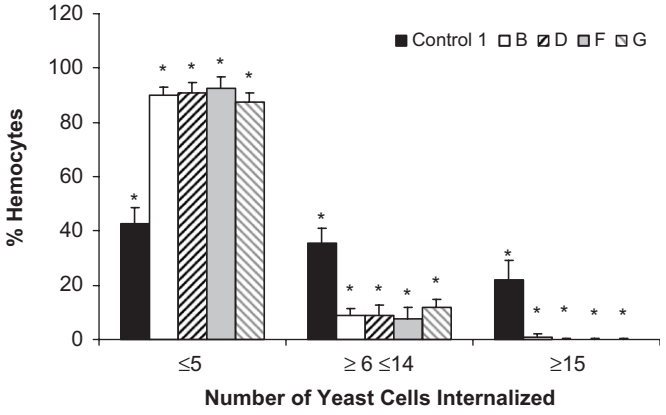

Fig. 5. Number of internalized yeast cells of *Rhodnius prolixus* fifth instar larvae hemolymph from insects treated with ethanol and treated with physalins at 5 days after feeding. After treatment, hemocyte monolayers were incubated with 1×10^7 FITC conjugated yeast cells/ml for 60 min. Control 2—ethanol treated insects inoculated with sterile culture medium. B, D, F and G—insects treated with physalins and inoculated with bacteria. Values represent means ± SD of 10 monolayers. Significant differences between phagocytosis from groups control 1 and treated with physalins B, D, F and G are indicated by an asterisk *. *p*-Values for these differences were $p<0.0001$ (hemocytes phagocytosing ⩽5 yeast cells); $p<0.0001$ (hemocytes phagocytosing ⩾6⩽14 yeast cells); $p<0.001$ (hemocytes phagocytosing ⩾15 yeast cells).

when compared to ethanol treated, non-inoculated insects (control 1) (not shown). A similar significant result was obtained for internalization of yeast cells: more than 20% of hemocytes phagocytosed 15 or more yeast cells per hemocyte in the control group, whereas in the case of insects previously treated with physalins, only a few yeast cells were internalized per hemocyte ($p<0.001$) (Fig. 5). Because the consistently high percentage of hemocytes on a monolayer ingesting five or less yeast cells per hemocyte in the groups of insects that fed blood with physalin B, F, G and D, these groups differed significantly from the ethanol treated insects non-inoculated (control 1) ($p<0.0001$) (Fig. 5). Considering the dataset representing 6–14 internalized cells per hemocyte on a monolayer, the results were very similar but there were significant differences among the groups treated with physalins and the ethanol treated, non-inoculated insects ($p<0.0001$) (Fig. 5). There was no significant difference in cell viability between monolayers prepared with hemocytes of physalins B, D, F and G treated insects and those from control insects.

4. Discussion

Several compounds found in plants, often called secondary chemicals, interfere with specific physiological functions in *R. prolixus*, including feeding, development and reproduction (Garcia and Azambuja, 2004). In the present paper we demonstrated that physalins B, D, F and G administered by oral route at a concentration of 1 μg/ml blood meal did not alter the amount of blood ingested. Similarly, exposure of the insects to all physalins, inoculated or not with sterile culture medium, did not influence development, processes associated with ecdysis, mortality or hemocyte morphology. However, physalins B, D, F and G caused diverse effects on humoral and cellular immune responses and mortality of fifth-instar larvae of *R. prolixus* challenged with the bacteria *E. cloacae* β-12. The latter observation confirms and extends our first studies on physalin B in *R. prolixus* infected with the parasite *T. rangeli* (Garcia et al., 2006).

To assess the effects of physalins on humoral immune reactions to bacterial infection of *R. prolixus*, we estimated lysozyme and antibacterial activities in the inoculated hemolymph. There was significant effect of physalins B and F treatments on lysozyme activity in response to treatment with *E. cloacae* β12 at day 6. While physalins B, F and G

had no effect on antibacterial activity at either 3 or 6 days after infection, prior exposure to physalin D significantly reduced antibacterial activity in the insect hemolymph at day 6 not at day 3.

The immune depression by physalins was evidenced by the inhibition of hemocyte microaggregation and phagocytosis by some types of physalins. Physalins B, F, and G drastically blocked the hemocyte microaggregation induced by inoculation of *E. cloacae* β-12, while physalin D had no effect on this cellular reaction. On the other hand, physalin B, D, F and G significantly inhibited hemocyte internalization of yeast particles, indicating that hemocyte microaggregation may have different regulatory pathways for physalin D, but not phagocytosis. The extra hydroxyl group presented in physalin D structure might explain the differences in its action.

Hemocyte microaggregation responses in insects are triggered by eicosanoids (Miller and Stanley, 2004) and the platelet-activating factor (PAF) (Machado et al., 2006), thus the effects of physalins B, F, and G could be explained by a direct action of these substances on hemocytes, in some way blocking the effects of eicosanoids and PAF. Since also hemocyte phagocytosis is dependent on eicosanoids (Winter et al., 2007) and PAF (Figueiredo et al., 2007), a similar hypothesis could be extended to the effect of physalins B, D, F, and G on hemocyte phagocytosis. The activities of physalins described in this and in a previous work (Garcia et al., 2006) suggest that some of the effects are in part due to a general action of these secosteroids within the immune system of animals (Soares et al., 2003, 2006; Vieira et al., 2005).

Although in this paper we did not attempt to distinguish the mechanism of hemocyte microaggregation and phagocytosis utilized by the insects against bacteria, there is evidence, for example, that physalins could act through a mechanism similar of dexamethasone (Soares et al., 2006). This issue is currently under investigation.

In conclusion, our results indicate that physalin administration may be a useful tool to study the relevance of the humoral and cellular immune response for the insects. Its use will be especially relevant since these phytosteroids have no toxic effects *per se* and only express their effects after infection with some microorganisms.

Acknowledgments

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) to E.S.G. and P.A., Fundação Oswaldo Cruz (Papes project to P.A.), and Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ, to P.A.). E.S.G. and P.A. are CNPq Senior Research Fellows. D.P.C. is a post-graduate student with FAPERJ scholarship and M.B.F. is a post-graduate student from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

References

Asgari, S., Schmidt, O., 2003. Is cell surface calreticulin involved in phagocytosis by insect hemocytes? Journal of Insect Physiology 49, 545–550.

Azambuja, P., Garcia, E.S., 1997. Care and maintenance of triatomine colonies. In: Crampton, J.M., Beard, C.B., Louis, C. (Eds.), Molecular Biology of Insect Disease Vectors: A Method Manual. Chapman & Hall, London, pp. 56–64.

Azambuja, P., Garcia, E.S., Ratcliffe, N.A., Warthen Jr., J.D., 1991. Immune-depression in *Rhodnius prolixus* induced by the growth inhibitor, Azadirachtin. Journal of Insect Physiology 37, 771–777.

Azambuja, P., Feder, D., Mello, C., Gomes, S., Garcia, E.S., 1999. Immunity in *Rhodnius prolixus*: trypanosomatid-vector interactions. Memórias Instituto Oswaldo Cruz 94 (Suppl. 1), 219–222.

Chiang, H.C., Jaw, S.M., Cehn, P.M., 1992a. Inhibitory effects of physalin B and physalin F on various human leukemia cells in vitro. Anticancer Research 23, 1155–1162.

Chiang, H.C., Jaw, S.M., Cehn, P.M., Kan, W.S., 1992b. Antitumor agent, physalin F from *Physalis angulata* L. Anticancer Research 12, 837–843.

Damu, A.G., Kuo, P.C., Su, C.R., Kuo, T.H., Chen, T.H., Bastow, K.F., Lee, K.H., Wu, T.S., 2007. Isolation, structures, and structures–cytotoxic activity relationships of withanolides and physalins from *Physalis angulata*. Journal of Natural Products 70, 1146–1152.

Di Stasi, L.C., Santos, E.M.G., Moreira dos Santos, C., Hiruma, C.A., 1989. In Plantas Medicinais da Amazônia; Editora Unesp: São Paulo, pp. 45–46.

Figueiredo, M.B., Castro, D.P., Nogueira, N.F.S., Garcia, E.S., Azambuja, P., 2006. Cellular immune response in *Rhodnius prolixus*: role of ecdysone in hemocyte phagocytosis. Journal of Insect Physiology 52, 711–716.

Figueiredo, M.B., Garcia, E.S., Azambuja, P., 2007. Blockades of phospholipase A(2) and platelet-activating factor receptors reduce the hemocyte phagocytosis in *Rhodnius prolixus*: in vitro experiments. Journal of Insect Physiology.

Garcia, E.S., Azambuja, P., 2004. Lignoids in insects: chemical probes for the study of ecdysis excretion and *Trypanosoma cruzi*–triatominae interactions. Toxicon 44, 431–440.

Garcia, E.S., Azambuja, P., Contreras, V.T., 1984. Large-scale rearing of *Rhodnius prolixus* and production of metacyclic trypomastigotes of *Trypanosoma cruzi*. In: Morel, C.M. (Ed.), Genes and Antigens of Parasites, A Laboratory Manual. UNDP/World Bank/WHO and Fundação Oswaldo Cruz, Rio de Janeiro, pp. 43–46.

Garcia, E.S., Machado, E.M., Azambuja, P., 2004a. Effects of eicosanoid biosynthesis inhibitors on the prophenoloxidase-activating system and microaggregation reactions in the hemolymph of *Rhodnius prolixus* infected with *Trypanosoma rangeli*. Journal of Insect Physiology 50, 157–165.

Garcia, E.S., Machado, E.M., Azambuja, P., 2004b. Inhibition of hemocyte microaggregation reactions in *Rhodnius prolixus* larvae orally infected with *Trypanosoma rangeli*. Experimental Parasitology 107, 31–38.

Garcia, E.S., Castro, D.P., Ribeiro, I.M., Tomassini, T.C., Azambuja, P., 2006. *Trypanosoma rangeli*: effects of physalin B on the immune reactions of the infected larvae of *Rhodnius prolixus*. Experimental Parasitology 112, 37–43.

Garcia, E.S., Ratcliffe, N.A., Whitten, M.M., Gonzalez, M.M., Azambuja, P., 2007. Exploring the role of insect host factors in the dynamics of *Trypanosoma cruzi–Rhodnius prolixus* interactions. Journal of Insect Physiology 53, 11–21.

Gomes, S.A.O., Feder, D., Garcia, E.S., Azambuja, P., 2003. Suppression of the prophenoloxidase system in *Rhodnius prolixus* orally infected with *Trypanosoma rangeli*. Journal of Insect Physiology 49, 829–837.

Januário, A.H., Filho, E.R., Pietro, R.C., Kashima, S., Sato, D.N., Franç a, S.C., 2002. Antimycobacterial physalins from *Physalis angulata* L. (Solanaceae). Phytotherapy Research 16, 445–448.

Kuo, P.C., Kuo, T.H., Damu, A.G., Su, C.R., Lee, E.J., Wu, T.S., Shu, R., Chen, C.M., Bastow, K.F., Chen, T.H., Lee, K.H., 2006. Organic Letters 8, 2953–2956.

Lin, Y.S., Chiang, H.C., Kan, W.S., Hone, E., Shih, S.J., Won, M.H., 1992. Immunomodulatory activity of various fractions derived from *Physalis angulata* L extract. American Journal Chinese Medicine 20, 233–243.

Machado, E.M., Azambuja, P., Garcia, E.S., 2006. Web 2086, a platelet-activating factor antagonist, inhibits prophenoloxidase-activating system and hemocyte microaggregation reactions induced by *Trypanosoma rangeli* infection in *Rhodnius prolixus* hemolymph. Journal of Insect Physiology 52, 685–692.

Miller, J.S., Stanley, D.W., 2004. Lipopolysaccharide evokes microaggregation reactions in hemocytes isolated from tobacco hornworms, *Manduca sexta*. Comparative Biochemistry and Physiology Part A 137, 285–295.

Moncayo, A., Ortiz Yanine, M.I., 2006. An update on Chagas disease (human American trypanosomiasis). Annual Tropical Medical Parasitology 100, 663–677.

Rohloff, Lutz-H., Wiesner, A., Götz, P., 1994. A fluorescence assay demonstrating stimulation of phagocytosis by haemolymph molecules of *Galleria mellonella*. Journal of Insect Physiology 40, 1045–1049.

Schofield, C.J., Jannin, J., Salvatella, R., 2006. The future of Chagas disease control. Trends Parasitology 22, 583–588.

Soares, M.B., Bellintani, M.C., Ribeiro, I.M., Tomassini, T.C., Ribeiros dos Santos, R., 2003. Inhibition of macrophage activation and lipopolysaccharide-induced death by seco-steroids purified from *Physalis angulata* L. European Journal of Pharmacology 459, 107–112.

Soares, M.B., Brustolim, D., Santos, L.A., Bellintani, M.C., Paiva, F.P., Ribeiro, Y.M., Tomassini, T.C., Ribeiro Dos Santos, R., 2006. Physalins B, F and G, seco-steroids purified from *Physalis angulata* L., inhibit lymphocyte function and allogeneic transplant rejection. International Immunopharmacology 6, 408–414.

Vieira, A.T., Pinho, V., Lepsch, L.B., Scavone, C., Ribeiro, I.M., Tomassini, T., Ribeiro-dos-Santos, R., Soares, M.B., Teixeira, M.M., Souza, D.G., 2005. Mechanisms of the anti-inflammatory effects of the natural secosteroids physalins in a model of intestinal ischaemia and reperfusion injury. Brazilian Journal Pharmacology 146, 244–251.

Winter, P., Rayne, R.C., Coast, G.M., 2007. The effects of intracellular signaling pathway inhibitors on phagocytosis by haemocytes of *Manduca sexta*. Journal of Insect Physiology 53, 975–982.

Journal of Insect Physiology 55 (2009) 532–537

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

Physalin B inhibits *Rhodnius prolixus* hemocyte phagocytosis and microaggregation by the activation of endogenous PAF-acetyl hydrolase activities

D.P. Castro [a], M.B. Figueiredo [a], F.A. Genta [a,c], I.M. Ribeiro [b], T.C.B. Tomassini [b], P. Azambuja [a,c,*], E.S. Garcia [a,c]

[a] *Laboratório de Bioquímica e Fisiologia de Insetos, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Av. Brasil 4365, Rio de Janeiro, 21045-900, RJ, Brazil*
[b] *FarManguinhos, Fiocruz, Av. Brasil 4365, Rio de Janeiro, 21045-900, RJ, Brazil*
[c] *Instituto Nacional de Entomologia Molecular, CNPq, Brazil*

ARTICLE INFO

Article history:
Received 27 October 2008
Received in revised form 12 January 2009
Accepted 26 January 2009

Keywords:
Rhodnius prolixus
Physalis angulata
Hemocyte
Microaggregation
Physalin
Phagocytosis
Arachidonic acid
PAF
Phospholipase A_2

ABSTRACT

The effects of physalin B (a natural secosteroidal chemical from *Physalis angulata*, Solanaceae) on phagocytosis and microaggregation by hemocytes of 5th-instar larvae of *Rhodnius prolixus* were investigated. In this insect, hemocyte phagocytosis and microaggregation are known to be induced by the platelet-activating factor (PAF) or arachidonic acid (AA) and regulated by phospholipase A_2 (PLA_2) and PAF-acetyl hydrolase (PAF-AH) activities. Phagocytic activity and formation of hemocyte microaggregates by *Rhodnius* hemocytes were strongly blocked by oral treatment of this insect with physalin B (1 μg/mL of blood meal). The inhibition induced by physalin B was reversed for both phagocytosis and microaggregation by exogenous arachidonic acid (10 μg/insect) or PAF (1 μg/insect) applied by hemocelic injection. Following treatment with physalin B there were no significant alterations in PLA_2 activities, but a significant enhancement of PAF-AH was observed. These results show that physalin B inhibits hemocytic activity by depressing insect PAF analogous (iPAF) levels in hemolymph and confirm the role of PAF-AH in the cellular immune reactions in *R. prolixus*.

1. Introduction

Insects are constantly exposed to microbial infections and to protect themselves they have developed powerful immune defense mechanisms similar to the innate immune responses of vertebrates. These mechanisms involve two broad categories: cellular and humoral immune responses. The former consists of encapsulation, nodulation, hemocyte microaggregation and phagocytosis of aggressive microorganisms by hemocytes. Humoral response comprises factors related to the recognition of invading microorganisms, melanization and coagulation and killing factors such as antimicrobial peptides (AMPs), reactive oxygen species and reactive nitrogen intermediates, including nitric oxide, prostaglandins and eicosanoids (Boman, 1998; Stanley, 2006).

Several aspects of physiological processes in mammals are affected by physalins, which are substances with secosteroidal chemical structures found in the plant *Physalis angulata* (Solanaceae) (Soares et al., 2003; Vieira et al., 2005). Physalins are described as having anti-inflammatory activities in macrophages, as assessed by a marked inhibitory action on NO production and prevention of lethality associated with lipopolysaccharide injections in mice (Soares et al., 2003). The *in vivo* anti-inflammatory actions of physalins are mostly due to the activation of the glucocorticoid receptor (Vieira et al., 2005) and these compounds inhibit lymphocyte function and allogeneic transplant rejection (Soares et al., 2006).

In *Rhodnius prolixus* we previously observed that physalin B, D, F, and G affected some immune reactions in responses to bacterial and *Trypanosoma rangeli* infections. We have shown that physalin B caused a reduction in hemocyte microaggregation and nitric oxide production in *R. prolixus* larvae with a systemic infection of *T. rangeli* (Garcia et al., 2006) and that physalins B, D, F and G significantly interfere in the cellular immune responses that occur when the insects were challenged by bacterial infection, so that the number of circulating hemocytes is depressed, and hemocyte microaggregation and phagocytosis are reduced (Castro et al., 2008).

Hemocyte microaggregation responses in insects are triggered by eicosanoids (Miller and Stanley, 2004; Stanley, 2006; Garcia et al., 2004a,b) and the platelet-activating factor (PAF) (Machado

* Corresponding author at: Laboratório de Bioquímica e Fisiologia de Insetos, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Av. Brasil 4365, Rio de Janeiro, 21045-900, RJ, Brazil. Tel.: +55 21 3865 8134; fax: +55 21 2590 3495.
E-mail address: azambuja@ioc.fiocruz.br (P. Azambuja).

0022-1910/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jinsphys.2009.01.013

et al., 2006). Hemocyte phagocytosis is also dependent on both eicosanoids (Winter et al., 2007; Figueiredo et al., 2008a) and PAF pathways (Figueiredo et al., 2008b). The first step in the biosynthesis of several lipid mediators, which are pivotal to the development of effective cellular immune responses, is catalyzed by phospholipase A_2 (PLA_2) which selectively liberates arachidonic acid and lyso-phospholipids that in the case of 1-O-alkyl-lyso-glyceryl-3-phosphocholine (lyso-PAF) is acetylated by the acetyl-coenzyme A (CoA)/lyso-PAF-acetyl transferase to generate PAF (Gijón et al., 2000; Rubin et al., 2006; Castro Faria Neto et al., 2005).

Recently, we showed that physalins significantly interfere with microaggregation of *Rhodnius* hemocytes when these insects are challenged by bacterial infection and that physalin B can also inhibits other cellular immune reactions such as the hemocyte phagocytosis (Castro et al., 2008). So we decided to investigate whether the reduction in hemocyte phagocytosis and microaggregate formation in *R. prolixus* treated by the oral route with physalin B is related to a potential inhibition of eicosanoid and PAF pathways. We observed using *in vitro* experiments that both cellular immune reactions are depressed in insects treated with physalin B. The inhibition induced by this compound is reverted by the addition of arachidonic acid and PAF. Interestingly, there is no inhibition of PLA_2 but PAF-acetyl hydrolase (PAF-AH) activity is enhanced by the treatment with physalin B. These data provide strong evidence in support of the hypothesis that the immunosuppressor effect of physalin B on the hemocyte phagocytosis and microaggregation is due primarily to an increase in PAF-AH and not by the inhibition of PLA_2 activity.

2. Materials and methods

2.1. Insects

Fifth-instar *R. prolixus* larvae were used throughout these studies. After molting, insects were starved for 15–20 days and were randomly chosen when required. All insects were raised and maintained as previously described (Garcia et al., 1984) being fed on defibrinated rabbit blood through a membrane feeding apparatus (Azambuja and Garcia, 1997).

2.2. Reagents

Platelet-activating factor (Sigma) was dissolved in 0.25% (w/v) bovine serum albumin (BSA) in insect saline (18 g L^{-1} D-glucose, 12.2 g L^{-1} KCl, 0.36 g L^{-1} $NaHCO_3$, 380 mOsm, pH 7.8) (Whitten et al., 2001), at a concentration of 10 μM. Arachidonic acid (5,8,11,14-eicosatetranoic acid) purchased from Sigma was dried, suspended in sodium bicarbonate 150 mM, and diluted in Grace's Insect Medium (GIM, Gibco-BRL) plus 0.25% (w/v) BSA dissolved in insect saline (1:1) to give a final concentration of 10 μM. The phospholipid substrates 1-palmitoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine (C_{12}-NBD-PC, Avanti Polar Lipids) and 1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-*sn*-glycero-3-phosphocoline (C_6-NBD-PC, Avanti Polar Lipids) were prepared above their critical micellar concentrations (Wittenauer et al., 1984). Vesicles were formed by the addition of ethanol (75 μl) to 2 mg of substrate followed by flushing with a Hamilton syringe into 2 mL of 0.16 M KCl (Batzri and Korn, 1973).

2.3. Microorganism

Saccharomyces cerevisiae, dried baker's yeast cells kindly donated by Dr. Norman Ratcliffe, were diluted at a concentration of 5% (w/v) in phosphate buffer-saline (PBS), autoclaved at 120 °C for 15 min and kept at 4 °C for a maximum period of 30 days prior to usage. For the experiments heat-killed yeast cell suspensions were prepared as described by Figueiredo et al. (2006).

2.4. Physalin purification

Physalin B was purified from stems of dried *P. angulata* plants collected in Belém do Pará, Brazil. *P. angulata* was identified by Dr. Lucia Carvalho from the Rio de Janeiro Botanical Garden. A voucher specimen is held under number RFA 23907/8 at the Federal University of Rio de Janeiro, Brazil. Purification was carried out according to the previously described methodology (Soares et al., 2003). The purity of physalin B was determined by HPLC and the average range was between 96% and 98% for the *seco*-ergostane derivatives. Physalin B is stable at room temperature (30–33 °C). It was kept in an amber flask and is soluble in ethyl acetate and methanol. Preparations of pure physalin B were dissolved in dimethyl sulfoxide (DMSO, Sigma) at 2 mg/mL and diluted in the blood meal to a final concentration of 1 μg/mL.

2.5. Insect hemocelic inoculation

R. prolixus 5th-instar larvae weighing 33.7 ± 3.1 mg were allowed to feed on blood containing physalin B (1 μg/mL). A control group was fed with blood containing 1 μL DMSO per mL of blood. All experiments were performed only with fully gorged insects weighting 194.2 ± 10.5 mg. Five days after feeding, the insects were inoculated via the thorax with 10 μg of arachidonic acid or 1 μg of PAF (prepared as described in Section 2.2) using a 10-μL Hamilton syringe.

2.6. Preparation of FITC-labeled yeast for phagocytosis assay

The method described by Rohloff et al. (1994) and modified by Figueiredo et al. (2006) was used. Basically, autoclaved yeast cells suspended in insect saline were washed three times with the same sterile solution and the pellet was finally resuspended in carbonate buffer (Na_2CO_3 0.2 M, $NaHCO_3$ 0.2 M, pH 9.4). Suspensions were then adjusted to a concentration of 5×10^8 yeast cells/mL in carbonate buffer containing fluorescein isothiocyanate (FITC) 0.1 mg/mL and incubated for 30 min in the dark at room temperature (range 22–27 °C) in a rotary mixer. After that, cells were washed three times with insect saline to remove all traces of free FITC and then the conjugated FITC-yeasts were resuspended in sterile GIM (Gibco-BRL) at a concentration of 1×10^8 cells/mL and stored at −20 °C until use.

2.7. Hemocyte monolayers and phagocytosis assay

Hemocyte monolayers were prepared as previously described by Figueiredo et al. (2006). Basically, hemolymph from arachidonic acid or PAF inoculated insects previously treated with physalin B or ethanol was collected with a glass micropipette and applied onto 12 mm diameter round glass cover slips, containing sterile insect saline with traces of phenylthiourea (PTU) and located at the bottom of 24-well plates (Corning), which were then incubated at room temperature (22–27 °C) for 60 min. Each cover slip contained 20 μL of hemolymph (pool from 2 insects) and the same volume of insect saline–PTU solution. The monolayer was gently rinsed three times with 300 μL of sterile insect saline to remove non-attached cells. Immediately after washing, 300 μL of FITC-labeled yeast suspension in GIM solution (1×10^8 cells/mL) was added to each well and incubated for 1 h at room temperature (22–27 °C). The non-adherent yeast cells were rinsed from the cover slips three times using insect saline. The non-internalized yeast cells were quenched with insect saline containing 1.4 mg/mL trypan blue for 15 min according to the method described by Asgari and Schmidt

(2003). After washing three times with insect saline the cell monolayers were fixed with 2.5% (w/v) glutaraldehyde in insect saline for 60 min at room temperature (22–27 °C). The cover slips were washed three times in insect saline and kept in a humid chamber before being inverted onto glass slides and studied microscopically.

2.8. Hemocyte microaggregation assay

Hemocyte microaggregation was assayed *in vitro* following the method of Miller and Stanley (2001) as adapted by Machado et al. (2006). In brief, a sterile 96-well, flat bottom polystyrene microtiter plate was used for each experiment (Becton, Dickinson and Co., Lincoln, NJ). Each well was preloaded with 25 μL of cold insect saline and then 20 μL of hemolymph suspension (approximately 6.4×10^5 cells, pool from 2 insects), which was collected with a glass micropipette rinsed with traces of PTU, was added to each well. The plate was maintained on ice. The hemolymph was taken after 3 h of arachidonic acid or PAF inoculation in control insects or insects previously fed with physalin B. The hemocyte preparations were then challenged by adding 5 μL of yeast cells suspended in GIM solution (1×10^5 cells/mL). Then the plate was incubated for 90 min at 26 °C on a rotary shaker at 100 rpm. After incubation, 10 μL of the hemocyte preparation was applied to a hemocytometer. The number of hemocyte microaggregates (operationally defined as a cluster of five or more cells larger than 100 μm in diameter) for each sample was determined by direct counting with phase-contrast optical microscopy immediately after yeast introduction (0 time) and again after 90 min of incubation.

2.9. Phospholipase A2 and PAF-acetyl hydrolase assays

Here the method of Kitsiouli et al. (1999) as modified by Figueiredo et al. (2008b) was used. Basically, C_{12}-NBD-PC and C_6-NBD-PC were used in a coupled assay as substrates for PLA_2 and PAF-AH activities, respectively. Samples containing 50 μL of crude hemolymph from the 5th-instar *R. prolixus* nymphs were combined with 1 mL ringer solution (7.6 g L^{-1} NaCl, 0.75 g L^{-1} KCl, 0.22 g L^{-1} $CaCl_2$, 0.19 g L^{-1} $MgCl_2$, 0.37 g L^{-1} $NaHCO_3$, 0.48 g L^{-1} NaH_2PO_4) and centrifuged at $5000 \times g$ for 2 min at 4 °C. Supernatants (cell-free hemolymph) were collected and the pelleted hemocytes were resuspended in 2 mL of 10 mM Tris–HCl buffer pH 8.0, containing 2 mM $CaCl_2$. The hemocytes were then sonicated with a tip sonifier for 15 s (Branson 450, output 1) and both samples (cell-free hemolymph and hemocyte lysate) were directly used in enzymatic assays. The centrifugation above may result in degranulation or loss of some hemocyte populations, and our supernatants may have contaminants from cellular origin. To check this possibility all hemocyte preparations were observed directly in a hemocytometer chamber by phase contrast optical microscopy. As no difference was observed in hemocyte number and morphology after centrifugation, we consider this contamination as negligible.

Aliquots of 0.2 mL of each sample were incubated in white 96-well microplates for 5 min at 37 °C and mixed with 10 μL of the C_{12}-NBD-PC solution (final concentration of 5 μM). Then fluorescence (excitation 475 nm, emission 535 nm) was measured at 1-min intervals for 30 min using a Gemini XPS Reader (Molecular Devices; cutoff 530 nm, PMT medium) (total PLA_2 activity). After this, 50 μL of an EDTA solution was added (final EDTA concentration of 10 mM) and readings were performed for an additional 30 min ($iPLA_2$ activity). Then, 10 μL of the C_6-NBD-PC solution was added (final concentration of 5 μM) and fluorescence was monitored for 30 min (PAF-AH activity). Ca^{2+}-dependent PLA_2 activity was calculated by the difference between total PLA_2 and $iPLA_2$ activities. Assays were carried out under conditions where activity was proportional to protein concentration and time. Controls showed that our hemocyte preparations do not show auto-fluorescence, and that Physalin B does not interfere with these measurements. The initial burst in fluorescence was not used in the calculations (Kitsiouli et al., 1999). Protein concentrations in samples were determined with a Bicinchoninic Acid Protein Assay Kit (PIERCE, Cat. No. 23225) and specific activities were expressed as Units/mg protein (U/mg), where each unit corresponds to the amount of enzyme that increases the fluorescence at a ratio of one Relative Fluorescence Unit (RFU) per minute.

2.10. Statistical analyses

For all experiments conducted, the results were analyzed using ANOVA and the Student–Newman–Keuls (SNK) multiple range test of comparison of means according to the SPSS Version 12.0 for Windows. Differences between samplings were considered statistically significant at a probability less than 5% ($p < 0.05$). Probability levels are specified in the text.

3. Results

3.1. Preliminary observations

Initially, we observed that in hemocyte preparations from *R. prolixus* larvae previously fed with blood containing only DMSO and then inoculated with the GIM plus 0.25% (w/v) BSA dissolved in insect saline (1:1) or insect saline plus BSA, the numbers of yeast cells internalized were similar to preparations from non-inoculated insects (not shown). When the percentage of phagocytosis by control hemocytes was significantly different from those obtained in our routine experiments, the hemocyte preparations were discarded. Also, PAF and arachidonic acid, prepared as described in Section 2 that was inoculated in the control insects or insects fed with physalin B, did not alter significantly the viability of the cells (not shown).

3.2. Inhibition of hemocyte phagocytosis by physalin B and its reversion by arachidonic acid and PAF

At first, we checked the effects of feeding *R. prolixus* with physalin B on the phagocytic activity of the hemocytes (Fig. 1). Considering a quantity equal to or higher than 15 yeast cells phagocytosed per hemocyte, there was a significant decrease in the group treated with physalin B alone ($5 \pm 2\%$) when compared to the control group ($53 \pm 6\%$) ($p < 0.0001$) (Fig. 1).

Administration of arachidonic acid or PAF counteracts the effects of physalin treatment, as here we could see percentages of highly phagocyting hemocytes (more than 15 yeast cells internalized) in these groups that are more similar to the control group ($40 \pm 6\%$ and $25 \pm 5\%$, respectively) than the physalin-treated insects (Fig. 1). Also, these percentages are significantly different from the values observed after the treatment with physalin B alone ($p < 0.001$).

Given the consistently high percentage of hemocytes on a monolayer ingesting 15 or more yeast particles in the three other groups, only in the insects treated with physalin B was there a significant number of hemocytes internalizing 5 or less yeast cells (59%) ($p < 0.0001$) (Fig. 1). Considering the number of yeast cells ingested by hemocytes on a monolayer of 6–14 cells, there were no significant differences among all groups ($p > 0.05$) (Fig. 1).

3.3. Inhibition of hemocyte microaggregation by physalin B and its reversion by arachidonic acid and PAF

Experiments in which *R. prolixus* larvae were previously fed blood plus physalin B demonstrated that the total free circulating

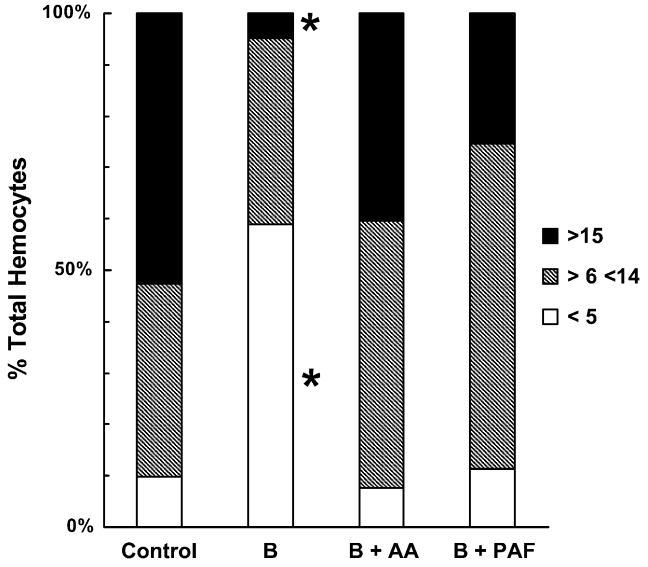

Fig. 1. Number of internalized yeast cells by hemocytes of *Rhodnius prolixus* 5th-instar larvae hemolymph from insects treated with DMSO or treated with physalin B and inoculated with arachidonic acid or PAF at 5 days after feeding. After treatment, hemocyte monolayers were incubated with 1×10^7 FITC-conjugated yeast cells/mL for 60 min. Control—DMSO-treated insects; B—insects treated with physalin B; AA—insects treated with physalin B and inoculated with arachidonic acid; PAF—insects treated with physalin B and inoculated with PAF. Values represent means of 10 monolayers. S.E.M. values are described in the text. Percentages significantly different from the respective other groups ($p < 0.05$) are marked with an asterisk.

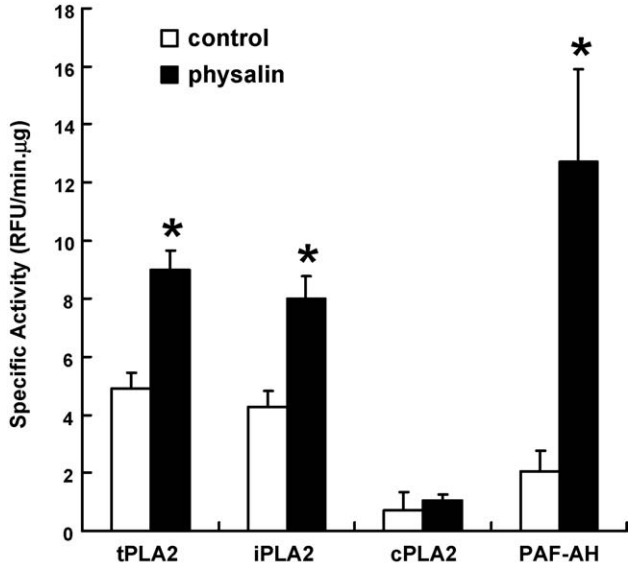

Fig. 3. Specific activities of total PLA2 (tPLA2), Ca^{2+}-independent PLA2 (iPLA2) and Ca^{2+}-dependent PLA2 (cPLA2) and PAF-acetyl hydrolase (PAF-AH) of samples from cell-free hemolymph of *R. prolixus* 5 days after regular blood feeding (control) or feeding with blood plus physalin B (for details see Section 2). Values are means ± S.E.M. from 10 samples containing cell-free hemolymph from three insects each. Values significantly different from controls ($p < 0.05$) are marked with an asterisk.

hemocytes count did not differ when compared to insects fed with blood containing DMSO, both inoculated with arachidonic acid or PAF, at any time (not shown).

The groups that had blood containing physalin B or which received blood with DMSO and were not challenged with yeast cells resulted in negligible numbers of hemocyte microaggregates ($p > 0.05$) (not shown). However, the group of insects fed with blood plus solvent and challenged with yeast cells and the groups of larvae treated with physalin B injected with arachidonic acid or PAF and then challenged with yeast cells, resulted in significantly higher numbers of hemocyte microaggregates ($2.2–2.6 \times 10^4$ cells/mL) than in the groups of insects fed with blood containing physalin B and challenged with yeast cells (0.8×10^4 cells/mL) ($p < 0.05$ for all three groups) (Fig. 2).

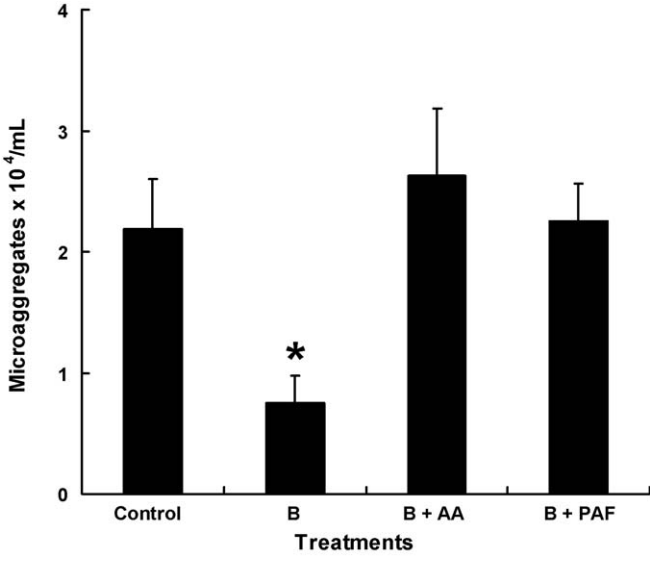

Fig. 2. Microaggregates of *Rhodnius prolixus* hemolymph from insects treated with DMSO or treated with physalin B and inoculated with arachidonic acid or PAF at 5 days after feeding. After treatment, hemocyte preparations were incubated with 1×10^5 yeast cells/mL for 60 min. Control—DMSO-treated insects; B—insects treated with physalin B; AA—insects treated with physalin B and inoculated with arachidonic acid; PAF—insects treated with physalin B and inoculated with PAF. Values represent means + S.E.M. of the hemocyte microaggregation, per milliliter of hemolymph of 10–15 insects per each point. Values significantly different from other groups ($p < 0.05$) are marked with an asterisk.

3.4. Phospholipase A_2 and PAF-AH activities in R. prolixus treated with physalin B

We measured the activities of PLA_2 and PAF-AH in cell-free plasma and hemocytes of control and physalin fed *R. prolixus* nymphs. Total plasmatic PLA_2 activity showed a significant ($p < 0.001$) increase (1.5×) with physalin treatment, mainly due to the increase (2×, $p < 0.001$) in the $iPLA_2$ form, as the $cPLA_2$ did not change significantly ($p > 0.05$) (Fig. 3). PAF-AH showed a dramatic increase in insects that were fed with physalin (6×, $p < 0.005$).

Surprisingly, the hemocyte PLA_2 activity was not affected by the physalin treatment (Fig. 4). Neither the total PLA_2 nor any of PLA_2 isoforms ($iPLA_2$ or $cPLA_2$) showed any significant differences between insects treated with physalin and the controls ($p > 0.05$) (Fig. 4). In these hemocyte samples a very low PAF-AH activity was detected and this enzyme was more active (3×) in the physalin-treated insects when compared to the controls ($p < 0.05$) (Fig. 4).

4. Discussion

Physalins have been described to have suppressive activities on immune systems of insects and mammals (Soares et al., 2003, 2006; Vieira et al., 2005; Garcia et al., 2006; Castro et al., 2008). Thus, in the present paper, we attempted to understand the mechanism of action of physalins as immune depression compounds using *R. prolixus* as the animal model. Our results confirm previous findings (Castro et al., 2008) that physalin B administered orally through an insect blood meal inhibited cellular

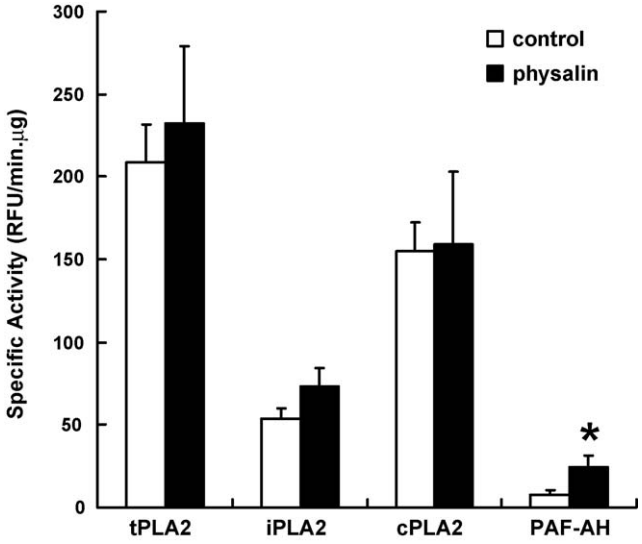

Fig. 4. Specific activities of total PLA2 (tPLA2), Ca^{2+}-independent PLA2 (iPLA2) and Ca^{2+}-dependent PLA2 (cPLA2) and PAF-acetyl hydrolase (PAF-AH) of samples from hemocytes of *R. prolixus* 5 days after regular blood feeding (control) or feeding with blood plus physalin B (for details see Section 2). Values are means ± S.E.M. from 10 samples containing hemocytes from three insects each. Values significantly different from controls ($p < 0.05$) are marked with an asterisk.

immune reactions, such as hemocyte microaggregate formation and hemocyte phagocytosis. Additionally, we also report that these inhibitions were counteracted with injection of arachidonic acid and PAF in *R. prolixus* hemocele. Thus, these data are in agreement with the hypothesis that eicosanoid and PAF pathways are mediators of the cellular immune reactions in *R. prolixus* (Garcia et al., 2004a,b; Machado et al., 2006; Figueiredo et al., 2008a,b).

The involvement of arachidonic acid and iPAF (insect PAF analogous) in these inhibitions was also investigated by measuring PLA_2 and PAF-AH activities. PLA_2 activity was not decreased in the hemocytes and cell-free hemolymph provided by insects previously fed with blood containing physalin B. However, PAF-AH levels in cell-free hemolymph were strongly elevated in insects treated with physalin B. These observations indicate a physiologically important role for PAF-AH, but not for PLA_2, in the insect cellular defense reactions in *R. prolixus* treated with physalin B.

Working with an *in vivo* model of gastrointestinal ischemia/reperfusion in mice, Vieira et al. (2005) proposed that physalin B binds to the glucocorticoid receptor as dexamethasone does, lowering the circulating levels of Tumor Necrosis Factor α (TNFα). Soares et al. (2006), working with cultured mice splenocytes, proposed that physalin B binds to an endogenous steroid receptor, different from the glucocorticoid receptor targeted by dexamethasone. Jacobo-Herrera et al. (2006) suggested that the effects of physalins B and F in cultured HeLa cells are mainly due to the inhibition of the NF-κB cascade, whose activation by PMA (phorbol 12-myristate-13-acetate) or TNFα is blocked after exposure to these physalins. More recently, Vandenberghe et al. (2008) showed that physalin B is a strong inhibitor of the ubiquitin–proteasome pathway and that this property is responsible for the inhibition of the NF-κB cascade.

Some effects of physalin B in the cellular immune responses of *R. prolixus* can be interpreted in the light of the information above. Hemocyte PLA_2 activities are apparently regulated by the glucocorticoid receptor (Figueiredo et al., 2008b), and the finding that physalin B does not affect hemocyte PLA_2 levels points to a mechanism similar to that observed in mammalian cells, based on the inhibition of the NF-κB cascade. In fact, physalins down-regulate the expression of antibacterial factors in *R. prolixus* (Castro et al., 2008), a type of humoral response strongly related to activation of κB-responsive elements (Ferrandon et al., 2007). It is also possible that the inhibition of the ubiquitin–proteasome response interferes with other intracellular signaling pathways, but if physalin B is really specific for the NF-κB cascade (as it does not interfere with the eicosanoid pathway) it could be an interesting tool to study insect humoral immunity.

