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**Imunidade inata e imunopatogenia nas
leishmanioses experimentais**

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LISTA DE ABREVIATURAS

CR-1	Receptor de macrófago 1 para fração C3b do complemento
CR-3	Receptor de macrófago 3 para fração iC3b do complemento
EGF	Fator de crescimento da epiderme
GM-CSF	Fator de estimulação de colônia de monócitos e granulócitos
gp63	Metaloprotease, glicoproteína de massa molecular 63
H ₂ O ₂	Peróxido de hidrogênio
IGF	‘Insulin-like growth factor’ = fator de crescimento insulina-símile
IFN- γ	Interferon gama
IL	Interleucina
LPG	Lipofosfoglicano
LV	Leishmaniose visceral
NK	“Natural killer cells” = célula citotóxica natural
NO	Óxido nítrico
iNOS / NOS ₂	Síntase induzível de NO
TGF- β	Fator de transformação de crescimento beta
TNF- α	Fator de necrose de tumor alfa
IP-10	Proteína 10 induzível por interferon

Resumo

A tese é apresentada como uma compilação crítica dos trabalhos oriundos de pesquisa desenvolvida nos últimos quinze anos, aproximadamente, e de outros em andamento dentro da área de imunidade inata e imunopatologia das leishmanioses, especialmente na leishmaniose visceral. Dados inéditos e de peso foram obtidos em diferentes projetos na área de elementos inespecíficos e estes permitem concluir que: a) o complemento in vivo, estudo que continua inédito, constitui-se num fator de evasão dos parasitos que determinam a doença visceral; b) a avaliação do papel de células NK é complexa pela sua implicação estreita com o complemento; c) polimorfonucleares neutrófilos in vivo aparenta não ter papel decisivo na determinação da evolução da infecção; d) fator de crescimento insulina-símile (IGF)-I é um fator promotor de crescimento para leishmânia e importante na leishmaniose, pioneiramente estudado por nós, e que necessita ser explorado na interação com o macrófago e com componentes da resposta imune específica. Quanto à imunossupressão na leishmaniose visceral em hamster, é específica ao antígeno de leishmânia e parece ser ela conseqüente a uma superativação prévia dos linfócitos e não por anergia ou apoptose de linfócitos. Além disso, macrófagos são protegidos de apoptose quando infectados por leishmânia, favorecendo o parasitismo, dado somente conhecido em experimentos in vitro antes da nossa observação. Na evolução, no entanto, as amastigotas entram em apoptose nas fases finais da infecção, mais uma vez uma observação inédita in vivo. Estudando aspectos patológicos e imunopatogênicos das lesões que ocorrem na leishmaniose visceral, contradizendo o conceito corrente de mecanismo por imunocomplexo, sugerimos mecanismos inéditos com a participação de imunoglobulinas, linfócitos T e moléculas de adesão.

Abstract

The thesis is a compilation of data from studies developed during last fifteen years approximately and others ongoing in the field of innate immunity and immunopathology in visceral leishmaniasis. Unique and front line data were obtained in different projects in the area of non specific elements that led us to following conclusions: a) complement in vivo is an important factor for evasion of the parasite of viscerotropic strain in a study that still maintains its originality; b) evaluation of the role of NK cells is complex due to the close connection to the complement system; c) polymorphonuclear neutrophils in vivo seemingly do not have decisive role in the evolution of visceral leishmaniasis; d) insulin-like growth factor (IGF)-I is a growth promoting factor for *Leishmania* and it has an important contribution in leishmaniasis, (pioneer studies developed by us), and deserves careful further studies on interaction with macrophages and with components of specific immune response. Immunosuppression was studied in visceral leishmaniasis in hamsters that was *Leishmania* antigen-specific, and apparently was due to overstimulation of lymphocytes rather than anergy or apoptosis. Further, macrophages were apparently protected from apoptosis by *Leishmania* infection favouring the parasite survival, data only known in in vitro experiments before our observation. During infection in hamsters, in the latter phases, amastigotes undergo apoptosis, again another original in vivo observation. Studying pathology and immunopathogenesis of lesions present in visceral leishmaniasis, contradicting the current view, we suggested an alternative mechanism with participation of immunoglobulins, T lymphocytes and adhesion molecules.

A. Introdução e revisão da literatura

A1. Introdução

As leishmanioses são causadas por protozoários do gênero *Leishmania* que se estabelecem em células do sistema fagocítico mononuclear, determinando formas clínicas tegumentar e visceral da doença. No Brasil, tanto a leishmaniose tegumentar quanto a visceral são endêmicas e em franca expansão. A prevalência elevada, dificuldades na intervenção no ciclo de transmissão e a repercussão sócio-econômica fazem das leishmanioses um grande problema de saúde pública no Brasil e no mundo.

Dentre as áreas de pesquisa, a de imunidade e imunopatologia são importantes na busca de medidas de intervenção terapêutica e preventiva, abarcando desde questões relacionadas à suscetibilidade ou resistência à infecção por *Leishmania* até mecanismos que determinam as alterações da resposta imune e lesão tecidual na evolução das leishmanioses. Temos desenvolvido nos últimos quinze anos várias linhas de pesquisa dentro de diversos campos da imunidade inata e imunopatologia das leishmanioses, especialmente na leishmaniose visceral. Embora o grande objetivo seja o entendimento dos mecanismos de infecção/doença no homem, realizamos estudos experimentais. Realizamos estudos in vitro, mas, vários projetos são direcionados para a utilização de modelos experimentais, entendendo que a compreensão do papel dos elementos no contexto da interação in vivo, embora complexa na abordagem, traz contribuições essenciais para entender os complexos mecanismos que estejam presentes no desenvolvimento da infecção ou doença no homem.