Administration of physalin B significantly raises the activity of PAF-AH in *R. prolixus* plasma and hemocytes. It is possible that the inhibition of NF-κB cascade results in the blockage of the production of an inhibitor of this enzyme, but as the regulation of PAF-AH gene is poorly understood in insects, we cannot rule out the presence of other mechanisms in the regulation of PAF-AH expression by physalins. In any case, the control of iPAF levels in plasma seems to be an important mechanism of activation of *R. prolixus* cellular immune responses and it is probably related to other humoral and cellular defense mechanisms. It is interesting to notice that PAF is an important activator of the NF-κB pathway (Im et al., 1997) and that the elimination of plasmatic PAF by the activation of PAF-AH might have an inhibitory feedback effect on the suppression of the NF-κB responses.

The effects of physalins on phagocytosis and microaggregate formation seem to be more complex, as we observed reversal of physalin B action by both arachidonic acid and PAF injections. It is possible that iPAF modulates these hemocyte responses (Figueiredo et al., 2008a,b) and in this case depletion of iPAF levels caused by the activation of PAF-AH might explain the diminished hemocyte activity after physalin B treatment. Nevertheless, the reversal of the effect of physalin B by arachidonic acid suggests that phagocytosis and microaggregation are regulated by more than one pathway, as physalin B apparently does not affect PLA_2 activity and arachidonic acid levels. It is possible that the activation of the glucocorticoid receptor counteracts the inhibition of the NF-κB cascade. These two routes probably converge in general cellular responses as hemocyte aggregation or phagocytosis and more research is necessary to clarify this point.

In conclusion, the present study provides the first experimental evidence that physalin B modulates the PAF pathway involved in hemocyte phagocytosis and hemocyte microaggregation formations. More precisely, this report strongly suggests that physalin B plays an important role in the inhibition of iPAF activity. In addition to other known inhibitory effects of physalins on cellular and humoral immune reactions (Garcia et al., 2006; Castro et al., 2008), the reduced hemocyte phagocytic response and hemocyte microaggregation contributed to the immunodepression shown by physalin-treated *R. prolixus*. Finally, this system constitutes an attractive model for further research into the regulation of the immune responses of insect species.

Acknowledgments

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) to E.S.G. and P.A., Fundação Oswaldo Cruz (Papes project to P.A.), and Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ, to P.A. and F.A.G.). E.S.G. and P.A. are CNPq Senior Research Fellows. D.P.C. is a post-graduate student with a FAPERJ scholarship and M.B.F. is a post-graduate student from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

References

Asgari, S., Schmidt, O., 2003. Is cell surface calreticulin involved in phagocytosis by insect hemocytes? Journal of Insect Physiology 49, 545–550.

Azambuja, P., Garcia, E.S., 1997. Care and maintenance of triatomine colonies. In: Crampton, J.M., Beard, C.B., Louis, C. (Eds.), Molecular Biology of Insect Disease Vectors: A Method Manual. Chapman & Hall, London, pp. 56–64.

Batzri, S., Korn, E.D., 1973. Single bilayer liposomes prepared without sonication. Biochimica et Biophysica Acta 298, 1015–1019.
Boman, H., 1998. Gene-encoded peptide antibiotics and the concept of innate immunity: an update review. Scandinavian Journal of Immunology 48, 15–25.
Castro, D.P., Figueiredo, M.B., Ribeiro, I.M., Tomassini, T.C.B., Azambuja, P., Garcia, E.S., 2008. Immune depression in *Rhodnius prolixus* by *seco*-steroids, physalins. Journal of Insect Physiology 54, 555–562.
Castro Faria Neto, H., Stafforini, D.M., Prescott, S.M., Zimmerman, G.A., 2005. Regulating inflammation through the anti-inflammatory enzyme platelet-activating factor acetylhydrolase. Memórias do Instituto Oswaldo Cruz 100 (Suppl. I), 83–91.
Ferrandon, D., Imler, J.-L., Hetru, C., Hoffmann, J., 2007. The Drosophila systemic immune response: sensing and signalling during bacterial and fungal infections. Nature Reviews Immunology 7, 862–874.
Figueiredo, M.B., Castro, D.P., Nogueira, N.F.S., Garcia, E.S., Azambuja, P., 2006. Cellular immune response in *Rhodnius prolixus*: role of ecdysone in hemocyte phagocytosis. Journal of Insect Physiology 52, 711–716.
Figueiredo, M.B., Garcia, E.S., Azambuja, P., 2008a. Blockades of phospholipase A(2) and platelet-activating factor receptors reduce the hemocyte phagocytosis in *Rhodnius prolixus*: in vitro experiments. Journal of Insect Physiology 54, 344–350.
Figueiredo, M.B., Genta, F.A., Garcia, E.S., Azambuja, P., 2008b. Lipid mediators and vector infection: *Trypanosoma rangeli* inhibits *Rhodnius prolixus* hemocyte phagocytosis by modulation of phospholipase A2 and PAF-acetylhydrolase activities. Journal of Insect Physiology 54, 1528–1537.
Garcia, E.S., Azambuja, P., Contreras, V.T., 1984. Large-scale rearing of *Rhodnius prolixus* and production of metacyclic trypomastigotes of *Trypanosoma cruzi*. In: Morel, C.M. (Ed.), Genes and Antigens of Parasites, A Laboratory Manual. UNDP/World Bank/WHO and Fundação Oswaldo Cruz, Rio de Janeiro, pp. 43–46.
Garcia, E.S., Machado, E.M.M., Azambuja, P., 2004a. Effects of eicosanoid biosynthesis inhibitors on the prophenoloxidase-activating system and microaggregation reactions in the hemolymph of *Rhodnius prolixus* infected with *Trypanosoma rangeli*. Journal of Insect Physiology 50, 157–165.
Garcia, E.S., Machado, E.M.M., Azambuja, P., 2004b. Inhibition of hemocyte microaggregation reactions in *Rhodnius prolixus* larvae orally infected with *Trypanosoma rangeli*. Experimental Parasitology 107, 31–38.
Garcia, E.S., Castro, D.P., Ribeiro, I.M., Tomassini, T.C., Azambuja, P., 2006. *Trypanosoma rangeli*: effects of physalin B on the immune reactions of the infected larvae of *Rhodnius prolixus*. Experimental Parasitology 112, 37–43.
Gijón, M.A., Spencer, D.M., Siddiqi, A.R., Bonventre, J.V., Leslie, C.L., 2000. Cytosolic phospholipase A2 is required for macrophage arachidonic acid release by agonists that do and do not mobilize calcium. Journal of Biological Chemistry 30, 20146–20156.
Im, S.Y., Han, S.J., Ko, H.M., Choi, J.H., Chun, S.B., Lee, D.G., Ha, T.Y., Lee, H.K., 1997. Involvement of nuclear factor-κB in platelet activating factor-mediated tumor necrosis factor-α expression. European Journal of Immunology 27, 2800–2804.
Jacobo-Herrera, N.J., Bremner, P., Márquez, N., Gupta, M.P., Gibbons, S., Muñoz, E., Heinrich, M., 2006. Physalins from *Witheringia solanacea* as modulators of the NF-κB cascade. Journal of Natural Products 69, 328–331.
Kitsiouli, E.I., Nakos, G., Lekka, L.E., 1999. Differential determination of phospholipase A2 and PAF-acetylhydrolase in biological fluids using fluorescent substrates. Journal of Lipid Research 40, 2346–2356.
Machado, E.M., Azambuja, P., Garcia, E.S., 2006. Web 2086, a platelet-activating factor antagonist, inhibits prophenoloxidase-activating system and hemocyte microaggregation reactions induced by *Trypanosoma rangeli* infection in *Rhodnius prolixus* hemolymph. Journal of Insect Physiology 52, 685–692.
Miller, J.S., Stanley, D.W., 2001. Eicosanoids mediate microaggregation reactions to bacterial challenge in isolated hemocyte preparations. Journal of Insect Physiology 47, 1409–1417.
Miller, J.S., Stanley, D.W., 2004. Lipopolysaccharide evokes microaggregation reactions in hemocytes isolated from tobacco hornworms, *Manduca sexta*. Comparative Biochemistry and Physiology Part A 137, 285–295.
Rohloff, Lutz-H., Wiesner, A., Götz, P., 1994. A fluorescence assay demonstrating stimulation of phagocytosis by haemolymph molecules of *Galleria mellonella*. Journal of Insect Physiology 40, 1045–1049.
Rubin, B.B., Downey, G.P., Koh, A., Degousee, N., Ghomashchi, F., Nallan, L., Stefanski, E., Harkin, D.W., Sun, C., Smart, B.P., Lindsay, T.F., Cherepanov, V., Vachon, E., Kelvin, D., Sadilek, M., Brown, G.E., Yaffe, M.B., Plumb, J., Grisntein, S., Glogauer, M., Gelb, M.H., 2006. Cytosolic phospholipase A2 is necessary for platelet-activating factor biosynthesis, efficient neutrophil-mediated bacterial killing, and the innate immune response to pulmonary infection. Journal of Biological Chemistry 280, 7519–7529.
Soares, M.B., Bellintani, M.C., Ribeiro, I.M., Tomassini, T.C., Ribeiros dos Santos, R., 2003. Inhibition of macrophage activation and lipopolysaccharide-induced death by *seco*-steroids purified from *Physalis angulata* L. European Journal of Pharmacology 459, 107–112.
Soares, M.B., Brustolim, D., Santos, L.A., Bellintani, M.C., Paiva, F.P., Ribeiro, Y.M., Tomassini, T.C., Ribeiro Dos Santos, R., 2006. Physalins B, F and G, *seco*-steroids purified from *Physalis angulata* L., inhibit lymphocyte function and allogeneic transplant rejection. International Immunopharmacology 6, 408–414.
Stanley, D.W., 2006. Prostaglandins and other eicosanoids in insects: biological significance. Annual Review of Entomology 51, 25–44.
Vandenberghe, I., Créancier, L., Vispé, S., Annereau, J.-P., Barret, J.-M., Pouny, I., Samson, A., Aussagues, Y., Massiot, G., Ausseil, F., Bailly, C., Kruczynski, A., 2008. Physalin B, a novel inhibitor of the ubiquitin–proteasome pathway, triggers NOXA-associated apoptosis. Biochemical Pharmacology 76, 453–462.
Vieira, A.T., Pinho, V., Lepsch, L.B., Scavone, C., Ribeiro, I.M., Tomassini, T., Ribeiro-dos-Santos, R., Soares, M.B., Teixeira, M.M., Souza, D.G., 2005. Mechanisms of the anti-inflammatory effects of the natural secosteroids physalins in a model of intestinal ischaemia and reperfusion injury. Brazilian Journal Pharmacology 146, 244–251.
Whitten, M.M.A., Mello, C.B., Gomes, S.A.O., Nigam, Y., Azambuja, P., Garcia, E.S., Ratcliffe, N.A., 2001. Role of superoxide and reactive nitrogen intermediates in *Rhodnius prolixus* (Reduviidae)/*Trypanosoma rangeli* interactions. Experimental Parasitology 98, 44–57.
Winter, P., Rayne, R.C., Coast, G.M., 2007. The effects of intracellular signaling pathway inhibitors on phagocytosis by haemocytes of *Manduca sexta*. Journal of Insect Physiology 53, 975–982.
Wittenauer, L.A., Shirai, K., Jackson, R.L., Johnson, D., 1984. Hydrolysis of a fluorescent phospholipid substrate by phospholipase A2 and lipoprotein lipase. Biochemical and Biophysical Research Communications 118, 894–901.

4. DISCUSSÃO

A maioria dos compostos extraídos de plantas utilizados atualmente no controle de insetos atua em funções fisiológicas impedindo ou prejudicando a muda, alterando o comportamento (com ação repelente ou fagoinibidora) ou a reprodução. Porém, o mecanismo envolvido na ação da azadirachtina é mais complexo, pois atua sobre hormônios que acarretam em mudanças fisiológicas e imunológicas dos insetos (Rembold, 1983; Garcia e Rembold, 1984; Garcia e Azambuja, 2004). Já as fisalinas, como observado no presente trabalho, atuam apenas no sistema imunológico, principalmente no que diz respeito às respostas celulares (microagregação e fagocitose) bloqueando a via de sinalização de PAF (Garcia et al., 2006 – Anexo 1; Castro et al., 2008 – Anexo 3; Castro et al., 2009 – Anexo 4).

A alimentação é essencial para o disparo de respostas fisiológicas e imunológicas de *R. prolixus* tanto pelos nutrientes da digestão sanguínea como pela dilatação do intestino médio anterior (Feder et al., 1997). Sendo assim, deve-se avaliar se a droga administrada junto ao sangue possui ação fagoinibidora a fim de certificar que as informações obtidas dos ensaios relacionados à fisiologia e imunologia do inseto são conseqüência da droga ingerida e não da deficiência alimentar. Assim de acordo com os resultados obtidos, as fisalinas não possuem efeito fagoinibidor nas doses inferiores a 15 μg/ml de sangue, e também não influenciam a ecdise e a sobrevivência de *R. prolixus* nas doses aplicadas (Garcia et al., 2006 – Anexo 1; Castro et al., 2008 – Anexo 3).

Entretanto, insetos alimentados com sangue contendo fisalina B e inoculados com formas epimastigotas curtas de *T. rangeli* apresentaram altas taxas de mortalidade. Adicionalmente, insetos previamente tratados com as fisalinas B, D, F, e G e infectados por *E. cloacae* β12 também exibiram taxas de mortalidade elevadas. Portanto, apesar das fisalinas não afetarem a fisiologia dos insetos, quando estes são infectados ocorre aumento da mortalidade o que indica que as fisalinas inibem seu sistema imunológico. Como esse resultado foi observado tanto com inoculação de parasitas como de bactérias a ação das fisalinas nas respostas do sistema imune demonstra ser ampla e complexa (Garcia et al., 2006 – Anexo 1; Castro et al., 2008 – Anexo 3).

Estes dados confirmam os dados reportados por Garcia et al. (2004a) sobre a alta mortalidade de *R. prolixus* alimentados com sangue contendo inibidores da biossíntese de eicosanóides e inoculados com *T. rangeli*. Estes autores também constataram um

aumento da parasitemia e diminuição da contagem de microagregados de hemócitos na hemolinfa (Garcia et al., 2004a). Assim como inibidores da via dos eicosanóides afetaram a mortalidade dos insetos inoculados com os parasitas, as fisalina B, D, F e G também afetaram a viabilidade de insetos infectados.

Os resultados sugerem que as fisalinas atuam sobre o sistema de sinalização dos insetos bloqueando as respostas imunológicas responsáveis por eliminar tanto parasitas como bactérias da hemocele.

No presente estudo, ninfas de *R. prolixus* tratadas com a fisalina B produziram menos reativos de nitrogênio quando desafiadas pela inoculação de *T. rangeli* (Garcia et al., 2006 – Anexo 1). O efeito de fisalinas sobre a produção de nitrito e nitrato também foi observado por Soares et al. (2003) com macrófagos estimulados por lipopolissacarídeo e interferon e tratados com fisalina B. Estes autores demonstraram que as células reduziram a produção de óxido nítrico em comparação aos macrófagos não tratados (Soares et al. 2003).

Além do resultado relacionado aos reativos de nitrogênio, foi observado que *R. prolixus* tratados com a droga e inoculados com o parasita formaram menos microagregados de hemócitos, o que acarretou em alta parasitemia na hemolinfa (Garcia et al., 2006 – Anexo 1). O autor Takle (1988) demonstrou que a formação de agregados de hemócitos é a principal resposta celular responsável pela eliminação de *T. rangeli* da hemolinfa de *R. prolixus* quando ocorre aumento de parasitas livres. Em *Periplaneta americana* e *Schistocerca gregaria* a eliminação de *T. rangeli* da hemolinfa ocorre em intervalo de tempo mais curto do que em *R. prolixus*. Porém a resposta celular responsável pela eliminação do parasita em *P. americana* e *S. gregaria* também é a formação de microagregados de hemócitos que são encontrados em grande quantidade na hemolinfa (Takle, 1988).

Portanto, a redução das respostas imunológicas de microagregação e produção de nitrito/nitrato causadas pelo tratamento de insetos com fisalina B e posteriormente infectados por epimastigotas de *T. rangeli* e consequentemente o maior número de parasitas presentes na hemolinfa, reforçam o papel desse composto como modulador da resposta imune e a função dessas respostas na eliminação de tripanosomatídeos da hemolinfa.

Adicionalmente, o tratamento com as fisalinas B, D, F e G também afetou outras respostas imunológicas de *R. prolixus* inoculados com *E. cloacae* β12. De uma maneira

geral, a maior interferência das drogas foi observada sobre as respostas imunológicas do tipo celular, tais como formação de microagregados e atividade fagocítica. As fisalinas B, F, e G reduziram drasticamente a formação de microagregados e as fisalinas B e F causaram redução na contagem de hemócitos livres na hemolinfa de insetos infectados pela bactéria. Em contrapartida todas as fisalinas testadas, B, D, F e G, inibiram significativamente a internalização de partículas de leveduras por hemócitos, considerado como atividade fagocítica (Castro et al., 2008 – Anexo 3). Também a azadirachtina, quando administrada junto ao sangue alimentar de *R. prolixus* diminui a capacidade hemocítica de adesão e internalização de leveduras, resultando em menor atividade fagocítica (Figueiredo et al. 2006 – Anexo 2).

Assim, as fisalinas B e F demonstraram maior potencial inibitório das respostas imunológicas mediadas por hemócitos, com efeitos não só de redução da atividade fagocítica em ensaios de monocamada de hemócitos incubados com leveduras, como também na formação de microagregados e contagem total de hemócitos circulantes nos insetos desafiados por *E. cloacae* β12.

Avaliamos possíveis alterações na resposta imune humoral pela administração das diferentes fisalinas ao sangue através dos ensaios de ativação da profenoloxidase, estimativa da dosagem de lisozima e de atividade antibacteriana na hemolinfa dos insetos. Não foram observadas alterações na ativação da cascata de profenoloxidase na hemolinfa de insetos tratados oralmente com sangue acrescido de fisalina B quando comparados com insetos tratados apenas com sangue contendo solvente, ambos infectados com *T. rangeli* (Garcia et al., 2006 – Anexo 1). O tratamento de *R. prolixus* com fisalinas B e F causou menor queda da atividade de lisozima medida na hemolinfa de insetos tratados com as drogas seis dias após a inoculação de *E. cloacae* β12 (Castro et al., 2008 – Anexo 3). Feder et al (1997) observaram pico de atividade de lisozima no terceiro dia após inoculação de *E. cloacae* β12 e queda da atividade no sexto dia em ninfas de *R. prolixus* alimentadas com sangue, assim como observamos em nossos ensaios para insetos controle.

Em relação à atividade antibacteriana, apenas insetos tratados com fisalina D e inoculados com *E. cloacae* β12 apresentaram diminuição do diâmetro do halo de inibição de crescimento de *Escherichia coli* em testes com hemolinfa coletada após seis dias de inoculação da bactéria (Castro et al., 2008 – Anexo 3). Em *R. prolixus* , a cinética de atividade antibacteriana após inoculação com *E. cloacae* β12 segue o padrão

encontrado na atividade de lisozima, com pico de atividade a partir do terceiro dia e queda no sexto dia após inoculação (Azambuja et al., 1986, Feder et al., 1997).

Apesar da fisalina D afetar a atividade antibacteriana, a droga não apresentou efeito sobre as demais respostas imunológicas analisadas em *R. prolixus* (Castro et al., 2008 – Anexo 3). Soares et al. (2003, 2006) também observaram menor efeito inibidor da fisalina D em respostas imunológicas de macrófagos e linfócitos de camundongos, quando comparada a outras fisalinas também testadas.

As fisalinas apresentam diferentes atividades na resposta imune de *R. prolixus* provavelmente devido a diferenças na estrutura química de cada composto. A fisalina B é a mais simples e dela derivam as outras fisalinas D, F e G. A principal diferença entre essas fisalinas está nos substituintes presentes no anel B. A insaturação presente no anel B da fisalina B, sofre reações com acréscimo de uma hidroxila na fisalina G, duas hidroxilas na fisalina D e um grupamento epóxi na fisalina F. Essas pequenas alterações podem afetar a ligação desses compostos a receptores das células de *R. prolixus* ocasionando diferentes alterações na resposta imune.

Em resumo, a resposta imunológica de *R. prolixus* tratados com as fisalinas B, F e G e posteriormente infectados por parasitas ou bactérias demonstra imunossupressão principalmente com inibição da resposta imune celular.

O efeito imunomodulatório das fisalinas tem sido observado em testes que utilizam células de mamíferos. Soares et al. (2003) demonstraram que em macrófagos estimulados com lipopolissacarídeos e interferon, as fisalinas reduzem a formação de nitrito/nitrato e produzem baixos níveis de fator de necrose de tumor (TNF), interleucina-6 e interleucina-12. Recentemente os mesmos autores observaram efeito inibidor de reações inflamatórias em linfócitos desafiados com Con-A e em transplante de órgãos em camundongos tratados com as fisalinas B, F e G (Soares et al., 2006). Experimentos in vivo com camundongos apresentando isquemia intestinal e reperfusão demonstraram inibição de reação inflamatória quando os animais eram tratados com fisalinas B e F (Vieira et al. 2005).

Nesses mesmos trabalhos, os autores descrevem os efeitos de fisalinas no sistema imune de mamíferos comparando-os com o efeito da dexametasona (Soares *et al.*, 2003, 2006; Vieira *et al.*, 2005). A dexametasona, um glucocorticóide com ação inibitória sobre a enzima fosfolipase A_2 (PLA_2) é utilizado na regulação de respostas imune (Nakano et al., 1990). A PLA_2 é fundamental para a biossíntese de eicosanóides e

PAF, responsáveis pelo processo inflamatório. As fisalinas e a dexametasona são ambas derivadas dos esteróides sendo a primeira seco-esteróide e a segunda glucocorticóide (Tomassini *et al*., 2000). Apesar da mesma origem bioquímica, o mecanismo de ação das fisalinas parece ocorrer de maneira diferente da dexametasona, porém também por via de receptores de glucocorticóides (Soares *et al.*, 2003, 2006; Vieira *et al.*, 2005).

Em *R. prolixus*, o tratamento com ácido araquidônico dos insetos previamente alimentados com sangue contendo fisalina B reverteu a inibição das atividades fagocítica e de formação de microagregados tanto nos ensaios *in vitro*, por meio da aplicação direta sobre a monocamada de hemócitos, como também *in vivo* pela inoculação na hemocele. Já o tratamento dos insetos com fator de ativação de plaquetas (PAF) reverteu a inibição da fagocitose e formação de microagregados apenas nos ensaios *in vivo*, em que a droga era inoculada diretamente na hemocele do inseto (Castro et al., 2009 – Anexo 4). Estes dados sugerem que o PAF não tem ação direta sobre a inibição da fisalina necessitando da presença do corpo gorduroso, ou de outro fator, presente na hemocele para reverter a inibição.

Entretanto, os resultados obtidos na reversão do efeito inibidor da fisalina B após inoculação de PAF sugerem que as respostas imunes de microagregação de hemócitos e fagocitose são mediadas pela via PAF. Alguns trabalhos também relatam que as respostas de microagregação de hemócitos e fagocitose em insetos são mediadas pelo PAF (Machado et al., 2006; Figueiredo et al., 2008a e 2008b).

Como o efeito da fisalina B é revertido tanto com o tratamento com ácido araquidônico como com PAF sua ação poderia ser sobre a via dos eicosanóides e PAF através da inibição da enzima PLA_2. Entretanto os níveis de PLA_2 na hemolinfa, tanto de hemócitos como do plasma, dos insetos tratados com a fisalina B não se alteraram em relação aos níveis dessa enzima nos insetos controle (Castro et al., 2009 – Anexo 4). Assim, apesar da semelhança no anel esteroidal presentes tanto na estrutura química da dexametasona como nas fisalinas, não foi observado efeito da fisalina na atividade da enzima PLA_2. Portanto, as fisalinas não possuem mecanismo de ação similar ao da dexametasona em *R. prolixus*.

Todavia os níveis de PAF-AH dos insetos tratados com fisalina B se elevaram em relação aos dos insetos controle (Castro et al., 2009 – Anexo 4). A PAF-AH degrada PAF acarretando em menores reações imunológicas (Derewenda e Ho, 1999; Arai, 2002). Embora a fisalina B não tenha interferido na atividade da PLA_2, a qual promove a formação de LISO-PAF e em seguida de PAF, esta droga atuou sobre a enzima PAF-

AH, a qual tem ação sobre a hidrolise de PAF. Com taxas elevadas de PAF-AH as moléculas de PAF formadas pela ação da PLA_2 são degradadas, assim inibindo as respostas moduladas pela via PAF.

Como a fisalina B não resultou em efeito sobre os níveis de PLA_2 de hemócitos de *R. prolixus*, seu mecanismo de ação pode ser similar ao observado em mamíferos em que ocorre inibição da cascata do fator nuclear-κappaB (NF-kB). Jacobo-Herrera *et al.* (2006) observaram que fisalinas B e F bloqueiam a ação de forbol-12-miristato-13-acetato (PMA) ou TNFα, impedindo a ativação da cascata de NF-kB em culturas de células HeLa. Recentemente, Vandenberghe *et al.* (2008) demonstraram que a fisalina B é um forte inibidor da via ubiquitina-proteassoma, ocasionando a inibição da cascata NF-κB.

O principal regulador da imunidade inata é o sistema NF-kB, uma via de sinalização comum a insetos e vertebrados (Salminen et al, 2007). Responsável pela regulação da expressão de um grande número de genes, exerce função essencial como intermediário das respostas imunes e inflamatórias (Lopes et al., 2008). Os fatores NF-kB estão presentes no citosol em sua forma inativa associados a proteínas inibitórias IkBs. Essas IkBs são degradadas após estímulo através de fosforilação o que resulta na translocação de NF-kB para o núcleo aonde ocorre a transativação de genes que codificam várias citocinas proinflamatórias (Kopp et al., 1995). Glucocorticóides podem estimular a transcrição das proteínas inibitórias IkBα (Auphan et al., 1995; Scheinman et al., 1995) impedindo a translocação de fatores nucleares-kB para o núcleo ou podem também se ligar aos receptores de NF-kB (Heck et al., 1997; Wissink et al., 1997; De Bosscher et al., 2003; Lowenberg et al., 2008).

Os fatores NF-kB também estão relacionados com a ação de PAF (Im et al., 1997; Han et al., 1999; Han et al., 2002; Ko et al., 2002; Choi et al., 2001; Choi et al., 2007). A eliminação de PAF do plasma pelo excesso de PAF-AH estimulado pela administração de fisalina B ao inseto pode ser um efeito indireto relacionado à supressão de respostas relacionadas a cascata NF-kB como observado em mamíferos (Jacobo-Herrera *et al.* 2006; Vandenberghe *et al.* 2008). Os resultados sugerem que em *R. prolixus* a inibição da cascata NF-kB pela fisalina B resulta no bloqueio da produção de um inibidor da enzima PAF-AH. Entretanto, pouco se conhece sobre os genes relacionados a PAF-AH em insetos, o que dificulta a compreensão do mecanismo de ação de fisalinas relacionados a expressão da PAF-AH. De qualquer forma, os níveis de

PAF presentes no plasma parecem ser importantes no mecanismo de ativação de respostas imune celulares em *R. prolixus* e provavelmente está relacionado também a mecanismos de defesa humoral.

Uma vez que o efeito inibitório da fagocitose e microagregação foi revertido não só pelo PAF mas também pelo ácido araquidônico, o mecanismo de ação das fisalinas parece ser mais complexo, afetando mais de uma via de sinalização (Castro et al., 2009 – Anexo 4). O ácido araquidônico está intimamente ligado a PLA_2, sendo liberado pela hidrólise de fosfolipídeo de membrana (Stanley 2000). Em seguida o ácido araquidônico sofre diferentes reações formando os eicosanóides, responsáveis pela sinalização de respostas imune nos insetos (Stanley 2000, 2006b; Shrestha e Kim, 2008). Entretanto, em *R. prolixus* a fisalina B não atuou sobre a atividade da enzima PLA_2, a qual tem sido descrita como uma enzima responsável pela liberação do ácido araquidônico.

A administração de ácido araquidônico aos insetos indicou um aumento da formação de eicosanóides paralelamente à ação da PLA_2. Para as vias cicloxigenase, lipoxigenase e epoxigenase, a adição do ácido araquidônico, como substrato destas vias, aumentou a taxa de reação produzindo, provavelmente, mais eicosanóides.

Assim, a fisalina B possui ação supressora sobre as respostas celulares e da produção de nitrito e nitrato em *R. prolixus* infectados com *T. rangeli* ou *E. cloacae* β12, causando alta mortalidade do inseto. Esse composto atua na enzima PAF-acetilhidrolase (PAF-AH) aumentando seus níveis tanto nos hemócitos como no plasma, o que resulta na degradação de PAF e conseqüente inibição do sistema de defesa celular e humoral do inseto. Seu efeito é revertido pela adição de PAF e/ou ácido araquidônico aos insetos tratados.

Fatores que interferem no desenvolvimento de tripanosomatídeos em *Rhodnius prolixus*:

II-*Serratia marcescens* isolada da microbiota intestinal.

5. REVISÃO DE LITERATURA

5.1 Insetos vetores e sua microbiota

Os vertebrados e invertebrados possuem em seu sistema corporal uma grande variedade de bactérias patogênicas ou não. Muitas se associam em simbiose com o hospedeiro, como no caso da maioria das bactérias que habitam o trato digestivo. Algumas das espécies de bactérias podem ajudar na digestão e evitar a proliferação de micróbios patogénicos no hospedeiro enquanto que esse fornece habitat adequado e nutriente para as bactérias. Quando o hospedeiro possui apenas uma fonte de alimentação, como no caso de alguns insetos-vetores, a microbiota intestinal (bactérias do trato digestivo) se torna essencial, pois além de ajudar na digestão, fornece vitaminas, nutrientes e enzimas, os quais são deficientes ao inseto (Terra, 1990; Beard et al., 2002; Genta et al., 2006).

Triatomíneos são insetos hematófagos que se alimentam exclusivamente de sangue em todo seu ciclo de vida e necessitam de simbiontes para o fornecimento do complexo de vitaminas B (Wigglesworth, 1929; Dasch et al., 1984). O triatomíneo *Rhodnius prolixus*, mantêm associação simbiótica com a bactéria *Rhodococcus rhodnii,* inicialmente caracterizada como *Nocardia rhodnii,* (Harington, 1960; Auden, 1972; Dasch et al., 1984) que é essencial para seu crescimento e desenvolvimento. Essas bactérias crescem no intestino e alcançam números próximos a 10^9 unidade formadora de colônia-CFU/ml a partir da ingestão de sangue alimentar. São transmitidas dos adultos para as fases jovem através da ingestão de fezes, comportamento comum entre os triatomíneos. Insetos que não se tornam colonizados pela bactéria apresentam altas taxas de mortalidade e não conseguem trocar de estádio (Harington, 1960; Nyirady, 1973; Hill et al., 1976; Dasch et al., 1984).

A especificidade de bactérias simbiontes em *R. prolixus* e *Triatoma infestans* foi revisada por Harington (1960), o qual observou a presença de outras bactérias presentes no trato digestivo desses insetos em trabalhos de diversos autores (Goodchild, 1955; Bewing e Schwartz, 1956 citados por Harington, 1960). Atualmente, sabe-se que o *R. prolixus*, assim como outros insetos, é colonizado por inúmeras espécies de bactérias diferentes e não apenas pela espécie descrita inicialmente, *R. rhodnii*. As espécies de bactérias que formam a microbiota do inseto variam com seu tipo de alimentação, distribuição geográfica entre outros fatores diversos.

Recentemente foram isolados de oito espécies de triatomíneos, capturados no campo ou criados em laboratórios, inúmeras bactérias de gêneros diferentes (Figueiredo *et al.* 1995). As bactérias mais freqüentemente encontradas em *T. infestans, T. pseudomaculata, Panstrongylus megistus* e *R. prolixus* são: *Enterobacter cloacae*, *Serratia marcescens* e *Enterococcus faecalis* (Figueiredo 1995; Figueiredo *et al.* 1995). No laboratório, os triatomíneos podem contaminar-se com bactérias através do contato com as fezes de outros insetos ou através de animais (pombos, camundongos, ratos, coelhos) utilizados na alimentação, ou ainda, quando for o caso, através do alimentador artificial (Brecher e Wigglesworth, 1944).

Para melhor compreensão da biologia dos insetos é necessário estuda-los em um contexto ecológico, tendo os microorganismos como um importante componente do sistema (Steinhaus, 1960). Assim, a caracterização da microbiota dos insetos é essencial bem como estabelecer o tipo de relacionamento, simbiose ou parasitismo, que ocorre entre as espécies em estudo.

Entretanto, a microbiota, principalmente de insetos coletados em campo, é composta por uma grande variedade de espécies de bactérias que na grande maioria não são cultiváveis em laboratório o que dificulta sua identificação. Contudo, o avanço das técnicas de biologia molecular propiciou a detecção apurada da presença de material genético de bactérias cultiváveis ou não em todos os órgãos dos insetos e consequentemente a sua identificação (Dillon et al, 2008).

5.2 Interações entre microbiota, parasita e insetos vetores

Além das bactérias, os insetos, principalmente os vetores de agentes patogênicos, albergam em seus tratos digestivos grandes comunidades de diversos microorganismos além de parasitas. Porém, pouco se sabe sobre as interações que ocorrem nos nichos ocupados tanto por bactérias como por parasitas nos insetos (Dillon e Dillon, 2004). Em humanos e outros mamíferos, como exemplo de interação, foi demonstrado que a microbiota intestinal tem papel fundamental no processo conhecido como "resistência de colônia" no qual a bactéria previne a colonização do intestino por patógenos (Dillon e Dillon, 2004).

Alguns trabalhos relatam a influência da microbiota intestinal de insetos vetores no ciclo de vida de parasitas. Infecções intestinais de bactérias em mosquitos podem inibir significativamente o desenvolvimento esporogônico de *Plasmodium vivax* (Pumpuni et al., 1996; Gonzalez-Ceron et al., 2003). Um fenômeno similar é observado

no intestino de flebótomos infectados com protozoário *Leishmania major* (Schlein et al., 1985), e no intestino médio de *R. prolixus* infectado com *Trypanosoma cruzi*, o agente causador da Doença de Chagas (Azambuja et al., 2004, 2005; Azambuja e Garcia, 2005). A interação existente entre insetos, microbiota e patógenos é extremamente complexa e necessita maiores estudos.

Azambuja *et al.* (2004) isolaram do intestino do *R. prolixus* a bactéria identificada como *S. marcescens* biotipo A1a (referenciada como *R. prolixus* human, RPH). Neste trabalho os autores observaram que *S. marcescens* apresenta alta atividade hemolítica e tripanolítica em experimentos *in vitro* com eritrócitos e parasitas. Assim pode-se dizer que *S. marcescens* ajuda na digestão sanguínea e na regulação do desenvolvimento dos parasitas presentes no intestino do inseto vetor, *R. prolixus*. Porém, a colonização de *S. marcescens,* dependendo da espécie de inseto pode ser nociva, como no caso da mosca *Drosophila melanogaster* em que essa bactéria atravessa a membrana do intestino e invade a hemocele, demonstrando resistência às respostas imune. Quando inoculadas diretamente na hemocele da mosca causa morte dos insetos em apenas um dia (Mallo et al., 2002; Kurz et al., 2003; Dillon et al., 2005; Nehme et al., 2007).

Entretanto, um aspecto prático relacionado ao conhecimento da microbiota de insetos vetores é o uso de bactérias simbiontes transformadas geneticamente. Estas podem expressar e liberar produtos transgênicos tóxicos nos tecidos de insetos vetores de parasitas patogênicos, eliminando o parasita sem, entretanto matar o inseto, o que pode significar uso potencial em controle alternativo da doença (Durvasula *et al*., 1997, 2008; Beard et al., 2001; Dotson et al., 2003). Esta abordagem representa, potencialmente, uma poderosa forma de controle biológico.

Além das interações com a microbiota do inseto, os parasitas enfrentam vários outros fatores que influenciam no estabelecimento da infecção nos insetos vetores. Em amostras de trato digestivo de *R. prolixus* foram identificados fatores líticos induzidos pela alimentação sanguínea por Azambuja et al. (1983) e lectinas com capacidades aglutinantes de eritrócitos e tripanosomatídeos por Mello *et al.* (1996) e Volf *et al.* (2001). Estas moléculas foram caracterizadas como determinantes importantes nos processos de suscetibilidade às infecções e enfatizam a necessidade de investigar as reações entre a microbiota intestinal e o desenvolvimento dos tripanosomatídeos em seus hospedeiros intermediários.

5.3 Glicosilinositolfosfolipídeos (GIPLs) na superfície de tripanossomatídeos

Para os tripanosomatídeos a adesão ao hospedeiro é processo fundamental para completar seu ciclo de vida. Por isso as moléculas presentes na sua superfície desempenham papel fundamental no seu ciclo de vida. A membrana das formas de multiplicação no vetor (epimastigotas) de *T. cruzi* e *T. rangeli* é majoritariamente composta por glicosilinositolfosfolipídeos (GIPLs) que são responsáveis por promover o ancoramento de proteínas à bicamada lipídica da membrana atuando como sistema de defesa do parasita, adesão à células hospedeiras e promovendo invasão. As GIPLs possuem em comum nos parasitas a estrutura central de Manα1-2Manα1-6Manα1-4GlcNα1-6inositolfosfolipídeo (Lederkremer e Bertello, 2001).

Inúmeros trabalhos relatam a importância das GIPLs para os tripanosomatídeos. Em relação a sua fase de desenvolvimento no inseto, as GIPLs são responsáveis pela adesão dos parasitas à membrana intestinal, fase essencial para sua sobrevivência e no caso de *T. rangeli* fundamental para penetração na parede do tubo digestivo e invasão da hemocele (Mello et al., 1996; Alves et al., 2007). Em vários trabalhos relata-se a importância de resíduos de manose na GIPL de *T. cruzi* como importante fator de adesão celular (de Araújo-Jorge e de Souza, 1986; Boschetti et al., 1987; Kahn et al., 1995, 1996; Vray et al., 2004).

***5.4** Serratia marcescens*

Trata-se de uma espécie de bactéria Gram-negativa, pertencente à família Enterobacteriaceae, facultativamente anaeróbia. Apresenta-se em forma de bastonete, sendo comumente encontrada no solo, água, alimentos e amostras clínicas. Pode causar sério problema de infecção hospitalar por colonizar equipamentos e ser proeminente patógeno oportunista para pacientes imunodeprimidos.

Algumas variantes dessa espécie são produtoras de pigmento vermelho intenso conhecido como prodigiosina, 2-metil-3-amil-6-metoxiprodigiosene. A prodigiosina é um metabólito secundário de baixo peso molecular com máxima produção na fase estacionária de desenvolvimento da cultura de *S. marcescens* nas temperaturas de 28 a 30ºC (Williams 1973). Esse composto é responsável por inúmeras propriedades apoptóticas em vários tipos celulares tumorais (Llagostera et al. 2003; Díaz-Ruiz et al. 2001; Montaner e Pérez-Tomás 2001; Montaner et al. 2000), possui efeitos citotóxicos em *P. falciparum* (Isaka et al. 2002) e atividade tripanolítica (Melo et al. 2000).

Além da atividade citotóxica da prodigiosina, diferentes espécies de bactérias do gênero *Serratia* produzem também substâncias hemolíticas (Poole *et al.* 1988, Braun *et al.* 1985, 1987; Walker et al, 2004; Hertle 2005). Alguns autores sugerem a facilitação da digestão sanguínea pela atividade hemolítica de bactérias que colonizam o trato digestivo de insetos hematófagos (Fouda et al., 2001; Azambuja et al., 2005b). Bactérias do gênero *Serratia* são frequentemente encontradas no intestino médio de insetos como *Aedes triseriatus*, *Culex pipiens*, *Psoropora columbiae* (Demaio et al. 1996), *Anopheles gambiae*, *A. funestus* (Straif et al. 1998), *A. albimanus* (Gonzalez-Ceron et al. 2003), *P. papatasi* (Dillon et al. 1996), *Lutzomya longipalpis* (Oliveira et al. 2001), *Schistocerca gregaria* (Dillon et al., 2005).

S. marcescens é uma enterobacteriácea que se adapta muito bem às condições do aparelho digestivo de insetos, pois possui estruturas em sua superfície, conhecidas como fimbriae, que são responsáveis pela adesão à célula hospedeira (Grimont and Grimont, 1978).

5.5 Adesão bacteriana: fimbriae.

A principal função das fimbriae é a adesão tanto às superfícies bióticas como abióticas. A adesão às células hospedeiras disponibiliza nutriente, facilita a liberação de toxinas aos tecidos e penetração nestes. Após a adesão, a bactéria pode produzir e liberar toxinas, invadir células e formar biofilmes. Como a adesão é um fator extremamente importante para ativação de fatores de virulência das bactérias, inúmeros trabalhos têm focado na cura de doenças através do tratamento com carboidratos impedindo a adesão bacteriana à célula hospedeira (revisão Sharon, 2006).

Fimbriae são longos filamentos protéicos delgados e flexíveis presentes na superfície bacteriana que projetam lectinas. Geralmente em sua extremidade, e algumas vezes ao longo da estrutura, possuem proteínas distintas, denominadas *adesinas*, as quais medeiam a adesão específica da célula bacteriana a diferentes substratos (de Greve et al., 2007). As fimbriae são constituídas de subunidades protéicas idênticas e repetitivas da proteína pilina. Cada subunidade da proteína tem aproximadamente vinte quilodaltons e é empacotada em um arranjo helicoidal para formar uma longa estrutura cilíndrica. Em observações utilizando a microscopia eletrônica, as fimbriae são vistas como uma cobertura difusa envolvendo a bactéria (Soto e Hultgren, 1999).

A adesão ocorre quando sítios de ligação localizados nas extremidades das fimbriae reconhecem e reagem com sítios receptores específicos presentes na superfície

de células eucarióticas (Old, 1972). A colonização pode estar relacionada a associações simbióticas mutualistas ou a processos infecciosos. Neste último caso, as fímbrias atuam como fatores de virulência (Pizarro-Cerdá e Cossart, 2006).

Algumas bactérias são capazes de produzir diferentes tipos de fímbrias (Finlay e Falkow, 1997; Soto e Hultgren, 1999). A fimbria do tipo 1 é encontrada em várias espécies de bactérias da família Enterobacteriaceae como *Escherichia coli* (Salit et al., 1977), *Salmonella typhimurium* (Korhonen et al., 1980), *S. marcescens* (Old et al., 1983) e *E. cloacae* (Hornick et al., 1991). Em *E. coli*, bactéria patogência a animais e humanos, as fimbrias são bem conhecidas tendo sido recentemente caracterizadas.

Estruturalmente, a fimbria tipo 1 (7nm de largura 8-9nm) é formada por uma haste cilíndrica de repetições de subunidades de FimA imunoglobulinas like (Ig-like) (peso molecular 17kDa) que são arranjadas em forma helicoidal. Essa haste está conectada a uma pequena ponta de 3nm de largura que consiste de duas proteínas adaptadoras, FimF e FimG e um terceiro tipo de proteína, FimH (Horst et al., 2001; Bouckaert et al., 2006). A fimbria tipo 1 é codificada pelo operon *fim* que compreende nove genes (*fim*A, *fim*B, *fim*C, *fim*E, *fim*F, *fim*G, *fim*H e *fim*I) (Moreira et al., 2003; Capitani et al., 2006). As subunidades FimA, FimF, FimG e FimH são transportadas para membrana externa através da formação de complexo com a chaperona FimC e interação com a proteína FimD (Vetsch et al., 2004) (Fig 5).

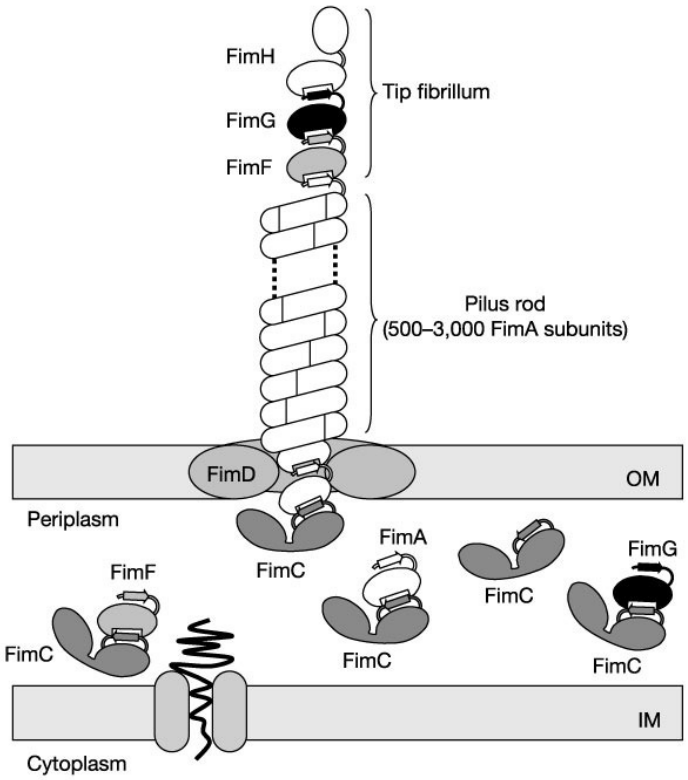

Figura 5. Esquema da formação da fimbria. IM, membrana interna; OM, membrana externa (Vetsch et al., 2004).

Os genes que codificam as fimbrias de *S. marcescens* e *E. coli* são homólogos e tem alta similaridade entre si (Mizunoe et al., 1988). Das bactérias estudadas que possuem a fimbria tipo 1 existe uma pequena variação nas seqüências de alelos, porém a subunidade FimH é altamente conservada (Girardeau e Bertin, 1995; Gerlach et al., 1989; Bouckaert et al., 2006). A proteína FimH é a única subunidade que possui o sítio de ligação à carboidratos sendo portanto, responsável pela propriedade de adesão bacteriana do tipo manose sensível (Horst et al., 2001; Bouckaert et al., 2006).

Na subunidade FIMH, as adesinas medeiam a aderência às superfícies de células eucarióticas, através do reconhecimento de carboidratos específicos presentes na superfície de células glicoconjugadas (Ofek and Sharon, 1990). *S. marcescens* possui duas classes de lectinas do tipo adesinas, conhecidas como manose sensível (MS) e manose resistente (MR). *S. marcescens* e *E. cloacae* possuem fimbria do tipo 1 manose sensível e portanto, de reconhecimento específico para resíduos de manose as quais são determinantes na adesão bacteriana a diferentes células de mamíferos. (Rumelt et al., 1988; Pan et al., 1997). Já a MR é subdividida em manose resistente *Klebsiella*-"like"

(fimbria tipo 3) responsável pela aglutinação de eritrócitos tratados com ácido tânico e manose-resistente *Proteus*-"like" (fimbria tipo MRP) responsável pela aglutinação de eritrócitos não tratados com ácido tânico (Old, 1972; Adegbola e Old, 1982; Old et al., 1983; Hejazi e Falkiner, 1977).

Recentemente autores têm demonstrado que o gene da adesina da fimbria tipo 1 entre outros genes responsáveis pelo "quorum sensing" e produção de exopolissacarídeos são importantes para a adesão de *S. marcescens* MG1 em células epiteliais da córnea (Labbate et al., 2007). Hejazi and Falkiner, em 1997 já haviam demonstrado que a fimbria MS de *S. marcescens* também é responsável pelo início da formação de biofilmes que são agregados de bactérias e secreções extracelulares polissacarídicas.

5.6 Biofilme

A maior parte da atividade bacteriana na natureza ocorre, não com as células individualizadas, mas com as bactérias organizadas em comunidades sob a forma de biofilme. Esses biofilmes são constituídos por uma comunidade estruturada de células aderidas a uma superfície inerte (abiótica) ou viva (biótica), embebidas numa matriz de exopolissacárido que as protege e serve como reserva de nutrientes (Donlan, 2002; Battin et al., 2007; Van Houdt et al., 2007).

O padrão de desenvolvimento de um biofilme envolve várias etapas: a adesão inicial à superfície, seguida da formação de microcolônias e, na maioria dos casos, a diferenciação das microcolônias em macrocolônias envolvidas numa matriz de exopolissacárido, formando biofilmes maduros (Stoodley et al., 2002; Rice et al., 2005; Battin et al., 2007). A adesão de uma bactéria a uma superfície abiótica é, geralmente, mediada por interações inespecíficas (e.g., forças hidrofóbicas), enquanto que a adesão a um tecido vivo é normalmente mediada por mecanismos moleculares específicos de "ancoragem", através de lectinas ou adesinas (Donlan, 2002; Jansen et al., 2004).

Estudos recentes de biofilme de *S. marcescens* têm demonstrado a importância de vários genes que fazem parte da via de adesão a superfícies abióticas e bióticas dependentes ou não do "quorum sensing" (Labbate et al., 2004, 2007; Shanks et al., 2007). A formação de biofilmes envolve genes relacionados ao sistema de secreção tipo 1, a produção de exopolissacarídeos bem como genes que controlam o "quorum sensing" (Labbate et al., 2004; 2007). Porém a formação do biofilme pode também ser

mediada por outros fatores como disponibilidade de nutrientes não dependendo do "quorum-sensing" (Déziel et al., 2001; Rice et al., 2005).

"Quorum-sensing" ou comunicação célula-célula é o processo pelo qual várias bactérias coordenam a expressão de genes de acordo com a densidade da população local através da produção de moléculas de sinalização. Essas moléculas de sinalização são conhecidas como "autoinducers" e através da detecção de sua concentração no meio as bactérias "calculam" a densidade populacional e então passam a expressar genes de acordo com o meio ambiente (Diggle et al, 2007; Bassler e Losick 2006; Williams et al. 2007).

As estruturas maduras de um biofilme conferem maior resistência das bactérias às condições de estresse ao meio ambiente ou àquelas geradas por respostas de hospedeiros constituindo, portanto, numa forma de proteção, fomentando, dependendo do caso, relações simbióticas e permitindo a sobrevivência em ambientes hostis (Costerton et al., 1999; Mah e O´Toole, 2001; Jarrett et al., 2004; Rice et al., 2005; Weitere et al., 2005; Queck et., 2006). Segundo Matz et al. (2004), *Pseudomonas aeruginosa* forma microcolônias de biofilme como um mecanismo de proteção à predação do flagelado *Rhynchomonas nasuta*. De maneira similar, Queck et al (2006) observaram que *S. marcescens* forma biofilme para se proteger dos protozoários *Bodo saltans* e *Acanthamoeba polyphaga*.

Em ecossistemas aquáticos, mais de 99,9% das bactérias crescem em biofilmes associadas a uma grande variedade de superfícies. Nos humanos, a variedade de infecções bacterianas crônicas, envolvendo biofilmes é bastante significativa. Estas comunidades podem ser formadas por uma ou mais espécies, alterando o quadro infeccioso do paciente. O elevado nível de resistência das bactérias a antibióticos e a alteração da resposta imune do hospedeiro, são as principais características para o biofilme bacteriano ser prejudicial ao organismo humano.

5.7 Fatores de virulência

Baseando-se no mecanismo de virulência, as bactérias podem ser agrupadas em fatores de virulência constituídos por proteínas de membrana, cápsulas de polissacarídeos, proteínas secretoras, parede celular, componentes externos da membrana entre outros (Wu et al., 2008).

As proteínas da membrana são responsáveis pela adesão, colonização e invasão de células hospedeiras. Já as proteínas secretoras, como as toxinas, são responsáveis

pela interação entre bactérias e células hospedeiras modificando as condições favoráveis da célula. Bactérias patogênicas utilizam sistemas secretores diferentes, comumente tipos I-IV, para transportar as proteínas tóxicas do citoplasma para a célula hospedeira (Wu et al., 2008).

Cápsulas de polissacarídeos que envolvem a célula bacterina possuem propriedades que impedem a atividade fagocítica, enquanto a parede celular (lipopolissacarídeo), como barreira física, protegem a bactéria das células hospedeiras. Como outros fatores de virulência, são citadas as proteínas formadoras de biofilme e os sideróforos. A formação de biofilme confere uma vantagem seletiva para sobrevivência sob diversas condições ambientais, garantindo maior resistência a agentes antimicrobianos e facilitando a colonização do hospedeiro (Wu et al., 2008).

Já os sideróforos são pequenas moléculas que apresentam alta afinidade por metais, principalmente ferro. São responsáveis por seqüestrar ferro de outras células e formar complexos de alta estabilidade. Esses complexos são internalizados pela célula bacteriana que utiliza ferro em seu metabolismo.

A maior classe de toxinas produzidas por bactérias é conhecida como toxinas formadoras de poros. O aspecto mais fascinante dessas toxinas é a habilidade de formar canais entre as membranas da célula alvo levando à sua ruptura. As proteínas secretadas pela bactéria são altamente solúveis, se ligam à membrana através de receptores específicos, em seguida penetram na membrana e formam os canais (Iacovache et al., 2008; Gonzalez et al., 2008).

Em 1982, Mercado e Colón-Whitt, utilizando de *Pseudomonas fluorescens* identificaram um fator citotóxico constituído por proteínas e lipopolissacarídeos capaz de lisar formas tripomastigotas de *T. cruzi* isoladas de sangue de camundongo infectado. Mudanças ultraestruturais no *T. cruzi* semelhantes aos efeitos causados por ânion superóxido e peróxido de hidrogênio foram observadas após incubação com esse fator tripanomicida por Mercado et al. (1986).

S. marcescens tem sido reportada como secretora de vários fatores de virulência como hemolisinas, nuclease, quitinase, metaloprotease, serino proteases, sideroforos e lipases (Hejazi e Falkiner, 1977; Hines et al., 1998; Marty et al., 2002; Carbonell et al., 2004; Fineran et al., 2007). Os autores, Marty et al (2002), caracterizaram um fator citotóxico de uma metaloprotease de peso molecular de 56kDa, em filtrados de cultura de *S. marcescens*. Vários autores têm purificado citotoxinas de *S. marcescens* e

demonstrado seus efeitos tóxicos em células humanas, porém essas são diferentes das toxinas já identificadas em *E. coli* (Hertle et al., 1999; Carbonell et al., 2003, 2004).

A fimbria manose-sensível de *S. marcescens* estimula a produção de superóxido em células hospedeiras causando danos aos tecidos (Mizunoe et al., 1995). Recentemente Shanks et al. (2007), relataram que o fator de transcrição que regula a via de estresse oxidativo regula também a expressão de fimbria na formação de biofilme de *S. marcescens.*

6. OBJETIVOS

Geral

Avaliar o efeito da bactéria *Serratia marcescens* presente no trato digestivo de *Rhodnius prolixus* sobre a atividade hemolítica e tripanolítica bem como seu modo de ação.

Específicos

I. Investigar o efeito de diferentes carboidratos na atividade hemolítica e tripanolítica de diferentes variantes de *Serratia marcescens*.

II. Avaliar os efeitos de variantes de *S. marcescens* sobre o *Trypanosoma cruzi* utilizando as ferramentas de microscopia eletrônica de transmissão e varredura.

III. Analisar o processo de adesão de variantes de *S. marcescens* sobre o *T. cruzi* e posterior formação de biofilme bem como o efeito de carboidratos sobre a adesão.

7. RESULTADOS

ARTIGOS:

Anexo 5 - Inhibitory effects of d-mannose on trypanosomatid lysis induced by *Serratia marcescens.* Castro DP, Moraes CS, Garcia ES, Azambuja P. Experimental Parasitology 2007; 115(2):200-4.

Anexo 6 - *Trypanosoma cruzi*: Ultrastructural studies of adhesion, lysis and biofilm formation by *Serratia marcescens*. Castro DP, Seabra SH, Garcia ES, de Souza W, Azambuja P. Experimental Parasitology 2007; 117(2):201-7.

Available online at www.sciencedirect.com
ScienceDirect
Experimental Parasitology 115 (2007) 200–204
Experimental Parasitology
www.elsevier.com/locate/yexpr

Inhibitory effects of D-mannose on trypanosomatid lysis induced by *Serratia marcescens*

Daniele P. Castro, Caroline S. Moraes, Eloi S. Garcia, Patrícia Azambuja *

Departamento de Bioquímica e Biologia Molecular, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Avenida Brasil 4354, Manguinhos, CEP 21045-900, Rio de Janeiro, RJ, Brazil

Received 29 May 2006; received in revised form 27 July 2006; accepted 2 August 2006
Available online 20 September 2006

Abstract

Studies were carried out on the effects of different carbohydrates on the lysis of *Trypanosoma cruzi*, *Trypanosoma rangeli* and erythrocytes caused by the bacteria *Serratia marcescens* variants SM 365 and RPH. High concentrations of D-mannose were found to protect *T. cruzi* and *T. rangeli* markedly diminishing the lysis caused by *S. marcescens*. However, this carbohydrate is unable to interfere with the hemolysis induced by SM 365 and RPH variants. These results showed that the trypanolytic effect induced by *S. marcescens* SM 365 and RPH variants is dependent on D-mannose and distinct from the hemolytic activity, strongly suggesting that bacterial fimbriae are relevant to *S. marcescens* in lysis of parasites.

Index Descriptors and Abbreviations: D-mannose; *Trypanosoma cruzi*; *Trypanosoma rangeli*; Hemolysis; *Serratia marcescens*

1. Introduction

Many trypanosomatids develop their life cycles or part of them in the digestive tube of vector insects (Lehane, 1991). *Trypanosoma cruzi* and *Trypanosoma rangeli* develop within the digestive tube of the invertebrate triatomine host. The parasites are taken in the blood meal of the insect as trypomastigotes, which differentiate into epimastigotes that multiply extracellularly in the midgut of reduviid insects (Brener, 1973; Watkins, 1971; Azambuja and Garcia, 2005). There are, however, differences in the biological cycles between both parasites: *T. rangeli*, but not *T. cruzi*, invades the hemocoel (Watkins, 1971; Hecker et al., 1990; Azambuja and Garcia, 2005) and completes its development in the salivary glands, where metacyclogenesis takes place (Hecker et al., 1990; Garcia et al., 1994). In the rectum, *T. cruzi* transforms into infective nondividing metacyclic trypomastigotes, which are released in the feces and urine (Garcia and Azambuja, 1991; Kollien and Schaub, 2000). Finally, *T. cruzi* is the causative agent of Chagas' disease whereas *T. rangeli*, which is apparently harmless to humans, can be pathogenic to the insect vector (Garcia and Azambuja, 1991; Kollien and Schaub, 2000; Azambuja et al., 2005a; Azambuja and Garcia, 2005).

Several factors are believed to influence the establishment of trypanosomes in the gut of the insect vector. Among these factors, few molecules have been identified in the invertebrate host *Rhodnius prolixus* such as a stomach lytic factor (Azambuja et al., 1983), lectins (Mello et al., 1996; Ratcliffe et al., 1996) and nitric oxide and superoxide (Whitten et al., 2001; Ratcliffe and Whitten, 2004) that in some way are involved in the population dynamics of trypanosomes in the insect gut.

Recently, Azambuja et al. (2004) examined the infections of *T. cruzi* DM28c clone and Y strain in the gut of *R. prolixus* after infective human blood ingestion. In the stomach, two days after the blood meal, the number of bacteria increased about 10.000-fold and, simultaneously, there was a rapid increase in lysis of red blood cells and the Y strain of parasites. A bacterium, *Serratia marcescens* biotype A1a (referenced as *R. prolixus* human, RPH), a

* Corresponding author. Fax: +55 21 2590 3495.
E-mail address: azambuja@ioc.fiocruz.br (P. Azambuja).

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doi:10.1016/j.exppara.2006.08.001

producer of the pigment prodigiosin, was isolated from the gut of *Rhodnius* (Azambuja et al., 2004). *In vitro* incubation of *S. marcescens* RPH or a non-prodigiosin-producing strain, *S. marcescens* DB11, with erythrocytes showed that both bacterial variants caused hemolysis but that only prodigiosin-producing RPH killed the *T. cruzi* Y strain (Azambuja et al., 2004, 2005b). Azambuja et al. (2004) demonstrated that the killing mechanism of *S. marcescens* SM365, also a prodigiosin-producing variant, and RPH involve the rapid attachment of many bacteria to the parasite membrane followed by damage of the *T. cruzi* Y strain.

Many adhesins, that are multiple bacterial adherence factors, act as lectins, recognizing specific carbohydrate moieties on host cell surface glycoconjugates and are considered essential factors in Gram-negative bacterial virulence (Ofek and Sharon, 1990). We therefore investigated the effects of different carbohydrates on the lytic activities caused by *S. marcescens* SM365 and RPH variants in *T. cruzi*, *T. rangeli* and erythrocytes.

2. Materials and methods

2.1. Parasites

Trypanosoma cruzi strain Y and *T. rangeli* strain H14 were supplied by Dr. M.A. Sousa (Trypanosomatid Collection of the Oswaldo Cruz Institute, CT-IOC, Fiocruz, Brazil). *T. cruzi* were grown in brain heart infusion (BHI) medium (DIFCO Laboratories, Detroit, MI) containing folic acid (30 mg/L), hemin (25 mg/L), and supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 28 °C. The parasites used in our experiments were taken from 4 day cultures of epimastigotes from exponential growth phase. *T. rangeli* were grown in liver infusion tryptose (LIT) medium (DIFCO) supplemented with 20% FBS. The short epimastigotes of *T. rangeli* (90% purity) were harvested from exponential growth phase four days culture. To eliminate all carbohydrates of the culture medium, *T. cruzi* suspensions were washed three times in sterile phosphate-buffered saline (0.15 M NaCl and 0.01 M phosphate buffer, pH 7.2), and *T. rangeli* in sterile phosphate-buffered saline (0.18 M NaCl and 0.01 M phosphate buffer, pH 7.2) at 300*g* for 15 min.

2.2. Bacteria

Serratia marcescens, prodigiosin pigment producer variants SM 365 (supplied by Dr. Cecile Wandersman, Pasteur Institute, Paris, France) and RPH, isolated from the stomach of *R. prolixus* (Azambuja et al., 2004) were used in these experiments. The two variants of *S. marcescens* were cultured into stationary phase for 24 h at 28 °C in liquid BHI medium by aseptically adding 1 ml of liquid medium into 4 ml of sterile BHI. The colony-forming units (CFU) were estimated by comparing bacteria culture turbidity by optical density with $BaSO_4$ solutions of McFarland standards (Jousimies-Somier and Summanen, 2002).

2.3. Carbohydrates

All carbohydrates (D-mannose, D-mannosamine, *N*-acetyl mannosamine, D-glucose and D-galactose) (Sigma Chemical, St. Louis, MO) were diluted in phosphate-buffered saline (PBS, 0.15 M NaCl and 0.01 M phosphate buffer, pH 7.2) at concentration of 2 M and filtered in 0.22 μm pore Millipore membranes.

2.4. Trypanolytic assays

To quantify the trypanolytic activity, 10 μl of *S. marcescens* SM 365 and RPH variants in concentration of 1×10^8 CFU/ml were added to 80 μl of *T. cruzi* and *T. rangeli* epimastigote suspensions of 250×10^4 parasites/ml in Eppendorf tubes, and incubated at 28 °C for 30, 60 and 120 min. In some experiments, to a number of tubes 10 μl of one of the carbohydrate solutions (2 M) were added to 80 μl of parasites (250×10^4 epimastigotes/ml), and then followed immediately by 10 μl suspensions of the *S. marcescens* variants (1×10^8 CFU/ml). As controls in each assay, 10 μl of phosphate-buffered saline was added instead of the bacteria suspensions or carbohydrate solutions. After completing the final volumes of 100 μl, the mixtures were incubated at 28 °C up to 120 min. In each assay, the number of living parasites, recognized by the integral morphology and some slight movements of the flagellum in the medium, was estimated by direct counting in a Neubauer hemocytometer chamber under a light microscope.

2.5. Hemolytic activity

For *in vitro* hemolysis, citrated human blood was centrifuged for 10 min at 1500*g* and the plasma discarded. After five washes with PBS under sterile conditions, the erythrocytes were suspended at 10% in the same buffer and stored at 4 °C until use (no more than 72 h). Aliquots of 240 μl of erythrocyte suspension (10%) were incubated with 30 μl of bacteria suspension (final concentration of 1×10^8 CFU/ml). For carbohydrate hemolysis inhibition, 30 μl of each carbohydrate solution (final concentration of 0.2 M) were added to 240 μl of erythrocytes, and then 30 μl suspensions of *S. marcescens* variants (1×10^8 CFU/ml). For controls, 30 μl of phosphate-buffered saline were added instead of the bacteria suspension or carbohydrate solution to complete the 300 μl of solution. After incubation of the mixtures at 30 °C for 4 h, aliquots of 15 μl of the supernatants obtained by centrifugation at 1500*g* for 10 min where added to 100 μl of Drabkin's reagent (Sigma Chemical Co, St. Louis, MO) in 96-well cell culture microtiter plates and read on an Elisa reader at 550 nm. Hemoglobin release was calculated as a percentage of the total hemoglobin concentration in the sample (Azambuja et al., 2004).

2.6. Statistical analysis

Data were compared using the Student–Newman–Keuls (SNK) test according to Stats Direct Statistical Software, version 2.2.7 for Windows 98. Data are reported as means ± SD. Differences between the groups were considered not statistically significant when $p > 0.05$. Probability levels are specified in the text.

3. Results

3.1. Trypanolytic and hemolytic of S. marcescens: effects of carbohydrates

In this investigation, we examined the lysis of *T. cruzi* Y strain, *T. rangeli* H14 strain and human erythrocytes caused by *S. marcescens* SM 365 and RPH and compared with the data obtained by the addition of different carbohydrates in the incubation medium. The results are displayed in Table 1. *T. cruzi* and *T. rangeli* were drastically lysed by both variants of *S. marcescens* in the absence of sugars. The results of the incubation of *T. cruzi* and *T. rangeli* with *S. marcescens* SM 365 and RPH in the presence of different carbohydrates demonstrated that while 0.2 M of D-mannose inhibited the lyses of *T. cruzi* and *T. rangeli*, D-mannosamine, *N*-acetyl mannosamine, D-glucose and D-galactose did not protect the parasites against the trypanolytic effects caused by both variants of bacteria. The hemolysis induced by *S. marcescens* variants SM 365 and RPH was not inhibited in the presence of D-mannose, D-mannosamine, *N*-acetyl mannosamine, although a decrease in the hemolytic activities in the samples incubated with D-glucose and D-galactose was observed (Table 1).

3.2. Kinetics of lysis of T. cruzi Y strain and T. rangeli H14 strain by S. marcescens variants SM 365 and RPH: effects of D-mannose

The kinetics of lysis of *T. cruzi Y strain* by *S. marcescens* SM 365 and RPH in the presence of 0.2 M of D-mannose is shown in Fig. 1A. From the initial number of parasites (i.e., 250×10^4 flagellates/ml) by 60 min incubation the number of parasites had significantly been reduced and by 120 min, the number of surviving Y strain parasites in the incubation medium containing the variants SM 365 and RPH declined to the limit of parasite detection/ml. In contrast, the number of parasites decreased only approximately 2-fold in the incubation medium containing D-mannose for both variants of bacteria and this is significantly different to the massive parasite reductions recorded above without incubation with mannose (Student's test, $p < 0.001$ for both bacteria) (Fig. 1A).

The lysis of *T. rangeli* H14 following incubation with *S. marcescens* variants SM 365 and RPH is displayed in Fig. 1B. The population densities of H14 strain rapidly decreased in the incubation medium when incubated with SM 365 and RPH variants. By 60 min, the number of parasites was significantly reduced and at 120 min after starting the incubation with *S. marcescens* SM 365 and RPH, very few H14 parasites were observed and, in some cases, below the limit of detection of parasites. In contrast, the *T. rangeli* strain H14 maintained the number of flagellate

Table 1
Effects of different carbohydrates on the lytic activities of *Trypanosoma cruzi* strain Y, *Trypanosoma rangeli* strain H14 and human erythrocytes induced by *Serratia marcescens* variants SM 365 and RPH

Carbohydrates[a]	*S. marcescens*[b] variants	Parasites number ($\times 10^4$/ml)[c]		Hemolyis (%)[d]
		T. cruzi	*T. rangeli*	
Without	SM 365	12 ± 4	12 ± 5	59.4 ± 5.3
	RPH	4 ± 1	19 ± 9	67.1 ± 3.3
	No bacteria	240 ± 18	232 ± 13	0.5 ± 0.02
D-mannose	SM 365	123 ± 15	138 ± 27	56.1 ± 6.3
	RPH	115 ± 31	135 ± 22	52.9 ± 6.6
	No bacteria	203 ± 24	238 ± 12	1.5 ± 0.1
D-mannosamine	SM 365	19 ± 4	19 ± 10	64.6 ± 8.4
	RPH	14 ± 6	22 ± 8	73.8 ± 11.0
	No bacteria	235 ± 1	242 ± 7	2.6 ± 0.1
N-acetyl mannosamine	SM 365	5 ± 3	6 ± 3	59.0 ± 6.2
	RPH	2 ± 1	4 ± 2	53.1 ±3.6
	No bacteria	241 ± 12	225 ± 22	1.2 ± 0.2
D-glucose	SM 365	8 ± 5	3 ± 1	34.8 ± 5.4
	RPH	5 ± 4	16 ± 5	35.5 ± 1.3
	No bacteria	217 ± 15	216 ± 29	0.9 ± 0.1
D-galactose	SM 365	18 ± 5	13 ± 5	29.6 ± 5.9
	RPH	5 ± 3	14 ± 2	36.7 ± 5.1
	No bacteria	203 ± 14	218 ± 17	1.6 ± 0.3

[a] 0.2 M carbohydrates were incubated with parasites before the addition of *S. marcescens*. Each point represents the means ± SD of 4 experiments.
[b] Fresh stationary cultures of *S. marcescens* added in concentration of 1×10^8 CFU/ml according the McFarland Standard.
[c] Epimastigotes of *T. cruzi* and *T. rangeli* estimated by Neubauer hemocytometer chamber after 120 min of incubation at 28 °C.
[d] Percentage of hemolysis was estimated by the release of free hemoglobin measured by Drabkin's reagent after 120 min of incubation at 28 °C.

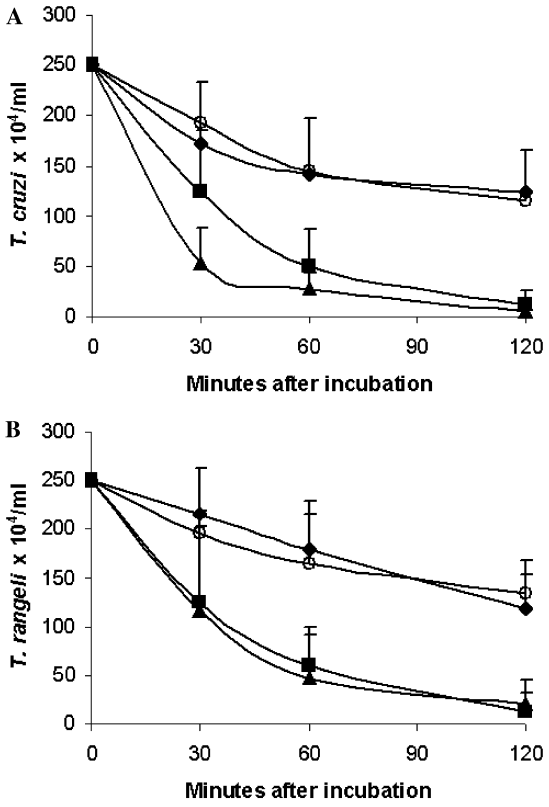

Fig. 1. Temporal course of *T. cruzi* Y strain (A) and *T. rangeli* strain H14 (B) lysis at different time of incubation with *S. marcences*. Epimastigotes (250×10^4 parasites/ml) were incubated with different strains of *S. marcescens* (approximately 1.0×10^8 CFU/ml) and counted in a Neubauer hemocytometer at different times of incubation. *S. marcescens* variants used were SM365 (■), RPH (▲) and tested also in presence of mannose 0.2 M for SM365 (◆) and RPH (◇). Each point represents the means ± SD of 10 experiments.

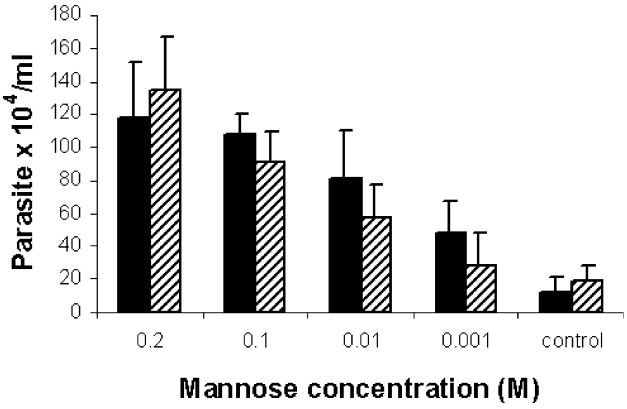

Fig. 2. Lysis of *T. rangeli* H14 strain incubated with *S. marcescens* at different concentration of Mannose. Epimastigotes (250×10^4 parasites/ml) were incubated with different concentration of Mannose and *S. marcescens* (1.0×10^8 CFU/ml). Parasites numbers were estimated by counting in Neubauer hemocytometer after 120 min of incubation (28 °C). The *S. marcescens* variants used were SM365 (■) and RPH (▨). Each point represents the means ± SD of least 8 experiments.

survivors when the incubation medium contained SM 365 and RPH variants in addition to D-mannose (Fig. 1B) ($p < 0.001$ for 60 and 120 min of incubation for both variants).

Fig. 2 shows that D-mannose concentrations from 10 to 200 mM protected *T. cruzi* and *T. rangeli* epimastigotes against lysis induced by the two variants of *S. marcescens*. Even the concentration of 1 mM of D-mannose was able to reduce the lysis of about 50% of the parasites (Fig. 2).

4. Discussion

The main results in the present work can be stated as follows. In vitro trypanolytic proprieties of Gram-negative enterobacteria, *S. marcescens* variants SM 365 and RPH, performed with epimastigotes of *T. cruzi* strain Y and *T. rangeli* strain H14 *are* distinct from hemolytic action of the bacteria and dependent on D-mannose concentration in the medium. With regard to the role of carbohydrates in trypanolytic and hemolytic activities of *S. marcescens*, the results demonstrated that only D-mannose inhibits the lyses of *T. cruzi* and *T. rangeli* caused by *S. marcescens*, although this carbohydrate was unable to interfere with the lyses of human erythrocytes induced by *S. marcescens*. The fact that D-glucose and D-galactose did not protect the parasites indicates the trypanolytic action of *S. mascescens* is distinct from the hemolytic effect. The trypanolytic effect of both variants of bacteria tested with *T. cruzi* and *T. rangeli* decreased with addition of D-mannose in concentrations higher than 0.1 M.

Many papers have described the importance of glycosylphosphatidyllinositol (GPI) membrane anchors and glycoproteins in the parasite life cycle. It is well known that mannose is an important carbohydrate present in GPI membrane anchors on *T. cruzi* and *T. rangeli* (Bonay and Fresno, 1995; Nok et al., 2000; Bonay et al., 2001; Barboza et al., 2005). Bonay et al. (2001) reported that epimastigotes and trypomastigotes of *T. cruzi* parasites express a carbohydrate binding protein (CBP) that can bind D-mannose residues and that CBP from epimastigotes displays strong affinity for higher D-mannose oligomers. Azambuja et al. (2004, 2005b) using Y strain of *T. cruzi* demonstrated that *S. marcescens* rapidly attaches to the parasite membrane to kill the flagellate. The presence of fimbriae on a bacterium is known to be associated with adhesive properties on the cellular membrane surface (Duguid et al., 1966). *S. marcescens* possesses mannose sensitive fimbriae which mediate adherence to different eukaryotic cells and consequently virulence (Reid and Sobel, 1987). Analyses of the fimbriae of this bacterium showed at least two main types of fimbriae, one causing mannose resistant (MR) and another causing mannose sensitive (MS) hemagglutination of guinea-pig or chicken erythrocytes (Hejazi and Falkner, 1997). Since the treatment of *T. cruzi* and *T. rangeli* with D-mannose systematically resulted in inhibiting the trypanolytic activity induced by *S. marcescens* variants SM 365 and RPH, we suggest that MS fimbriae are relevant to the mechanisms of trypanosome–bacteria associations. i.e., D-mannose binds to *S. marcescens* MS fimbriae

avoiding the attachment to CBP found in the surface of *T. cruzi* and *T. rangeli*. In contrast, as expected, D-mannose was unable to block the hemolysis caused by *S. marcescens* variants SM 365 and RPH, indicating a different trypanolytic mechanism.

An association between a protozoan and bacteria can also be observed in *Shigella flexneri* that expresses mannose-binding surface lectins, attaching to mannose molecules on the surface of *Entomoeba histolytica* (Verdon et al., 1992). The association of some Gram-negative bacteria, including *Pseudomonas aeroginosa*, with their target cells is also important for the release of toxic bacterial proteins into the cytosol of these cells (Cornelis, 1998). Recently, Hejazi and Falkner (1997) demonstrated that *S. marcescens* possesses mannose-dependent fimbriae and can adhere to biological surfaces and induce the production of reactive oxygen molecules by cells. It is worth to speculate that such molecules might be very toxic to parasites even within the digestive tract of the insect-vector, where *S. marcescens* are found in the microbiota (Azambuja et al., 2004, 2005b).

The present paper hopefully provides a sound basis for future work on protozoan–bacteria interaction models focused on the action of virulence factors on parasites, which might yield a novel vector control strategy.

Acknowledgments

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (to P.A.), Fundação Oswaldo Cruz (Fiocruz) (Papes project to P.A.), and Fundação de Amparo a Pesquisa do Estado do Rio de Janeiro (Faperj) (to P.A.) P.A. and E.S.G. are CNPq Senior Research Fellows, and D.P.C. is Ph.D. student with fellowship from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior).

References

Azambuja, P., Garcia, E.S., 2005. *Trypanosoma rangeli* interactions within the vector *Rhodnius prolixus*—a mini review. Memorias do Instituto Oswaldo Cruz 100, 567–572.

Azambuja, P., Guimarães, J.A., Garcia, E.S., 1983. Hemolytic factor from the stomach of *Rhodnius prolixus*: evidence and partial characterization. Journal of Insect Physiology 29, 833–837.

Azambuja, P., Feder, D., Garcia, E.S., 2004. Isolation of *Serratia marcescens* in the midgut of *Rhodnius prolixus*: impact on the establishment of the parasite *Trypanosoma cruzi* in the vector. Experimental Parasitology 107, 89–96.

Azambuja, P., Ratcliffe, N.A., Garcia, E.S., 2005a. Towards an understanding of the interactions of *Trypanosoma cruzi* and *Trypanosoma rangeli* within the reduviid insect host *Rhodnius prolixus*. Anais da Academia Brasileira de Ciências 77, 397–404.

Azambuja, P., Garcia, E.S., Ratcliffe, N.A., 2005b. Gut microbiota and parasite transmission by insect vectors. Trends in Parasitology 21, 568–572.

Barboza, M., Duschak, V.G., Fukuyama, Y., Nonami, H., Erra-Balsells, R., Cazzulo, J.J., Couto, A.S., 2005. Structural analysis of the *N*-glycans of the major cysteine proteinase of *Trypanosoma cruzi*. Identification of sulfated high-mannose type oligosaccharides. FEBS Journal 272 (15), 3803–3815.

Bonay, P., Fresno, M., 1995. Characterization of carbohydrate binding proteins in *Trypanosoma cruzi*. The Journal of Biological Chemistry 270 (19), 11062–11070.

Bonay, P., Molina, R., Fresno, M., 2001. Binding specificity of mannose-specific carbohydrate-binding protein from the cell surface of *Trypanosoma cruzi*. Glycobiology 21 (9), 719–729.

Brener, Z., 1973. Biology of *Trypanosoma cruzi*. Annual Review of Microbiology 27, 347–382.

Cornelis, G.R., 1998. The *Yersinia* deadly kiss. Journal of Bacteriology 180, 5495–5504.

Duguid, J.P., Anderson, E.S., Campbell, I., 1966. Fimbriae and adhesive properties in salmonellae. Journal of Pathology and Bacteriology 92, 107–137.

Garcia, E.S., Azambuja, P., 1991. Development and interactions of *Trypanosoma cruzi* within the insect vector. Parasitology Today 7, 240–244.

Garcia, E.S., Mello, C.B., Azambuja, P., Ribeiro, J.M.C., 1994. *Rhodnius prolixus*: salivary anti-hemostatic components decrease with *Trypanosoma rangeli* infection. Experimental Parasitology 78, 287–293.

Hecker, H., Schwarzenbach, M., Rudin, W., 1990. Development and interactions of *Trypanosoma rangeli* in and with the reduviid bug, *Rhodnius prolixus*. Parasitology Research 76, 311–318.

Hejazi, A., Falkner, F.R., 1997. *Serratia marcescens*. Journal of Medical Microbiology 46, 903–912.

Jousimies-Somier, H., Summanen, P., 2002. Recent taxonomic changes and terminology update of clinically significant anaerobic gram-negative bacteria (excluding spirochetes. Clinical and Infection Diseases 35, 17–21.

Kollien, A.H., Schaub, G.A., 2000. The development of *Trypanosoma cruzi* in Triatominae. Parasitology Today 16, 381–387.

Lehane, M.J., 1991. Transmission of parasites by blood sucking insects. Biology of Blood Sucking Insects. University Press, Cambridge, pp. 143–192.

Mello, C.B., Azambuja, P., Garcia, E.S., Ratcliffe, N.A., 1996. Differential in vivo behaviour of three strains of *Trypanosoma cruzi* in the gut and hemolymph of *Rhodnius prolixus*. Experimental Parasitology 82, 112–121.

Nok, A.J., Shuaibu, M.N., Kanbara, H., 2000. Purification and partial characterization of α-mannosidase from *Trypanosoma rangeli*. Parasitology Research 86, 923–928.

Ofek, I., Sharon, N., 1990. Adhesins as lectins: specificity and role in infection. Current Topics on Microbiology and Immunology 151, 91–113.

Ratcliffe, N.A., Whitten, M.M.A., 2004. In: Gillespie, S.H. (Ed.), Vector Immunity in Microbe–vector Interactions. SGM Symposium. Cambridge University Press, Cambridge, pp. 199–262.

Ratcliffe, N.A., Nigan, Y., Mello, C.B., Garcia, E.S., Azambuja, P., 1996. *Trypanosoma cruzi* and eryhrocyte agglutinins: a comparative study of ocurrence and properties in the gut and hemolymph of *Rhodnius prolixus*. Experimental Parasitology 83, 83–93.

Reid, G., Sobel, J.D., 1987. Bacterial adherence in the pathogenesis of urinary tract infection: a review. Review of Infection Diseases 9, 470–487.

Verdon, R., Mirelman, D., Sansonetti, P.J., 1992. A model of the interaction between *Entamoeba histolytica* and *Shigella flexneri*. Research in Microbiology 143, 67–74.

Watkins, R., 1971. Histology of *Rhodnius prolixus* infected with *Trypanosoma rangeli*. Journal of Invertebrate Pathology 17, 59–66.

Whitten, M.M., Mello, C.B., Gomes, S.A.O., Nigam, Y., Azambuja, P., Garcia, E.S., Ratcliffe, N.A., 2001. Role of superoxide and reactive nitrogen intermediates in *Rhodnius prolixus* (Reduviidae)/*Trpanosoma rangeli* interactions. Experimental Parasitology 98, 44–57.

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Experimental Parasitology 117 (2007) 201–207
Experimental Parasitology
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Trypanosoma cruzi: Ultrastructural studies of adhesion, lysis and biofilm formation by *Serratia marcescens*

Daniele P. Castro [a], Sergio H. Seabra [b], Eloi S. Garcia [a], Wanderley de Souza [c], Patrícia Azambuja [a,*]

[a] *Departamento de Bioquímica e Biologia Molecular, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Avenida Brasil 4354, Manguinhos, Rio de Janeiro, CEP 21045-900 RJ, Brazil*
[b] *Departamento de Pesquisa e Extensão, Centro Universitário Estadual da Zona Oeste, Rio de Janeiro, CEP 21070-200 RJ, Brazil*
[c] *Laboratório de Ultraestrutura Celular Hertha Meyer, Instituto de Biofísica Carlos Chagas Filho, Edifício do Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Ilha do Fundão, Rio de Janeiro, CEP 21941-590 RJ, Brazil*

Received 16 January 2007; received in revised form 26 April 2007; accepted 26 April 2007
Available online 22 May 2007

Abstract

A few days after blood meal the number of bacteria in the anterior midgut (stomach) of *Rhodnius prolixus*, a vector of *Trypanosoma cruzi*, the causative agent of Chagas' disease, increases dramatically. Many of the bloodstream trypomastigotes of the pathogenic protozoan as well as ingested erythrocytes are lysed in the stomach. Incubation of *T. cruzi* with *Serratia marcescens* variant SM365, lead to parasite lysis. In the present study, this bacterium rapidly adhered to the protozoan surface through D-mannose recognizing fimbriae and rapidly induced its complete lysis. In contrast, the DB11 variant of the same bacterial species did not adhere and did not induce protozoan lysis. Scanning and transmission electron microscopy revealed that following bacteria–protozoan attachment there is an assembly of long filamentous structures, identified as a biofilm, which connect the protozoan to the bacteria forming bacterial clusters. We conclude that parasite lysis and biofilm formation mechanisms are important for understanding parasite–microbiota interactions in the gut of insect vectors of trypanosomatids.

Index Descriptors and Abbreviations: *Trypanosoma cruzi*; *Serratia marcescens*; Adhesion; Lysis; Biofilm formation

1. Introduction

Little is known about the relative niches occupied at the same time by parasites and bacteria. One of these niches, for example, is the gut of many insects that contains large communities of diverse microorganisms (Dillon and Dillon, 2004). In human and other mammals the gut microbiota play a vital role in a process termed "colony resistance" in which bacteria prevent colonization of the gut by pathogens (Dillon and Dillon, 2004). There are reports concerning the influence of insect vector gut microbiota on parasite life cycle. In mosquitoes midgut infections with bacteria can significantly inhibit sporogonic development (Pumpini et al., 1996; Gonzalez-Ceron et al., 2003). A similar phenomenon is observed in the guts of sandflies infected by the protozoan *Leishmania major* (Schlein et al., 1985), and in the midgut of *Rhodnius prolixus* infected with *Trypanosoma cruzi*, the causative agent of Chagas' disease (Azambuja et al., 2004, 2005).