Embora a imunidade específica seja importante nas leishmanioses, entendendo que os eventos iniciais que ocorrem imediatamente após a transmissão de promastigotas pelo inseto vetor, ainda pouco elucidados, sejam cruciais no

estabelecimento da infecção, desenvolvemos estudos nas leishmanioses em relação a complemento, células “natural killer”, polimorfonucleares neutrófilos e fator de crescimento insulina-símile.

Embora na leishmaniose visceral, as pesquisas na imunidade específica se voltem principalmente para os mecanismos de ativação ou supressão da resposta imune celular e participação de citocinas, entendemos que uma outra abordagem dos mecanismos de suscetibilidade/imunossupressão na leishmaniose visceral seria de interesse, dentro da óptica de morte e sobrevivência de diferentes populações celulares. Desenvolvemos assim estudos de apoptose de diferentes populações celulares, a de linfócitos que teria um provável papel na imunossupressão, a de macrófagos que interferiria na evolução da infecção e a de amastigotas, no controle do parasitismo.

No campo da imunopatogenia propriamente dita, o mecanismo patogênico aceito na literatura é o de deposição de imunocomplexos, porém, diante de dados que indicavam a necessidade de estudos mais aprofundados, desenvolvemos estudos explorando a participação de linfócitos T e imunoglobulinas, estas por um mecanismo alternativo, nas lesões teciduais, principalmente no rim, na leishmaniose visceral.

A revisão da literatura abarca os tópicos referentes a essas diferentes áreas de pesquisa e os resultados são apresentados, de forma segmentada por áreas, acompanhados de artigos publicados e dados recentes em forma de manuscritos, alguns preliminares, para o acompanhamento da evolução das linhas de pesquisa.

A abrangência e a diversidade dos estudos apresentados nesta tese são em parte consequência das colaborações estabelecidas e das características dos pesquisadores e alunos envolvidos em cada linha de pesquisa.

A2. Leishmânia e leishmanioses

As leishmanioses são causadas por protozoários da ordem Kinetoplastida, família Trypanosomatidae, gênero *Leishmania*. A infecção se estabelece nas células do sistema fagocítico mononuclear e conhecem-se as formas clínicas tegumentar e visceral (Pearson, 1993). A leishmaniose tegumentar pode manifestar-se por lesão cutâneo-mucosa ou somente cutânea que pode ser única, múltipla ou disseminada, dependendo da espécie do parasito e da resposta imune do hospedeiro (Pearson, 1993). A leishmaniose visceral caracteriza-se por proliferação de parasitos principalmente no baço, fígado e medula óssea, mas podendo determinar lesões em praticamente todos os órgãos (Duarte, 2000) e pode manifestar-se sob diferentes formas, dependendo da resposta imune do hospedeiro: a) assintomática caracterizada por sorologia positiva para leishmânia sem nenhuma manifestação clínica, b) oligossintomática caracterizada por sorologia positiva e presença de sinais e/ou sintomas discretos como febre, hepatomegalia e/ou esplenomegalia de pequeno grau ou c) forma clássica ou doença plenamente manifesta caracterizada por hepatoesplenomegalia volumosa, febre, pancitopenia, hipergamaglobulinemia e com grande comprometimento do estado geral (Badaró et al., 1986).

As principais espécies de leishmânia causadoras de leishmanioses são classificadas em dois subgêneros: *Leishmania* e *Viannia*. O subgênero *Viannia* compreende quatro complexos bem definidos: *Leishmania brasiliensis*, *Leishmania guyanensis*, *Leishmania naiffi*, *Leishmania lainsoni*. O subgênero *Leishmania* compreende onze complexos: *Leishmania (Leishmania) hertigi*, *L. (L.) mexicana*, *L. (L.) amazonensis*, *L. (L.) enrietti*, *L. (L.) arabica*, *L. (L.) aethiopica*, *L. (L.) gerbilli*, *L.*

(*L.*) *major*, *L. (L.) tropica*, *L. (L.) donovani* e *L. (L.) infantum* (Thomaz-Soccol et al., 1993). Na classificação de Lainson e Shaw (1987) constam ainda as espécies *L.(V.) panamensis* e *L.(V.) peruviana* que pertenceriam ao subgênero *Viannia* e *L.(L.) chagasi*, *L.(L.) aristidesi* e *L.(L.) venezuelensis*, ao subgênero *Leishmania*. No Brasil, a leishmaniose tegumentar é causada, principalmente, pelas espécies *Leishmania (Viannia) braziliensis*, *Leishmania (Leishmania) amazonensis* e *Leishmania (Viannia) guyanensis* e a leishmaniose visceral, pela *Leishmania (Leishmania) chagasi* (Lainson e Shaw, 1987).

Durante o ciclo biológico, as leishmânias apresentam-se sob duas formas morfollogicamente distintas. No hospedeiro vertebrado, no interior de macrófagos, comportando-se como parasita intracelular obrigatório, encontram-se as formas amastigotas, arredondadas e sem flagelo. Estas formas transformam-se em formas promastigotas, no tubo digestivo do inseto vetor, alongada, com flagelo externo, que evoluem para forma metacíclica, que é altamente infectiva (Sacks, 1989, Walters, 1993). A transmissão do parasito ocorre durante o repasto sanguíneo do vetor infectado que inocula promastigotas no hospedeiro. Estas são fagocitadas por macrófagos dos órgãos do sistema fagocítico mononuclear, transformam-se em amastigotas e proliferam desencadeando o processo patológico no hospedeiro (Pearson, 1993).

Para a ocorrência da infecção ou doença é fundamental a presença do vetor na área geográfica de transmissão. Os vetores são flebotomíneos, sendo importantes para a transmissão das leishmânias, as espécies dos subgêneros *Phlebotomus* no Velho Mundo e *Lutzomyia* no Novo Mundo (Lewis e Ward, 1987).