Azambuja et al. (2004) investigated the fate of the protozoan *T. cruzi* Dm28c clone and Y strain in the gut of the triatomine vector *R. prolixus* after ingestion of an infected blood meal. Two days following blood feeding, the number of stomach bacteria had enhanced ~10,000-fold and, simultaneously, there was a rapid increase in lysis of both erythrocytes and the Y strain protozoan. The Gram-negative bacterium *Serratia marcescens*, a biotyped A1a referenced as *R. prolixus* human (RPH) and a known producer of the pigment prodigiosin, was isolated from the

* Corresponding author. Fax: +55 21 2590 3495.
E-mail address: azambuja@ioc.fiocruz.br (P. Azambuja).

0014-4894/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.exppara.2007.04.014

gut of *Rhodnius*. Following in vitro incubations of *S. marcescens* RPH or a nonprodigiosin-producing form, *S. marcescens* DB11, with erythrocytes and *T. cruzi* Y strain, both bacterial variants lysed the erythrocytes but only the RPH strain killed the parasite (Azambuja et al., 2004, 2005).

Serratia marcescens is an opportunistic pathogen (Grimont and Grimont, 1978). Fimbriae are among the virulent cell surface structures of *S. marcescens* which contain the adhesins mediating adherence to eukaryotic cell surfaces, via recognition of specific carbohydrate moieties on the host cell surface glycoconjugates (Ofek and Sharon, 1990). Hejazi and Falkiner (1997) demonstrated that *S. marcescens* mannose-dependent fimbriae can adhere to biological surfaces and form biofilms which are aggregations of bacteria and extracellular secretions (Queck et al., 2006). Recent experiments carried out in our laboratory demonstrated that D-mannose protects the *T. cruzi* and *T. rangeli* from lysis caused by *S. marcescens* variants SM365 and RPH. However, this carbohydrate was unable to interfere with the hemolysis induced by SM365 and RPH variants. Thus, D-mannose bacterial fimbriae are relevant to *S. marcescens* in lysis of parasites (Castro et al., 2007).

In view of the fact that bacteria-induced *T. cruzi* lysis may explain the dramatic reduction in the number of viable parasites in the initial segments of the insect gut, it was decided to analyze the interaction process of *S. marcescens* with *T. cruzi* further. Here, the authors present ultrastructural evidence, for the first time, that colonization and toxicity of a pathogenic protozoan by *S. marcescens* is a highly dynamic process involving adhesion, lysis and production of biofilms, and that D-mannose recognizing bacterial fimbriae are probably involved in this process.

2. Materials and methods

2.1. Parasites

Trypanosoma cruzi strain Y (supplied by Dr. M.A. Sousa, Trypanosomatid Collection of the Oswaldo Cruz Institute, CT-IOC, Fiocruz, Brazil) was grown in brain heart infusion (BHI) medium (DIFCO Laboratories, Detroit, MI) containing folic acid (30 mg/L), hemin (25 mg/L), and supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 28 °C. After 4 days in cultures, the parasites were collected, washed three times in sterile phosphate-buffered saline (PBS 0.15 M NaCl and 0.01 M phosphate buffer, pH 7.2) at 300 g for 15 min and resuspended in PBS. Parasites were quantified and used for bacteria interaction experiments.

2.2. Bacteria

Two variants of *Serratia marcescens* were used, a prodigiosin pigment producer variant SM365, and a nonpigment variant DB11 (Kurz et al., 2003) as a control (both supplied by Dra. Cecile Wandersman, Pasteur Institute, Paris, France). Both variants of *S. marcescens* were cultured into a stationary phase for 24 h at 28 °C in liquid BHI medium by aseptically adding 1 ml of liquid medium into 4 ml of sterile BHI. The colony-forming units (CFU) were estimated by comparing bacteria culture turbidity by optical density matches with $BaSO_4$ solutions of McFarland standard (Jousimies-Somier and Summanen, 2002).

2.3. Carbohydrate

D-Mannose (Sigma Chemical, St. Louis, MO) was diluted in phosphate-buffered saline (0.15 M sodium chloride in 0.001 M phosphate buffer, pH 7.2) at a concentration of 2 M and filtered in 0.22 μm pore Millipore membranes.

2.4. Bacteria adhesion and lysis to parasite

Interactions were performed for 30, 60 and 120 min at 28 °C using 10:1, 25:1 and 50:1 bacteria:parasite cell number ratio. Quantification of the interaction was assayed by randomly counting at least 200 parasites for each coverslip in quadruplicates under a Zeiss Axioplan Microscope using a 100× oil immersion objective. An adhesion index was determined as follows: adhesion index = percentage of parasites with adhered bacteria × mean number of bacteria adhered per parasite (Seabra et al., 2004). This procedure was performed with the same microscope equipped with a camera to record a video (video microscopy). Photographs were also taken from the video microscopy.

To quantify the trypanolytic activity, *S. marcescens* SM365 and DB11 variants were incubated in the presence of *T. cruzi* epimastigotes using a bacteria/protozoa cell density ratio of 10:1–50:1 times in Eppendorf tubes, and incubated according to Castro et al. (2007). In some experiments 0.2 M D-mannose was added to the incubation medium.

2.5. Electron microscopy

Following bacteria–parasite interaction as described above, the cells were nixed for 2 h in 0.1 M cacodylate buffer, pH 7.2, containing 2.5% glutaraldehyde and 4% freshly prepared formaldehyde, and washed twice with the same buffer. In scanning electron microscopy, cells were post-fixed in a solution of 1% OsO_4 for 40 min at room temperature and washed twice with cacodylate buffer 0.1 M. The cells were dehydrated in graded series of acetone (50–100%), critical point dried using CO_2, mounted on metal stubs and coated with gold (5–30 nm) for observation in a scanning electron microscope (JSM5310). For transmission electron microscopy, after washing, the cells were scraped off the coverslips with a rubber policeman. Centrifugation at 300*g* for 10 min was followed by washing with 0.1 M cacodylate buffer. Cells were post-fixed in a solution of 1% OsO_4 for 40 min at room temperature and washed twice with 0.1 M cacodylate buffer, pH 7.2. The cells were

dehydrated in graded series of acetone (50–100%), and embedded in Epoxy resin. Thin sections were stained with uranyl acetate and lead citrate and observed in a Zeiss 900 transmission electron microscope (TEM). (DaMatta et al., 1998).

3. Statistical analysis

Data were compared using the Student–Newman–Keuls (SNK) test according to Stats Direct Statistical Software, version 2.2.7 for Windows 98. Data are reported as means ± standard deviation. Differences between the groups were considered not statistically significant when $p > 0.05$. Probability levels are specified in the text.

4. Results

4.1. Interaction of Serratia marcescens with T. cruzi

Background experiments using video microscopy recorded *T. cruzi* epimastigote behavior when incubated with *S. marcescens* SM365. Usually the parasite present fast flagellar movement before bacteria attachment (Fig. 1, 10 and 30 s), and after bacteria bind to different regions of the epimastigote body surface the parasite is immobilized (Fig. 1, 1 min). Some bacteria attached to the cell body while others to the protozoan flagellum, and in both cases the parasite started to change the shape (Fig. 1, 5 min) and before lysing the parasite presented round forms (Fig. 1, 10 and 20 min).

4.2. Adhesive and lytic properties of Serratia marcescens in T. cruzi

Serratia marcescens variants SM365 and DB11 were tested for adhesive properties and for lysis against *T. cruzi* strain Y. The adhesion of SM365 to the *T. cruzi* epimastigote surface was dependent on bacteria concentration but not on the time allowed for adhesion (Fig. 2a). With the lower concentration of bacteria (bacteria:parasite ratio of 10:1) in the incubation medium for 15 min, the number of bacteria that adhered to the parasite surface was not significantly different to 120 min (Fig. 2a). In a bacteria:parasite ratio of 25:1 the adhesion index was double of 10:1, while a 50:1 ratio gave an even higher adhesion index. Even using the 50:1 ratio, the DB11 variant of *S. marcescens* did not adhere to the parasite surface.

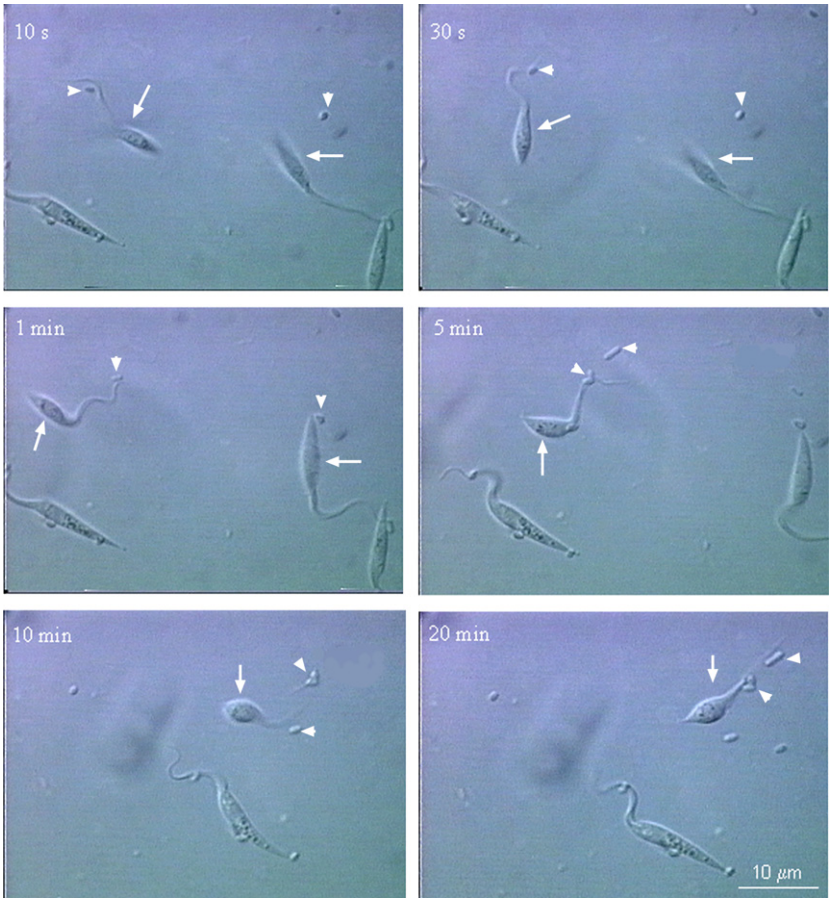

Fig. 1. Video microscopy of initial contact (10 and 30 s), adhesion and immobilization (1 min) of *Trypanosoma cruzi* strain Y by *Serratia marcescens* variant SM365 and initial parasite morphology alteration (5 min) and induction of parasite round forms before lysing (10 and 20 min). Arrowheads indicate bacteria and arrows the parasite.

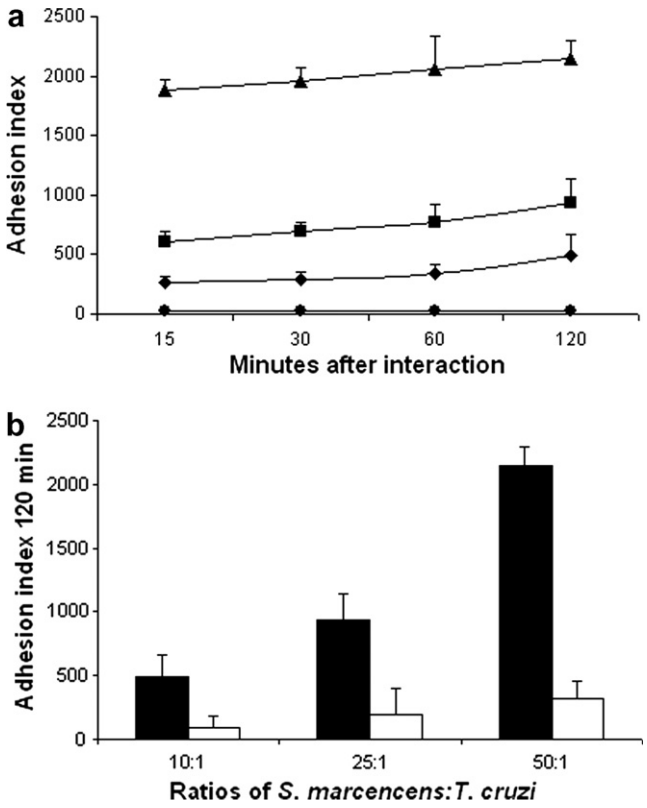

Fig. 2. Adhesion index of *Serratia marcescens* to epimastigotes of *Trypanosoma cruzi* Y strain. (a) Temporal course of adhesion ratios of *S. marcescens*: *T. cruzi* were 10:1 of SM365 (◆), 25:1 SM365 (■), 50:1 SM365 (▲) and 50:1 DB11 (●). (b) Adhesion of *S. marcescens* to *T. cruzi* in incubation medium containing D-mannose (black column) or not (control) (white column). The adhesion index was calculated multiplying the mean number of attached bacteria per epimastigote by the percentage of parasites with adhered bacteria. Each point represents the means ± SD of 5 experiments.

In the presence of 0.2 M D-mannose and after incubation of 120 min, all three bacteria:parasite ratios were significantly diminished ($p < 0.05$, $p < 0.001$ and $p < 0.0001$ for 10:1, 25:1 and 50:1 ratios, respectively) (Fig. 2b).

In the assays of protozoan lysis induced by *S. marcescens* SM365, after incubation for 30 min the number of surviving parasites was reduced approximately 2-fold when the bacteria:parasite ratios were of 25:1 and 50:1. Using a ratio of 10:1 no significant difference was detected in parasite lysis when incubation was carried out in the presence of the SM365 or DB11 variants (not shown). As expected, addition of D-mannose to the incubation medium inhibited the lysis of *T. cruzi* strain Y caused by *S. marcescens* variant SM365 (not shown).

4.3. Structural analysis of interactions

Using scanning electron microscopy, as early as after 1 min of bacteria–parasite interaction (ratio of 10:1), the SM365 bacteria dispersed around and rapidly started adhesion to the surface of *T. cruzi* strain Y (Fig. 3a and b). Some images were indicative of the formation of bacteria 0.2 μm protrusions and a 45.5 nm thin structure, which seem to establish continuity with the protozoan surface (Fig. 3b). At bacteria:parasite ratio of 20:1, a larger number of bacteria was seen on the parasite surface and after a few minutes they modified the shape of the flagellate body (Fig. 3c and d). After 30 min, the bacteria had almost disintegrated the parasite (Fig. 3e), and anomalous rounded parasite shape forms with a large number of bacteria adhered to the protozoan flagellum (Fig. 3e) were frequently observed. Interestingly, after 120 min of incubation, a large number of 23.8 nm (range of 19.0–28.6 nm) thin structures were seen interconnecting bacteria or connecting bacteria to the parasite surface. At this time structured colonies, composed of bacteria connected by these filaments, formed structures which resemble the so called biofilms over the lysed parasites (Fig. 3f–h).

In transmission electron microscopy, the adhesion was a tight apposition of the bacteria and protozoan membranes (Fig. 4A and B). Interestingly, an electron dense material accumulation between *T. cruzi* and *S. marcescens* variant SM365 in the adhesion region was observed (Fig. 4B and C). The filamentous material that connected bacteria to protozoan was also seen (Fig. 4D). The adhesive capacity of variant DB 11 was lower (Fig. 4E) and weaker then variant 365 (Fig. 4F).

5. Discussion

Since the initial studies on *T. cruzi* interaction with its insect vector, it has been recognized that a significant number of parasites ingested during blood meal are destroyed in the early anterior portions of the midgut (Dias, 1934). It is even possible that such a process is responsible for the fact that the protozoan does not develop well in all Reduviidae species (Garcia and Azambuja, 1991; Garcia et al., 1984). Previous studies by the authors have shown that following blood feeding a dramatic increase in the number of bacteria in the insect anterior midgut takes place leading to lysis of erythrocytes and *T. cruzi* strain Y (Azambuja et al., 2004, 2005). Our present observations in vitro show that *S. marcescens* SM365 adheres to the *T. cruzi* surface in a process where cell surface exposed carbohydrate residues seem to be involved.

Many papers describe the importance of the glycosylphosphatidylinositol membrane anchor and glycoproteins in the parasite life cycle and that mannose is an important carbohydrate present on *T. cruzi* (Bonay and Fresno, 1995; Nok et al., 2000; Bonay et al., 2001; Barboza et al., 2005). Epimastigotes and trypomastigotes of *T. cruzi* express a carbohydrate binding protein that can bind D-mannose residues and this binding protein from epimastigotes displays strong affinity for higher D-mannose oligomers (Bonay et al., 2001). *S. marcescens,* mannose sensitive fimbriae (MS) are associated with bacteria virulence (Duguid, 1959; Reid and Sobel, 1987; Hejazi and Falkiner, 1997), and the trypanolytic activity induced by *S. marcescens* variant 365 against *T. cruzi* is inhibited by D-mannose (Castro

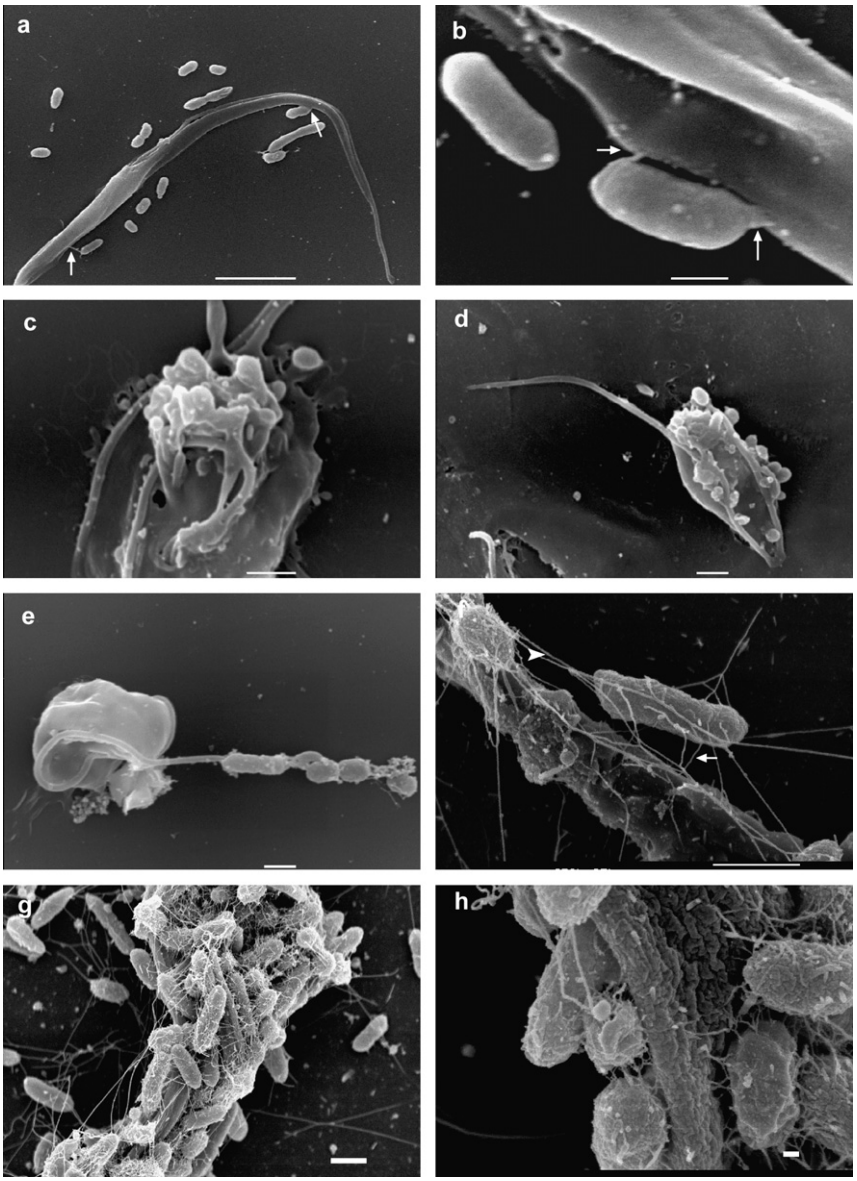

Fig. 3. Scanning electron microscopy of *Serratia marcescens* variant SM365 adhered to *Trypanosoma cruzi*. (a) Adhesion of *S. marcescens* to *T. cruzi* using a 10:1 ratio after 1 min of incubation (arrows). (b) Filaments are seen connecting bacteria to the parasite after 1 min of incubation (arrows). (c and d) Dense concentration of bacteria adhered to flagellate shows modified parasite form after 5 min of incubation using a 20:1 bacteria:parasite ratio. (e) Parasite almost disintegrated after 30 min of incubation in 20:1 ratio. (f) Filaments interconnecting bacteria (arrowhead) and connecting bacteria to the parasite surface (arrow) after 120 min of incubation. (g and h) After 120 min of incubation biofilms were seen adhering to and distributed across parasite surface or membrane fragments. Bars, 5 μm (a), 0.5 μm (b), 1 μm (c–g) and 100 nm (h).

et al., 2007). Since the treatment with D-mannose resulted also in inhibiting the bacteria binding to parasites (present paper), the authors suggest that the MS fimbriae act as adhesins, promoting bacteria attachment to the parasite surface, a fundamental step for the subsequent destruction of parasite.

Some investigations have analyzed the mechanisms which are involved in the survival of many bacteria in nature (for review see Queck et al., 2006). One of the most common reactions of bacteria against protozoa is the formation of a bacterial community, biofilm (Matz et al., 2004; Weitere et al., 2005; Queck et al., 2006). In natural ecosystems, bacteria frequently form biofilms, which are recognized as the predominant form of development in the environmental life cycle of bacteria (Watnick and Kolter, 2000; Matz et al., 2004; Rice et al., 2005; Weitere et al., 2005; Queck et al., 2006). Biofilms correspond to assemblages of single or multiple populations that are attached to abiotic or biotic surfaces through extracellular polyanionic substances. In the present work, scanning and transmission electron microscopies showed that *S. marcescens* variant SM365 adheres to *T. cruzi* strain Y epimastigotes or their surface fragments and that thin filaments connected bacteria to each other and to the parasite surface (Fig. 3f and g). This formation suggests the structure

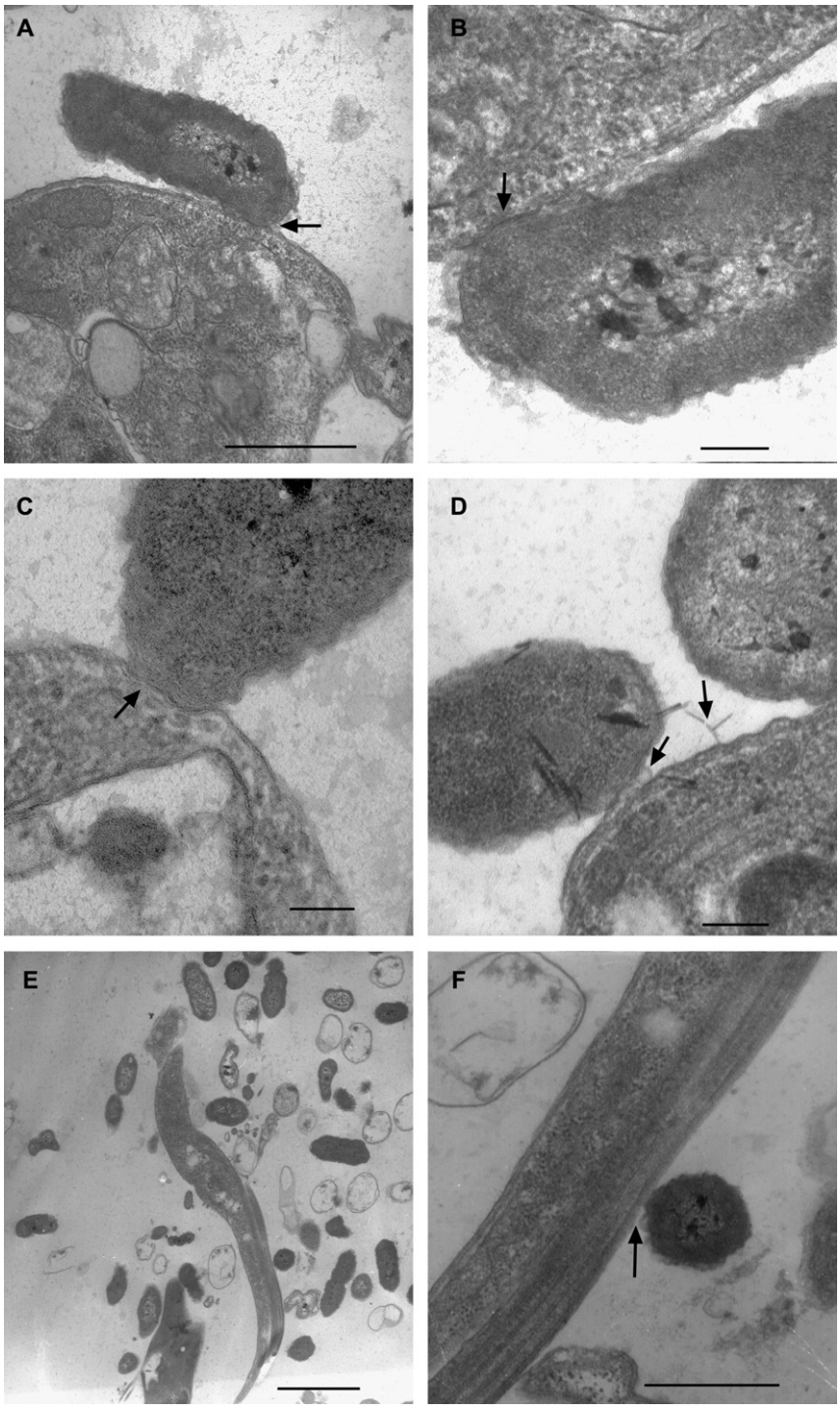

Fig. 4. Transmission electron microscopy of *Serratia marcescens* variant SM365 and DB11 adhered to *Trypanosoma cruzi* after 120 min of incubation. (A and B) Adhesion of *S. marcescens* to *T. cruzi* showing closely apposed plasma membranes (arrows). (C) Electron dense material is seen in the adhesion region (arrow). (D) Bacteria filaments and thin structures connected to parasite membrane (arrows). (E) Low adhesive capacity of *S. marcescens* variant DB11. (F) Weak DB11 variant adhesion (arrow). Bars, 5 μm (A), 1 μm (B and C), 10 μm (D) and 5 μm (E).

of biofilm production through a series of defined stages that culminate in a highly porous, filamentous biofilm composed of cell chains, filaments and cell clusters (Fig. 3f and g). These are the same characteristics as a biofilm formation described for many Gram-negative bacteria in nature (Rice et al., 2005; Labbate et al., 2004; Matz et al., 2004).

Finally, further detailed study of the parasite lysis and biofilm formation mechanism would be extremely useful for understanding the biological cycle of *T. cruzi* in the gut of its insect vector. We believe that the present work is an important first step towards future investigations on protozoa–bacteria interaction models focusing on patho-

genic parasites, which could yield an effective and novel strategy for disease transmission control.

Acknowledgments

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (to P.A. and W.S.), Fundação Oswaldo Cruz (Fiocruz) (Papers project to P.A.) and Fundação de Amparo a Pesquisa do Estado do Rio de Janeiro (Faperj) (to P.A.). P.A., E.S.G. and W.S. are CNPq Senior Research Fellows, and D.P.C. is Ph.D. student with fellowship from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior).

References

Azambuja, P., Feder, D., Garcia, E.S., 2004. Isolation of *Serratia marcescens* in the midgut of *Rhodnius prolixus*: impact on the establishment of the parasite *Trypanosoma cruzi* in the vector. Experimental Parasitology 107, 89–96.

Azambuja, P., Garcia, E.S., Ratcliffe, N.A., 2005. Gut microbiota and parasite transmission by insect vectors. Trends in Parasitology 21, 568–572.

Barboza, M., Duschak, V.G., Fukuyama, Y., Nonamo, H., Erra-Balsells, R., Cazzulo, J.J., Couto, A.S., 2005. Structural analysis of the *N*-glycans of the major cysteine proteinase of *Trypanosoma cruzi*. Identification of sulfated high-mannose type oligosaccharides. FEBS Journal 272, 3803–3815.

Bonay, P., Fresno, M., 1995. Characterization of carbohydrate binding proteins in *Trypanosoma cruzi*. Journal of Biological Chemistry 270, 11062–11070.

Bonay, P., Molina, R., Fresno, M., 2001. Binding specificity of mannose-specific carbohydrate-binding protein from the cell surface of *Trypanosoma cruzi*. Glycobiology 11, 719–729.

Castro, D.P., Moraes, C.S., Garcia, E.S., Azambuja, P., 2007. Inhibitory effects of D-mannose on trypanosomatid lysis induced by *Serratia marcescens*. Experimental Parasitology 115, 200–204.

DaMatta, R.A., Seabra, S.H., de Souza, W., 1998. Further studies on the phagocytic capacity of chicken thrombocytes. Journal of Submicroscopy Cytology and Pathology 30, 271–277.

Dias, E., 1934. Estudos sobre o *Schizotrypanum cruzi*. Memórias do Instituto Oswaldo Cruz 8, 1–115.

Dillon, R.J., Dillon, V.M., 2004. The gut bacteria of insects: nonpathogenic interactions. Annual Review of Entomology 49, 71–92.

Duguid, J.P., 1959. Fimbriae and adhesive properties in *Klebsiella* strains. Journal of General Microbiology 21, 271–286.

Garcia, E.S., Azambuja, P., 1991. Development and interactions of *Trypanosoma cruzi* within the insect vector. Parasitology Today 7, 240–244.

Garcia, E.S., Vieira, E., Gomes, J.E.P.L., Gonçalves, A.M., 1984. Molecular biology of the interaction *Trypanosoma cruzi* invertebrate host. Memórias do Instituto Oswaldo Cruz 29, 33–37.

Gonzalez-Ceron, L., Santillan, F., Rodríguez, M.H., Mendez, D., Hernández-Avila, J.E., 2003. Bacteria in midguts of field-collected *Anopheles albimanus* block *Plasmodium vivax* sporogonic development. Journal of Medical Entomology 40, 371–374.

Grimont, P.A.D., Grimont, F., 1978. The genus *Serratia*. Annual Review of Microbiology 32, 221–248.

Hejazi, A., Falkiner, F.R., 1997. Serratia marcescens. Journal of Medical Microbiology 46, 903–912.

Jousimies-Somier, H., Summanen, P., 2002. Recent taxonomic changes and terminology update of clinically significant anaerobic gram-negative bacteria (excluding spirochetes). Clinical Infectious Diseases 35, 17–21.

Kurz, C.L., Chauvet, S., Andres, E., Aurouze, E., Vallet, M., Michel, G.P.F., Uh, M., Celli, J., Filloux, A., De Bentzmann, S., Steinmetz, I., Hoffmann, J.A., Finlay, B.B., Gorvel, J.P., Ferrandon, D., Ewbank, J.J., 2003. Virulence factors of the human opportunistic pathogen *Serratia marcescens* identified by in vivo screening. EMBO Journal 22, 1451–1460.

Labbate, M., Queck, S.Y., Koh, K.S., Rice, S.A., Givskov, M., Kjelleberg, S., 2004. Quorum sensing-controlled biofilm development in *Serratia liquefaciens* MG1. Journal of Bacteriology 186, 692–698.

Matz, C., Bergfeld, T., Rice, S.A., Kjelleberg, S., 2004. Micro-colonies, quorum sensing and cytotoxiy determine the survival of *Pseudomonas aeruginosa* biofilms exposed to protozoan grazing. Environmental Microbiology 6, 218–226.

Nok, A.J., Shuaibu, M.N., Kanbara, H., Yanagi, T., 2000. Purification and partial characterization of alpha-mannosidase from *Trypanosoma rangeli*. Parasitology Research 86, 923–928.

Ofek, I., Sharon, N., 1990. Adhesins as lectins: specificity and role in infection. Current Topics in Microbiology and Immunology 151, 91–113.

Pumpini, C.B., DeMaio, J., Kent, M., Davis, J.R., Beier, J.C., 1996. Bacterial population dynamics in three anopheline species: impact on *Plasmodium* sporogonic development. American Journal of Tropical Medicine and Hygiene 54, 214–218.

Queck, S.Y., Weitere, M., Moreno, A.M., Rice, S.A., Kjelleberg, S., 2006. The role of quorum sensing mediated developmental traits in the resistance of *Serratia marcescens* biofilms against protozoan grazing. Environmental Microbiology 8, 1017–1025.

Reid, G., Sobel, J.D., 1987. Bacterial adherence in the pathogenesis of urinary tract infection: a review. Review of Infectious Diseases 9, 470–487.

Rice, A.S., Koh, K.S., Queck, S.Y., Labbate, M., Lam, K.W., Kjelleberg, S., 2005. Biofilm formation and sloughing in *Serratia marcescens* are controlled by quorum sensing and nutrient cues. Journal of Bacteriology 187, 3477–3485.

Schlein, Y., Polacheck, I., Yuval, B., 1985. Mycoses, bacterial infections and antibacterial activity in sandflies (Psychodidae) and their possible role in the transmission of leishmaniasis. Parasitology 90, 57–66.

Seabra, S.H., DaMatta, R.A., de Mello, F.G., de Souza, W., 2004. Endogenous polyamine levels in macrophages is sufficient to support growth of *Toxoplasma gondii*. Journal of Parasitology 90, 455–460.

Watnick, P., Kolter, R., 2000. Biofilm, city of microbes. Journal of Bacteriology 182, 2675–2679.

Weitere, M., Bergfeld, T., Rice, S.A., Matz, C., Kjelleberg, S., 2005. Grazing resistance of *Pseudomonas aeruginosa* biofilms depends on type of protective mechanism, developmental stage and protozoan feeding mode. Environmental Microbiology 7, 1593–1601.

8. DISCUSSÃO

O completo desenvolvimento dos parasitas no hospedeiro invertebrado é complexo e seu sucesso depende da adaptação entre as espécies envolvidas. Porém o inseto vetor, assim como a maioria dos seres vivos, possue microorganismos simbiontes em seu trato digestivo (Grimont e Grimont, 1978). Assim para que o parasita complete seu ciclo de vida no inseto, este deve enfrentar ambientes potencialmente hostis não apenas impostos pelas barreiras fisiológicas e imunológicas, mas também as diferentes conseqüências advindas das interações com a diversa variedade de bactérias resultantes da co-habitação com a microbiota de cada inseto (Azambuja et al., 2005). Essas interações entre inseto vetor, microbiota e parasita é extremamente complexa e influencia diretamente no sucesso da transmissão de uma doença. Quanto mais longa a co-evolução do parasita junto ao hospedeiro invertebrado e sua microbiota maior será a adaptação e portanto, a probabilidade de ser transmitido a hospedeiros vertebrados.

Em 2004, Azambuja et al. isolaram de *Rhodnius prolixus* mantidos em colônias de laboratórios a bactéria *Serratia marcescens*. Essa bactéria por sua vez demonstrou atividade hemolítica e tripanolítica em ensaios *in vitro* com diferentes cepas de tripanossomatídeos. Os autores também observaram que após a alimentação sanguínea ocorre um drástico aumento no número de bactérias presentes no trato digestório do inseto seguido de decréscimo do número de parasitas. Já era de conhecimento que um número significativo de *Trypanosoma cruzi* ingeridos durante a alimentação do barbeiro são destruídos nas primeiras porções do intestino médio anterior (Dias, 1934) e que isso acarreta na capacidade limitada de desenvolvimento desse parasita em insetos da espécie Reduviidae (Garcia e Azambuja, 1991; Garcia et al., 1984). Porém, até então não se cogitava a hipótese da microbiota presente no sistema digestório do inseto apresentar atividade tripanolítica que pudesse controlar o número de parasitas que colonizam o intestino.

Neste contexto, foi investigado os diversos efeitos e mecanismos envolvidos na interação dos parasitas presentes no inseto vetor, *R. prolixus*, com diferentes variantes da bactéria *S. marcescens* sendo uma delas isolada desse inseto (Azambuja et al., 2004).

No presente trabalho de tese, primeiramente, foi observado que apenas as variantes de *S. marcescens* RPH e SM365 (ambas produtoras de prodigiosina, o pigmento vermelho) possuem atividade lítica sobre *T. cruzi* e *T. rangeli* o que não ocorre com a variante DB11. As variantes SM365 e DB11 (colônia branca, não produtora de prodigiosina) foram cedidas pelo Institute Pasteur (Castro et al., 2007 – Anexo 5). Sabe-

se que a prodigiosina somente é produzida por essas bactérias em temperaturas em torno de 30ºC em fase estacionária de crescimento, mas não a 37ºC (Williams, 1973).

Apesar de a prodigiosina purificada apresentar efeito tripanolítico (Melo et al., 2000) os autores, Moraes et al. 2009 observaram que *S. marcescens* possui efeito lítico sobre *Leishmania braziliensis* independente da presença do pigmento prodigiosina. Uma vez que prodigiosina somente é produzida por essas bactérias em temperaturas em torno de 30ºC em fase estacionária de crescimento (Williams, 1973), os autores cultivaram a cepa SM365 de *S. marcescens* a 30 e 37ºC. Como esperado, na temperatura mais alta (37ºC) não ocorre produção de prodigiosina, e ambas apresentaram atividade lítica sobre *L. braziliensis*. Além disso, em infecções hospitalares são encontradas apenas cepas de *S. marcescens* não produtoras de prodigiosina (Phillips e King, 1977; Carbonell et al., 2000), indicando que a prodigiosina não parece ser um fator de virulência para *T. cruzi* e *Leishmania* (Hejazi e Falkner, 1997).

A cinética de lise das cepas SM365 e RPH demonstra que a atividade lítica dessas variantes é rápida eliminando em torno de 50% dos parasitas, *T. cruzi* e *T. rangeli*, presentes no meio em apenas 30 min de incubação. Após 120 min de incubação a presença de parasitas vivos no meio chega ao limiar de detecção da câmara de Neubauer. Nesse processo podemos observar rápida adesão das bactérias aos parasitas após a incubação, mudanças na morfologia seguida de lise da célula (Castro et al., 2007 – Anexo 5). Alterações semelhantes foram observadas pelos autores, Mercado e Colón-Whitt (1982), após incubação de *Pseudomonas fluorescens* com *T. cruzi*.

Para o sucesso da atividade tripanolítica as bactérias precisam primeiramente aderir à célula. A adesão bacteriana, por meio de adesinas (lectinas), ocorre pelo reconhecimento de carboidratos presentes na célula hospedeira (Duguid, 1959; Old, 1972; Soto e Hultgren, 1999; Garred et al., 2003; Sharon, 2006).

Na presente tese, correlacionando a importância da adesão bacteriana como fator de virulência descrita em inúmeros trabalhos (Duguid, 1959; Reid e Sobel, 1987; Hejazi e Falkiner, 1997; Wu et al., 2008), foram realizados ensaios de interação entre os microorganismos estudados com prévia incubação das variantes de *S. marcescens* com diferentes carboidratos. Observamos que na presença de D-manose as bactérias não aderiam ao parasita, impedindo a atividade lítica. Essa proteção à adesão das bactérias aos parasitas foi específica para D-manose, não tendo efeito nesse processo outros carboidratos derivados de manose. Old, em 1972, demonstrou que o carboidrato D-

manose e alguns de seus derivados são fortes inibidores da interação de fimbria tipo 1 de *Salmonella typhimurium* e *Shigella flexneri* com eritrócitos. De acordo com inúmeros trabalhos, a fimbria tipo 1 associada a lectina do tipo manose sensível de *S. marcescens* é um importante fator de adesão as células (Hejazi e Falkiner, 1997).

Ensaios de atividade lítica com diferentes concentrações de D-manose demonstraram que a proteção dada ao parasita pela incubação de *S. marcescens* com esse carboidrato é dose dependente. Acima de 0,1M de D-manose no meio ocorre inibição de mais de 50% da atividade lítica sobre *T. cruzi* e *T. rangeli* (Castro et al., 2007 – Anexo 5).

Além da presença de lectinas, denominadas de adesinas, do tipo manose sensível em *S. marcescens* vários trabalhos relatam a presença de resíduos de manose na superfície do *T. cruzi* e *T. rangeli*. As âncoras de glicosilfosfatidillinositol (GPI) e glicoproteínas presentes na membrana de tripanosomatídeos são extremamente importantes para seu ciclo de vida. Nessas âncoras de GPI da membrana de *T. cruzi* e *T. rangeli* são bem conhecidas as presenças de subunidades de manose na sua estrutura (Bonay e Fresno, 1995; Kahn et al., 1995; Mello et al., 1996; Nok et al., 2000; Bonay et al., 2001; Barboza et al., 2005; Mendonça-Previato et al., 2005). As subunidades de manose presentes na superfície de tripanosomatídeos têm sido também relatadas como importante fator de adesão à parede do trato digestivo dos insetos e de reconhecimento por células de mamíferos (Villalta e Kierszenbaum, 1983; Araújo-Jorge e de Souza, 1986; Kahn et al., 1995; Mello et al., 1996; Alves et al., 2007).

Em resumo, sabe-se da presença de manose na superfície da membrana dos parasitas *T. cruzi* e *T. rangeli*, a presença de fimbrias com adesinas sensíveis a manose nas variantes SM365 e RPH de *S. marcescens* e que D-manose inibe a atividade lítica dessas variantes. Assim, pode-se sugerir que a adesão bacteriana ao parasita através do reconhecimento de manose na sua superfície é de extrema importância para o início da atividade lítica da bactéria. Em outras palavras as fimbriae MS de *S. marcescens* se ligam ao carboidrato livre D-manose, evitando a adesão às GPIs presentes na superfície de *T. cruzi* e *T. rangeli* e, consequentemente, protegendo o parasita da destruição pela bacteria.

Similarmente, outros trabalhos enfocam o papel de adesinas manose-sensíveis no mecanismo de adesão de bactérias a células hospedeiras. A bactéria *Shigella flexneri* expressa lectinas que se ligam a manose e são responsáveis pelo reconhecimento desse carboidrato na superfície da célula do protozoário *Entamoeba histolytica* (Verdon et al.,

1992). Segundo os autores Pan et al. (1997) a adesão de *E. cloacae* a células de tumor intestinal envolve a participação das fimbriae manose-sensíveis da bactéria, nas quais se observam 50% de inibição da adesão quando adicionados ao meio D-manose ou derivados de manose. Esses mesmos autores isolaram quatro subunidades da fimbria manose-sensível de *E. cloacae* e demonstraram reconhecimento de Man9(GlcNac)2 pela subunidade de 35-kDa. O bloqueio ou inibição das lectinas presentes nas bactérias patogênicas através de carboidratos ou análogos na prevenção e tratamento de doenças microbianas é o alvo das pesquisas de terapia anti-adesão dessas doenças (Sharon, 2006).

Em relação à atividade hemolítica de *S. marcescens,* observada pela incubação *in vitro* com suspensão de eritrócitos humanos, a presença de D-manose e outros carboidratos testados não interferiu na lise de eritrócitos, indicando que a atividade hemolítica e tripanolítica dessas bactérias são processos independentes um do outro (Castro et al., 2007 – Anexo 5).

Em seguida foram observados os fatores que afetam o mecanismo de adesão da *S. marcescens* aos parasitas. Utilizando microscopia óptica através de contraste por interferência diferencial (DIC) foi observado com maiores detalhes o processo de adesão das bactérias, a fim de calcular o índice de adesão em diferentes condições. Assim, foi constatado que esse processo é dependente da concentração de células de bactérias por células de parasitas e que a adesão entre os microorganismos ocorre em poucos segundos da interação não variando em função do tempo de incubação. As variantes SM365 e RPH de *S. marcescens* possuem alto índice de adesão mesmo nas menores concentrações de bactéria por parasita, porém a variante DB11 não adere ao parasita. Na presença de D-manose a adesão bacteriana é inibida, como esperado, evidenciando a importância da fimbria manose sensível nessa interação (Castro el al., 2007 – Anexo 6). Cabe ressaltar que no genoma da variante DB11, recentemente sequenciado, não consta o gene que codifica fimbria manose sensível em contrapartida com o publicado para outras variantes dessa mesma bactéria (Pesquisa em Sanger Institute - http://www.sanger.ac.uk/cgi-bin/blast/submitblast/s_marcescens).

Moraes et al., (2008) observaram através de microscopia eletrônica de varredura a adesão *S. marcescens* à base do flagelo de *L. chagasi* correspondendo ao observado pela coloração do parasita com concanavalina A (ConA) conjugada com fluorocromo fluoresceína isotiocianato (FITC). A ConA possuindo afinidade à derivados de D-manose demonstrou a presença de manose preponderantemente na base do flagelo de *L.*

chagasi, onde foi observado maior adesão bacteriana. No caso de *T. cruzi* e *T. rangeli* a fluorescência do parasita com ConA-FITC A demonstrou sítios de presença de manose espalhados aleatoriamente pela superfície do corpo do parasita (dados não mostrados).

Corroborando esses resultados, a microscopia eletrônica de varredura dos tripanossomatídeos *T. cruzi* e *T. rangeli* interagindo com *S. marcescens* SM365 e RPH demonstrou adesão da bactéria em toda a superfície da membrana dos parasitas. Além de bactérias aderidas ao corpo do parasita em poucos minutos de interação observou-se estruturas conectando as bactérias aos parasitas. Com 30 minutos de incubação já foi possível observar rompimento da membrana dos parasitas e deformação em sua estrutura. Com maiores períodos de interação foram observados restos de membrana de parasitas na lâmina. Utilizando microscopia com maior resolução pode-se observar detalhes da adesão bacteriana que consiste de finos filamentos saindo das bactérias e aderindo aos parasitas (Castro et al., 2007– Anexo 6).

Mudanças na morfologia da célula do parasita também foram observadas através da microscopia de vídeo. Em apenas poucos segundos as bactérias se aderem ao parasita e o imobilizam. Em alguns minutos os parasitas começam a apresentar formas arredondadas e inchar, seguindo de rompimento da célula (Castro el al., 2007– Anexo 6). Alterações semelhantes em tripanosomatídeos advindas da interação com bactérias também foram observadas pelos autores Mercado e Colón-Whitt em 1982.

Em um período maior de interação, foram observados parasitas parcialmente danificados e envoltos por uma rede de bactérias com longos filamentos fazendo conexão entre si e com o parasita (Castro el al., 2007– Anexo 6). Essas estruturas, semelhantes às descritas por outros autores para bactérias Gram-negativas (Rice et al., 2005; Labbate et al., 2004; Matz et al., 2004), foram caracterizadas em *S. marcescens* e parasitas como biofilme. A formação de biofilme é uma das reações mais comuns que envolvem a sobrevivência de bactérias na natureza aumentando sua adaptação e resistência a condições adversas (Matz et al., 2004; Weitere et al., 2005; Queck et al., 2006). Nesse caso a formação de biofilme sugere a resposta de defesa contra os parasitas. Na complexa estrutura do biofilme as bactérias produzem e liberam ao meio uma grande quantidade de exopolissacarídeos que são considerados também fatores de virulência.

Ensaios de formação de biofilme foram realizados, porém não foi possível quantificar o biofilme formado pelas variantes de *S. marcescens* apenas sob a superfície dos parasitas. Nas técnicas utilizadas o biofilme se formava sob superfícies abióticas

contendo ou não parasitas. Em superfícies abióticas não ocorre atração de fimbriae manose-sensível ao substrato específico e sim atrações eletrostáticas através das fimbriae manose-resistentes (Donlan, 2002; Jansen et al., 2004). Assim a presença ou ausência de manose não interfere na formação de biofilme em superfícies abióticas.

Na microscopia de transmissão, a adesão bacteriana pode ser observada com mais detalhes demonstrando uma ligação forte entre as membranas da bactéria e do parasita. Na região da adesão bacteriana foi constatado um acúmulo de material elétron denso entre o parasita e a bactéria. A capacidade de adesão da variante DB11 foi menor e quando presente demonstrou apresentar uma ligação mais fraca à superfície do parasita (Castro el al., 2007 – Anexo 6).

Inúmeros trabalhos relatam fatores de virulência de *S. marcescens*, principalmente por ser uma bactéria patogênica, como hemolisinas, nuclease, quitinase, metaloprotease, serino proteases, sideroforos e lipases (Hejazi e Falkiner, 1977; Hines et al., 1998; Marty et al., 2002; Carbonell et al., 2004; Fineran et al., 2007). Vários autores têm purificado citotoxinas de *S. marcescens* e demonstrado seus efeitos tóxicos em células humanas (Hertle et al., 1999; Marty et al., 2002; Carbonell et al., 2003, 2004). Também tem sido relatado que essas bactérias induzem a produção de reativos de oxigênio nas células hospedeiras após o processo de adesão (Mizunoe et al., 1995; Hejazi e Falkner 1997; Shanks et al., 2007).

Técnicas de filtração e sonicação de cepas de *S. marcescens* foram realizadas com o propósito de identificar o fator de virulência responsável pela atividade tripanolítica dessas bactérias. Porém não foi observado nenhum efeito tóxico das frações obtidas sobre os tripanosomatídeos (dados não mostrados). Portanto, pode-se sugerir que o processo de adesão seja essencial para atividade lítica, havendo a necessidade de administrar possíveis toxinas dentro da célula hospedeira. Ensaios de quantificação de nitrito e nitrato também foram realizados nas amostras de *S. marcescens* incubadas com *T. cruzi* e *T. rangeli*, porém não foi observada nenhuma alteração significativa entre as amostras e o controle (dados não mostrados).

A identificação do fator de virulência de *S. marcescens* responsável pela atividade lítica sobre os parasitas, *T. cruzi* e *T. rangeli* é de extrema importância para descobrir novas estratégias de controle da doença de Chagas. Uma dessas estratégias é a utilização de toxinas produzidas pelas bactérias, ou moléculas sintetizadas a partir dessa toxina, no controle dos parasitas em humanos.

Em relação à associação de *R. prolixus* com os parasitas *T. cruzi* e *T. rangeli* e a bactéria *S. marcescens* pode-se inferir, de acordo com os resultados encontrados, como essas espécies interagem entre si. Provavelmente com a alimentação sanguínea a bactéria se multiplica, pois aumenta a fonte de nutrientes importantes para seu desenvolvimento e aumenta a oferta de heme necessária para produção de prodigiosina. Em contrapartida a bactéria colabora com o inseto ajudando na digestão sanguínea e fornecendo vitaminas importantes para seu desenvolvimento. Além disso, a bactéria com sua ação tripanolítica, dependendo da cepa do parasita, controla a população do protozoário ingerido durante o repasto sanguíneo obtido do hospedeiro vertebrado infectado. Já o parasita, como forma de sobrevivência, adere à membrana perimicrovilar do intestino do inseto se protegendo da atividade lítica da bactéria e dos fatores citotóxicos produzidos pelo inseto completando seu ciclo de vida e transmitindo a doença de Chagas para hospedeiros vertebrados.

CONCLUSÕES

Do estudo da ação de diferentes fisalinas sobre a resposta imune de *R. prolixus* e dos efeitos citotóxicos da *S. marcescens* sobre os parasitas transmitidos por este inseto-vetor foram obtidas as seguintes conclusões:

Parte I:

A administração oral de fisalinas B, D, F e G em doses de 1 a 10 µg/ml de sangue alimentar não altera a viabilidade e ecdise de 5º estádio de *Rhodnius prolixus*, porém causa aumento da mortalidade em insetos tratados com as drogas e infectados com *Tryapnosoma rangeli* ou *Enterobacter cloacae* β12.

R. prolixus inoculados com *T. rangeli* e alimentados com a fisalina B apresentam diminuição do número de microagregados de hemócitos e da produção de óxido nítrico.

Em insetos previamente tratados por via oral com as fisalinas B, F e G (1 µg/ml de sangue) e inoculados com *E. cloacae* β12, ocorre menor número de microagregados de hemócitos no terceiro e sexto dia após inoculação.

O tratamento dos insetos com fisalinas B e F (1 µg/ml) reduz o número de hemócitos circulantes na hemolinfa três dias após inoculação com *E. cloacae* β12.

R. prolixus tratados com as fisalinas B e F na dose de 1 µg/ml de sangue apresentam maior atividade de lisozima no sexto dia após inoculação de *E. cloacae* β12 e os tratados com a fisalina D (1 µg/ml) menor atividade antibacteriana testada contra *Escherichia coli* no sexto dia após inoculação.

A hemolinfa coletada de *R. prolixus* previamente tratados com fisalinas B, D, F e G (1 µg/ml de sangue) resulta na inibição da atividade fagocítica de hemócitos preparados em monocamada e desafiados com *Saccharomyces cerevisiae* conjugada a FITC.

A administração de fisalina B (1 µg/ml) ao sangue alimentar inibe a atividade fagocítica e de microagregação de hemócitos de *R. prolixus* preparados em monocamada e desafiados com leveduras *in vitro*. Ácido araquidônico quando

incubado em concentração de 10μM na monocamada de hemócitos ou inoculado na hemocele em concentração de 10 μg/inseto reverte o efeito inibitório da fisalina B. Já a administração de PAF (1 μg/inseto) reverte a inibição de fisalina B apenas através da inoculação na hemocele de *R. prolixus* tratados oralmente com a droga.

Hemolinfa coletada de *R. prolixus* tratados com a fisalina B (1 μg/ml) não apresenta alteração na atividade da enzima fosfolipase A_2, porém observou-se elevada atividade de PAF-AH.

Parte II:

As variantes SM365 e RPH de *Serratia marcescens* possuem atividades tripanolítica sobre os parasitas *Trypanosoma cruzi* e *Trypanosoma rangeli*.

O carboidrato D-manose (0,2M) protege *T. cruzi* e *T. rangeli* da atividade lítica de *S. marcescens*, porém não inibe a atividade hemolítica da bactéria testada contra eritrócitos humanos;

As variantes de *S. marcescens* SM365 e RPH aderem à membrana celular de *T.* cruzi em apenas alguns segundos após o ensaio de interação.

O índice de adesão bacteriano em ensaios de interação de *S. marcescens* SM365 e epimastigotas de *T. cruzi* é diretamente dependente da razão bactéria:parasita. O tempo de interação não interfere nos índices de adesão em observação de até 120 min. A variante DB11 de *S. marcescens* possui índices de adesão baixos mesmo na maior razão bactéria:parasita (50:1) testada.

A adição de D-manose na concentração de 0,2M nos ensaios de interação de *S. marcescens* SM365 e epimastigotas de *T. cruzi* reduziu o índice de adesão nas razões 10:1, 25:1 e 50:1 (bactéria:parasita) utilizadas.

A adesão bacteriana aos parasitos ocorre por finos filamentos conhecidos como fimbria as quais em *S. marcescens* são descritas como do tipo 1, manose sensíveis.

Após 120 min de ensaio de interação *S. marcescens* - *T. cruzi* ocorre a formação de biofilme sob a superfície da membrana do parasito que, em geral, já se encontra lisado.

TRABALHOS COM COLABORAÇÃO

ANEXO:

Leishmania (Leishmania) *chagasi* interactions with *Serratia marcescens*: ultrastructural studies, lysis and carbohydrate effects. Moraes CS, Seabra SH, Castro DP, Brazil RP, de Souza W, Garcia ES, Azambuja P.
Exp Parasitol. 2008 Apr;118(4):561-8.

Prodigiosin is not a determinant factor in lysis of *Leishmania* (Viannia) *braziliensis* after interaction with *Serratia marcescens* D-mannose sensitive fimbriae. Moraes CS, Seabra SH, Albuquerque-Cunha JM, Castro DP, Genta FA, de Souza W, Brazil RP, Garcia ES, Azambuja P.
Exp Parasitol. 2009 Jun;122(2):84-90.

Available online at www.sciencedirect.com
ScienceDirect
Experimental Parasitology 118 (2008) 561–568
Experimental Parasitology
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Leishmania (*Leishmania*) *chagasi* interactions with *Serratia marcescens*: Ultrastructural studies, lysis and carbohydrate effects

Caroline S. Moraes [a], Sergio H. Seabra [b], Daniele P. Castro [a], Reginaldo P. Brazil [a], Wanderley de Souza [c], Eloi S. Garcia [a], Patrícia Azambuja [a,*]

[a] *Department of Biochemistry and Molecular Biology, Laboratório de Bioquímica e Fisiologia de Insetos, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Avenida Brasil 4354, Manguinhos, CEP 21045-900, Rio de Janeiro, RJ, Brazil*
[b] *Departamento de Pesquisa e Extensão, Centro Universitário Estadual da Zona Oeste, Rio de Janeiro, CEP 21070-200, RJ, Brazil*
[c] *Laboratório de Ultraestrutura Celular Hertha Meyer, Instituto de Biofísica Carlos Chagas Filho, Edifício do Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Ilha do Fundão, Rio de Janeiro, CEP 21941-590, RJ, Brazil*

Received 9 August 2007; received in revised form 16 November 2007; accepted 22 November 2007
Available online 5 December 2007

Abstract

Studies on the lysis of *L. chagasi* caused by the bacteria *Serratia marcescens* were carried out. *In vitro* experiments demonstrated that *S. marcescens* variant SM 365, a prodigiosin pigment producer, lysed this species of *Leishmania* but variant DB11, a nonpigmented bacteria, was unable to lyse the parasite. High concentrations of D-mannose were found to protect *L. chagasi* markedly diminishing the lysis by *S. marcescens* SM 365. Promastigotes of *L. chagasi* bound the lectin Concanavalin A conjugated with FITC, the fluorescence was intensely found at the base of the flagellum (flagellar pocket). Scanning electron microscopy revealed that the bacteria adherence occurred mainly in the flagellar pocket. *S. marcescens* SM 365 formed filamentous structures, identified as biofilms, which connect the protozoan to the developing bacterial clusters, in low concentrations of bacteria after 30 min incubation time. We suggest that bacterial mannose-sensitive (MS) fimbriae are relevant to *S. marcescens* SM 365 in the lysis of *L. chagasi.*

Index Descriptors and Abbreviations: D-Mannose; *Leishmania* and insect-vector; *Serratia marcescens*; Lysis; Biofilms

1. Introduction

Zoonotic visceral leishmaniasis is a severe endemic disease caused by *Leishmania chagasi*/*Leishmania infantum* and it occurs in several countries of Central and South America, the Mediterranean basin and Asia (Gramiccia and Gradoni, 2005). Enzyme electrophoresis and schizodeme analysis indicate that *L. chagasi* is a synonym species of *L. infantum* in the New World (Momem et al., 1993; Maurício et al., 2000), however, this is still controversy (Shaw, 1994).

The biological life cycle of *Leishmania* parasites involves aflagellated amastigotes in the mammalian macrophage and flagellated promastigotes in the sandfly midgut. The parasite is transmitted by the vector bite which injects promastigotes into the vertebrate host (Turco and Descoteaux, 1992). In the insect–parasite interactions, the gut represents the first contact between ingested parasites and the vector epithelial surfaces. Once in the vector midgut, the parasite passes through many physiological modifications in order to survive the adverse conditions present, such as proteolytic enzymes secreted by the midgut epithelium (Borovsky and Schlein, 1987), and the formation of a peritrophic matrix (PM) that surrounds the blood meal (Pimenta et al., 1997). Thus, for successful transmission by the insect, *Leishmania* needs to escape from the PM before blood excretion. A chitinase released by the parasite is therefore required to degrade the PM before the adherence of *Leishmania* to the hindgut so that the parasite

* Corresponding author. Fax: +55 21 2590 3495.
E-mail address: azambuja@ioc.fiocruz.br (P. Azambuja).

0014-4894/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.exppara.2007.11.015

can subsequently migrate to the midgut and foregut (Schlein et al., 1992; Sacks et al., 1994).

Bacteria exist naturally in the midgut of wild and laboratory-reared mosquitoes (Chao and Wistreich, 1959, 1960; Fergunson and Micks, 1961; Vasanthi and Hoti, 1992; DeMaio et al., 1996; Pumpuni et al., 1996) and triatomines (Figueiredo, 1995; Figueiredo et al., 1995). Like most hematophagous insects, sandflies are also vulnerable to gut contamination by various microorganisms (Schlein et al., 1985; Dillon et al., 1996; Oliveira et al., 2000, 2001). Oliveira et al. (2000, 2001) identified, in *Lutzomya longipalpis* reared in the laboratory, many Enterobacteriaceae species including *Pantoea agglomerans*, *Serratia marcescens* and *Serratia rubidae*, *Hafnia alvei*, *Enterobacter aerogenes* and *Enterobacter cloacae* and the non-fermenters *Stenotrophomonas maltophilia*, *Acinetobacter baumanni* and *Pseudomonas putida*; while in sandflies from the field (Gruta da Lapinha, Minas Gerais) bacteria such as *Acinetobacter lowffii*, *Stenotrophomonas maltophihilia*, *P. putida* and *Flavimonas orizihabitans* and the non-fermenters, *E. cloacae* and *Klebsiella ozaenae*, and the Gram positive organisms *Bacillus thuringiensis* and *Staphylococcus* spp.

Many aspects of the sandfly—parasite interactions have been studied (Turco and Descoteaux, 1992; Sacks et al., 2000), but little is known about the niches occupied by the parasites and the microbiota of the vector digestive tract. There is thus a need to study if the colonized bacteria of the sandfly gut could potentially produce lytic compounds to kill *Leishmania*. Evidence of a possible influence of gut microbiota on the parasite life cycle has been reported in sandflies (Schlein et al., 1985). Microbial infections in the guts of sandflies, *Phlebotomus papatasi*, significantly reduced rates of infection by *Leishmania major* (Schlein et al., 1985).

Serratia marcescens is an opportunistic bacterium (Grimont and Grimont, 1978) with fimbriae among the virulent cell surface structures, which contain adhesins mediating adherence to eukaryotic cell surfaces, via recognition of specific carbohydrate moieties on the host cell surface glycoconjugates (Ofek and Sharon, 1990). *S. marcescens* mannose-dependent fimbriae can attach to biological surfaces and form biofilms (Queck et al., 2006; Castro et al., 2007a). Castro et al. (2007a,b) demonstrated that D-mannose protects *Trypanosoma cruzi* and *Trypanosoma rangeli* from attachment and lysis by *S. marcescens*, and concluded that type 1 mannose-sensitive fimbriae are relevant to bacteria lysis of these parasites.

Since knowledge of the *Leishmania*—sandflies interaction is wanting, we extended our findings observed in *Trypanosoma–Serratia* relationships to *Leshmania* sp. Thus, we developed a model for *in vitro* studies between bacteria and parasites using variants of *S. marcescens* (Kurz et al., 2003) and cultured promastigotes of *L. chagasi*. In this paper, we provide evidence that *L. chagasi* is lysed by *S. marcescens* SM 365 through carbohydrate-recognizing fimbriae and demonstrate that *Leishmania* bound FITC-conjugated ConA mainly at the base of the flagellum, that is the flagellar pocket. By scanning electron microscopy, the formation of a biofilm formed by long filamentous structures was also shown, which connect the protozoan to the bacteria forming bacterial clusters.

2. Materials and methods

2.1. Parasites

Leishmania chagasi L579 (strain MHOM/BR/1974/PP75) was supplied by Dr. E. Cupollilo (*Leishmania* Type Culture Collection of the Oswaldo Cruz Institute, Fiocruz, Brazil). *Leishmania* sp. was grown in Schneider's Insect Medium (Sigma Chemical, St. Louis, MO) supplemented with 20% heat-inactivated fetal bovine serum at 28 °C. The parasites used in our experiments were taken from 4 day old cultures of promastigotes in the exponential growth phase. To eliminate all carbohydrates from the culture medium the *L. chagasi* suspension was washed twice in sterile phosphate-buffered saline (PBS, 0.15 M NaCl and 0.01 M phosphate buffer, pH 7.2) at 300*g* for 15 min.

2.2. Bacteria

Two variants of *S. marcescens* were used, a prodigiosin pigment producer variant SM365, and a nonpigmented variant DB11 (Kurz et al., 2003) as a control (both supplied by Dr. Cecile Wandersman, Pasteur Institute, Paris, France). To obtain the stationary growth phase, variants of *S. marcescens* were incubated for 24 h at 28 °C in liquid BHI medium by aseptically adding 1 ml of liquid medium into 4 ml of sterile BHI. The colony-forming units (CFU) were estimated by comparing bacteria culture turbidity using optical density with $BaSO_4$ solutions of McFarland standards (Jousimies-Somier and Summanen, 2002).

2.3. Carbohydrates

All carbohydrates (D-mannose, D-mannosamine, *N*-acetyl-D-mannosamine, D-glucose and D-galactose) (Sigma Chemical, St. Louis, MO) were diluted in PBS at a concentration of 2 M and filtered through 0.22 μm pore Millipore membranes.

2.4. Lytic assays

To quantify the lytic activity, 10 μl of *S. marcescens* SM 365 or DB11 in concentrations of 1×10^8 CFU/ml were added to 80 μl of *L. chagasi* promastigote suspension of approximately 4×10^6 parasites/ml in Eppendorf tubes and incubated at 28 °C for 30, 60 and 120 min. To observe the lysis kinetics of *S. marcescens* SM 365 the parasite was incubated with different concentrations of bacteria (from 1×10^5 to 1×10^8 CFU/ml) for 120 min. In some experiments, 10 μl of one of the carbohydrate solutions (2 M), 80 μl of parasite (4×10^6 promastigotes/ml) were added to the Eppendorf tubes, and then followed immediately by

10 μl suspensions of the *S. marcescens* variants (1×10^8 CFU/ml). As controls in each assay, 10 μl of phosphate-buffered saline was added instead of the bacteria suspensions or carbohydrate solutions. After completing the final volumes of 100 μl, the mixtures were incubated at 28 °C for up to 120 min. In some experiments, parasites were pre-incubated with D-mannose at a final concentration of 0.2 M for 30 min. Subsequently, promastigotes suspension was washed as described above and incubated with *S. marcescens* SM365 variant for 120 min.

Before parasite counting the cell suspensions were mixed, the numbers of parasites estimated by direct counting in a Neubauer hemocytometer chamber under a light microscope, and the total parasite numbers were normalized in log. In each assay, apparently undamaged parasites were recognized by their integral morphology with live parasites having slight movements of their flagella in the medium.

2.5. Staining with Concanavalin A lectin conjugated with FITC

For staining the parasites, Mello et al. (1996) methodology was adapted. Basically, *L. chagasi* promastigotes were washed as described above and fixed with 2.0% paraformaldehyde in PBS for 120 min at 4 °C. After incubation, the parasites were washed three times in PBS at 300*g* for 15 min and incubated with Concanavalin A (Con A) lectin conjugated with fluorescein isothiocyanate (FITC, Sigma) at a final concentration of 0.2 mg/ml for 120 min at room temperature. As control, Con A (in same concentration) was pre incubated with D-mannose or D-glucose at final concentration 0.2 mg/ml for 120 min before incubation with parasites. Subsequently, promastigotes were washed again as above to eliminate all lectin present in the supernatant. The cells were observed under a Differential Interference Contrast (DIC) and fluorescence microscope (Nikon Eclipse E600).

2.6. Scanning electron microscopy

To evaluate the bacteria–parasite interactions by scanning electron microscopy, *S. marcescens* SM 365 was added in a concentration of 1×10^8 CFU/ml to promastigote suspensions of 1×10^7 parasites/ml (bacteria/protozoa cells density ratio of 10:1 times) in Eppendorf tubes and incubated at room temperature from 1 and 30 min. After these times, the samples were fixed in 0.1 M PBS, pH 7.2, containing 2.5% glutaraldehyde for 30 min. Then, the parasites were adhered on cover slips previously coated with 0.1% aqueous poly-L-lysine for 30 min at 37 °C. Subsequently, the cells were washed twice with 0.1 M cacodylate buffer, post-fixed in solution of 1% OsO_4 for 40 min at room temperature and washed twice again with 0.1 M cacodylate buffer. The cells were dehydrated in a graded series of ethanol (30–100%), critical point dried using CO_2, mounted on metal stubs and coated with gold (5–30 nm) for observation in a scanning electron microscope (JSM5310).

2.7. Statistical analysis

Data were compared using the ANOVA test according to Statistical Package for Social Sciences Software (SPSS), version 12.0 for Windows. Data are reported as means ± standard deviation. Differences between the groups were considered not statistically significant when $p > 0.05$. Probability levels are specified in the text.

3. Results

3.1. Kinetics of L. chagasi lysis during interactions

Leishmania chagasi was incubated with different concentrations of *S. marcescens* SM 365 for 120 min (Fig. 1). *S. marcescens* SM 365 at concentrations of 1×10^5, 1×10^6 and 1×10^7 CFU/ml decreased non-significantly the population densities of flagellates. However, at higher concentrations of bacteria (1×10^8 CFU/ml) the parasite was lysed intensely after 120 min incubation ($p < 0.001$). We also observed some clusters formed by living parasite and cellular debris of dead flagellates.

Serratia marcescens SM 365 and DB11 were tested for comparative lytic activities against *L. chagasi*. The result presented in Fig. 2 shows a significant reduction of the initial number of flagellates (i.e., 4×10^6 parasites/ml) when incubated with bacteria SM 365 (1×10^8 CFU/ml) ($p < 0.001$). In contrast, *S. marcescens* DB11 decreases the number of parasites similar to the control group after incubation of 120 min ($p > 0.05$) (Fig. 2).

3.2. Lytic activity of S. marcescens on L. chagasi: effects of carbohydrates

We also studied the effect of different carbohydrates incubation on the lysis of *L. chagasi* caused by *S. marcescens* SM365. The parasite was lysed by *S. marcescens* SM

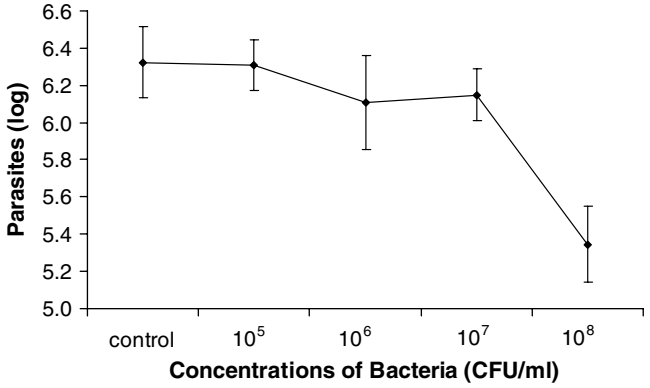

Fig. 1. Effects of different concentrations of *Serratia marcescens* SM365 on *Leishmania chagasi* L579 lysis. Promastigotes (4×10^6 parasites/ml) were incubated with different concentrations of *Serratia marcescens* SM365 (1.0×10^8 to 1.0×10^5 CFU/ml) or only PBS (control) and counted in a Neubauer hemocytometer after 120 min. Each point represents the mean ± SD of 5 experiments.

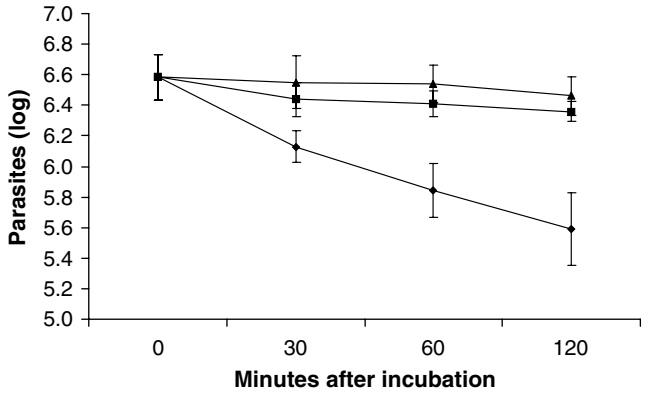

Fig. 2. Temporal course of *Leishmania chagasi* L579 strain lysis at different times of incubation with *Serratia marcescens*. Promastigotes (4.0×10^6 parasites/ml) were incubated with different strains of *Serratia marcescens* (approximately 1.0×10^8 CFU/ml) and counted in a Neubauer hemocytometer at different times of incubation. *Serratia marcescens* variants used were SM365 (◆), DB11 (■) and control without bacteria (▲). Each point represents the mean ± SD of 5 experiments.

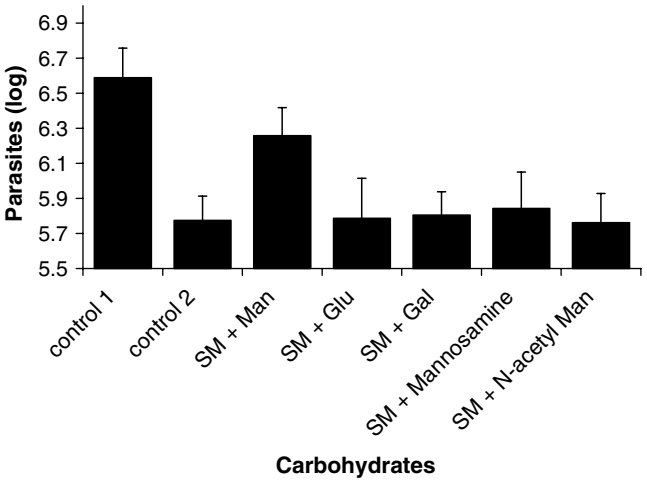

Fig. 3. Effects of different carbohydrates on the lysis of *Leishmania chagasi* L579 strain induced by *Serratia marcescens* SM365 variant. Promastigotes (4×10^6 parasites/ml) were incubated with *S. marcescens* (1.0×10^8 CFU/ml) plus D-mannose (SM + Man), D-glucose (SM + Glu), D-galactose (SM + Gal), D-mannosamine (SM + Mannosamine) or N-acetyl-D-mannosamine (SM + *N*-acetyl Man), or only PBS (control 1) or only bacteria (control 2). Parasites numbers were estimated by counting in Neubauer hemocytometer after 120 min of incubation (28 °C). Each point represents the mean ± SD of least 5 experiments.

365 in the absence of sugars ($p < 0.001$). The results of flagellates and bacteria incubation in the presence of different carbohydrates demonstrated that while 0.2 M of D-mannose significantly inhibited the lysis of this flagellate ($p < 0.001$), D-glucose, D-galactose, *N*-acetyl-D-mannosamine and D-mannosamine did not protect the parasite against the lytic effects caused by *S. marcescens* SM 365 ($p > 0.05$ for all cases) (Fig. 3). However, parasites pre-incubated with D-mannose did not inhibit the bacterial lytic activity. It was not possible to perform a similar D-mannose pre-incubation with *S. marcescens* alone because centrifugation disrupted the fimbriae, and even in the control the lytic effect was lost (data not shown).

The dose–response of parasite lysis inhibition with different concentrations of D-mannose, ranging from 0.01 to 0.2 M, demonstrated that only concentrations above 0.1 M completely protects *L. chagasi* against the lysis induced by *S. marcescens* SM 365 ($p < 0.005$) (Fig. 4). The concentration of 0.01 M only partially inhibited the parasites lysis ($p < 0.02$).

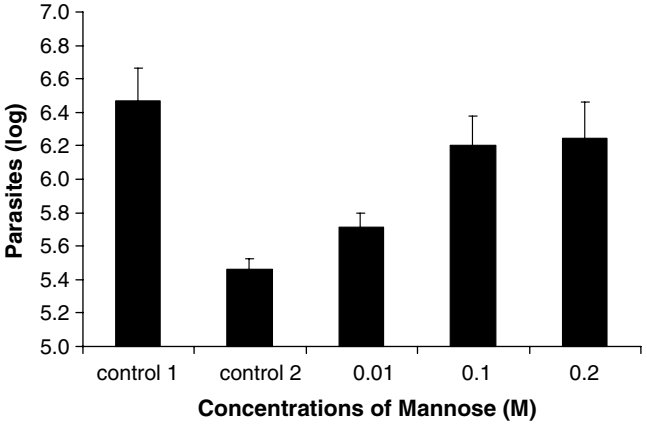

Fig. 4. Lysis of *Leishmania chagasi* L579 strain incubated with *Serratia marcescens* SM365 variant at different concentrations of D-mannose. Promastigotes (4×10^6 parasites/ml) were incubated with different concentrations of mannose and *Serratia marcescens* (1.0×10^8 CFU/ml), or only PBS (control 1) or only bacteria (control 2). Parasites numbers were estimated by counting in Neubauer hemocytometer after 120 min of incubation (28 °C). Each point represents the mean ± SD of least 5 experiments.

3.3. D-mannose surface in L. chagasi

The presence of D-mannose in promastigote surfaces of *L. chagasi* was investigated by fluorescent microscopy assay using Concanavalin A lectin conjugated with FITC. Our results showed that the lectin bound *Leishmania*, which displayed a similar fluorescent labeling throughout the cell surfaces (Fig. 5A and B). The fluorescence over the parasite promastigote surface was intensely found at the base of the flagellum, in the region of the flagellar pocket (Fig. 5B). In contrast, although parasites incubated with lectin and D-mannose or D-glucose present few or no fluorescence signals in the promastigote surface (data not shown), only D-mannose inhibited the bacteria lysis of the parasite.

3.4. Structural analysis of the interactions

The attachment of the bacteria *S. marcescens* SM 365 to the surface of *L. chagasi* was observed using scanning electron microscopy. After a few minutes of bacteria–parasite interactions the *S. marcescens* SM 365 started adhering to the *L. chagasi* (Fig. 6). Scanning electron microscopy indicates clearly that bacteria bound greater at the base of flagella of *Leishmania* before starting the modification of the

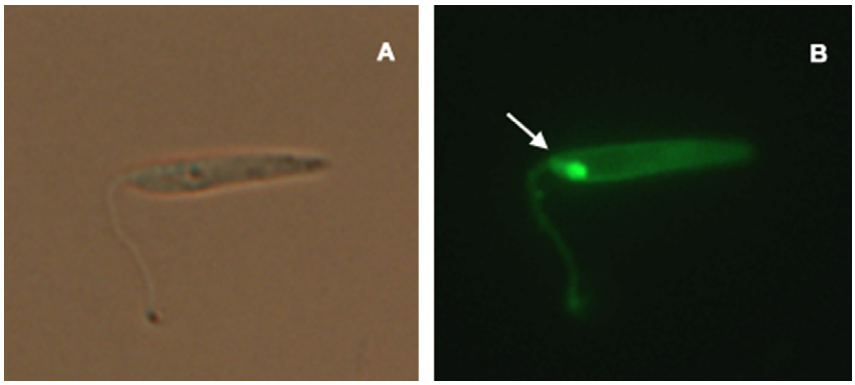

Fig. 5. Diferencial Interference Contrast (DIC) (A) and fluorescence microscopy (B) of promastigotes *Leishmania chagasi* incubated with FITC-conjugated Concanavalin A.

flagellate body shape (Fig. 6B) when compared with parasites without bacteria (control) (Fig. 6A). Interestingly, after 1 min of incubation, there was a formation of parasite cluster (Fig. 6C and D), and following 30 min, we observed a large number of thin filamentous structures interconnecting bacteria or connecting bacteria to *L. chagasi* surface or parasite surface fragments. The colonies, formed of bacteria interconnected by these filaments, formed structures similar to biofilms over the lysed parasites (Fig. 6E and F).

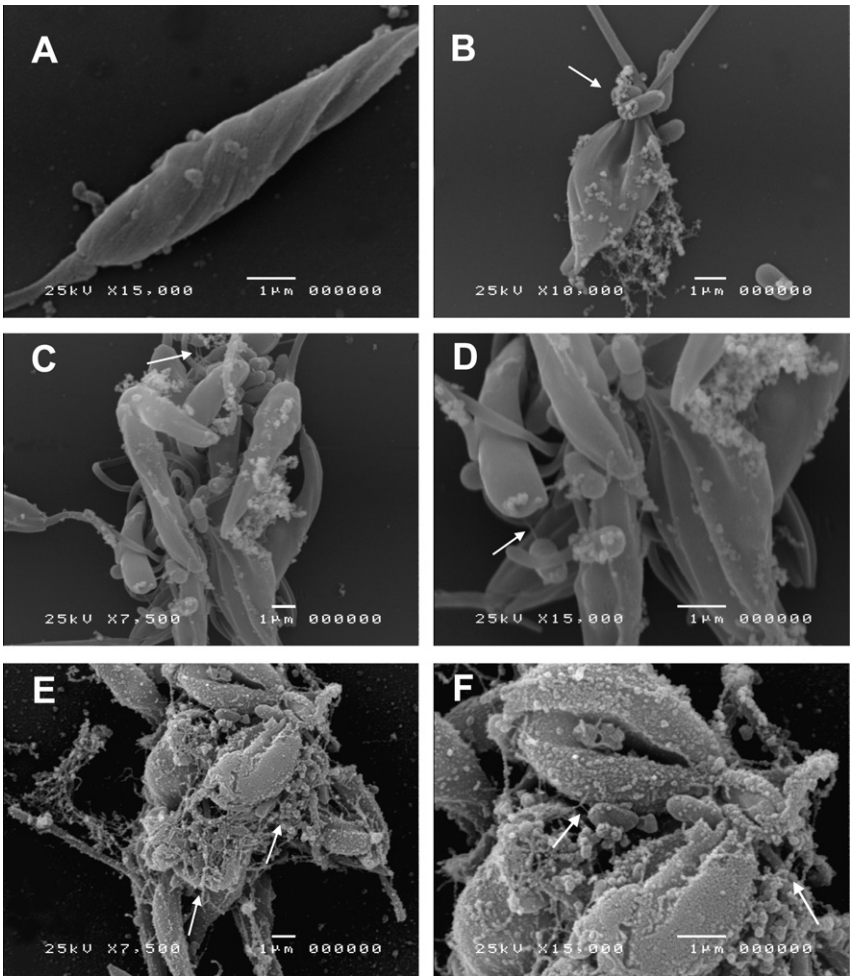

Fig. 6. Scanning electron microscopy of the interaction of *Leishmania chagasi* L579 strain and *Serratia marcescens* SM365 variant. (A) Control parasites without bacteria. (B) Adhered bacteria in flagellum pocket region (arrow) (1 min). (C and D) Agglomerate parasites and the start of biofilm formation (arrows) (1 min). (E and F) Biofilm formation (arrows) (30 min).

4. Discussion

Bacteria of the genus *Serratia* are frequently associated to insects of many orders (Grimont and Grimont, 1978), and some species have been found in the midgut bacterial flora of hematophagous insects such as mosquitoes (DeMaio et al., 1996; Straif et al., 1998; Gonzalez-Ceron et al., 2003), triatomines (Figueiredo, 1995; Figueiredo et al., 1995; Azambuja et al., 2004) as well as in sandflies (Dillon et al., 1996; Oliveira et al., 2000, 2001). Recently, it was shown that *S. marcescens* is lytic for *T. cruzi* and *T. rangeli*, two species of parasites that use triatomine insects as vectors (Azambuja et al., 2004; Castro et al., 2007a). We then speculated that *S. marcescens* could also lyse *L. chagasi*.

Firstly, we studied the effect of *S. marcescens* SM 365 and *S. marcescens* DB11 on the promastigote of *L. chagasi*. *In vitro* experiments showed a low susceptibility of this parasite to *S. marcescens* DB11 nonpigmented variant, but SM 365 pigmented variant was able to lyse the flagellate in concentrations of 1×10^8 CFU/ml. Otherwise, since there is no gene with high similarity to fimbriae (fimA and fimB subunits) in *S. marcescens* DB11 (search in Sanger Institute), we suggest that both fimbriae and/or prodigiosin are involved in the lysis of *L. chagasi* induced by SM 365.

Several papers have described the importance of parasite surface lipophosphoglycan (LPG) for sandfly–parasite interactions (Sacks et al., 1994, 2000; Soares et al., 2002, 2005). For example, recognition of binding sites in the epithelium by the LPG is a crucial step preventing the loss of the parasite during the excretion of the digested blood meal (Pimenta et al., 1994). LPG variations have been implicated in the specificity of different *Leishmania* species for development in different *Phlebotomus* species and thus for the development of vectorial competence in the invertebrate host (Pimenta et al., 1994; Kamhawi et al., 2000; Sacks and Kamhawi, 2001; Turco and Sacks, 2003). All LPGs have a conserved glycan core region of galactose (α1,6)galactose(α1,3)galactofuranose(β1,3)(glucose(α1)-PO4)-mannose(α1,3)mannose (α1,4)*N*-acetylglucosamine(α1) linked to a 1-0-alkyl-2-lyso-phosphatidyl-inositol anchor (Turco and Descoteaux, 1992). Although LPG is the major glycoconjugate at the parasite's surface, it cannot be ruled out that other relevant glycoconjugates (such as GPI-anchored glycoproteins, proteophosphoglycans and free GPI glycolipids) that compose the overall parasite surface glycocalix may also play a substantial role in bacteria–protozoa interaction (Mukhopadhyay and Mandal, 2006).

It has been shown that *L. chagasi* produces large amounts of LPG (Soares et al., 2005), which results in concentrations of carbohydrates on the flagellate surface. Thus, we hypothesized that the complexity and modification in the structure and distribution of LPG may be related to bacteria ligands responsible for the interaction between *Leishmania* sp. and *S. marcescens* SM 365.

It has been reported that mannose-sensitive (MS) fimbriae present in *S. marcescens* are associated with adhesive properties on the cellular membrane surface of different eukaryotic cells and with the bacterial virulence (Duguid et al., 1966; Reid and Sobel, 1987). In the present paper, we showed that *L. chagasi* promastigotes bind the Concanavalin A lectin mainly in the flagellar pocket and suggested that the carbohydrate-specific binding of the lectin to the surface of *Leishmania* occurs mainly via D-mannose. Interesting, *S. marcescens* SM 365 also preferentially bound to the base of the flagella in the parasite where D-mannose is found. Since the treatment of *L. chagasi* with D-mannose resulted in (i) inhibition of the lytic activity induced by *S. marcescens* SM 365 and (ii) parasites pre-incubated with D-mannose were not protected of the lysis induced by *S. marcescens* SM365, we suggest that MS fimbriae are relevant to the mechanism *Leishmania* spp.—bacteria associations, i.e., D-mannose binds to *S. marcescens* MS fimbriae avoiding the adhesion of the bacteria on the surface of the flagellates.

An association between a protozoan and bacteria can also be observed in *Shigela flexneri* that expresses mannose-binding lectins, attaching to mannose molecules on the surface of *Entomoeba histolytica* (Verdon et al., 1992) and in *T. cruzi* which expresses a carbohydrate binding protein (CBP) that can bind mannose residues (Bonay et al., 2001) of different organisms, including *S. marcescens* (Castro et al., 2007a).