Leishmanioses são originariamente zoonoses e vários reservatórios que estão envolvidos no seu ciclo são silvestres (Shaw e Lainson, 1987). Os hospedeiros vertebrados incluem uma grande variedade de mamíferos. As infecções mais comuns

ocorrem nos roedores e canídeos, sendo conhecidas também em edentados (tamanduá, bicho preguiça e tatu), marsupiais (gambá), procionídeos (mão pelada e quati) e primatas, inclusive o homem. No entanto, o avanço de novas fronteiras agrícolas e desmatamento para expansão da área urbana colocaram o homem nessa cadeia ecológica. Particularmente no ciclo da *Leishmania (L.) chagasi*, o cão doméstico passou a constituir-se no principal reservatório no peri e intradomicílio. A manutenção do ciclo de leishmânia depende de vários fatores relacionados às características epidemiológicas da área, do hospedeiro, do vetor e do parasito. A participação de animais silvestres e insetos no ciclo do parasito nas matas e florestas dificulta qualquer intervenção para o controle da transmissão. Mesmo no ciclo da *Leishmania (L.) chagasi* que em parte ocorre no peri-domicílio envolvendo cães, o controle não tem sido possível, em parte pela ocorrência de epidemia em áreas urbanas com densidade populacional alta, mas também pela resistência dos insetos aos inseticidas em uso atualmente.

Leishmanioses têm distribuição mundial, sendo mais prevalente nas regiões tropicais e subtropicais do globo terrestre. Estima-se que 350 milhões de pessoas em todo o mundo vivem em áreas de risco de transmissão da doença e atualmente 12 milhões de pessoas estão infectadas, com ocorrência de 400 mil casos novos ao ano. As leishmanioses são consideradas endêmicas em 88 países, 22 países no Novo Mundo e 66 no Velho Mundo, 16 países desenvolvidos e 72 em desenvolvimento e/ou subdesenvolvidos. Noventa por cento de todos os casos de leishmaniose visceral ocorrem em Bangladesh, Brasil, Índia, Nepal e Sudão e 90% dos casos de leishmaniose tegumentar ocorrem no Afeganistão, Arábia Saudita, Brasil, Bolívia, Irã, Peru e Síria (WHO, 1998). Em países desenvolvidos há também uma preocupação constante com esta doença visto que o turismo, operações de guerra e fluxo migratório

fazem com que ocorram casos da doença nesses países. Um outro fato preocupante é que, com o surgimento da síndrome da imunodeficiência adquirida, a leishmaniose tem ressurgido em regiões desenvolvidas como a Europa, comportando-se como doença oportunística, cujo tratamento tem-se mostrado difícil (Alvar et al., 1997).

No Brasil, a leishmaniose tegumentar ocorre em praticamente todo o território, estando em plena expansão. Ao longo dos anos, a leishmaniose tegumentar apresenta oscilações no número de casos, sendo que nos últimos 20 anos houve crescimento constante, descrevendo-se surtos nas regiões Nordeste, Norte, Centro-Oeste, Sudeste e Sul (Paraná), sendo notificado, em média, 28.000 casos por ano (FUNASA, 2003), com coeficientes de detecção (1985 a 2001) que oscilam entre 10,45 a 21,23 por 100 mil habitantes.

A leishmaniose visceral é endêmica no Brasil e ocorre em 17 estados da federação, atingindo quatro das cinco regiões brasileiras. Sua maior incidência encontra-se no Nordeste com 92% do total de casos, seguido pela região Sudeste (4%), região Norte (3%) e região Centro-Oeste (1%). Ocorre em zonas rurais, porém encontra-se também em centros urbanos, estando em franca expansão, como por exemplo em Teresina, São Luís, Fortaleza, Natal, Aracaju, Belo Horizonte, Santarém e Corumbá, além de sua introdução no estado de São Paulo, em regiões com características urbanas como Araçatuba e Marília. Anualmente são notificados cerca de 1.980 casos, com coeficiente de 20,4 casos por 100 mil habitantes em algumas localidades de estados nordestinos como Piauí, Maranhão e Bahia, com taxas de letalidade de 10 % (FUNASA, 2003).

Diante da prevalência elevada, expansão nas áreas tradicionalmente endêmicas, ressurgimento em áreas novas, dificuldade na intervenção no ciclo no ambiente onde ocorre a transmissão, resistência dos insetos a inseticidas e a repercussão sócio-

econômica fazem das leishmanioses um grande problema de saúde pública no mundo e também no Brasil. Desta forma é considerada pela Organização Mundial da Saúde entre as seis endemias prioritárias para investimento em pesquisa.

Dentre as áreas de pesquisa, a imunopatologia é uma área abrangente, abarcando desde questões relacionadas à suscetibilidade ou resistência à infecção por *Leishmania* até mecanismos que determinam as alterações da resposta imune e aqueles que determinam lesão tecidual na evolução das leishmanioses. Temos desenvolvido nos últimos quinze anos várias linhas de pesquisa nos diversos campos, dentro da imunopatologia das leishmanioses, especialmente na leishmaniose visceral. Realizamos estudos in vitro, mas, vários projetos são direcionados para a utilização de modelos experimentais, entendendo que a compreensão do papel dos elementos no contexto da interação in vivo, embora complexa na sua abordagem, traz contribuições essenciais para entender os intrincados mecanismos que estejam presentes no desenvolvimento da infecção ou doença no homem.

A3. Imunidade inata no estabelecimento da infecção e na evasão na leishmaniose

Embora a imunidade específica seja importante nas leishmanioses, os eventos iniciais, que ocorrem imediatamente após a transmissão de promastigotas pelo inseto vetor, ainda pouco elucidados, são cruciais no estabelecimento da infecção e envolvem a participação de complemento, de polimorfonucleares neutrófilos, células “natural killer” (NK), macrófagos e fatores de crescimento. São esses elementos, entre outros, que formam a primeira barreira à infecção, dificultando a sua instalação e que, ao mesmo tempo, durante o processo, constituem-se em elementos que auxiliam a evasão do parasito da resposta protetora do hospedeiro. Desenvolvemos estudos nas leishmanioses em relação a complemento, polimorfonucleares neutrófilos, células NK

e fator de crescimento insulina-símile, portanto, esses aspectos serão melhor detalhados a seguir.