Finally, one of the most common reactions of bacteria against Protozoa in nature is the formation of a bacterial community recognized as a biofilm (Watnick and Kolter, 2000; Matz et al., 2004; Weitere et al., 2005; Queck et al., 2006). Biofilms correspond to assemblages of single or multiple populations of bacteria that adhere to abiotic or biotic surfaces through extracellular polyanionic compounds. Castro et al. (2007b) demonstrated by scanning and transmission electron microscopy that *S. marcescens* SM 365 attaches to *T. cruzi* epimastigotes or their surface fragments and that thin filaments connected bacteria to each other and the flagellate surface. This was identical to the observations in the present paper with the interaction of *S. marcescens* SM 365 and *L. chagasi*. The biofilm formed is similar to previous structures described in many Gram-negative bacteria in nature (Labbate et al., 2004; Matz et al., 2004; Rice et al., 2005; Castro et al., 2007b).

Further detailed study of the parasite lysis and bacterial biofilm formation *in vivo* would be extremely useful for understanding the biological cycle of *Leishmania* spp. in the gut of sandflies. Otherwise, the parasite establishment in the midgut of sandfly can be interfered by the bacteria variant species present in the intestinal microbiota. Our present work is an important step toward future research in a parasite–bacteria interaction model for focusing on pathogenic protozoa, which could lead to new insights, strategies and a better knowledge concerning the transmission and control of leishmaniasis.

Acknowledgments

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (to P.A., E.S.G. and W.S.), Fundação Oswaldo Cruz (Fiocruz) (Papes project to P.A.) and Fundação Carlos Chagas Filho de Amparo a Pesquisa do estado do Rio de Janeiro (Faperj) (to P.A. and R.P.B.). P.A., E.S.G. and W.S. are CNPq Senior Research Fellows. D.P.C. and C.S.M. are Faperj Pos-Graduated Fellows. We thank Dr. Cécile Wandersman (Pasteur Institute, France) for the gift of *S. marcescens* SM 365 and DB11 variants.

References

Azambuja, P., Feder, D., Garcia, E.S., 2004. Isolation of *Serratia marcescens* in the midgut of *Rhodnius prolixus*: impact in the establishment of the parasite *Trypanosoma cruzi* in the vector. Experimental Parasitology 107, 89–96.

Bonay, P., Molina, R., Fresno, M., 2001. Binding specificity of mannose-specific carbohydrate-binding protein from the cell surface of *Trypanosoma cruzi*. Glycobiology 21, 719–729.

Borovsky, D., Schlein, Y., 1987. Trypsin and chymotrypsin-like enzymes of the sandfly *Phlebotomus papatasi* infected with *Leishmania* and their possible role in vector competence. Medical and Veterinary Entomology 1, 235–269.

Castro, D.P., Moraes, C.S., Garcia, E.S., Azambuja, P., 2007a. Inhibitory effects of D-mannose on trypanosomatid lysis induced by *Serratia marcescens*. Experimental Parasitology 115, 200–204.

Castro, D.P., Seabra, S.H., Garcia, E.S., de Souza, W., Azambuja, P., 2007b. *Trypanosoma cruzi*: ultrastructural studies of adhesion, lysis and biofilm formation by *Serratia marcescens*. Experimental Parasitology 117, 201–207.

Chao, J., Wistreich, G.A., 1960. Microorganisms from the midgut of larval and adult *Culex quinquefasciatus* Say. Journal of Insect Pathology 2, 220–224.

Chao, J., Wistreich, G.A., 1959. Microbial isolations from the midgut of *Culex tarsalis* Coquillett. Journal of Insect Pathology 1, 311–318.

DeMaio, J., Pumpuni, C.B., Ken, M., Beier, J.C., 1996. The midgut bacterial flora of wild *Aedes triseriatus*, *Culex pipiens*, and *Psorophora columbiae* mosquitoes. American Journal of Tropical Medicine and Hygiene 54, 219–223.

Dillon, R.J., El Kordy, E.L., Lanee, R.P., 1996. The prevalence of a microbiota in the digestive tract of *Phlebotomus papatasi*. Annals of Tropical Medicine and Parasitology 90, 669–673.

Duguid, J.P., Anderson, E.S., Campbell, I., 1966. Fimbriae and adhesive properties in *Salmonellae*. Journal of Pathology and Bacteriology 92, 107–137.

Fergunson, M.J., Micks, D.W., 1961. Microorganisms associated with mosquitoes: I. Bacteria isolated from the midgut of adult *Culex fatigans* Wiedermann.. Journal of Insect Pathology 3, 112–119.

Figueiredo, A.R., 1995. Isolamento da microbiota bacteriana de triatomíneos e persistência do *Enterobacter cloacae* em *Rhodnius prolixus* Stal, 1859. MSc Thesis. Instituto Oswaldo Cruz, 72 pp.

Figueiredo, A.R., Nunes, Z.G., Silva, A.A.L., Giordano-Dias, C.M.G., Coura, J.R., Hofer, E., 1995. Isolation of microorganisms of triatomines maintained in artificial and sylvatic conditions. Memórias do Instituto Oswaldo Cruz 90, 228.

Gonzalez-Ceron, L., Santillan, F., Rodrigue, M.H., Mendez, D., Hernández-Avila, J.E., 2003. Bacteria in midguts of field-collected *Anopheles albimanus* block *Plasmodium vivax* sporogonic development. Journal of Medical Entomology 40, 371–374.

Gramiccia, M., Gradoni, L., 2005. The current status of zoonotic leishmaniases and approaches to disease control. International Journal for Parasitology 35, 1169–1180.

Grimont, P.A., Grimont, F., 1978. The genus *Serratia*. Annual Review of Microbiology 32, 221–248.

Jousimies-Somier, H., Summanen, P., 2002. Recent taxonomic changes and terminology update of clinically significant anaerobic gram-negative bacteria (excluding spirochetes). Clinical and Infection Diseases 35, 17–21.

Kamhawi, S., Modi, G.B., Pimenta, P.F.P., Rpwton, E., Sacks, D.L., 2000. The vectorial competence of *Phlebotomus sergenti* is specific for *Leishmania tropica* and is controlled by species-specific, lipophosphoglycan-mediated midgut attachment. Parasitology 121, 2533.

Kurz, C.L., Chauvet, S., Andres, E., Aurouze, M., Vallet, I., Michel, G.P., Uh, M., Celli, J., Filloux, A., De Bentzmann, S., Steinmetz, I., Hoffmann, J.A., Finlay, B.B., Gorvel, J.P., Ferradon, D., Ewbank, J.J., 2003. Virulence factors of the human opportunistic pathogen *Serratia marcescens* identified by in vivo screening. EMBO Journal 22 (7), 1451–1460.

Labbate, M., Queck, S.Y., Koh, K.S., Rice, S.A., Givskov, M., Kjelleberg, S., 2004. Quorum sensing-controlled biofilm development in *Serratia liquefaciens* MG1. Journal of Bacteriology 186, 692–698.

Matz, C., Bergfeld, T., Rice, S.A., Kjelleberg, S., 2004. Micro-colonies, quorum sensing and cytotoxity determine the survival of *Pseudomonas aeruginosa* biofilms exposed to protozoan grazing. Environmental Microbiology 6, 218–226.

Maurício, I.L., Stothard, J.R., Miles, M.A., 2000. The strange case of *Leishmania chagasi*. Parasitology Today 16 (5), 188–189.

Mello, C., Azambuja, P., Garcia, E.S., Ratcliffe, N.A., 1996. Differential in Vitro and in Vivo Behavior of Three Strains of *Trypanosoma cruzi* in the Gut and Hemolymph of *Rhodnius* prolixus. Experimental Parasitology 82, 112–121.

Momem, H., Pacheco, R.S., Cupolillo, E., Grimaldi Júnior, G., 1993. Molecular evidence for the importation of Old World *Leishmania* into the Americas. Biol. Res. 26 (1-2), 249–255.

Mukhopadhyay, S., Mandal, C., 2006. Glycobiology of *Leishmania donovani*. Indian Journal of Medical Research 123, 203–220.

Ofek, I., Sharon, N., 1990. Adhesins as lectins: specificity and role in infection. Current Topics in Microbiology and immunology 151, 91–113.

Oliveira, S.M.P., Morais, B., Gonçalves, C.A., Giordano_Dias, K.M., Vilela, M.L., Brazil, R.P., D'Almeida, J.M., Asensi, M.D., Mello, R.P., 2001. Microbiota do trato digestivo de fêmeas de *Lutzomya longipalpis* (Diptera: Psychodidae) provenientes de colônia alimentada com sangue e com sangue e sacarose. Cadernos de Saúde Pública 17, 229–232.

Oliveira, S.M.P., Morais, B., Gonçalves, C.A., Giordano_Dias, C.M., Vilela, M.L., Brazil, R.P., D'Almeida, J.M., Asensi, M.D., Mello, R.P., 2000. Prevalência da microbiota no trato digestivo de fêmeas de *Lutzomya longipalpus* (Lutz and Neiva, 1912) (Diptera: Psychodidae) provenientes do campo. Revista da Sociedade Brasileira de Medicina Tropical 33, 319–322.

Pimenta, P.F.P., Modi, G.B., Pereira, S.T., Shahabuddin, M., Sacks, D.A., 1997. A novel role for the peritrophic matrix in protecting *Leishmania* from the hydrolytic activities of the sandfly midgut. Parasitology 115, 359–369.

Pimenta, P.F.P., Saraiva, E.M., Rowton, E., Modi, G.B., Garraway, L.A., Beverley, S.M., Turko, S.J., Sacks, D.L., 1994. Evidence that vectorial competence of phlebotomine sandflies for different species is controlled by structural polymorphisms in the surface of lipophosphoglycan. Proceedings of the National Academy of Sciences United States of America 91, 9155–9159.

Pumpuni, C.B., DeMaio, J., Kent, M., Davis, J.R., Beier, J.C., 1996. Bacterial population dynamics in three anopheline species: the impact on *Plasmodium* sporogonic development. American Journal of Tropical Medicine and Hygiene 54, 214–218.

Queck, S.Y., Weitere, M., Moreno, A.M., Rice, S.A., Kjelleberg, S., 2006. The role of quorum sensing mediated developmental traits in the resistance of *Serratia marcescens* biofilms against protozoan grazing. Environmental Microbiology 8, 1017–1025.

Reid, G., Sobel, J.D., 1987. Bacterial adherence in the pathogenesis of urinary tract infection: a review. Review of Infection Diseases 9, 470–487.

Rice, A.S., Koh, K.S., Queck, S.Y., Labbate, M., Lam, K.W., Kjelleberg, S., 2005. Biofilm formation and sloughing in *Serratia marcescens* are controlled by quorum sensing and nutrient cues. Journal of Bacteriology 187, 3477–3485.

Sacks, D.L., Kamhawi, S., 2001. Molecular aspects of parasite–vector and vector–host interaction in Leishmaniasis. Annual Review of Microbiolgy 55, 453–483.

Sacks, D.L., Modi, G., Rowton, E., Späth, G., Epstein, L., Turco, S.J., Beverley, S.M., 2000. The role of phosphoglycan in *Leishmania*–sand fly interactions. Proceedings of the National Academy of Sciences United States of America 97, 406–411.

Sacks, D.L., Saraiva, E.M., Rowton, E., Turco, S.J., Pimenta, P.F.P., 1994. The role of lipophosphoglycan of *Leishmania* in vector competence. Parasitology 908, 855–862.

Schlein, Y., Jacobson, R.L., Shlomai, J., 1992. Chitinase secreted by *Leishmania* functions in the sandfly vector. Proceedings of the Royal Society of London SerieB: Biological Sciences 245, 121–126.

Schlein, Y., Polacheck, I., Yuval, B., 1985. Mycoses, bacterial infections and antibacterial activity in sandflies (Psychodidae) and their possible role in the transmission of Leishmaniasis. Parasitology 90, 57–66.

Shaw, J.J., 1994. Taxonomy of genus Leishmania: present and future trends and their implications. Memórias do Instituto Oswaldo Cruz 89, 471–478.

Soares, R.P.P., Cardoso, T.L., Barron, T., Araújo, M.S.S., Pimenta, P.F.P., Turco, S.J., 2005. *Leishmania braziliensis*: a novel mechanism in the lipophosphoglycan regulation during metacyclogenesis. International Journal of Parasitology 25, 245–253.

Soares, R.P.P., Macedo, M.E., Ropert, C., Gontijo, N.F., Almeida, I.C., Gazzinelli, R.T., Pimenta, P.F.P., Turco, S.J., 2002. *Leishmania chagasi*: lipophosphoglycan characterization and binding to the midgut of the sand fly vector *Lutzomyia longipalpis*. Molecular and Biochemical Parasitology 121, 213–224.

Straif, S.C., Mbogo, C.N.M., Toure, A.M., Walker, E.D., Kaufman, M., Toure, Y.T., Beier, J.C., 1998. Midgut bacteria in *Anopheles gambiae* and *An. funestus* (Diptera: Culicidae) from Kenya and Mali. Journal of Medical Entomology 35, 222–226.

Turco, S.J., Sacks, D.L., 2003. Control of *Leishmania*–sand fly interactions by polymorphisms in lipophosphoglycan structure. Methods in Enzimology 363, 377–381.

Turco, S.J., Descoteaux, A., 1992. The lipophosphoglycan of Leishmania parasites. Annual Review of Microbiology 46, 65–94.

Vasanthi, V., Hoti, S.L., 1992. Microbial flora in gut of *Culex quinquefasciatus* breeding in cesspits. Southeast Asian Journal of Tropical Medicine and Public Health 23, 312–317.

Verdon, R., Mirelman, D., Sansonetti, P.J., 1992. A model of the interaction between *Entomoeba histolytica* and *Shigella flexneri*. Research in Microbiology 143, 67–74.

Watnick, P., Kolter, R., 2000. Biofilm, city of microbes. Journal of Bacteriology 182, 2675–2679.

Weitere, M., Bergfeld, T., Rice, S.A., Matz, C., Kjelleberg, S., 2005. Grazing resistance of *Pseudomonas aeruginosa* biofilms depends on type of protective mechanism, developmental stage and protozoan feeding mode. Environmental Microbiology 7, 1593–1601.

Experimental Parasitology 122 (2009) 84–90

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Prodigiosin is not a determinant factor in lysis of *Leishmania (Viannia) braziliensis* after interaction with *Serratia marcescens* D-mannose sensitive fimbriae

Caroline S. Moraes [a], Sergio H. Seabra [b], José Maurício Albuquerque-Cunha [a], Daniele P. Castro [a], Fernando A. Genta [a], Wanderley de Souza [c], Reginaldo P. Brazil [a], Eloi S. Garcia [a], Patrícia Azambuja [a,*]

[a] Laboratório de Bioquímica e Fisiologia de Insetos, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Avenida Brasil 4354, Manguinhos, CEP 21045-900, Rio de Janeiro, RJ, Brazil
[b] Departamento de Pesquisa e Extensão, Centro Universitário Estadual da Zona Oeste, Rio de Janeiro, CEP 21070-200, RJ, Brazil
[c] Laboratório de Ultraestrutura Celular Hertha Meyer, Instituto de Biofísica Carlos Chagas Filho, Edifício do Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Ilha do Fundão, Rio de Janeiro, CEP 21941-590, RJ, Brazil

ARTICLE INFO

Article history:
Received 7 August 2008
Received in revised form 9 December 2008
Accepted 6 March 2009
Available online 18 March 2009

Keywords:
D-mannose
Leishmania
Serratia marcescens
Prodigiosin
Lysis
Biofilm

ABSTRACT

In this paper, the lytic activity of two variants of *Serratia marcescens* against promastigotes of *Leishmania braziliensis* was studied. *In vitro* assays showed that *S. marcescens* variant SM365 lyses *L. braziliensis* promastigotes, while the variant DB11 did not. Scanning electron microscopy (SEM) revealed that *S. marcescens* SM365 adheres to all cellular body and flagellum of the parasite. Several filamentous structures were formed and identified as biofilms. After 120 min incubation, they connect the protozoan to the developing bacterial clusters. SEM also demonstrated that bacteria, adhered onto *L. braziliensis* promastigote surface, formed small filamentous structures which apparently penetrates into the parasite membrane. D-mannose protects *L. braziliensis* against the *S. marcescens* SM365 lytic effect in a dose dependent manner. SM365 variant pre cultivated at 37 °C did not synthesize prodigiosin although the adherence and lysis of *L. braziliensis* were similar to the effect observed with bacteria cultivated at 28 °C, which produce high concentrations of prodigiosin. Thus, we suggest that prodigiosin is not involved in the lysis of promastigotes and that adherence promoted by bacterial mannose-sensitive (MS) fimbriae is a determinant factor in the lysis of *L. braziliensis* by *S. marcescens* SM365.

1. Introduction

Leishmania (*Viannia*) *braziliensis* is a protozoan that belongs to the Trypanosomatidae family and is the causative agent of cutaneous and mucocutaneous leishmaniasis, endemic diseases in many South American countries and a major public health concern in Brazil (Ambrosio and De Messias-Reason, 2005). *Leishmania braziliensis* has a digenetic life cycle, alternating between promastigote forms, which are the flagellated stage found in the sand fly midgut and the amastigote forms, present in the phagolysosome compartment of mammalian macrophages (Kamhawi, 2006).

The parasite passes through many physiological modifications in order to survive the adverse conditions in the vector midgut, such as proteolytic enzymes secreted by the midgut epithelium (Borovsky and Schlein, 1987) and the formation of a peritrophic matrix (PM) that surrounds the blood meal (Borovsky and Schlein, 1987; Pimenta et al., 1997). For successful transmission by the insect, *Leishmania* needs to escape from the PM before blood excretion. A chitinase released by the parasite is therefore required to degrade the PM before the adherence of *Leishmania* to the hindgut so that the parasite can subsequently migrate to the midgut and foregut (Sacks et al., 1994). The parasite is transmitted by the vector bite which injects promastigotes into the vertebrate host (Turco and Descoteaux, 1992).

Although several aspects of the sandfly–parasite interactions have been studied (Sacks et al., 2000), little is known about the niches occupied by the parasites and the role of the vector gut microbiota during these interactions. Further research is required to investigate if colonized species of bacteria in the sandfly gut produce lytic compounds able to kill *Leishmania spp*. Evidence of the possible influence of gut microbiota on the parasite life cycle has been reported in sandflies. Gut microbial infections in sandflies, *Phlebotomus papatasi*, significantly reduces the rate of infection by *Leishmania major* (Schlein et al., 1985). Many papers have already described the gut microbiota capacity of affect the development of parasites in the insect vectors (for review see Azambuja et al., 2005).

Several Enterobacteriaceae species were identified in laboratory reared *Lutzomyia longipalpis*, including the opportunistic bacterium *Serratia marcescens* (Oliveira et al., 1999, 2001). In *S. marcescens*, fimbriae are the virulent cell surface structures. Adhesins present in fimbriae mediate adherence to eukaryotic cell surfaces, via recognition of specific carbohydrate moieties on the host cell

* Corresponding author. Fax: +55 21 2590 3495.
E-mail address: azambuja@ioc.fiocruz.br (P. Azambuja).

0014-4894/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.exppara.2009.03.004

surface glycoconjugates (Ofek and Sharon, 1990). *Serratia marcescens* mannose-dependent fimbriae can attach to biological surfaces and form biofilms (Queck et al., 2006; Castro et al., 2007b). Castro et al. (2007a) demonstrated that D-mannose protects *Trypanosoma cruzi* and *Trypanosoma rangeli* from attachment and lysis by *S. marcescens* and concluded that type 1 mannose-sensitive fimbriae are relevant to bacterial lysis of these parasites. Recently, Moraes et al. (2008) studied the lysis of *L. chagasi* caused by the bacteria *S. marcescens* and demonstrated that bacterial mannose-sensitive (MS) fimbriae are relevant to this lytic effect.

Some variants of *S. marcescens* produce a pink pigment with small molecular-weight, prodigiosin (2-methyl-3-amyl-6-methoxyprodigiosene) (Williamson et al., 2006; Van Houdt et al., 2007). Prodigiosin induces apoptosis in cancer cell lines (Montaner et al., 2000), exhibits cytotoxic activity on V79 fibroblast cell line and on *T. cruzi* (Melo et al., 2000), has antimalarial activity (Isaka et al., 2002) as well as anticancer properties (Manderville, 2001). In this context, Azambuja et al. (2004) described the effects of different of *S. marcescens* variants on trypanosomatids, showing that some prodigiosin-producing forms lyse *T. cruzi* and *T. rangeli* and that the DB11 variant, which is not producer of the pigment, did not kill the parasites.

Castro et al. (2007a,b) reported that *S. marcescens* rapidly adhered to the *T. cruzi* surface through D-mannose recognizing fimbriae and rapidly induced its complete lysis. Scanning and transmission electron microscopy studies revealed that following bacteria–protozoan attachment there is an assembly of long filamentous structures, identified as biofilm, which connect the protozoan to the bacteria, which form clusters (Castro et al., 2007b). Recently, Moraes et al. (2008) demonstrated *in vitro* that *S. marcescens* SM365 lyses *Leishmania chagasi*, but DB11 variant is unable to lyse the parasite. They also reported that the lectin Concanavalin A (conjugated with FITC) bind *L. chagasi* promastigotes, resulting in intense fluorescence at the base of the flagellum (flagellar pocket). Also, D-mannose protects *L. chagasi,* markedly diminishing the lysis by *S. marcescens* SM365. Scanning electron microscopy revealed that the bacteria adherence occurred mainly in the flagellar pocket. Besides that, *S. marcescens* SM365 formed filamentous structures (identified as biofilm) on this protozoan species (Moraes et al., 2008).

In this paper, we extend anterior findings (Moraes et al., 2008) on the interactions between *Leishmania* and *S. marcescens*. We show that *S. marcescens* adheres to *L. braziliensis* membrane in a distinct manner when compared to *L. chagasi*, and that D-mannose-recognizing fimbriae from *S. marcescens* also is involved in the lysis of *L. braziliensis*. We suggest, for the first time, that prodigiosin is not related to the lysis of *Leishmania* induced by *S. marcescens* SM365 variant. These results point to a new mechanism of toxicity against trypanosomatids, based on cellular interactions instead of the action of isolated drugs.

2. Materials and methods

2.1. Leishmania braziliensis

Leishmania braziliensis L566 (strain MHOM/BR/1974/M209) was supplied by *Leishmania* Type Culture Collection of the Oswaldo Cruz Institute, Fiocruz, Brazil. *Leishmania* was grown in Schneider's Insect Medium (Sigma Chemical, St. Louis, MO) supplemented with 20% (v/v) heat-inactivated fetal bovine serum at 28 °C. The parasites used in all experiments were taken from 4-day-old cultures of promastigotes in the exponential growth phase. *Leishmania braziliensis* suspension was washed twice in sterile phosphate-buffered saline (PBS, 0.15 M NaCl and 0.01 M phosphate buffer, pH 7.2) and then centrifuged at 1800 rpm for 15 min.

2.2. Bacteria

Two variants of *S. marcescens* were used, a prodigiosin pigment producer variant SM365 and a non-pigmented variant DB11 (Kurz et al., 2003) (both supplied by Dr. Cecile Wandersman, Pasteur Institute, Paris, France). To obtain the stationary growth phase, variants of *S. marcescens* were incubated for 24 h at 28 °C in sterile liquid BHI (Difco) medium by aseptically adding 1 mL of liquid medium into 4 mL of sterile BHI. The colony-forming units (CFU) were estimated by comparing bacteria culture turbidity using optical density with $BaSO_4$ solutions of McFarland standards (Jousimies-Somier and Summanen, 2002), where the equivalent scale of 0.4 (Ba/mL) corresponds to 1.2×10^9 CFU/mL.

2.3. Carbohydrates

All carbohydrates (D-mannose, D-mannosamine, *N*-acetyl-D-mannosamine, D-glucose and D-galactose) (Sigma Chemical, St. Louis, MO) were diluted in PBS at concentrations of 2, 1 and 0.1 M and filtered through Millipore membranes with 0.22 μm pores.

2.4. Lytic assays

To measure the lytic activity, 10 μL of *S. marcescens* SM365 or DB11 in concentrations of 1×10^9 CFU/mL were added to 80 μL of *L. chagasi* promastigote suspension (final concentration of approximately 4.10^6 parasites/mL) in Eppendorf tubes and incubated at 28 °C for 30, 60 and 120 min. To analyze the lysis kinetics of *S. marcescens* SM365 the parasite was incubated with different concentrations of bacteria (from 1×10^5 to 1×10^8 CFU/mL) for 120 min. In some experiments, 10 μL suspensions of the *S. marcescens* variants (1×10^8 CFU/mL) cultured at 28 °C were pre-incubated with 10 μL of one of the carbohydrate solutions (2 M) in Eppendorf tubes at room temperature for 10 min. After this time, 80 μL of parasites (4.10^6 promastigotes/mL) were added and then the samples were incubated for 120 min. As controls for each assay, 10 μL of phosphate-buffered saline was added instead of the bacteria suspensions or carbohydrate solutions. After completing the final volumes to 100 μL, the mixtures were incubated at room temperature for 120 min. In some experiments, parasites were pre-incubated with D-mannose at a final concentration of 0.2 M for 30 min. Subsequently, the promastigote suspension was washed as described above and incubated with *S. marcescens* SM365 variant for 120 min. Before parasite counting the cell suspensions were mixed, the numbers of parasites estimated by direct counting in a Neubauer hemocytometer chamber under a light microscope, and the total parasite numbers were normalized on a log scale. In each assay, apparently undamaged *L. braziliensis* promastigotes were considered those parasites with integral morphology having slight movements of their flagella in the medium.

2.5. Scanning electron microscopy

To evaluate the bacteria–parasite interactions by scanning electron microscopy, *L. braziliensis* promastigotes in a suspension of 1×10^7 parasites/mL were adhered onto cover slips previously coated with 0.1% (w/v) aqueous poly-L-lysine for 30 min at 37 °C. Subsequently, *S. marcescens* SM365 was added in a concentration of 1×10^8 CFU/mL to parasites (bacteria/protozoa cell density ratio of 10:1 or 1:50 times) and the samples were incubated at room temperature from 1 to 120 min. After these times, the cover slips were washed in 0.1 M PBS, pH 7.2 and the adhered cells fixed with 2.5% (w/v) glutaraldehyde overnight. Then, the samples were washed again in PBS, post-fixed in solution of 1% (w/v) OsO_4 for 40 min at room temperature and washed twice with 0.1 M

cacodylate buffer. The cells were dehydrated in a graded series of acetone (30–100%, v/v), critical point dried using CO_2, mounted on metal stubs and coated with gold (5–30 nm) for observation in a scanning electron microscope (Jeol JSM6490LV).

2.6. Extraction, quantification and influence of prodigiosin on the lytic activity on the parasite

To measure the prodigiosin production of *S. marcescens* SM365 on *L. braziliensis* we adapted the method described by Witney et al. (1977). As negative control (blank) the *S. marcescens* DB11 variant that is known as a non-prodigiosin producer was used.

Basically, 1 mL of *S. marcescens* suspension was diluted in 20 mL of BHI medium and grown aerobically at 28 °C (temperature at which the variant SM365 produces prodigiosin), or at 37 °C (temperature that blocks bacterial prodigiosin synthesis) (Williams, 1973) for 48 h. After incubation, 10 mL of the bacteria cultures were harvested by centrifugation at 10,000 rpm for 10 min and the supernatant discarded. Then, the bacteria were resuspended into 900 μL of methanol and 40 μL of HCl (1 N) and incubated for 30 min at room temperature under constant agitation. The samples were then centrifuged at 10,000 rpm for 20 min and the supernatant was used for absorbance measurements at 550 nm in a 96-well plate reader (Elx800, Biotek). Prodigiosin production was expressed as 100 × Abs. 550 nm. The values corresponding to absorbances of the SM365 variant cultured at 28 or 37 °C were subtracted from the values obtained with the DB11 variant.

To evaluate the lytic activity of the *S. marcescens* variants grown at different temperatures, 10 μL suspensions of the *S. marcescens* variants (1×10^8 CFU/mL) described above were pre-incubated with PBS (control) and mannose solution (2 M) in Eppendorf tubes at room temperature for 10 min. After this time, 80 μL of parasites (4.10^6 promastigotes/mL) were added and then the samples were incubated for 120 min.

2.7. Statistical analysis

Data were compared using the ANOVA test according to Statistical Package for Social Sciences Software (SPSS), version 12.0 for Windows. Data are reported as means ± standard deviation. Differences between the groups were considered statistically significant when $p < 0.05$. Probability levels are specified in the text.

3. Results

3.1. Kinetics of L. braziliensis lysis during interactions with S. marcescens

In vitro experiments demonstrated that the number of *L. braziliensis* promastigotes decreased proportionally with time during incubation with *S. marcescens* SM365. After 30 min incubation the number of promastigotes was greatly reduced when compared to the initial concentration of parasites (Fig. 1A). After 60 min the density of parasites had diminished even further and after 120 min this difference was highly significant ($p < 0.001$) (Fig. 1A). *Serratia marcescens* DB11 variant incubated with *L. braziliensis* did not show any lytic effect (Fig. 1A). In other experiments, *S. marcescens* SM365 at concentrations of 1×10^5, 1×10^6, 1×10^7 and 1×10^8 CFU/mL were tested for comparative lytic activities against *L. braziliensis*. We observed that the concentrations of the variant SM365 of 1×10^5 and 1×10^6 CFU/mL did not significantly decrease the population densities of flagellates after 120 min incubation (Fig. 1B). However, the results presented in Fig. 1B show that at higher concentrations of bacteria (1×10^7 and 1×10^8 CFU/mL) the promastigotes were lysed intensely after 120 min incubation ($p < 0.001$) (Fig. 1B).

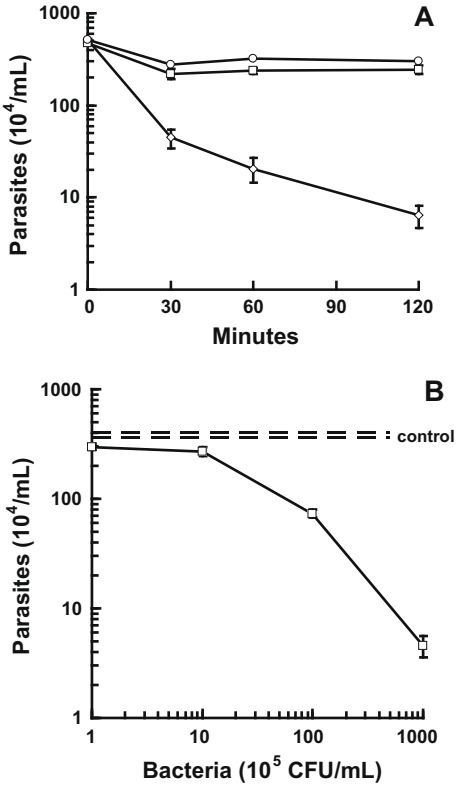

Fig. 1. (A) Temporal course of *Leishmania braziliensis* L566 strain lysis at different times of incubation with *Serratia marcescens*. Promastigotes (4×10^6 parasites/mL) were incubated with two variants of *Serratia marcescens* SM365 (◇) and DB11 (□) (approximately 1.0×10^8 CFU/mL) and counted in a Neubauer hemocytometer at different times of incubation. Control without bacteria (○). Each point represents the means ± SEM of five experiments. (B) Effects of different concentrations of *Serratia marcescens* SM365 on *Leishmania braziliensis* L566 lysis. Promastigotes (4×10^6 parasites/mL) were incubated with *Serratia marcescens* SM365 at concentrations from 1.0×10^5 to 1.0×10^8 CFU/mL and counted in a Neubauer hemocytometer after 120 min. Dotted lines show mean ± SEM of control experiments where parasites were incubated without bacteria. Each point represents the means ± SEM of five experiments.

3.2. Effects of carbohydrates on the lytic activity of S. marcescens against L. braziliensis

We examined the lysis of *L. braziliensis* by *S. marcescens* SM365 in the presence of different carbohydrates in the incubation medium. The promastigotes were strongly lysed by *S. marcescens* SM365 in the absence of sugars ($p < 0.001$, Fig. 2A, control 2). The results of flagellates and bacteria incubation in the presence of 0.2 M of different carbohydrates demonstrated that while of D-mannose significantly inhibited the lysis of this flagellate ($p < 0.001$), D-glucose, D-galactose, *N*-acetyl-D-mannosamine and D-mannosamine did not protect the parasite against the lytic effects caused by *S. marcescens* SM365 (Fig. 2A). In the last cases no difference was observed between all treatments and the group incubated with no carbohydrate, control 1 ($p > 0.05$) (Fig. 2A). Background experiments demonstrated that incubation with carbohydrates did not change significantly the parasite densities in *L. braziliensis* suspensions, when comparing with promastigotes incubated only with PBS (control 1) (data not shown). Also, pre-incubation of *L. braziliensis* with D-mannose did not inhibit the *S. marcescens* variant SM365 lytic activity on parasites (data not shown).

D-mannose inhibits parasite lysis in a dose dependent manner (Fig. 2B). Concentrations from 0.1 to 0.3 M block the lysis of *L. braziliensis* induced by *S. marcescens* SM365, in comparison to the

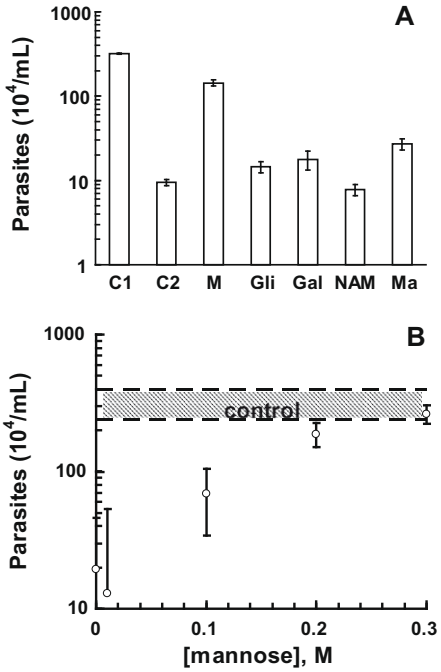

Fig. 2. (A) Effects of different carbohydrates on the lysis of *Leishmania braziliensis* L566 strain induced by *Serratia marcescens* SM365. Promastigotes (4×10^6 parasites/mL) were incubated with *S. marcescens* (1.0×10^8 CFU/mL) plus D-mannose (M), D-glucose (Glu), D-galactose (Gal), D-mannosamine (Ma) or *N*-acetyl-D-mannosamine (NAM) at final concentration of 0.2 M of all carbohydrates, or only PBS (C1) or bacteria (C2). Parasite numbers were estimated by counting in Neubauer hemocytometer after 120 min of incubation at 28 °C. Each point represents the means ± SEM of at least five experiments (B) Lysis of *Leishmania braziliensis* L566 strain incubated with *Serratia marcescens* SM365 variant with D-mannose. Promastigotes (4×10^6 parasites/mL) were incubated with D-mannose at different concentrations and *Serratia marcescens* (1.0×10^8 CFU/mL). Dotted lines show mean ± SD of control experiments where parasites were incubated without bacteria. Parasite numbers were estimated by counting in Neubauer hemocytometer after 120 min of incubation at 28 °C. Each point represents the means ± SD of at least five experiments.

parasite counting in the presence of bacteria without carbohydrate (control 2) ($p < 0.001$). The concentration of 0.01 M did not inhibited the parasite lysis ($p > 0.05$) (Fig. 2B).

3.3. Structural analysis of interactions between L. braziliensis and S. marcescens

Using scanning electron microscopy, as early as 1 min after bacteria–parasite mixing (ratio of 10:1), the *S. marcescens* SM365 dispersed around and rapidly started adhesion to the cellular body and flagellum of *L. braziliensis* (Fig. 3A–B). After 15 min incubation, the images show the formation of a large number of thin filamentous structures interconnecting bacteria or connecting bacteria to the surface of *L. braziliensis* or to parasite fragments (Fig. 3C). These thin structures increased in number after 30 min incubation (data not shown). At this point a large number of bacteria were seen on the parasite surface and a few minutes later the parasites were modified to an anomalous rounded form, evidenced mainly by the loss of the common spiral body shape (data not shown). At bacteria: parasite ratio of 50:1, after 120 min incubation the bacteria had almost totally disintegrated the parasites. *Serratia marcescens* SM365 were seen interconnected by the filaments, forming structures similar to biofilms over the rounded form of the parasites or disrupted cellular membranes (Fig. 3D). In controls alterations in the parasite morphology were not observed (Fig. 3A). Previous to lysis, the adhesion of bacteria onto the parasite surface is mediated by cylindrical structures present on the bacteria surface, which apparently penetrate the *L. braziliensis* body (Fig. 4).

3.4. Effects of prodigiosin on the lysis of L. braziliensis

The variant SM365 cultured for 48 h at 28 or 37 °C was treated with a methanol–HCl solution and the pigment extracted was used for quantification of prodigiosin. Table 1 shows that $A_{550} \times 100$ of the SM365 variant cultured at 37 °C ($p > 0.05$) gave no significant difference when compared with the absorbance of the DB11 variant, a non-prodigiosin producer which was considered as negative control. Only the SM365 grown at 28 °C produced the red pigment ($p < 0.001$). Lytic activities against *L. braziliensis* by the *S. marcescens* SM365 variant were observed for both bacteria cultured at 28 or 37 °C, but not in the presence of mannose ($p > 0.001$). The numbers of viable parasites incubated with variant DB11 were similar to control without bacteria (Table 1). Furthermore, there was no significant difference between the lysis of promastigotes incubated with the *S. marcescens* SM365 cultured at 28 or 37 °C as well as between the group incubated with mannose, control group (with no bacteria) and parasites incubated with the variant DB11 ($p > 0.05$). Controls of *S. marcescens* SM365 grown at 37 or 28 °C were kept for 24 h at room temperature, and after that no prodigiosin was produced by first group, only by the second. Also, we did not see significant difference (data not shown) between lysis of *L. braziliensis* by *S. marcescens* SM365 when the bacteria was incubated with parasites at 28 or 37 °C, for the two bacterial growing conditions (28 or 37 °C). Thus, we can conclude that parasite lysis is more related to the presence of mannose-sensitive fimbriae than to the production of prodigiosin by these bacteria.

4. Discussion

Bacteria of the genus *Serratia* are frequently associated to insects of many orders (Grimont and Grimont, 1978) and some species have been found in the midgut microbiota of hematophagous insects (Straif et al., 1998; Gonzalez-Ceron et al., 2003; Figueiredo et al., 1995; Dillon et al., 1996; Oliveira et al., 2001; Azambuja et al., 2004). Recently, it was shown that *S. marcescens* is lytic for *T. cruzi* and *T. rangeli*, two species of parasites that use triatomine insects as vectors (Azambuja et al., 2004; Castro et al., 2007a), and also to *L. chagasi* (Moraes et al., 2008). In this study, we confirmed some previous data obtained by the incubation of *S. marcescens* with *L. chagasi* extending our investigation to *L. braziliensis*.

Azambuja et al. (2004) opened up an exciting research area by evaluating the effects of resident bacteria in the stomach of *Rhodnius prolixus* on *T. cruzi* infection. These authors demonstrated that *S. marcescens* isolated from the gut of *R. prolixus* destroys some strains of *T. cruzi*, but they could not discard the possibility that the pigment produced by these bacteria, prodigiosin, was the factor responsible for the lysis of the parasites. Furthermore, these authors had demonstrated the importance of the adherence of *S. marcescens* to *T. cruzi* and *T. rangeli* and that D-mannose-dependent receptors are involved in the bacteria attachment and lysis of *T. cruzi* (Castro et al., 2007a,b) or *L. chagasi* (Moraes et al., 2008). Moraes et al. (2008) studied the lytic effect of *S. marcescens* SM365 and DB11 variants on promastigotes of *L. chagasi*. *In vitro* experiments demonstrated that the *S. marcescens* non-pigmented variant DB11 did not lyse the parasites, while the prodigiosin producer SM365 did. Since *S. marcescens* DB11 does not have genes coding fimbriae subunits fimA and fimB (search in Sanger Institute), Moraes et al. (2008) suggested that both D-mannose fimbriae and/or prodigiosin are involved in the lysis of *L. chagasi* induced by SM365. In this paper, we demonstrated conclusively that

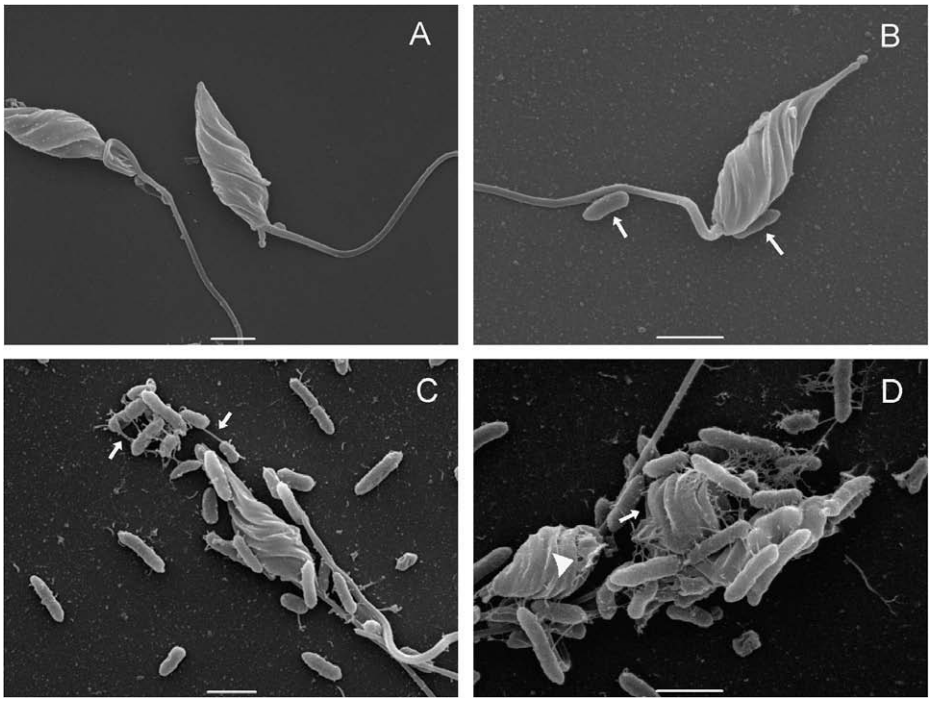

Fig. 3. Scanning electron microscopy of *Leishmania braziliensis* L566 strain and *Serratia marcescens* SM365 variant interaction. (A) Control, parasites without bacteria. (B) Bacteria (arrows) adhered on promastigote at bacteria–parasite ratio of 10:1 after 1 min of incubation. (C) After 15 min of incubation at bacteria–parasite ratio of 10:1, note the presence of thin filaments interconnecting bacteria and parasite (arrows). (D). Biofilm formation after 120 min (in concentration of 50:1). Note parasite membrane fragmentation (head arrow) and morphologically changed parasite (arrow). Bar (A–D) 2 μm.