A interação da leishmânia com o complemento e os fagócitos se dá pela ligação com várias moléculas de superfície. Duas moléculas se destacam pela abundância e pelos efeitos identificados na interação com vários elementos do hospedeiro, lipofosfoglicano (“lipophosphoglycan” = LPG) constituído de uma âncora de lípido fosfoinositol, núcleo glicano, região repetitiva sacarídeo-fosfato e um oligossacarídeo (Sacks, 1992, Turco e Descoteaux, 1992) e glicoproteína de massa molecular 63 (gp63) (Colomer-Gould et al., 1985) que é uma endopeptidase dependente de zinco (Bouvier et al., 1989).

3.1. Participação do complemento

Complemento é um dos primeiros fatores com o qual o protozoário interage ao ser inoculado na pele do hospedeiro vertebrado. Nas leishmanioses, o complemento tem três efeitos biológicos importantes: efeito leishmanicida pela fixação dos seus componentes terminais que levam à lise, opsonização pela fixação de C3b que favorece a ligação e evasão do parasito no macrófago e a liberação de fragmentos quimiotáticos C3a e C5a que promovem inflamação.

Efeito lítico do soro humano normal sobre promastigotas de *Leishmania* já era conhecido há muito tempo, mas, o sistema complemento foi implicado neste processo em 1968 quando se verificou a termolabilidade do fator sérico (Ulrich et al., 1968). A maioria das espécies de leishmânia, *Leishmania major*, *L. enrietti*, *L. mexicana*, *L. amazonensis* e *L. braziliensis*, ativa a via alternativa do complemento (Mosser e Edelson, 1984, Mosser et al., 1986). Por outro lado, *L. donovani* ativa a via clássica

(Mosser et al., 1986, Pearson e Steigbigel, 1980) e pensou-se inicialmente na participação de anticorpos naturais. No entanto, com a observação da ligação de promastigotas a proteína C reativa (Pritchard et al, 1985) e a proteína sérica de ligação a manana (Green et al., 1994^b) aventa-se uma outra possibilidade que é a da ativação pela terceira via do complemento ou a via das lectinas que consomem componentes da via clássica à semelhança do que ocorre na ativação mediada pelo anticorpo (Claus et al., 1977, Ikeda et al., 1987). Dados mais recentes vêm aumentar ainda mais esta controvérsia e contestam de uma maneira geral esses dados obtidos in vitro em condições não fisiológicas. Em experimentos que se supõem ser em condições mais próximas às fisiológicas, utilizando sangue total ou concentração alta de soro ($\geq 25\%$), retoma-se a importância de anticorpos naturais anti-leishmânia, presentes no soro de vertebrados, e enfatizando a importância da via clássica na lise de leishmânia, mesmo de espécies relatadas como ativadoras da via alternativa em outros estudos (Dominguez et al., 2003).

Independentemente da via utilizada, a clivagem do C3 e a deposição do fragmento C3b na superfície do parasito constituem-se na etapa crucial de ativação do complemento. As moléculas mais abundantes na superfície de leishmânia, LPG e gp63, são identificados como aceptores de C3 e mais estudados (Puentes et al., 1988, Russell, 1987), embora outras moléculas são ainda referidas como tendo interação com o sistema complemento (Nunes et al., 1997). Após a ativação e geração de C3b, a reação pode prosseguir por um dos dois caminhos: a formação de C5 convertase com subsequente formação de complexo de ataque de membrana C5b-C9 que leva à lise do parasito ou a inativação do C3b com geração do fragmento iC3b. Sabendo-se que a infectividade de promastigotas é diferente nas várias fases evolutivas no inseto, tornando-se infectiva com a metaciclogênese (Sacks, 1989) e com observação de que o

aparecimento de forma metacíclica ocorre na fase estacionária de crescimento in vitro de promastigotas, a suscetibilidade ao ataque pelo complemento foi analisada nas diferentes fases de crescimento in vitro. Observou-se que promastigotas de *L. donovani* e *L. panamensis* na fase estacionária são mais resistentes à lise pelo complemento que os parasitos em fase logarítmica de crescimento (Franke et al., 1985). Esta resistência à lise foi atribuída à modificação na molécula de LPG que se torna mais longa na fase metacíclica (Puentes et al., 1988), impedindo com isso a ligação do C5b-C9 à membrana do parasito (Puentes et al., 1990). Sabe-se por outro lado que gp63 também aumenta a sua expressão na forma metacíclica (Kweider et al., 1989) e seu papel na resistência ao ataque pelo complemento foi estudado. Utilizando *L. amazonensis* deficiente em gp63 transfectado com variante de gp63, foi possível determinar a correlação entre a expressão de gp63 e a resistência à lise pelo complemento, o mecanismo seria a transformação de C3b à sua forma inativa iC3b pela atividade enzimática de gp63 (Brittingham et al., 1995), o que impediria a formação de C5 convertase e do complexo de ataque de membrana. Um outro fator referido também é a quinase de proteína C, aumentada na forma metacíclica, e que fosforila vários componentes da cadeia do complemento, C3, C5 e C9 (Hermoso et al., 1991), levando à inibição tanto da via clássica quanto da alternativa.

A maioria dos estudos na literatura é com promastigotas, mas, num estudo, amastigotas de *L. donovani* foram consideradas não suscetíveis à lise pelo complemento (Pearson e Steigbigel, 1980). No entanto, posteriormente a lise pelo complemento foi comprovada, mas, comparativamente, *L. donovani* era pelo menos dez vezes menos suscetível que *L. major* (Hoover et al., 1984).

Uma outra consequência da ativação do complemento é a geração de fragmentos C3a e C5a, quimiotáticos, que promovem inflamação tecidual. As células

atraídas para o local da infecção, principalmente fagócitos polimorfonucleares neutrófilos e monócitos/macrófagos, fagocitam leishmânias com interação direta de ligantes do parasito, entre eles LPG e gp63, ou parasitos opsonizados por fragmentos do complemento e diversos receptores de macrófagos. Embora outros ligantes devam ser efetivos na promoção da fagocitose, os parasitos opsonizados parecem ter papel predominante neste processo; C3b liga-se ao receptor CR1 e iC3b a CR3 de macrófago (Mosser e Brittingham, 1997). Veremos na sequência também que a opsonização pelo complemento contribui no estabelecimento da infecção e/ou na evasão do parasito no macrófago.

Percebe-se nesta revisão que praticamente não existem estudos in vivo do papel do complemento. Um dos trabalhos apresentados nesta tese, vem suprir esta lacuna, com estudo do papel do complemento no modelo de leishmaniose visceral em hamsteres.

3.2. Papel de polimorfonucleares neutrófilos (PMN)

PMN são potencialmente uma das primeiras barreiras do hospedeiro vertebrado à invasão do parasito, mas, crescem dados que os apontam também como elemento de evasão.

PMN são atraídos ao local da infecção pelos peptídeos resultantes da ativação do complemento mas também pela liberação de fator quimiotático de granulócito por promastigota de *Leishmania* (van Zandbergen et al., 2002). Há estudos que mostram diferenças na dinâmica de migração tanto de neutrófilos quanto de macrófagos nas linhagens suscetíveis e resistentes a infecção por *L. major* e migração maior de

macrófagos imaturos em linhagens suscetíveis, sugerindo que estas diferenças estejam ligadas ao desenvolvimento da imunidade (Beil et al., 1992; Sunderkötter et al., 1993).

Estudos iniciais mostram PMN com poder leishmanicida de amastigotas e promastigotas de *L. donovani* pela geração de produtos reativos de oxigênio, principalmente peróxido de hidrogênio (Chang, 1981, Pearson e Steigbigel, 1981). Em leishmanioses cutânea e visceral em camundongo, utilizando anticorpos anti-granulócitos para depletar PMN, seu papel no controle da infecção na fase inicial foi observada (Lima et al, 1998, Rousseau et al, 2001), embora esse modelo de depleção de PMN leve à ativação maciça de complemento que tem interferência significativa na interação leishmânia-hospedeiro.

Existem outros dados, no entanto, que reforçam também seu papel na evasão, sendo sugerido, inicialmente, o PMN como alvo seguro (Mirkovich et al, 1986) e mais recentemente reforçado por outros dados. A protease gp63, fosfatase de *Leishmania* e fator excretado de promastigotas inibem a atividade oxidativa de PMN in vitro (El-On et al., 1990, Remaley et al., 1984, Sorensen et al., 1994). Em cão com leishmaniose visceral observou-se também menor atividade oxidativa em PMN do sangue periférico (Brandonisio et al., 1996). Esses efeitos provavelmente favorecem a sobrevivência do parasito em PMN, observado in vitro e in vivo (Laufs et al., 2002). Além disso, observou-se inibição ou retardo da apoptose de PMN quando infectado por *Leishmania major*, prolongando o tempo de manutenção de leishmânia no seu interior, possibilitando a sua transmissão para macrófagos que geralmente tem seu influxo posterior ao de PMN; PMN entrariam em apoptose retardada e estes albergando parasitos seriam fagocitados por macrófagos, completando e contribuindo na transmissão (Aga et al., 2002).

O influxo inicial de PMN foi relacionado também à expressão de IL-4 e portanto na indução da resposta TH2 na leishmaniose cutânea por *L. major* (Tacchini-Cottier et al., 2000) mas, por outro lado, num outro estudo, observou secreção de IFN- γ por neutrófilos que estimulariam os macrófagos para uma resposta anti-leishmânia efetiva (Venuprasad et al. 2002).

Repete-se também aqui a escassez de estudos in vivo do papel de PMN. De fato existem dois estudos realizados in vivo em leishmanioses visceral e cutânea (Lima et al, 1998, Rousseau et al, 2001), porém, como os experimentos foram realizados com o uso de anticorpos para depletar PMN, é difícil ignorar o fato deste procedimento levar a uma ativação maciça de complemento que, como vimos anteriormente, tem interferência marcante na interação leishmânia-hospedeiro, com possível alteração dos resultados. Diante desta ponderação, iniciamos um estudo apresentado nesta tese, ainda em andamento, que acreditamos venha a esclarecer o papel dos PMN na leishmaniose visceral. Para isso, lançamos mão de linhagens de camundongos que desenvolvem resposta inflamatória aguda de intensidades variadas, mínima ou máxima. Essas linhagens de camundongos foram selecionadas para reatividade inflamatória aguda (“acute inflammatory reactivity” = AIR) máxima (*AIR_{max}*) e mínima (*AIR_{min}*), pelo Laboratório de Imunogenética do Instituto Butantan, a partir de uma população F₀, geneticamente heterogênea, resultante do inter cruzamento de linhagens isogênicas (A, DBA/2, P, SWR, SJL, CBA, BALB/c e C57BL/6), e que prosseguiu com acasalamentos entre animais com fenótipos extremos semelhantes, de alta ou de menor resposta (Ibañez et al., 1992). Na reação no sítio inflamatório no subcutâneo após injeção de Biogel ocorre predomínio de PMN com presença de leucócitos monucleares constituídos por monócitos de diversos tamanhos e um número insignificante de linfócitos (Ibañez et al., 1992; Stiffel et al., 1987). Na geração F₁₉

analisada, a população de PMN permaneceu ao redor de 95% nos *AIRmax* e de 45% nos *AIRmin*, sendo que na linhagem *AIRmin* ocorreu menor quantidade de PMN no exsudado inflamatório (Araujo et al., 1996; Ibañez et al., 1992). Nessas linhagens extremas não foram observadas diferenças em relação à reação de hipersensibilidade tardia e potencialidade para produção de anticorpos com doses ótimas de antígeno (ovoalbumina, eritrócito de carneiro ou *Salmonella typhimurium*) (Araujo, 1996).