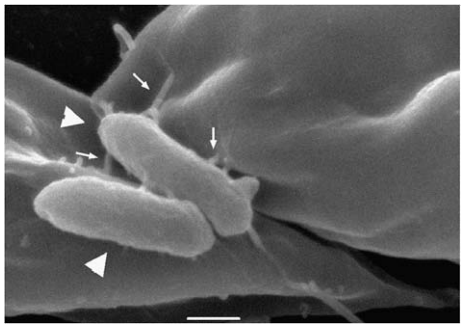

Fig. 4. Scanning electron microscopy of *Serratia marcescens* SM365 variant attached to *Leishmania braziliensis* L566 strain. Note the bacterial adhesion on the parasite surface (head arrow) connected to the parasite through cylindrical structures (arrows). Bar 0.5 μm.

prodigiosin, a pigment synthesized by *S. marcescens*, is not involved in the lysis of *Leishmania*. Melo et al. (2000) described a potent trypanocidal activity for prodigiosin against *T. cruzi*, and as this compound showed a lower cytotoxicity against mammal cells, they proposed that the *in vivo* action of this compound should be investigated. In fact, our studies suggest that during the interaction between vector midgut bacteria and *Leishmania* promastigotes, prodigiosin is not a determinant factor of possible blockage of infection by gut microbiota.

Herein, we confirmed the importance of fimbriae in the binding of *S. marcescens* SM365 to parasites as described by Castro et al. (2007a,b) and Moraes et al. (2008), by the demonstration that D-mannose also protects *L. braziliensis* against the lysis caused by bacteria. We also showed that when *S. marcescens* SM365 interacts with *L. braziliensis,* the attachment occurred on the cellular body and flagellum of the parasite, suggesting that carbohydrate-specific binding, in this case, is found on all membrane surfaces instead of only at the flagellar pocket, as observed in *L. chagasi* promastigotes (Moraes et al., 2008). It is possible that lipophosphoglycan (LPG) and/or other relevant glycoconjugates such as glycosylphosphatidylinositol (GPI)-anchored glycoproteins, proteophosphoglycans (PPG) and free GPI glycolipids (Mukhopadhyay and Mandal, 2006; Kamhawi, 2006), which result in high concentrations of carbohydrates distributed on the parasite surface are the ligands responsible for the interaction of *S. marcescens* SM365

Table 1
Effect of D-mannose on lysis of *L. braziliensis* L566 strain incubated with *S. marcescens* variants pre cultivated at 28 and 37 °C.

Serratia marcescens strain	Temperature cultivation of bacteria (°C)	Presence of red pigment	Prodigiosin absorbances[a] ($A_{550} \times 100$)	D-Mannose[b] (0.2 M)	Number of parasites (log)[c]
	28	+	35.0 ± 14.5	–	5.0 ± 0.5
SM365	37	–	0.0	–	4.9 ± 0.5
	37	–	0.0	+	6.2 ± 0.2
DB11	28	–	0.0	–	6.4 ± 0.2
Control	(No bacteria)	–	–	–	6.5 ± 0.1

[a] Prodigiosin was extracted from *S. marcescens* and measured according to Witney et al. (1977).
[b] The signals (+) indicate presence and (–) absence of prodigiosin.
[c] The number of parasites was counted in Neubauer chamber after 120 min incubation. Mean ± SD of five experiments. After pre-cultivating bacteria strains at 28 or 37 °C for 48 h, lytic effects were immediately evaluated by incubation of the bacteria culture with the parasite at 28 °C for 120 min.

with *Leishmania chagasi* as well as *L. braziliensis*. It is interesting to note that the same type of fimbriae in *E. cloacae* strongly recognizes the glycidic moiety $Man_9(GlcNAc)_2$ present in glycoproteins (Pan et al., 1997). As these surface molecules (specially the gp63 protein) are one of the major surface motifs of trypanosomatids, they are interesting candidates of *S. marcescens* binding to these parasites. This is coherent with the initial observation that *S. marcescens* does not adhere to *Toxoplasma gondii* cells (Moraes, data not published) and it is a hypothesis in current investigation.

The most common reactions of bacteria against Protozoa in nature are the formation of a bacterial community recognized as a biofilm (Watnick and Kolter, 2000; Weitere et al., 2005; Queck et al., 2006). Castro et al. (2007b) using scanning and transmission electron microscopy reported that *S. marcescens* SM365 attaches to *T. cruzi* epimastigotes or their surface fragments forming biofilms. Identical observations were demonstrated by Moraes et al. (2008) when *S. marcescens* SM365 is incubated with *L. chagasi*. Our present results also demonstrate thin filaments connecting bacteria to each other and to the *L. braziliensis* promastigote surface. This filamentous structure, described as a biofilm formation, is similar to previous structures described by several authors (Matz et al., 2004; Rice et al., 2005; Castro et al., 2007b; Moraes et al., 2008).

Finally, we showed for the first time that prodigiosin is not directly related to the lysis of promastigotes of *L. braziliensis*. Although *in vitro* experiments on cancer cell lines and parasites demonstrated that the purified pigment prodigiosin has toxic effects (Montaner et al., 2000; Montaner and Pérez-Tomás, 2001; Díaz-Ruiz et al., 2001; Llagostera et al., 2003), our results clearly demonstrated that *S. marcescens* SM365 cultured at 37 °C, a temperature which blocks the synthesis of prodigiosin by the bacteria (Williams, 1973), was able to attach and lyse the parasites. Azambuja et al. (2004) hypothesized that prodigiosin could have importance in the lysis of parasites by *S. marcescens*, but we suggest that in spite of this prodigiosin toxic effect some other factor is responsible for the lytic effect of on *Leishmania*.

This paper provides a sound basis for future work on protozoan-bacteria interactions, focusing on the effects of parasite virulence factors, which might yield a novel strategy for insect vector control transmission. Traditional work on the development of drugs against trypanosomatids focuses on the research of isolated molecules with toxic effects against these organisms. The data presented here suggests that the control of trypanosomatid populations in natural environments can be extremely dependent on cellular interactions and on the mechanism of toxin delivery to the parasite cell. Detailed knowledge of this machinery could be of great relevance to the research on new strategies for control and therapy of this class of disease.

Acknowledgments

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (PA, ESG and WS), Fundação Oswaldo Cruz (Fiocruz) (Papes), and Fundação Carlos Chagas Filho de Amparo a Pesquisa do Estado do Rio de Janeiro (Faperj) (PA, RPB, FAG). PA, ESG and WS are Senior Research Fellows (CNPq), DPC and CSM are Postgraduation Fellows (Faperj) and José Mauricio is a PostDoc Fellow (CNPq). We thank Dr. Cecile Wandersman (Instituto Pasteur, France) for kindly supplying the variants SM365 and DB11 of *S. marcescens*, Dr. Suzana C. Real (Instituto Oswaldo Cruz) for helping us with scanning microscopy techniques and Dr. Maria de Fátima Madeira for helping us with cultures.

References

Ambrosio, A.R., De Messias-Reason, I.J., 2005. *Leishmania* (*Viannia*) *braziliensis*: interaction of mannose-binding lectin with surface glycoconjugates and complement activation. An antibody-independent defense mechanism. Parasite Immunology 27 (9), 333–340.

Azambuja, P., Garcia, E.S., Ratcliffe, N.A., 2005. Gut microbiota and parasite transmission by insect vectors. Trends Parasitology 21, 568–572.

Azambuja, P., Feder, D., Garcia, E.S., 2004. Isolation of *Serratia marcescens* in the midgut of *Rhodnius prolixus*: impact on the establishment of the parasite *Trypanosoma cruzi* in the vector. Experimental Parasitology 107, 89–96.

Borovsky, D., Schlein, Y., 1987. Trypsin and chymotrypsin-like enzymes of the sandfly *Phlebotomus papatasi* infected with *Leishmania* and their possible role in vector competence. Medical and Veterinary Entomology 1, 235–269.

Castro, D.P., Moraes, C.S., Garcia, E.S., Azambuja, P., 2007a. Inhibitory effects of D-mannose on trypanosomatid lysis induced by *Serratia marcescens*. Experimental Parasitology 115, 200–204.

Castro, D.P., Seabra, S.H., Garcia, E.S., de Souza, W., Azambuja, P., 2007b. *Trypanosoma cruzi*: ultrastructural studies of adhesion, lysis and biofilm formation by *Serratia marcescens*. Experimental Parasitology 117, 201–207.

Díaz-Ruiz, C., Montaner, B., Pérez-Tomás, R., 2001. Prodigiosin induces cell death and morphological changes indicative of apoptosis in gastric cancer cell line HGT-1. Histology and Histopathology 16, 415–421.

Dillon, R.J., El Kordy, E.L., Lanee, R.P., 1996. The prevalence of a microbiota in the digestive tract of *Phlebotomus papatasi*. Annals of Tropical Medicine and Parasitology 90, 669–673.

Figueiredo, A.R., Nunes, Z.G., Silva, A.A.L., Giordano-Dias, C.M.G., Coura, J.R., Hofer, E., 1995. Isolation of microorganisms of triatomines maintained in artificial and sylvatic conditions. Memórias do Instituto Oswaldo Cruz 90, 228.

Gonzalez-Ceron, L., Santillan, F., Rodríguez, M.H., Mendez, D., Hernández-Avila, J.E., 2003. Bacteria in midguts of field-collected *Anopheles Albimanus* block *Plasmodium vivax* sporogonic development. Journal of Medical Entomology 40, 371–374.

Grimont, P.A., Grimont, F., 1978. The genus *Serratia*. Annual Review of Microbiology 32, 221–248.

Isaka, M., Jaturapat, A., Kramyu, J., Tanticharoen, M., Thebtaranonth, Y., 2002. Potent in vitro antimalarial activity of metacycloprodigiosin isolated from *Streptomyces spectabilis* BCC 4785. Antimicrobial Agents and Chemotherapy 46, 1112–1113.

Jousimies-Somier, H., Summanen, P., 2002. Recent taxonomic changes and terminology update of clinically significant anaerobic gram-negative bacteria (excluding spirochetes). Clinical and Infection Diseases 35, 17–21.

Kamhawi, S., 2006. *Phlebotomine* sand flies and *Leishmania* parasites: friends or foes? Trends in Parasitology 22, 439–445.

Kurz, C.L., Chauvet, S., Andrés, E., Aurouze, M., Vallet, I., Michel, G.P., Uh, M., Celli, J., Filloux, A., De Bentzmann, S., Steinmetz, I., Hoffman, J.Á., Finlay, B.B., Gorvel, J.P., Ferradon, D., Ewbank, J.J., 2003. Virulence factors of the human opportunistic pathogen *Serratia marcescens* identified by *in vivo* screening. EMBO Journal 22, 1451–1460.

Llagostera, E., Soto-Cerrato, V., Montaner, B., Pérez-Tomás, R., 2003. Prodigiosin induces apoptosis by acting on mitochondria in human lung cancer cells. Annals of the New York Academy of Sciences 1010, 178–181.

Manderville, R., 2001. Synthesis, proton-affinity and anti-cancer properties of the prodigiosin-group natural products. Current Medicinal Chemistry Anticancer Agents 1, 195–218.

Matz, C., Bergfeld, T., Rice, S.A., Kjelleberg, S., 2004. Micro-colonies, quorum sensing and cytotoxity determine the survival of *Pseudomonas aeruginosa* biofilms exposed to protozoan grazing. Environmental Microbiology 6, 218–226.

Moraes, C.S., Seabra, S.H., Castro, D.P., Brazil, R.P., de Souza, W., Garcia, E.S., Azambuja, P., 2008. *Leishmania* (*Leishmania*) *chagasi* interactions with *Serratia marcescens*: ultrastructural studies, lysis and carbohydrate effects. Experimental Parasitology 118, 561–568.

Melo, P.S., Duran, N., Haun, M., 2000. Cytotoxicity of prodigiosin and benzinidazole on V79 cells. Toxicology Letters 116, 237–242.

Montaner, B., Pérez-Tomás, R., 2001. Prodigiosin-induced apoptosis in human colon cancer cells. Life Sciences 68, 2025–2036.

Montaner, B., Navarro, S., Pique, M., Vilaseca, M., Martinell, M., Giralt, E., Gil, J., Pérez-Tómas, R., 2000. Prodigiosin from the supernatant of *Serratia marcescens* induces apoptosis in haematopoietic cancer cell lines. Brazilian Journal of Pharmacology 131, 585–593.

Mukhopadhyay, S., Mandal, C., 2006. Glycobiology of *Leishmania donovani*. Indian Journal of Medical Research 123, 203–220.

Ofek, I., Sharon, N., 1990. Adhesins as lectins: specificity and role in infection. Current Topics in Microbiology and immunology 151, 91–113.

Oliveira, S.M.P., Morais, B., Gonçalves, C.A., Giordano-Dias, C.M., Vilela, M.L., Brazil, R.P., D'Almeida, J.M., Asensi, M.D., Mello, R.P., 2001. Digestive tract microbiota in females of *Lutzomyia longipalpis* (Diptera: Psychodidae) feeding on blood meal and sucrose plus blood meal. Cadernos de Saúde Pública 17, 229–232.

Oliveira, S.M.P., Loureiro, M.M., Leandro, M.J.F., Giordano-Dias, C.M., Brazil, R.P., D'Almeida, J.M., Asensi, M.D., Mello, R.P., 1999. Caracterização da microbiota do trato digestório, em fêmeas de *Lutzomyia longipalpis* (Lutz & Neiva, 1912) (Diptera: Psychodidae) alimentadas com frutose. Entomologia y Vectores 6, 166–179.

Pan, Y.T., Xu, B., Rice, K., Smith, S., Jackson, R., Elbein, A.D., 1997. Specificity of the high-mannose recognition site between Enterobacter cloacae pili adhesin and HT-29 cell membranes. Infection and Immunology 65, 4199–4206.

Pimenta, P.F.P., Modi, G.B., Pereira, S.T., Shahabuddin, M., 1997. A novel role for the peritrophic matrix in protecting *Leishmania* from the hydrolytic activities of the sand fly midgut. Parasitology 115, 359–369.

Queck, S.Y., Weitere, M., Moreno, A.M., Rice, S.A., Kjelleberg, S., 2006. The role of quorum sensing mediated developmental traits in the resistance of *Serratia*

marcescens biofilms against protozoan grazing. Environmental Microbiology 8, 1017–1025.

Rice, A.S., Koh, K.S., Queck, S.Y., Labbate, M., Lam, K.W., Kjelleberg, S., 2005. Biofilm formation and sloughing in *Serratia marcescens* are controlled by quorum sensing and nutrient cues. Journal of Bacteriology 187, 3477–3485.

Sacks, D.L., Modi, G., Rowton, E., Späth, G., Epstein, L., Turco, S.J., Beverley, S.M., 2000. The role of phosphoglycan in *Leishmania*–sand fly interactions. Proceedings of the National Academy of Sciences United States of America 97, 406–411.

Sacks, D.L., Saraiva, E.M., Rowton, E., Turco, S.J., Pimenta, P.F.P., 1994. The role of lipophosphoglycan of *Leishmania* in vector competence. Parasitology 908, 855–862.

Schlein, Y., Polacheck, I., Yuval, B., 1985. Mycoses, bacterial infections and antibacterial activity in sandflies (Psychodidae) and their possible role in the transmission of Leishmaniasis. Parasitology 90, 57–66.

Straif, S.C., Mbogo, C.N.M., Toure, A.M., Walker, E.D., Kaufman, M., Toure, Y.T., Beier, J.C., 1998. Midgut bacteria in *Anopheles gambiae* and *An. funestus* (Diptera: Culicidae) from Kenya and Mali. Journal of Medical Entomology 35, 222–226.

Turco, S.J., Descoteaux, A., 1992. The lipophosphoglycan of *Leishmania* parasites. Annual Review of Microbiology 46, 65–94.

Van Houdt, R., Givskov, M., Michiels, C.W., 2007. Quorum sensing in *Serratia*. FEMS Microbiology Reviews 31, 407–424.

Watnick, P., Kolter, R., 2000. Biofilm, city of microbes. Journal of Bacteriology 182, 2675–2679.

Weitere, M., Bergfeld, T., Rice, S.A., Matz, C., Kjelleberg, S., 2005. Grazing resistance of *Pseudomonas aeruginosa* biofilms depends on type of protective mechanism, developmental stage and protozoan feeding mode. Environmental Microbiology 7, 1593–1601.

Williams, R.P., 1973. Biosynthesis of prodigiosin, a secondary metabolite of *Serratia marcescens*. Applied Microbiology 25, 396–402.

Williamson, N.R., Fineran, P.C., Leeper, F.J., Salmond, G.P.C., 2006. The biosynthesis and regulation of bacterial prodiginines. Nature Reviews Microbiology 4, 887–899.

Witney, F.R., Failla, M.L., Weinberg, E.D., 1977. Phosphate inhibition of secondary metabolism in *Serratia marcescens*. Applied Environmental Microbiology 33, 1042–1046.

REFERÊNCIAS BIBLIOGRÁFICAS

Adegbola RA, Old DC. New fimbrial hemagglutinin in *Serratia* species. Infect Immun. 1982; 38:306-315.

Aderem A, Underhill DM. Mechanisms of phagocytosis in macrophages. Annu Rev Immunol. 1999; 17:593-623.

Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD. Molecular Biology of the Cell. 3rd ed. New York: Garland Publishing; 1994.

Alves CR, Albuquerque-Cunha JM, Mello CB, Garcia ES, Nogueira NF, Bourguingnon SC, de Souza W, Azambuja P, Gonzalez MS. *Trypanosoma cruzi*: attachment to perimicrovillar membrane glycoproteins of *Rhodnius prolixus*. Exp Parasitol. 2007; 116:44-52.

Andersen SO. Sclerotization and tanning of the cuticle. In: Kerkut GA, Gilbert LI, editors. Comprehensive Insect Physiology, Biochemical Pharmacology. Oxford:Pergamon Press; 1985. p. 59-74.

Arai H, Koizumi H, Aoki J, Inoue K. Platelet-Activating Factor Acetylhydrolase (PAF-AH). J Biochem. 2002; 131:635-40.

Arai H. Platelet-activating factor acetylhydrolase. Prostaglandins Other Lipid Mediat. 2002; 68–69:83–94.

Ashida M, Brey PT. Recent advances on the research of the insect prophenoloxidase cascade. In: Brey PT, Hultmark D, editors. Molecular Mechanisms of Immune Responses in Insects. London: Chapman & Hall; 1998, p. 135-172.

Au BT, Teixeira MM, Collins PD, Williams TJ. Blockade of PAF receptors controls interleukin-8 production by regulating the activation of neutrophil CD11/CD18. Eur J Pharmacol. 2001; 425: 65-71.

Auden, DT. Studies on the development of *Rhodnius prolixus* and the effects of its symbiote *Nocardia rhodnii*. J Med Entomol. 1974; 11:68-71.

Azambuja P, Feder D, Garcia ES. Isolation of *Serratia marcescens* in the midgut of *Rhodnius prolixus*: impact in the establishment of the parasite *Trypanosoma cruzi* in the vector. Exper Parasitol 2004; 107:89-96.

Azambuja P, Feder D, Mello CB, Gomes SAO, Garcia E. Immunity in *Rhodnius prolixus*: trypanosomatid-vector interactions. Mem Inst Oswaldo Cruz 1999; (94Suppl) 1:219-22.

Azambuja P, Freitas CC, Garcia ES. Evidence and partial characterization of an inducible antibacterial factor in the haemolymph of *Rhodnius prolixus*. J Insect Physiol. 1986; 32:807-12.

Azambuja P, Garcia ES, Ratcliffe NA, Warthen Jr JD. Immune-depression in *Rhodnius prolixus* induced by the growth inhibitor, Azadirachtin. J Insect Physiol. 1991; 37:771-7.

Azambuja P, Garcia ES, Ratcliffe NA. Gut microbiota and parasite transmission by insect vectors. Trends Parasitol. 2005b; 21:568-572.

Azambuja P, Garcia ES. Care and maintenance of triatomine colonies. In: Crampton JM, Beard CB, Louis C, editors. Molecular biology of insect disease vectors: a methods manual. London: Chapman & Hall; 1997, p. 56-64.

Azambuja P, Garcia ES. Characterization of inducible lysozyme activity in the hemolymph of *Rhodnius prolixus*. Brazilian J Med Biol Research. 1987; 20:539 – 48.

Azambuja P, Garcia ES. *Trypanosoma rangeli* interactions within the vector *Rhodnius prolixus*: a mini review. Mem Inst Oswaldo Cruz. 2005; 100: 567-72.

Azambuja P, Guimarães JA, Garcia ES. Hemolytic factor from the stomach of *Rhodnius prolixus*: evidence and partial characterization. J Insect Physiol. 1983; 29:833-837.

Azambuja P, Mello CB, Feder D, Garcia ES. Influência do sistema de defesa celular e humoral dos triatomíneos no desenvolvimento dos tripanossomatídeos. In: Carcavallo RU, Girón IG, Jurberg J, Lent H. Atlas dos Vetores da Doença de Chagas nas Américas, Rio de Janeiro: Fiocruz, 1997.

Azambuja P, Ratcliffe NA, Garcia ES. Towards an understanding of the interactions of *Trypanosoma cruzi* and *Trypanosoma rangeli* within the reduviid insect host *Rhodnius prolixus*. An Acad Bras Cien. 2005a; 77:397-404.

Azambuja P, Ribeiro JM, Garcia ES. Effects of ecdysone on the metamorphosis and ecdysis prevention induced by precocene II in *Rhodnius prolixus*. Gen Comp Endocrinol. 1981; 45:100-4.

Azambuja P, S BW, Ribeiro JM, Souza E. Antifeedant activity of precocenes and analogs on *Rhodnius prolixus*. Experientia. 1982; 38:1054-5.

Barboza M, Duschak VG, Fukuyama Y, Nonami H, Erra-Balsells R, Cazzulo JJ, Couto AS. Structural analysis of the N-glycans of the major cysteine proteinase of *Trypanosoma cruzi.* Identification of sulfated high-mannose type oligosaccharides. FEBS J. 2005; 272:3803-15.

Basanova AV, Baskova IP, Zavalova LL.Vascular-platelet and plasma hemostasis regulators from bloodsucking animals. Biochemistry (Mosc). 2002; 67:143-50.

Bassler BL, Losick, R. Bacterially speaking. Cell. 2006; 125:237-246.

Battin TJ, Sloan WT, Kjelleberg S, Daims H, Head IM, Curtis TP, Eberl L. Microbial landscapes: new paths to biofilm research. Nat Rev Microbiol. 2007; 5:76-81.

Beard CB, Cordon-Rosales C, Durvasula RV. Bacterial symbionts of the triatominae and their potential use in controlo of Chagas Disease transmission. Annu Rev Entomol. 2002; 47:123-141.

Beard CB, Dotson EM, Pennington PM, Eichler S, Cordon-Rosales C, Durvasula RV. Bacterial symbiosis and paratransgenic control of vector-borne Chagas disease. Int J Parasitol. 2001; 31:621-7.

Beato M, Klug J. Steroid hormone receptors: an update. Hum Reprod Updat. 2000; 6:225-236.

Bewig F, Schwartz W. Untersuchungen über die symbiose von tieren mit pilzen und bakterien. VII Über die physiologie der symbiose bei einigen blutsaugenden insekten. Arch Mikrobiol. 1956; 24:174.

Beyaert R. Nuclear Factor KB: Regulation and Role in Disease. Ed. Springer, 2003 p. 426.

Blander JM, Medzhitov R. Regulation of phagosome maturation by signals from toll-like receptors. Sci. 2004; 304:1014–1018.

Boeke SJ, Boersma MG, Alink GM, van Loon JJ, van Huis A, Dicke M, Rietjens IM. Safety evaluation of neem (Azadirachta indica) derived pesticides. J Ethnopharmacol. 2004; 94:25-41.

Bonay P, Fresno M. Characterization of Carbohydrate Binding Proteins in *Trypanosoma cruzi*. J Biol Chem. 1995; 270:11062-70.

Bonay P, Molina R, Fresno M. Binding specificity of mannose-specific carbohydrate-binding protein from the cell surface of *Trypanosoma cruzi*. Glycobiol. 2001; 21:719-29.

Boschetti MA, Piras MM, Henríquez D, Piras R. The interaction of a Trypanosoma cruzi surface protein with Vero cells and its relationship with parasite adhesion. Mol Biochem Parasitol. 1987; 24:175-84.

Bouckaert J, Mackenzie J, de Paz JL, Chipwaza B, Choudhury D, Zavialov A,

Mannerstedt K, Anderson J, Piérard D, Wyns L, Seeberger PH, Oscarson S, de Greve H, Knight SD. The affinity of the fimH fimbrial adhesion is receptor-driven and quasi-independet of *Escherichia coli* pathotypes. Mol Microbiol. 2006; 61:1556-68.

Bowman AS, Gengler CL, Surdick MR, Zhu K, Essenberg RC, Sauer JR, Dillwith JW. A novel phospholipase A2 activity in saliva of the lone star tick, *Amblyomma americanum* (L.). Exp Parasitol. 1997; 87:121–132.

Brahmachari G. Neem--an omnipotent plant: a retrospection. Chembiochem. 2004; 5(4):408-21. Review

Braun V, Günther H, Neuss B, Tautz C. Hemolytic activity of *Serratia marcescens*. Arch Microbiol. 1985; 141:371-6.

Braun V, Neuss B, Ruan Y, Schiebel E, Schöffler H, Jander G. Identification of the *Serratia marcescens* hemolysin determinant by cloning into *Escherichia coli*. J Bacteriol. 1987; 169:2113-20.

Brecher G, Wigglesworth VB. The transmission of *Actinomyces rhodniii* Erikson in *Rhodnius prolixus* Stal (Hemiptera) and its influence on the growth of the host. Parasitol. 1944; 35:220.

Brey PT, Hultmark D. Molecular mechanism of immune responses in insects, Ed. Chapman & Hall, New York, 1998 28-35.

Brookman JL, Rowley AF, Ratcliffe NA. Studies on nodule formation in locust following injection of microbial products. J Invert Pathol. 1989; 53:315-323.

Bussolino F, Fischer E, Turrini F, Kazatchkine MD, Arese P.Platelet-activating factor enhances complement-dependent phagocytosis of diamide-treated erythrocytes by human monocytes through activation of protein kinase C and phosphorylation of complement receptor type one (CR1). J Biol Chem. 1989; 264:21711-21719.

Büyükgüzel E, Tunaz H, Stanley D, Büyükgüzel K. Eicosanoids mediate *Galleria mellonella* cellular immune response to viral infection. J Insect Physiol. 2007; 53:99–105.

Capitani G, Eidam O, Glockshuber R, Gru¨tter MG. Structural and functional insights into the assembly of type 1 pili from *Escherichia coli*. Microb Infect. 2006; 8:2284-90.

Carbonell GV, Amorim CRN, Furumura MT, Darini ALC, Fonseca BAL, Yano T. Biological activity of *Serratia marcescens* cytotoxin. Brazilian J Med Biol Res. 2003; 36:351-9.

Carbonell GV, Della Colleta HH, Yano T, Darini AL, Levy CE, Fonseca BA. Clinical revelance and virulence factors of pigmented *Serratia marcescnes*. FEMS Immunol Med Microbiol. 2000; 28:143-9.

Carbonell GV, Falcón R, Yamada AT, da Fonseca BA. Morphological and intracellular alterations induced by *Serratia marcescens* cytotoxin. Res Microbiol. 2004; 155:25-30.

Carvalho RU, Girón IG, Jurberg J, Lent H. Influência do sistema de defesa celular e humoral dos triatomíneos no desenvolvimento dos tripanossomatídeos. In: Atlas dos vetores da Doença de Chagas nas Américas. Rio de Janeiro, Ed. Fiocruz, vol II, 1997 p.715-727.

Cerenius L, Soderhall K. The prophenoloxidase-activating system in invertebrates. Immunol Rev. 2004; 198:116–126.

Chao J, Wistreich GA. Microorganisms from the midgut of larval and adult Culex quinquefasciatus Say. J Insect Pathol. 1960; 2:220-4.

Cheeseman MT, Bates PA, Crampton JM. Preliminary characterisation of esterase and platelet-activating factor (PAF)-acetylhydrolase activities from cat flea

(*Ctenocephalides felis*) salivary glands. Insect Biochem Mol Biol. 2001: 31:157–164.

Chiang HC, Jaw SM, Cehn PM. Inhibitory effects of physalin B and physalin F on various human leukemia cells in vitro. In: Anticancer Res 1992; 12(4):1155-62.

Choi JH, Choi EK, Park SJ, Ko HM, Kim KJ, Han SJ, Choi IW, Im SY. Impairment of p38 MAPK-mediated cytosolic phospholipase A2 activation in the kidneys is associated with pathogenicity of Candida albicans. Immunol. 2007; 120(2):173-81.

Choi JH, Ko HM, Kim JW, Lee HK, Han SS, Chun SB, Im SY. Platelet-activating factor-induced early activation of NF-kappa B plays a crucial role for organ clearance of Candida albicans. J Immunol. 2001; 166(8):5139-44.

Cociancich S, Bulet P, Hetru C, Hoffmann JA. The inducible antibacterial peptides of insects. Parasitol Today. 1994; 10:132-139.

Cornelis GR. The *Yersinia* deadly kiss. J Bacteriol. 1998; 180:5495-504.

Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Sci. 1999; 284:1318-22.

Cuba Cuba A. Review of the biologic and diagnostic aspects of *Trypanosoma* (Herpetosoma) *rangeli*. Rev Soc Bras Med Trop 1998; 31(2): 207-20. Review.

Cuba Cuba C, Morales N, Fernandez E, Fernandez W. Hallazgo de *Rhodnius ecuadorienses* Lent & León, 1958 infectado por tripanosomas similares a *Trypanossoma rangeli* Tejera, 1920 en caseíros del distrito de Cascas, Contumazá, Dpto de Cajamarca, Peru. Rev Inst Med Trop Sao Paulo 1972; 14: 191-202.

Da Matta RA, Seabra SH, de Souza W. Further studies on the phagocytic capacity of chicken thrombocytes. J Submicrosc Cytol Pathol. 1998; 30:271-7.

D'Alessandro A. Biology of *Trypanosoma* (Herpetosoma) *rangeli* Tejera, 1920. p. 327-404. In: LumsdenWH & Evans DA (eds.). Biology of the kinetoplastida. Academic Press, London 1976.

D'Alessandro-Bacigalupo A, Gore-Saraiva N. *Trypanosoma rangeli*. In: JP Kreier, Parasitic Protozoa VW, Academic Press, San Diego, New York, Boston, London, Sydney, Tokyo, Toronto, 1992 p.1-54.

D'Alessandro-Bacigalupo A, Gore-Saraiva N. *Trypanosoma rangeli*. In: Gilles HM, Protozoal Diseases, Oxford University Press, Oxford, 1999 p. 398-412.

Danon A, Assouline G. Inhibition of prostaglandin biosynthesis by corticosteroids requires RNA and protein synthesis. Nat. 1978; 273:552-4.

Dasch GA, Weiss E, Chang, K. Endosymbionts of insects. In: Bergey´s Manual of Systematic Bacteriology, NR Krieg, 1984, p. 811-833.

De Araújo-Jorge TC, de Souza W. Interaction of Trypanosoma cruzi with macrophages: effect of previous incubation of the parasites or the host cells with lectins. Z Parasitenkd. 1986; 72:153-71.

De Bosscher K, Berghe WV, Haegeman G. The Interplay between the Glucocorticoid Receptor and Nuclear Factor-kB or Activator Protein-1: Molecular Mechanisms for Gene Repression. Endocr Rev. 2003; 24(4):488-522. Review.

De Greve H, Wyns L, Bouckaert J. Combining sites of bacterial fimbriae. Curr Opin Struct Biol. 2008; 17:506-12.

De Maio J, Pampuni CB, Ken M, Beier JC. The midgut bacterial flora of wild *Aedes triseriatus*, *Culex pipiens*, and *Psorophora columbiae* mosquitoes. J Trop Med Hyg. 1996; 54:219-23.

Dennis EA. The growing phospholipase A_2 superfamily of signal transduction enzymes. Trends Biochem Sci. 1997; 22:1-2.

Derewenda ZS, Ho YS.PAF-acetylhydrolases. Biochim Biophys Acta., 1441, 229-236. Review.

Déziel E, Comeau Y, Villemur R. Initiation of biofilm formation by *Pseudomonas aeruginosa* 57RP correlates with emergence of hyperpiliated and highly adherent phenotypic variants deficient in swimming, swarming, and twitching motilities. J Bacteriol. 2001; 183:1195-204.

Dias E. Estudos sobre o *Schizotrypanum cruzi*. Mem Inst Oswaldo Cruz 1934; 8:1-115.

Díaz-Ruiz C, Montaner B, Pérez-Tomás R. Prodigiosin induces cell death and morphological changes indicative of apoptosis in gastric cancer cell line HGT-1. Histol Histopathol. 2001; 16:415-21.

Diggle SP, Crusz A, Cámara M. Quorum sensing. Curr Biol. 2007; 17:907-10.

Dillon RJ, Dillon VM. The gut bacteria of insects: nonpathogenic interactions. Ann Rev Entomol. 2004; 49:71-92.

Dillon RJ, El Kordy EL, Lanee RP. The prevalence of a microbiota in the digestive tract of *Phlebotomus papatasi*. Ann Trop Med Parasitol. 1996; 90:669-73.

Dillon RJ, Vennard CT, Buckling A, Charnley AK. Diversity of locust gut bacteria protects against pathogen invasion. Ecol Lett. 2005; 8:1291-8.

Dillon RJ, Webster G, Weightman AJ, Dillon VM, Blanford S, Charnley AK.Composition of Acridid gut bacterial communities as revealed by 16S rRNA gene analysis. J Invertebr Pathol. 2008; 97(3):265-72.

Donlan R. Biofilms: microbial life on surfaces. Emerg Infect Dis. 2002; 8:881-90.

Dotson EM, Plikaytis B, Shinnick TM, Durvasula RV, Beard CB. Transformation of *Rhodococcus rhodnii*, a symbiont of the Chagas disease vector *Rhodnius prolixus*, with integrative elements of the L1 mycobacteriophage. Infect Genet Evol. 2003; 3:103-9.

Duguid JP. Fimbriae and adhesive properties in *Klebsiella* strains. J Gen Microbiol. 1959; 21:271-86.

Dunn PE 1986. Biochemical aspects of insect immunology. Annu Rev Entomol. 1999; 31:321-339.

Durmus Y, Büyükgüzel E, Terzi B, Tunaz H, Stanley D, Büyükgüzel K. Eicosanoids mediate melanotic nodulation reactions to viral infection in larvae of the parasitic wasp, *Pimpla turionellae*. J Insect Physiol. 2008; 54:17-24.

Durvasula RV, Gumbs A, Panackal A, Kruglov O, Aksoy S, Merrifield RB, Richards FF, Beard CB. Prevention of insect-borne disease: an approach using transgenic symbiotic bacteria. Proc Natl Acad Sci USA. 1997; 94:3274-8.

Durvasula RV, Sundaram RK, Kirsch P, Hurwitz I, Crawford CV, Dotson E, Beard CB. Genetic transformation of a Corynebacterial symbiont from the Chagas disease vector Triatoma infestans. Exp Parasitol. 2008; 119:94-8.

Engström P, Carlsson A, Engström A, Tao ZJ, Bennich H. The antibacterial effect of attacins from the silk moth *Hyalophora cecropia* is directed against the outer membrane of Escherichia coli. EMBO J. 1984; 3:3347-3351.

Faria Neto HC, Stafforini DM, Prescott SM, Zimmerman GA. Regulation inflammation through the anti-inflamatory enzyme platelet-activating factor-acetylhydrolase. Mem Inst Oswaldo Cruz. 2005; 100(Suppl 1): 83-91.

Feder D, Mello CB, Garcia ES, Azambuja P. Immune Responses in *Rhodnius prolixus*: Influence of Nutrition and Ecdysone. J Insect Physiol. 1997; 43:513-519.

Ferrandon D, Imler JL, Hetru C, Hoffmann JA. The Drosophila systemic immune response: sensing and signalling during bacterial and fungal infections. Nat Rev Immunol. 2007; 7(11):862-74. Review

Figueiredo AR, Nunes ZG, Silva AAL, Giordano-Dias CMG, Coura JR, Hofer E. Isolation of microorganisms of triatomines maintained in artificial and sylvatic

conditions. Mem Inst Oswaldo Cruz. 1995; 90:228.
Figueiredo AR. Isolamento da microbiota bacteriana de triatomíneos e persistência do *Enterobacter cloacae* em *Rhodnius prolixus* Stal, 1859. MSc Thesis. Instituto Oswaldo Cruz. 1995, 72 pp.
Figueiredo MB, Castro DP, Nogueira NFS, Garcia ES, Azambuja P. Cellular immune response in *Rhodnius prolixus*: role of ecdysone in hemocyte phagocytosis. J Insect Physiol. 2006; 52:711-716.
Figueiredo MB, Garcia ES, Azambuja P. Blockades of phospholipase A(2) and platelet-activating factor receptors reduce the hemocyte phagocytosis in *Rhodnius prolixus*: In vitro experiments. J Insect Physiol. 2008a; 54:344-350.
Figueiredo MB, Genta FA, Garcia ES, Azambuja P. Lipid mediators and vector infection: *Trypanosoma rangeli* inhibits *Rhodnius prolixus* hemocyte phagocytosis by modulation of phospholipase A_2 and PAF-acetylhydrolase activities. J Insect Physiol doi: 10.1016/j.jinsphys.2008.08.013 2008b.
Fineran PC, Williamson NR, Lilley KS, Salmond GP. Virulence and prodigiosin antibiotic biosynthesis in *Serratia* are regulated pleiotropically by the GGDEF/EAL domain protein, PigX. J Bacteriol. 2007; 189:7653-62.
Finlay BB, Falkow S. Comon themes in microbial pathogenicity revisited. Microbiol Mol Biol Rev. 1997; 61:136-69.
Fouda MA, Hassan MI, Al-Daly AG, Hammad KM. Effect of midgut bacteria of Culex pipiens L. on digestion and reproduction. J Egypt Soc Parasitol. 2001; 31(3):767-80.
Francischetti IM, Andersen JF, Ribeiro JM.Biochemical and functional characterization of recombinant Rhodnius prolixus platelet aggregation inhibitor 1 as a novel lipocalin with high affinity for adenosine diphosphate and other adenine nucleotides. Biochem. 2002; 41:3810-8.
Freiburghaus F, Kaminsky R, Nkunya MHH, Brun R. Evaluation of african medicinal plants for their in vitro trypanocidal activity. J Ethnopharmacol. 1996; 55:1-11.
Garcia ES, Azambuja P, Contreras VT. Large-scale rearing of *Rhodnius prolixus* and preparation of metacyclic trypomastigotes of *Trypanosoma cruzi*. In: Morel CM. Genes and Antigens of Parasites, A Laboratory Manual, 2nd Ed. Fundação Oswaldo Cruz, World Health Organization, Rio de Janeiro 1984 pp. 43-46.
Garcia ES, Azambuja P. Development and interactions of *Trypanosoma cruzi* within the insect vector. Parasitol Today. 1991; 7:240-244.
Garcia ES, Azambuja P. Lignoids in insects: chemical probes for the study of ecdysis, excretion and *Trypanosoma cruzi*-triatomine interactions. Toxicon. 2004; 44:431-40. Review.
Garcia ES, Macarini JD, Garcia ML, Ubatuba FB. Feeding of *Rhodnius prolixus* in the laboratory. An Acad Bras Cienc. 1975; 47:537-545.
Garcia ES, Machado EMM, Azambuja P. Effects of eicosanoid biosynthesis inhibitors on the prophenoloxidase-activating system and microaggregate formation in *Rhodnius prolixus* infected with *Trypanosoma rangeli*. J Insect Physiol. 2004a; 50:157-165.
Garcia ES, Machado EMM, Azambuja P. Inhibition of hemocyte microaggregation reactions in *Rhodnius prolixus* larvae orally infected with *Trypanossoma rangeli*. Exp Parasitol. 2004b; 107:31-38.
Garcia ES, Rembold H. Effects of azadirachtin on ecdysis of *Rhodnius prolixus*. J Insect Physiol. 1984; 30:939-941.
Garcia ES, Subrahmanyam B, Müller T, Rembold H. Absortion, storage, organ distribution and excretion of dietary [22,23-3H2] dihidroazadirachtin a in the blood-feeding bug, *Rhodnius prolixus*. J Insect Physiol. 1989; 35:743-749.

Garcia ES, Uhl M, Rembold H. Azadirachtin, a chemical probe for the study of moulting in *Rhodnius prolixus*. Z Naturf. 1986; 41:771-775.

Garcia ES, Vieira E, Gomes JEPL, Gonçalves AM. Molecular biology of the interaction *Trypanosoma cruzi* invertebrate host. Mem Inst Oswaldo Cruz. 1984; 29:33-7.

Garred P, Strøm JJ, Quist L, Taaning E, Madsen HO. Association of mannose-binding lectin polymorphisms with sepsis and fatal outcome, in patients with systemic inflammatory response syndrome. J Infect Dis. 2003; 188:1394-403.

Genta FA, Dillon RJ, Terra WR, Ferreira C. Potential role for gut microbiota in cell wall digestion and glucoside detoxification in *Tenebrio molitor* larvae. J Insect Physiol. 2006; 52:593-601.

Gerlach GF, Clegg S, Ness NJ, Swenson DL, Allen BL, Nichols WA. Expression of type 1 fimbriae and mannose-sensitive hemagglutin by recombinant plasmids. Infect Immun. 1989; 57:764-770.

Gillespie JP, Kanost MR. Biological Mediators of Insect Immunity. Annu Rev Entomol. 1997; 42:611-643.

Giovannoni G, Land JM, Keir G, Thompson EJ, Heales SJR. Adaptation of the nitrate reductase and Griess reaction methods for the measurement of serum nitrate plus nitrate levels. Ann Clin Biochem. 1997; 34:193-198.

Girardeau JP, Bertin Y. Pilins of fimbrial adhesins of different member species of Enterobacteriaceae are structurally similar to the C-terminal half of adhesin proteins. FEBS Lett. 1995; 357:103-8.

Gomes SAO, Feder D, Garcia ES, Azambuja P. Supression of the prophenoloxidase system in *Rhodnius prolixus* orally infected with *Trypanossoma rangeli*. J Insect Physiol. 2003; 49: 829-837.

Gomes SAO, Feder D, Thomas NE, Garcia ES, Azambuja P. *Rhodnius prolixus* infected with *Trypanosoma rangeli*: in vivo and in vitro experiments. J Invertebr Pathol. 1999; 73:289-293.

Gonzalez MR, Bischofberger M, Pernot L, van der Goot FG, Frêche B. Bacterial pore-forming toxins: the (w)hole story? Cell Mol Life Sci. 2008; 65:493-507.

Gonzalez-Ceron L, Santillan F, Rodrigue MH, Mendez D, Hernández-Avila JE. Bacteria in midguts of field-collected *Anopheles albimanus* block *Plasmodium vivax* sporogonic development. J Med Entomol. 2003; 40:371-374.

Goodchild APJ. The bacteria associated with *Triatoma infestans* and some other species of Reduviidae. Parasitol. 1955; 45:441.

Götz P & Boman HG. Insect immunity. In: GA Kerkut, LI Gilbert, Comprehesive Insect Physiology, Biochemistry and Pharmacology. Pergamon, New York, 1985 p.453-485.

Granger DL, Taintor RR, Boockvar KS, Hibbs JBJr. Measurement of nitrate and nitrite in biological samples using nitrate reductase and Griess reaction. Meth Enzimol. 1996; 268:142-151.