3.3. Papel do macrófago

Macrófago é o habitat principal do parasito no hospedeiro vertebrado, mas, sendo este possuidor de maquinaria voltada à atividade microbicida, a sobrevivência da leishmânia no seu interior depende de uma série de recursos para evadir-se desses mecanismos destrutivos. Esta célula atua tanto na fase inespecífica da resposta imune quanto da específica, como efetora da imunidade mediada por células e como célula apresentadora de antígeno, mas nesta parte atemos basicamente à sua participação como elemento inespecífico, embora vários desses mecanismos provavelmente atuem nos macrófagos no seu papel como efetores da resposta específica.

No contato inicial de promastigota com macrófago, ocorrem várias interações entre moléculas de superfície como LPG, gp63, outras moléculas como produtos de glicosilação avançada e moléculas opsonizadas, todas do parasito, com vários receptores de membrana do macrófago, como o de fibronectina, de manose, de b-glucana e os receptores de fragmentos do complemento, CR1 e CR3 (Mosser e Brittingham, 1997). Os receptores participantes na interação de amastigotas com macrófago são menos estudados, CR3 e receptores Fc são considerados importantes mas não os de manose e C3 (Guy e Belosevic, 1993, Peters et al., 1995).

No processo fagocítico, leishmânias localizam-se inicialmente em vacúolo parasitóforo circundado pela membrana da célula hospedeira que a seguir funde-se com lisossomo, que contém enzimas proteolíticas capazes de lisar parasitos, formando o fagolisossomo. Além disso, com a ativação, são gerados no macrófago elementos efetores contra leishmânias, produtos reativos de oxigênio (principalmente peróxido de hidrogênio = H_2O_2) e nitrogênio (óxido nítrico = NO), com a participação de NADPH oxidase e superóxido dismutase na geração de produtos reativos de oxigênio e sintase induzível de óxido nítrico (iNOS ou NOS2) na geração dos reativos de nitrogênio (Green et al., 1990, Haidaris e Bonventre, 1982, Liew et al., 1990, Murray, 1981, Stenger et al., 1994, Wei et al., 1995, Zarley et al., 1991). Analisando a participação desses dois mecanismos leishmanicidas, no entanto, os produtos reativos de oxigênio parecem ser importantes somente nas fases iniciais de infecção (Murray e Nathan, 1999). Os principais mecanismos de evasão da leishmânia agem sobre esses diferentes passos e mecanismos.

Várias moléculas de leishmânia atuam contribuindo para a evasão do parasito. LPG foi apontada como importante na sobrevivência do parasito no macrófago (Handman et al., 1986, McNeely e Turco, 1990). Neste papel, LPG inibe a fusão do fagossomo ao lisossomo, favorecendo possivelmente a transformação de promastigota em amastigota (Desjardins e Descoteaux, 1997), e ainda seqüestra radicais hidroxila e ânions superóxido (Chan et al., 1989). Outra molécula abundante na superfície do parasito, gp63, é também descrita pela sua ação enzimática (Chaudhuri et al., 1989) com papel protetor do parasito da citólise e da degradação no interior do fagolisossomo (Seay et al., 1996), ao lado do fator excretado (Eilan et al., 1985, Handman et al., 1986). Proteinases de cisteína de leishmânia também parecem contribuir na sobrevivência do parasito no macrófago (Mottram et al., 1996).

Na fase inicial de infecção, é importante para a sobrevivência do parasito que o macrófago não seja ativado eficientemente. Há fatores de crescimento como TGF- β e GM-CSF que contribuem suprimindo o macrófago ou auxiliando a sobrevivência do parasito, como veremos adiante. Além disso, observa-se inibição da produção de citocinas pró-inflamatórias como IL-1 e IL-12 (Carrera et al., 1996, Reiner, 1987, Reiner et al., 1994) e a expressão de cerca de 40% de genes conhecidos de macrófagos é diminuída na infecção por *L. donovani* (Buates e Matlashewski, 2001).

Diminuição da produção de produtos reativos de oxigênio foi observada pela infecção de macrófago por leishmânias (Buchmüller-Rouiller e Mauel, 1987, Passwell et al., 1994) e particularmente com a molécula gp63 (Sorensen et al., 1994). A opsonização pelos fragmentos C3b e iC3b também tem papel importante nesses mecanismos, observando-se diminuição significativa de atividade oxidativa quando a fagocitose ocorre por receptores CR1 e CR3, dos parasitos opsonizados por fragmentos do complemento (Blackwell, 1985, Wright e Silverstein, 1983). A fagocitose de amastigotas induz atividade oxidativa menor em macrófagos que promastigotas (Channon et al., 1984).

Vários estudos mostram que a produção de NO também é modulada pela infecção. Glicoinositolfosfolípide de leishmânia inibe a atividade de NOS com conseqüente redução da atividade leishmanicida (Proudfoot et al., 1995). Fosfatase de tirosina, SHP-1, no macrófago contribui na sobrevivência do parasito, atenuando o mecanismo microbicida dependente e independente de NO (Forget et al., 2001). Amastigotas de lesão de pele, exibem fosfatidil serina na sua superfície, mimetizando apoptose de célula, que resulta na diminuição da produção de NO pelo macrófago (Balanco et al., 2001). O papel de NO é considerado principalmente no contexto da imunidade específica, mas, é muito importante na resposta inespecífica, com a sua

atuação nas primeiras 24 horas de infecção, observada em camundongo deficiente na atividade de NOS2 (Bogdan et al., 2000, Diefenbach et al., 1998).