Grimont PAD, Grimont F. The genus Serratia. Annu Rev Microbiol. 1978; 32:221-248. Review

Guhl F, Vallejo GA. *Trypanosoma* (Herpetosoma) *rangeli* Tejera, 1920 – an update review. Mem Inst. Oswaldo Cruz. 2003; 98(4):435-442.

Gupta C, Katsumata M, Goldman AS, Herold R, Piddington R.Glucocorticoid-induced phospholipase A2-inhibitory proteins mediate glucocorticoid teratogenicity in vitro. Proc Natl Acad Sci USA. 1984; 81:1140-3.

Han SJ, Choi JH, Ko HM, Yang HW, Choi IW, Lee HK, Lee OH, Im SY. Glucocorticoids prevent NF-kappaB activation by inhibiting the early release of

platelet-activating factor in response to lipopolysaccharide. Eur J Immunol. 1999; 29(4):1334-41.

Han SJ, Ko HM, Choi JH, Seo KH, Lee HS, Choi EK, Choi IW, Lee HK, Im SY. Molecular mechanisms for lipopolysaccharide-induced biphasic activation of nuclear factor-kB (NF-kB). J Biol Chem 2002; 277:44715–21.

Harrington JS. Studies on *Rhodnius prolixus*: growth and development of normal and sterile bugs, and the symbiotic relationship. Parasitol. 1960; 50:279-286.

Hejazi A, Falkiner FR. *Serratia marcescens.* J Med Microbiol. 1997; 46:903-912.

Herthe R, Hilger M, Weingardt-Kocher S, Walev I. Cytotoxic action of *Serratia marcescens* hemolysin on human epithelial cells. Infect Immunol. 1999; 67:817-825.

Hertle R. The family of *Serratia* type pore forming toxins. Curr Protein Pept Sci. 2005; 6:313-25. Review.

Hill P, Campbell JA, Petrie IA. *Rhodnius prolixus* and its symbiotic actinomycete: a microbiological, physiological and behavioural study. Proc R Soc London B Biol Sci. 1976; 194:501-525.

Hines DA, Saurugger, PN, Ihler GM, Benedik MJ. Genetic analysis of extracellular proteins of *Serratia marcescens*. J Bacteriol. 1988; 170:4141-4146.

Hirata F, Schiffmann E, Venkatasubramanian K, Salomon D, Axelrod J. A phospholipase A2 inhibitory protein in rabbit neutrophils induced by glucocorticoids. Proc Natl Acad Sci USA. 1980; 77:2533-6.

Hoffmann JA, Hoffmann D. The inducible antibacterial peptides of dipteran insects. Res Immunol. 1990; 141:910-918.

Hoffmann JA. The immune response of *Drosophila*. Nat. 2003; 426:33-38.

Hornick DB, Allen BL, Horn MA, Clegg S. Fimbrial types among respiratory isolates belonging to the family Enterobacteriaceae. J Clin Microbiol. 1991; 29:1795-800.

Horst AK, Kötter S, Lindhorst TK, Ludwig A, Brandt E, Wagener C. Binding inhibition of type 1 fimbriae to human granulocytes: a flow cytometric inhibition assay using trivalent cluster mannosides. Med Microbiol Immunol. 2001; 190:145-149.

Hultmark D, Engstron A, Andersson K, Steiner H, Bennich H, Boman HG. Insect imunity. Attacins, a family of antibacterial proteins from *Hyalophora cecropia*. EMBO J. 1983; 2:571-576.

Hwang JK, Shim JS, Chung JY. Anticariogenic activity of some tropical medicinal plants against *Streptococcus mutans*. Fitoterapia. 2004; 75(6):596-8.

Iacovache I; van der Goot FG, Pernot L. Pore formation: an ancient yet complex form of attack. Biochim Biophys Acta. 2008; 1778(7-8):1611-23. Review.

Ichinose M, Hara N, Sawada M, Maeno T.A flow cytometric assay reveals an enhancement of phagocytosis by platelet activating factor in murine peritoneal macrophages. Cell Immunol. 1994; 156:508-518.

Im SY, Han SJ, Ko HM, Choi JH, Chun SB, Lee DG, Ha TY, Lee HK. Involvement of nuclear factor-kappa B in platelet-activating factor-mediated tumor necrosis factor-alpha expression. Eur J Immunol. 1997; 27(11):2800-4.

Isaka M, Jaturapat A, Kramyu J, Tanticharoen M, Thebtaranonth Y. Potent in vitro antimalarial activity of metacycloprodigiosin isolated from *Streptomyces spectabilis* BCC 4785. Antimicrob Agents Chemother. 2002; 46:1112-3.

Ishii S, Shimizu T.Platelet-activating factor (PAF) receptor and genetically engineered PAF receptor mutant mice. Prog Lipid Res. 2000; 39:41-82. Review.

Jacobo-Herrera NJ, Bremner P, Márquez , Gupta MP, Gibbons S, Muñoz E, Heinrich M. Physalins from Witheringia solanaceae as modulators of the NF-kB cascade. J Nat Prod. 2006; 69: 328-331.

Jansen AM, Lockatell V, Johnson DE, Mobley HLT. Mannose-resistant proteus-like fimbriae are produced by most Proteus mirabilis strains infecting the urinary tract, dictate the in vivo localization of bacteria, and contribute to biofilm formation. Infect Immun. 2004; 72:7294-7305.

Januario AH, Filho ER, Pietro RC, Kashima S, Sato DN, Franca SC. Antimycobacterial physalins from *Physalis angulata* L. (Solanaceae). Phytother Res. 2002; 16(5):445-8.

Jarrett CO, Derek E, Isherwood KE, Oyston PC, Fischer ER, Whitney AR, Kobayashi SD, DeLeo FR, Hinnebusch BJ. Transmission of Yersinia pestis from an infectious biofilm in the flea vector. J Infect Dis. 2004; 190:783-792.

Jiang H, Kanost MR. The clip-domain family of serine proteinases in arthropods. Insect Biochem Mol Biol. 2000; 30:95-105.

Jiang H, Wang Y, Ma C, Kanost MR. Subunit composition of pro-phenoloxidase from *Manduca sexta*: molecular cloning of subunit proPO-P1. Insect Biochem Mol Biol. 1997; 27:835-850.

Jousimies-Somier H, Summanen P. Recent taxonomic changes and terminology update of clinically significant anaerobic gram-negative bacteria (excluding spirochetes). Clin Infect Dis. 2002; 35:17-21.

Kahn S, Wleklinski M, Aruffo A, Farr A, Coder D, Kahn M. *Trypanosoma cruzi* amastigote adhesion to macrophages is facilitated by the mannose receptor. J Exp Med. 1995; 182:1243-58.

Kahn SJ, Wleklinski M, Ezekowitz AB, Coder D, Aruffo A, Farr A. The major surface glycoproteins of *Trypanosoma cruzi* amastigotes are ligands of the human serum mannose-binding protein. Infect Immun. 1996; 64:2649-2656.

Kitsiouli EI, Nakos G, Lekka LE. Differential determination of phospholipase A2 and PAF-acetylhydrolase in biological fluids using fluorescent substrates. J Lipid Res. 1999; 40:2346-2356.

Ko HM, Seo KH, Han SJ, Ahn KY, Choi IH, Koh GY, Lee HK, Ra MS, Im SY. NF-kB-dependency of platelet-activating factor-induced angiogenesis. Cancer Res 2002; 62:1809–14.

Kollien AH, Schaub GA. The development of *Trypanosoma cruzi* in Triatominae. Parasitol Today. 2000; 16:381-387.

Kopp E, Ghosh S. NF-kB and Rel proteins in innate immunity.Adv Immunol 1995; 58:1–27.

Kordan W, Strzezek J, Fraser L. Functions of platelet activating factor (PAF) in mammalian reproductive processes: a review. Poland J Vet Sci. 2003; 6:55-60.

Korhonen TK, Lounatmaa K, Ranta H, Kuusi N. Characterization of type 1 pili of *Salmonella typhimurium* LT2. J Bacteriol. 1980; 144:800-5.

Kurucz E, Vilmos P. Insect immunity: evolutionary roots of the mammalian innate immune system. Immunol Lett. 1998; 62:59-66. Review

Kurz CL, Chauvet S, Andres E, Aurouze M, Vallet I, Michel GP, Uh M, Celli J, Filloux A, De Bentzmann S, Steinmetz I, Hoffmann JA, Finlay BB, Gorvel JP, Ferradon D, Ewbank JJ. Virulence factors of the human opportunistic pathogen *Serratia marcescens* identified by in vivo screening. EMBO J. 2003; 22:1451-1460.

Labbate M, Queck SY, Koh KS, Rice SA, Givskov M, Kjelleberg S. Quorum sensing-controlled biofilm development in *Serratia liquefaciens* MG1. J Bacteriol. 2004; 186:692-698.

Labbate M, Shu H, Thung L, Bandara R, Larsen MR, Willcox MDP, Givskov M, Rice SA, Kjelleberg S. Quorum-sensing regulation of adhesion in *Serratia marcescens* MG1 is surface dependent. J Bacteriol. 2007; 189:2702-2711.

Lackie AM. Immune mechanisms in insects. Parasitol Today. 1988; 4:98-105.

Lake P, Friend WG. The use of artificial diets to determine some of the effects of Nocardia rhodnii on the development of Rhodnius prolixus. J Insect Physiol. 1968; 14:543-62.

Lambeau G, Gelb MH. Biochemistry and Physiology of Mammalian Secreted Phospholipases A(2). Annu Rev Biochem. 2008; 77:495-520. Review

Lavine MD, Strand MR. Insect hemocytes and their role in immunity. Insect Biochem Mol Biol. 2002; 32:1295-1309.

Lederkremer RM, Bertello LE. Glycoinositolphospholipids, free and as anchors of proteins, in *Trypanosoma cruzi*. Curr Pharm Dês. 2001; 7:1165-79.

Lee YK, Soh BS, Wu JH. Quantitative assessment of phagocytic activity of hemocytes in the prawn, *Penaeus merguiensis*, by flow cytometric analysis. Cytometry 2001; 43:82-85.

Lemaitre B, Hoffmann J. The host defense of *Drosophila melanogaster*. Ann Rev Immunol. 2007; 25:697-743.

Lin YS, Chiang HC, Kan WS, Hone E, Shih SJ, Won MH. Immunomodulatory activity of various fractions derived from *Physalis angulata* L. extract. Am J Chin Med. 1992; 20:233-243.

Llagostera E, Soto-Cerrato V, Montaner B, Pérez-Tomás R. Prodigiosin induces apoptosis by acting on mitochondria in human lung cancer cells. Ann N Y Acad Sci. 2003; 1010:178-81.

Lopes JP, Oliveira SM, Soares Fortunato J. Oxidative stress and its effects on insulin resistance and pancreatic beta-cells dysfunction: relationship with type 2 diabetes mellitus complications. Acta Med Port. 2008; 21(3):293-302.

Luckhart S, Vodovotz Y, Cui L, Rosenberg R. The mosquito *Anopheles stephensi* limits malaria parasite development with inducible synthesis of nitric oxide. Proc Natl Acad Sci USA. 1998; 95:5700-5.

Machado EM, Azambuja P, Garcia ES. Web 2086, a platelet - activating factor antagonist, inhibits prophenoloxidase – activating system and hemocyte microaggregation reactions induced by *Trypanosoma rangeli* infection in *Rhodnius prolixus* hemolymph. J Insect Physiol. 2006; 52:685-692.

Machado PE, Eger-Mangrich I, Rosa G, Koerich LB, Grisard EC, Steindel M. Differential susceptibility of triatomine of the genus *Rhodnius* to *Trypanossoma rangeli* strains from different geographical origins. Int J Parasitol. 2001; 31:631-633.

Mah TF, O´Toole GA. Mechanism of biofilm resistance to antimicrobial agents. Trends Microbiol. 2001; 9:34-39.

Mallo GV, Kurz CL, Couillault C, Pujol N, Granjeaud S, Kohara Y, Ewbank JJ. Inducible antibacterial defence system in C. elegans. Curr. Biol. 2002; 12:1209-1214.

Mandato CA, Diehl-Jones WL, Moore SJ, Downer RGH. The effects of eicosanoid biosynthesis inhibitors on prophenol activation, phagocytosis and cell spreading in *Galleria mellonella*. J Insect Physiol. 1997; 43:1-8.

Mareggiani G, Picollo M. I., Zerba E, Burton G, Tettamanzi MC, Benedetti-Doctorovich MOV, Veleiro AS. Antifeedant Activity of Withanolides from *Salpichroa origanifolia* on *Musca domestica*. J Nat Prod. 2000; 63:1113-1116.

Marty KB, Williams CL, Guynn LJ, Benedik MJ, Blanke SR. Characterization of a cytotoxic factor in culture filtrates of *Serratia marcescens*. Infect Immun. 2002; 70:1121-1128.

Matz C, Bergfeld T, Rice SA, Kjelleberg S. Micro-colonies, quorum sensing and cytotoxiy determine the survival of *Pseudomonas aeruginosa* biofilms exposed to protozoan grazing. Environ Microbiol. 2004; 6:218-226.

Melamed MR, Lindmo T, Mendelsohn MF. Flow cytometry and sorting. 2ª ed. New York: Wiley-Liss, 1990 p.397-414.

Mello CB, Garcia ES, Ratcliffe NA, Azambuja P. *Trypanosoma cruzi* and *Tryapnosoma rangeli*: interplay with hemolymph components of *Rhodnius prolixus*. J Invertebr Pathol. 1995; 65:621-268.

Mello CB, Azambuja P, Garcia ES, Ratcliffe NA. Differential in Vitro and in Vivo Behavior of Three Strains of *Trypanosoma cruzi* in the Gut and Hemolymph of *Rhodnius* prolixus. Experim Parasitol. 1996; 82:112-121.

Melo PS, Duran N, Haun M. Cytotoxicity of prodigiosin and benzinidazole on V79 cells. Toxicol Lett. 2000; 116:237-42.

Mendonça-Previato L, Todeschini AR, Heise N, Previato JO. Protozoan parasite-specific carbohydrate structures. Curr Op Struct Biol. 2005; 15:499-505.

Mercado TI, Butany JW, Ferrans VJ. *Trypanosoma cruzi*: ultrastructural changes produced by an anti-tripanosomal factor from *Pseudomonas fluorescens*. Experim Parasitol. 1986; 61:65-75.

Mercado TI, Colón-Whitt A. Lysis of *Trypanosoma cruzi* by *Pseudomonas fluorescens*. Antimicrob Agents Chemother. 1982; 22:1051-1057.

Merchant D, Ertl RL, Rennard SI, Stanley DW, Miller JS. Eicosanoids mediate insect hemocyte migration. J Insect Physiol. 2008; 54:215-221.

Miller JS, Nguyen T, Stanley-Samuelson DW. Eicosanoids mediate insect nodulation response to bacterial infection. Proc Nat Acad Sc. 1994; 91:12418-22.

Miller JS, Stanley DW. Eicosanoids nediate microaggregation reactions to bacterial challenge in isolated hemocyte preparations. J Insect Physiol. 2001; 47:1409-17.

Miller JS, Stanley DW. Lipopolysaccharide evokes microaggregation reactions in hemocytes isolated from tobacco hornworms, *Manduca sexta*. Comparat Bioch Physiol Part A. 2004; 137:285-295.

Miller JS. Eicosanoids influence in vitro elongation of plasmatocytes from the tobacco hornworm, *Manduca sexta*. Arch Insect Biochem Physiol. 2005; 59:42-51.

Mizunoe Y, Matsumoto T, Haraoka M, Sakumoto M, Kubo S, Kumazawa J. Effect of pili of *Serratia marcescens* on superoxide production and phagocytosis of human polymorphonuclear leukocytes. J Urol. 1995; 154:12227-1230.

Mizunoe Y, Nakabeppu Y, Sekiguchi M, Kawabata S, Moriya T, Amako K. Cloning and sequence of the gene encoding the major structural component of mannose-resistant fimbriae of *Serratia marcescens*. J Bacteriol. 1988; 170:3567-74.

Moncayo A, Ortiz Yanine MI. An update on Chagas Disease (human American trypanosomiasis). Ann Trop Med Parasitol. 2006; 100:663-677.

Montaner B, Navarro S, Pique M, Vilaseca M, Martinell M, Giralt E, Gil J, Pérez-Tómas R. Prodigiosin from the supernatant of *Serratia marcescens* induces apoptosis in haematopoietic cancer cell lines. Br J Pharmacol. 2000; 131:585-93.

Montaner B, Pérez-Tomás. Prodigiosin-induced apoptosis in human colon cancer cells. Life Sci. 2001; 68:2025-36.

Moraes CS, Seabra SH, Castro DP, Brazil RP, de Souza W, Garcia ES, Azambuja P. *Leishmania* (Leishmania) *chagasi* interactions with *Serratia marcescens*: ultrastructural studies, lysis and carbohydrate effects. Exp Parasitol. 2008; 118:561-8.

Moraes CS, Seabra SH, Castro DP, Brazil RP, de Souza W, Garcia ES, Azambuja P. Interação de *Leishmania* (Viannia) *braziliensis* com *Serratia marcescens*: estudo

ultraestrutural, efeito de carboidratos e prodigiosina sobre a atividade lítica bacteriana. Manuscrito em preparação.
Moreira CG, Carneiro SM, Nataro JP, Trabulsi LR, Elias WP. Role of type I fimbriae in the aggregative adhesion pattern of enteroaggregative *Escherichia coli*. FEMS Microbiol Lett. 2003; 226:79-85.
Moreira-Ferro CK, Daffre S, James AA, Marinott O. A lyzozyme in the salivary glands of the malaria vector *Anopheles darlingi*. Insect Biochem Mol Biol. 1998; 7(3):257-264.
Mordue AJ, Blackwell A. Azadirachtin: an update. J Insect Physiol 1993; 39:903-924.
Morishima I, Yamano Y, Inoue K, Matsuo N. Eicosanoids mediate induction of immune genes in the fat body of the silkworm, *Bombyx mori*. FEBS Lett. 1997; 419:83-86.
Morita A, Isawa H, Orito Y, Iwanaga S, Chinzei1 Y, Yuda M. Identification and characterization of a collagen-induced platelet aggregation inhibitor, triplatin, from salivary glands of the assassin bug, *Triatoma infestans* FEBS J. 2006; 273:2955–2962
Moshage H, Jansen PL. Adaptation of the nitrate and Griess reaction methods for the measurement of serum nitrate plus nitrate levels. Ann Clin Biochem. 1998; 35:154-5.
Mukherjee AB, Miele L, Pattabiraman N. Phospholipase A2 enzymes: regulation and physiological role. Biochem Pharmacol. 1994; 48:1-10.
Mulla MS, Su T. Activity and biological effects of neem products against arthropods of medical and veterinary importance.J Am Mosq Control Assoc. 1999;15:133-52. Review.
Nagafuji S, Okabe H, Akahane H, Abe F. Tripanocidal Constituents in Plants 4. 1) Withanolides from the aerial parts of *Physalis angulata*. Biol Pharm Bull. 2004; 27:193-197.
Nakano T, Ohara O, Teraoka H, Arita H. Glucocorticoids suppress group II phospholipase A2 production by blocking mRNA synthesis and post-transcriptional expression. J Biol Chem. 1990; 265:12745-8.
Nappi AJ, Vass E, Frey F, Carton Y.Nitric oxide involvement in *Drosophila* immunity. Nitric Oxide. 2000; 4:423-30.
Nappi AJ, Vass E, Frey F, Carton Y. Superoxide anion generation in *Drosophila* during melanotic encapsulation of parasites. Eur J Cell Biol. 1995; 68:450-456.
Nappi AJ, Vass E. Hydrogen peroxide production in immune – reactive *Drosophila melanogaster*. J Parasitol. 1998; 84(6):1150-1157.
Nappi AJ, Vass E. Melanogenesis and the generation of cytotoxic molecules during insect cellular immune reactions. Pigment Cell Res. 1993; 6:117-126.
Nappi AJ. The role of melanisation in the immune reaction of larvae of *Drosophila alganquin* against *Pseudeucala bochei*. Parasitol. 1973; 66:23-32.
Nehme NT, Liégeois S, Kele B, Giammarinaro P, Pradel E, Hoffmann JA, Ewbank JJ, Ferradon D. A model of bacterial intestinal infectious in *Drosophila melanogaster*. PLOS Pathog. 2007; 3:1694-1709.
Nok AJ, Shuaibu MN, Kanbara H. Purification and partial characterization of α-mannosidase from *Trypanosoma rangeli*. Parasitol Res. 2000; 86:923-928.
Nyirady SA. The germfree culture of three species of Triatominae: *Triatoma protracta* (Uhler), *Triatoma rubida* (Uhler) and *Rhodnius prolixus* Stal. J Med Entomol. 1973; 10:417-448.
Ofek I, Sharon N. Adhesins as lectins: specificity and role in infection. Curr Top Microbiol Immunol. 1990; 151:91-113. Review

Okada M, Satori S. Primary structure of sarcotoxin I, an antibacterial protein induced in the hemolymph of *Sarcophaga peregrine* (flesh-fly) larvae. J Biol Chem. 1985; 25:7174-7177.

Old DC. Inhibition of the interaction between fimbrial haemagglutinins and erythrocytes by d-mannose and other carbohydrates. J Gen Microbiol. 1972; 71:149-157.

Old DC, Adegbola RA, Scott SS. Multiple fimbrial haemagglutinins in *Serratia* Species. Med. Microbiol. Immunol. 1983; 172:107-15.

Oliveira SMP, Morais B, Gonçalves CA, Giordano Dias CM, Vilela ML, Brazil RP, D'Almeida JM, Asensi MD, Mello RP. Prevalência da microbiota no trato digestivo de fêmeas de *Lutzomya longipalpus* (Lutz and Neiva, 1912) (Diptera:Psychodidae) provenientes do campo. Revista da Sociedade Brasileira de Medicina Tropical. 2000; 33:319-322.

Oliveira SMP, Morais B, Gonçalves CA, Giordano Dias KM, Vilela ML, Brazil RP, D'Almeida JM, Asensi MD, Mello RP. Microbiota do trato digestivo de fêmeas de *Lutzomya longipalpis* (Diptera: Psychodidae) provenientes de colônia alimentada com sangue e com sangue e sacarose. Cadernos de Saúde Pública. 2001; 17:229-232.

Pan YT, Xu B, Rice K, Smith S, Jackson R, Elbein AD. Specificity of the high-mannose recognition site between *Enterobacter cloacae* pili adhesin and HT-29 cell membranes. Infect Immun. 1997; 65:4199-206.

Park Y, Kim Y, Putnam SM, Stanley DW. The bacterium *Xenorhabdus nematophilus* depresses nodulation reactions to infection by inhibiting eicosanoid biosynthesis in tobacco hormworms, *Manduca sexta.* Arch Insect Biochem Physiol. 2003; 52:71-80.

Park Y, Kim Y. Eicosanoids rescue *Spodoptera exigua* infected with *Xenorhabdus nematophilus*, the symbiotic bacteria to the entomopathogenic nematode *Steinernema carpocapsae*. J Insect Physiol. 2000; 46:1469-1476.

Park Y, Nor Aliza AR, Stanley DW. A secretory PLA_2 associated with tobacco hornworm hemocyte membrane preparations acts in cellular immune reactions. Arch Insect Biochem Physiol. 2005; 60:105-115.

Phelps PK, Miller JS, Stanley DW. Proataglandins, not lipoxygenase products, mediate insect microaggregation reactions to bacterial challenge in isolated hemocytes preparations. Comparative Bioch Physiol PartA. 2003; 136:409-416.

Phillips I, King A. Serratia marcescens in hospital practice. Lancet. 1977; 1:538.

Pidiyar VJ, Jangid K, Patole MS, Shouche YS. Studies on cultured and uncultured microbiota of wild *Culex quinquefasciatus* mosquito midgut based on 16s ribosomal RNA gene analysis. Am J Trop Med Hyg. 2004; 70:597-603.

Pizarro-Cerdá J, Cossart P. Bacterial adhesion and entry into host cells. Cell. 2006; 124:715-727.

Poole K, Braun V. Influence of growth temperature and lipopolysaccharide on hemolytic activity of *Serratia marcescens*. J Bacteriol. 1988; 170:5146-5152.

Poole K, Schiebel E, Braun V. Molecular characterization of the hemolysin determinant of Serratia marcescens. J Bacteriol. 1988; 170:3177-3188.

Powning RF, Davidson WJ. Studies on insect bacteriolytic enzymes – II Some physical and enzymatic properties of lysozyme from haemolymph of *Galleria mellonella*. Comp Biochem Physiol B. 1976; 55:221-228.

Prescott SM, Zimmerman GA, Stafforini DM, McIntyre TM. Platelet-activating and related lipid mediators. Annu Rev Biochem. 2000; 69:419-445.

Pumpuni CB, DeMaio J, Kent M, Davis JR, Beier JC. Bacterial population dynamics in three anopheline species: impact on *Plasmodium* sporogonic development. Am J

Trop Med Hyg. 1996; 54:214-218.
Queck SY, Weitere M, Moreno AM, Rice SA, Kjelleberg S. The role of quorum sensing mediated developmental traits in the resistance of *Serratia marcescens* biofilms against protozoan grazing. Environ Microbiol. 2006; 8:1017-1025.
Ratcliffe NA, Gagen SJ. Cellular defence reactions of insect hemocytes in vivo: nodule formation and development in *Galleria mellonella* and *Peris brassicae* larvae. J Invertebr Pathol. 1976; 28:373-382.
Ratcliffe NA, Gagen SJ. Studies on the "in vivo" cellular reactions of the insects: an ultrastrutural analysis of nodule formation in *Galleria mellonella*. Tissue Cell. 1977; 9:73-85.
Ratcliffe NA, Rowley AF. Insect responses to parasite and other pathogens. Immune responses in parasitic infections: immunology, immunopathology and immunoprofilaxis. Ed. Soulsby, EJL, vol 4, CRC Press, Boca Raton, Flórida, 1987 pp 232-271.
Ratcliffe NA, Rowley AF. Role of hemocytes in defence against biological agents. In: Gupta, A.P. (Ed.), Insect Hemocytes Development, Forms, Functions and Techniques. Cambridge University Press, Cambridge, UK, 1979 pp.331-414.
Ratcliffe NA, Walters JB. Studies on the in vivo cellular reactions of insects: Clearance of pathogenic and non-pathogenic bacteria in *Galleria mellonella* larvae. J Insect Physiol. 1983; 29:407-415.
Ratcliffe NA. Cellular defense reactions of insects. Fortsch Zool. 1982; 27:223-244.
Reid G, Sobel JD. Bacterial adherence in the pathogenesis of urinary tract infection: a review. Rev Infect Dis. 1987; 9:470-487.
Ribeiro JMC. Role of saliva in blood-feeding by arthropods. Annu Rev Entomol. 1987; 32:463-478.
Ribeiro JMC, Francischetti M. Platelet-activating-factor-hydrolyzing phospholipase C in the salivary glands and saliva of the mosquito *Culex quinquefasciatus*. J Exp Biol. 2001; 204:3887-3894.
Rice AS, Koh KS, Queck SY, Labbate M, Lam KW, Kjelleberg S. Biofilm formation and sloughing in *Serratia marcescens* are controlled by quorum sensing and nutrient cues. J Bacteriol. 2005; 187:3477-3485.
Rohloff LH, Wiesner A, Gotz P. A fluorescence assay demonstrating stimulation of phagocytosis by haemolymph molecules of *Galleria mellonella*. J Insect Physiol. 1994; 40(12):1045-1049.
Rumelt S, Metzger Z, Kariv N, Rosenberg M. Clearance *of Serratia marcescens* from blood in mice: role of hydrophobic versus mannose-sensitive interactions. Infect Immun. 1988; 56:1167-70.
Salit IE, Gotschlich EC. Hemagglutination by purified type I *Escherichia coli* pili. J Exp Méd. 1977; 146:1169-81.
Salminen A, Kauppinen A, Suuronen T, Kaarniranta K. SIRT1 longevity factor suppresses NF-kappaB -driven immune responses: regulation of aging via NF-kappaB acetylation? Bioessays. 2008; 30(10):939-42.
Salt G. Cellular defense mechanisms in insects. Defense reactions in insects. Ed. Cambridge University Press, 1967 1671-1674.
Santos JAA, Tomassini TCB, Xavier DCDX, Ribeiro IM, Silva MTG, Morais Filho ZB. Molluscidal activity of *Physalis angulata* L. extracts and fractions on *Biomphalaria tenagophila* (d´Orbigny, 1835) under laboratory conditions. Mem. Inst. Oswaldo Cruz. 2003; 98(3):425-428.

Schumutterer H. Properties and potential of natural pesticides from the neem tree. Annu Rev Entomol. 1990; 35:271-297.

Schlein Y, Polacheck I, Yuval B. Mycoses, bacterial infections and antibacterial activity in sandflies (Psychodidae) and their possible role in the transmission of leishmaniasis. Parasitol. 1985; 90:57-66.

Schleusener DR, Stanley-Samuelson DW. Phospholipase A_2 in hemocytes of the tobacco hornworm, *Manduca sexta*. Arch Insect Biochem Physiol. 1996; 33:63-74.

Schofield CJ, Jannin J, Salvatella R. The future of Chagas Disease control. Trends Parasitol. 2006; 22:583-588.

Seabra SH, DaMatta RA, de Mello FG, de Souza W. Endogenous polyamine levels in macrophages is sufficient to support growth of *Toxoplasma gondii* .J Parasitol. 2004; 90:455-460.

Shanks RMQ, Stella NA, Kalivoda EJ, Doe MR, O´Dee DM, Lathrop KL, Guo FL, Nau GJ. A *Serratia marcescens* OxyR homolog mediates surface attachment and biofilm formation. J Bacteriol. 2007; 189:7262-7272.

Sharon N. Carbohydrates as future anti-adhesion drugs for infectious diseases. Biochim Biophys Acta. 2006; 1760:527 – 37.

Sheffield PJ, Garrad S, Caspi M, Aoki T, Arai H, Derewenda U, Suter B, Reiner O, Derewenda ZS. Homologs of the H and I subunits of mammalian brain platelet-activating factor acetylhydrolase in the *Drsophila melanogaster* genome. Proteins. 2000; 39:1-8.

Showell HJ, Williams TJ. The neutrophil in inflammation. In: T.J. Rogers and S.C. Gilman, Editors, *Imunopharmacology*, The Telford Press, New York, 1989 pp. 23–39.

Shrestha S, Kim Y. An entomopathogenic bacterium, *Xenorhabdus nematophila*, inhibits hemocyte phagocytosis of *Spodoptera exigua* by inhibiting phospholipase A2. J Invertebr Pathol. 2007a; 96:64-70.

Shrestha S, Kim Y. Eicosanoids mediate prophenoloxidase release from oenocytoids in the beet armyworm *Spodoptera exigua*. Insect Biochem Mol Biol. 2008; 38:99-112.

Shrestha S, Kim Y. Factors affecting the activation of hemolymph prophenoloxidase of *Spodoptera exigua* (Lepidoptera:Noctuidae). J Asia-Pac Entomol. 2007b; 10:131-5.

Silveira AC, Feitosa VR, Borges R. Distribuição de triatomíneos capturados no ambiente peridomiciliar, no período de 1975/83, Brasil. Ver Brás Malariol Doenças Trop. 1984; 36:15-31.

Snyder F, Lee TC, Blank M, Malone B, Woodard D, Robinson M. Platelet-activating factor: alternate pathways of biosynthesis, mechanism of inactivation, and reacylation of lyso-PAF with arachidonate. Adv Prostaglandin Thromboxane Leukot Res. 1985; 15:693-696.

Soares MB, Brustolim D, Santos LA, Bellintani MC, Paiva FP, Ribeiro YM, Tomassini TC, Ribeiro Dos Santos R. Physalins B, F and G, seco-steroids purified from *Physalis angulata* L., inhibit lymphocyte function and allogeneic transplant rejection. Int Immunopharmacol. 2006; 6:408-414.

Soares MBP, Bellintani MC, Ribeiro IM, Tomassini TCB, Santos RR. Inhbition of macrophage activation and lipopolysaccaride – induced death by seco-steroids purified from *Physalis angulata* L. Europ J Pharmacol. 2003; 459:107-112.

Soderhall K, Cerenius L, Johansso MW. Role of the Prophenoloxidase-Activating System in Invertebrate Immunity. In: New Directions in Invertebrate Immunology, SOS Publications 1996.

Söderhäll K, Smith VJ. Profenoloxidase-activating cascade as a recognition and defence system in arthropods. In: Hemocytic and Humoral Immunity in Arthropods (Ed Gupta, A.P.), John Wiley and Sons, New York, 1986 pp.251-286.

Söderhäll K. Invertebrate immunity. Development Comparat Immunol. 1999; 23:263-266 (special issue).

Söderhäll K. Prophenoloxidase activating system and melanization- A recognition mechanim of arthropods? A review. Dev Comp Immunol. 1982; 6:601-611.

Soto GE, Hultgren SJ. Bacterial Adhesins: Common Themes and Variations in Architecture and Assembly. J Bacteriol. 1999; 181:1059-1071.

Stafforini DM, McIntyre TM, Zimmerman GA, Prescott SM. Platelet-activating factor, a pleiotrophic mediator of physiological and pathological processes. Crit Review Clin Lab Sci. 2003; 40:643-672.

Stanley DW, Miller JS. Eicosanoid actions in insect cellular immune functions. Entomologia Experimentalis et Applicata 2006; 119:1-13.

Stanley DW, Nor Aliza AR, Tunaz H, Putnam SM, Park Y, Bedick JC 2002. Eicosanoids in Insect Biology. Forum. Neotrop Entomol. 2002; 31:341-350.

Stanley DW, Shapiro M. Eicosanoid biosynthesis inhibitors increase the susceptibility of *Lymantria dispar* to nucleopolyhedrovirus LdMNPV. J Invertebr Pathol. 2007; 95:119-24.

Stanley DW. Eicosanoids in invertebrate signal transduction systems. Princeton, Princeton Univ Press, 2000 277p.

Stanley DW. Eicosanoids. In: Gilbert, L.I., Iatrou, K., Gill, S. (Eds.), Comprehensive Molecular Insect Science, vol. 4. Elsevier, Oxford, UK, 2005 pp. 307–339.

Stanley DW. Prostaglandins and other eicosanoids in insects: biological significance. Annu Rev Entomol. 2006b; 51:25-44.

Stanley DW. The non-venom insect phospholipases A_2. Biochim Biophys Acta. 2006a; 1761:1383-90.

Stanley-Samuelson DW, Pedibhotla VK, Rana RL, Nor Aliza AR, Hoback WW, Miller JS. Eicosanoids mediate nodulation responses to bacterial infections in larvae of the silkmoth, *Bombyx mori*. Comp Biochem Physiol. 1997; 118A:93-100.

Steinhaus EA. 1960. The importance of environmental factors in the insect-microbe ecosystem. Bacteriol. Rev. 24: 365– 73.

Stoodley P, Sauer K, Davies DG, Costerton JW. Biofilms as a complex differentiated communities. Annu rev Microbiol. 2002; 56:187-209.

Straif SC, Mbogo CNM, Toure AM, Walker ED, Kaufman M, Toure YT, Beier JC. Midgut bacteria in *Anopheles gambiae* and *An. funestus* (Diptera: Culicidae) from Kenya and Mali. J Med Entomol. 1998; 35:222-226.

Stuart LM, Ezekowitz RA. Phagocytosis and comparative innate immunity: learning on the fly. Nat Rev Immunol. 2008; 8(2):131-41. Review.

Sugiura T, Fukuda T, Miyamoto T, Waku K. Distribution of alkyl and alkenyl ether-linked phospholipids and platelet-activating factor-like in various species of invertebrates. Biochem Biophy Acta. 1992; 1126:298-308.

Sugumaran M. Roles of the insect cuticle in immunity. In: Soderhall, K., Iwanaga, S., Vasta, G.R. (Eds.), New Directions in Invertebrate Immunology. SOS Publications, Fair Haven, NJ, pp. 1996 355-374.

Takle GB. Studies on the cellular immune responses of insects toward the insect pathogen Trypanosoma rangeli. J Invertebr Pathol. 1988;51(1):64-72.

Terra WR. Evolution of digestive systems of insects. Ann Rev Entomol. 1990; 35:181-200.

Tobie EJ. Observations on the development of *Trypanosoma rangeli* in the hemocel of *Rhodnius prolixus*. J Invertebr Pathol. 1970; 15:118-125.

Tomassini TCB, Barbi NS, Ribeiro IM, Xavier DCD. Gênero *Physalis* – uma revisão sobre vitaesteróides. Química Nova. 2000; 23(1):47-57.

Tomassini TCB, Silva MTG, Sanches EG, Ribeiro IM. Boll Chim Farm. 1997; 136:154.

Tunaz H, Park Y, Buyukguzel K, Bedick JC, Nor Aliza AR, Stanley DW. Eicosanoids in insect immunity: bacterial infection stimulates hemocytic phospholipase A_2 activity in tobacco hornworms. Arch Insect Biochem Physiol. 2003; 52:1-6.

Tzou P, De Gregorio E, Lemaitre B. How Drosophila combats microbial infection: a model to study innate immunity and host-pathogen interactions. Curr Opin Microbiol. 2002; 5(1):102-10.

Uscian JM, Stanley-Samuelson DW. Phospholipase A_2 activity in the fat body of the tobacco hornworm *Manduca sexta*. Arch Insect Biochem Physiol. 1993; 24:187-201.

Vallejo GA. Estudios comparativos entre las secuencias de KADN em *Tripanosoma cruzi* y *Tripanosoma rangeli* y su aplicación en le diagnosis de la tripanosomiasis americana. Rev Actual Biol. 1998; 20:43-56.

Van HR, Givskov M, Michiels CW. Quorum sensing in *Serratia*. FEMS Microbiol Rev. 2007; 31:407-424.

Vandenberghe I, Créancier L, Vispé S, Annereau J-P, Barret J-M, Pouny I, Samson A, Aussagues Y, Massiot G, Ausseil F, Bailly C, Kruczynski A. Physalin B, a novel inhibitor of the ubiquitin-proteasome pathway, triggers NOXA-associated apoptosis. Bioch Pharmacol. 2008; 76: 453–462.

Verdon R, Mirelman D, Sansonetti PJ. A model of the interaction between *Entomoeba histolytica* and *Shigella flexneri*. Res Microbiol. 1992; 143:67-74.

Verheij HM, Slotboom AJ, de Haas GḤ.Structure and function of phospholipase A2. Rev Physiol Biochem Pharmacol. 1981; 91:91-203. Review.

Vetsch M, Puorger C, Spirig T, Grauschopf U, Weber-Ban EU, Glockshuber R. Pilus chaperones represent a new type of protein-folding catalyst. Nat. 2004; 431:329-333.

Vieira AT, Pinho V, Lepsch LB, Scavone C, Ribeiro IM, Tomassini T, Ribeiro-dos-Santos R, Soares MB, Teixeira MM, Souza DG. Mechanisms of the anti-inflammatory effects of the natural secosteroids physalins in a model of intestinal ischaemia and reperfusion injury. Brazilian J Pharmacol. 2005; 146:244-251.

Villalta F, Kierszenbaum F. Role of cell surface mannose residues in host cell invasion by Trypanosoma cruzi. Biochim Biophys Acta. 1983; 736:39-44.

Vizioli J, Richman AM, Uttenweiler S, Blass C, Bulet P. The defensin peptide of the malaria vector mosquito *Anopheles gambiae*: antimicrobial activities and expression in adult mosquitoes. Insect Biochem Mol Biol. 2001; 31:241-248.

Volf P, Svobodová M, Dvoráková E. Bloodmeal digestion and *Leishmania major* infections in *Phlebotomus duboscqi*: effect of carbohydrates inhibiting midgut lectin activity. Med Vet Entomol. 2001; 15:281-6.

Vray B, Camby I, Vercruysse V, Mijatovic T, Bovin NV, Ricciardi-Castagnoli P, Kaltner H, Salmon I, Gabius HJ, Kiss R. Up-regulation of galectin-3 and its ligands by Trypanosoma cruzi infection with modulation of adhesion and migration of murine dendritic cells. Glycobiol. 2004; 14:647-57.

Walker FA. Nitric oxide interaction with insect nitrophorins and thoughts on the electron configuration of the {FeNO}6 complex. J Inorg Biochem. 2005; 99:216-236.

Walker G, Hertle R, Braun V. Activation of *Serratia marcescens* hemolysin through a

conformational change. Infect Immun. 2004; 72:611-614.
Walters JB, Ratcliffe NA. Studies on the “in vitro” cellular reactions of insects: fate of pathogenic and non-pathogenic bacteria in *Galleria mellonella* nodule. J Insect Physiol. 1983; 29:417-424.
Watkins R. Histology of *Rhodnius prolixus* infected with *Trypanosoma rangeli*. J Invert Pathol. 1971; 17:59-66.
Weitere M, Bergfeld T, Rice SA, Matz C, Kjelleberg S. Grazing resistance of *Pseudomonas aeruginosa* biofilms depends on type of protective mechanism, developmental stage and protozoan feeding mode. Environ Microbiol. 2005; 7:1593-1601.
Whitten M, Sun F, Tew I, Schaub G, Soukou C, Nappi A, Ratcliffe N. Differential modulation of *Rhodnius prolixus* nitric oxide activities following challenge with *Trypanosoma rangeli*, *T. cruzi* and bacterial cell wall components. Insect Biochem Mol Biol. 2007; 37:440-452.
Whitten MM, Melo CB, Gomes SAO, Nigam Y, Azambuja P, Garcia ES, Ratcliffe NA. Role of superoxide and reactive nitrogen intermediates in *Rhodnius prolixus* (Reduviidae)/ *Trypanossoma rangeli* interactions. Exp Parasitol. 2001; 98:44-57.
Whitten MM, Ratcliffe NA. In vitro superoxide activity in the haemolymph of the west indian leaf cockroach, *Blaberus discoidalis*. J Insect Physiol. 1999; 45(7):667-675.
Wigglesworth VB. The principles of insects physiology. Ed. Chapman & Hall, 7th ed., London 1972.
Wigglesworth VB. Digestion in the tsetse-fly: A study of structure and function. Parasitol. 1929; 21:288-321.
Williams P. Quorum sensing, communication and cross-kingdom signalling in the bacterial world. Microbiol. 2007; 153:3923-3938.
Williams RP. Biosynthesis of prodigiosin, a secondary metabolite of *Serratia marcescens*. Applied Microbiol. 1973; 25:396-402.
Wu HJ, Wang AHJ, Jennings MP. Discovery of virulence factors of pathogenic bacteria. Curr Opin Chem Biol. 2008; 12:93-101.
Yajima M, Takada M, Takahashi N, Kikuchi H, Natori S, Oshima Y, Kurata S. A newly established in vitro culture using transgenic Drosophila reveals functional coupling between the phospholipase A2-generated fatty acid cascade and lipopolysaccharide-dependent activation of the immune deficiency (imd) pathway in insect immunity. Biochem J. 2003; 371(Pt 1):205-10.
Yu XQ, Kanost MR. Immulectin-2 a pattern recognition receptor that stimulates hemocyte encapsulation and melanization in the tobacco hornworm *Manduca sexta*. Dev Comp Immunol. 2004; 28:891-900.
Yuda M, Hirai M, Miura K, Matsumura H, Ando K, Chinzei Y. cDNA cloning, expression and characterization of nitric oxide synthase from the salivary glands of the blood-sucking insects *Rhodnius prolixus*. Eur J Biochem. 1996; 242:807-812.