Nesta tese, além do estudo do papel do complemento na evasão do parasito no macrófago, estamos desenvolvendo projeto para avaliar o efeito de fator de crescimento insulina-símile (que explanamos abaixo) nos mecanismos microbicidas de macrófagos.

3.4. Participação de célula “natural killer” (NK)

Célula natural killer é um dos elementos importantes da resposta inespecífica de defesa do hospedeiro. No entanto, na leishmaniose, estas células são estudadas principalmente como fonte de IFN- γ nas fases iniciais da infecção e que direcionaria a resposta imune com expansão da subpopulação TH1, subpopulação esta ligada à resistência na leishmaniose cutânea experimental por *Leishmania major* (Scharton e Scott, 1993, Scott, 1991). São poucos estudos que analisam o seu papel como elemento inespecífico da resposta imune.

Inicialmente, observaram-se alterações na atividade citotóxica contra células de linfoma em camundongo durante a evolução da infecção por *Leishmania donovani* (Kirkpatrick e Farrell, 1982, 1984^a). Na seqüência, o mesmo grupo mostrou o papel de células NK na recuperação da leishmaniose visceral utilizando camundongos bege, sabidamente deficientes na atividade NK (Kirkpatrick et al, 1985). Com a depleção transitória de células NK, utilizando anticorpo monoclonal anti-asialo FM1 ou anti-NK1.1, observou-se suscetibilidade aumentada a *L. major* em camundongos (Laskay et al., 1993). Além disso, células NK teriam um papel importante na fase inicial da infecção, na contenção do parasito no local da infecção (Laskay et al, 1995) e as células NK migrariam para a pele e o linfonodo regional direcionadas pela produção

local de quimiocina, a proteína 10 induzível por interferon (interferon-inducible protein 10 = IP-10) (Müller et al., 2001).

Com as células NK parece também ocorrer modulação da sua atividade durante a infecção que poderia favorecer a evolução desta; na leishmaniose visceral em camundongo, após um período inicial (sétimo dia de infecção) de aumento da atividade NK, ocorre depressão da sua resposta do dia 15 aos 90 dias de infecção que se caracteriza por insensibilidade ao IFN- γ e supressão da atividade por uma via independente de IFN- γ (Kirkpatrick e Farrell, 1984^a e 1984^b). A migração de células NK e a sua atividade são afetadas também por efeito do parasito sobre outros elementos nesta fase: ocorre inibição da produção de IP-10 por PMN diminuindo o influxo de células NK (van Zandbergen et al., 2002) e há diminuição da produção de NO que tem efeito na atividade NK (Diefenbach et al., 1998).

Neste campo, observamos que os modelos utilizados apresentavam problemas de ativação do complemento pela utilização de anticorpos para depleção de células NK ou pelo uso de camundongos bege com menor atividade NK, mas, que apresentam também outras alterações na função dos fagócitos. No trabalho apresentado nesta tese utilizamos camundongos depletados em células NK com injeção de estrôncio radioativo (⁹⁰Sr) para estudar a sua participação na infecção por *L. (L.) amazonensis*. Este tratamento provoca uma irradiação local intensa na medula óssea levando a aplasia severa na medula, provocando uma mielopoiese extramedular no baço (Gidlund et al., 1990, Haller e Wigzell, 1977). Neste processo, ocorre depleção severa e permanente de atividade NK no baço, no linfonodo e na periferia sem nenhuma alteração perceptível nos compartimentos de células T e B ou na capacidade induzida de produzir IL-2 ou interferon alfa (Gidlund et al., 1990).

3.5. Participação de fatores de crescimento

Os parasitos na sua evolução desenvolveram capacidade para adaptar-se e ao mesmo tempo utilizar-se dos diversos fatores do hospedeiro para a sua sobrevivência. O crescimento in vitro de tripomastigotas de *Trypanosoma brucei* é estimulado pelo fator de crescimento da epiderme ("Epidermal growth Factor" = EGF) (Sternberg e McGuigan, 1994) e os parasitos *Leishmania donovani* e *Trypanosoma brucei* por si expressam fator de crescimento de fibroblasto-simile básico ("basic Fibroblast Growth Factor like" = bFGF-like) com possível papel no ciclo de vida do parasita (Kardami et al, 1992).

Na leishmânia e nas leishmanioses, o fator estimulador de colônias de granulócitos e monócitos ("Granulocyte-monocyte-colony stimulating factor" = GM-CSF) é estudado em diferentes contextos. Este fator, na sua interação, induz proliferação de promastigotas de *L.(L.) amazonensis* a 28° C e protege da morte induzida por choque térmico a 34/37° C (Charlab et al., 1990, Barcinski et al., 1992). In vitro, GM-CSF aumenta a morte de leishmânia no macrófago (Handman e Burgess, 1979, Ho et al., 1990, Weiser et al., 1987). In vivo, na leishmaniose visceral experimental, observa-se aumento de até dez vezes na expressão de mRNA de GM-CSF no fígado e com papel no controle da infecção com atividade leishmanicida similar a IFN- γ e dependente de célula T (Murray et al., 1995). No entanto, em modelo utilizando *L. major*, o tratamento com rGM-CSF levou ao parasitismo maior na lesão (Greil et al, 1988) e camundongo deficiente de receptor em GM-CSF mostrou-se resistente à infecção (Scott et al., 2000).

O fator de transformação de crescimento - β ("Transforming Growth Factor β " = TGF- β) é um outro fator que tem participação importante na infecção por leishmânia. O seu efeito inativador de macrófago favorece a replicação do parasita intracelular, observado em estudos *in vitro* e *in vivo* (Barral et al., 1993, Barral-Neto et al., 1992, Li et al., 1999, Nelson et al., 1991, Wilson et al., 1998). O seu efeito no mecanismo leishmanicida no macrófago é exercido sobre a produção de NO (Li et al., 1999, Stenger et al., 1994, Green et al., 1994^a). TGF- β tem também papel na imunidade celular pelo seu efeito na expressão da molécula CTLA-4 de linfócitos T (Gomes et al., 2000). Os dados na sua maioria implicam a sua participação na supressão de uma resposta celular específica como observado na leishmaniose visceral (Gant et al., 2003, Rodrigues et al., 1998), mas a sua expressão pode ser afetada na fase bem precoce da infecção na dependência da ativação de NOS2 (Diefenbach et al., 1998).

Um outro fator de crescimento que vem despertando grande interesse nos últimos anos, por sua ação diversificada e pleiotropismo, são os fatores de crescimento insulina-símile ("Insulin-like Growth Factor" = IGF). Estas características despertaram o nosso interesse no estudo do seu efeito sobre leishmânias e nas leishmanioses. Na literatura conhecem-se principalmente estudos na biologia celular, no efeito endócrino e na patogenia de lesões teciduais. Além dos nossos estudos em leishmaniose, em infecções, conhece-se um estudo de IGF-II sobre *Giardia lamblia* (Lujan et al., 1994).

Resumimos a seguir algumas características estruturais e funcionais de IGFs (veja revisões excelentes de Cohick e Clemmons 1993, Jones e Clemmons, 1995, Kooijman et al., 1996). IGFs são polipeptídeos, filogeneticamente bem preservados, com massa molecular de aproximadamente 7,5 kDa, sendo conhecidas duas formas principais, IGF-I e IGF-II, que mostram 60% de similaridade na seqüência de

aminoácidos. Estão presentes na circulação e na maioria dos tecidos, são sintetizados por maioria das células, mas principalmente no fígado. A concentração sérica em adultos normais é de 250 ng/ml para IGF-I e 600 ng/ml para IGF-II. No soro e nos tecidos a maior parte do IGF encontra-se ligada às proteínas conhecidas como proteínas ligantes de IGF (“IGF-binding proteins” = IGF-BPs). IGF-I e IGF-II interagem com células ligando-se a respectivos receptores de superfície. Nas células humanas, IGF-I liga-se ao receptor tipo 1, pertence à subclasse da família de receptores tirosina quinase (RTK) e que apresenta 80% de similaridade estrutural ao receptor de insulina. IGF-II interage com o receptor tipo 2 que apresenta similaridade ao receptor de manose-6-fosfato. O receptor de IGF-I medeia a maioria das ações biológicas de IGF-I e IGF-II e está presente numa ampla variedade de tecidos e linhagens de células, com exceção do fígado, tendo sido identificado em células NK, monócitos, células B, células T CD4⁺ e CD8⁺, células mononucleares e polimorfonucleares neutrófilos. Os efeitos de IGF-I e IGF-II têm ações diferentes na promoção do crescimento de acordo com o tipo celular. Os efeitos de IGF-I são melhor conhecidos. In vitro, IGF-I promove síntese de DNA/proliferação, diferenciação e efeitos metabólicos compreendendo a captação de glicose e amino ácidos e síntese de proteína. In vivo IGF-I medeia o efeito de promoção de crescimento do hormônio de crescimento, funcionando como fator de crescimento de feto, além de efeitos metabólicos similares a insulina e participação na regeneração e cicatrização de tecidos.

IGF-I atua sobre várias células do sistema imune, inato e adquirido, embora seu efeito não seja tão evidente de imediato (ver revisões excelentes de Heemskerk et al., 1999, Kooijman et al., 1996). Especificamente em macrófagos, mRNA de IGF-I está presente em macrófagos de tecido de lesão de camundongos (Rappolee et al., 1988). Em macrófagos alveolares é encontrada em quantidade equivalente a de fígado

humano (Rom et al., 1988). Os macrófagos alveolares, peritoniais e linhagens macrofágicas PU5-IR e U937 expressam uma molécula de IGF-I de 26 kDa (Arkins et al. 1993, Nagaoka et al., 1990 e 1991, Rom et al., 1988). Vários fatores controlam a expressão de IGF-I em macrófagos: TNF- α na ausência de IFN- γ (Winston et al., 1999), prostaglandina E2 (Fournier et al., 1995), citocinas Th2 IL-4 e IL-13 (Wynes e Riches, 2003) estimulam a produção de IGF-I, enquanto que IFN- γ a inibe (Arkins et al., 1995). IGF-I por seu lado leva à produção de TNF- α pelo macrófago com provável atuação autócrina (Renier et al., 1996).

A4. Imunidade específica nas leishmanioses

Após a transmissão de promastigotas de *Leishmania* pelo inseto vetor, resposta imune específica e inespecífica do hospedeiro vertebrado são responsáveis pela evolução ou controle da doença. O controle exercido pela resposta imune específica é objeto de estudo pormenorizado nos modelos experimentais de leishmanioses cutânea e visceral, mas principalmente na leishmaniose cutânea utilizando *Leishmania major*. Como o foco da tese não é a resposta específica relacionada à resistência e suscetibilidade nas leishmanioses, apresento aqui um breve sumário dos conhecimentos nessa área para depois detalhar os aspectos relacionados às pesquisas por nós desenvolvidas.

Nas leishmanioses em geral a resposta imune celular é considerada a mais importante tanto na resistência quanto na suscetibilidade. Na leishmaniose cutânea experimental em camundongos isogênicos infectados com *L. (L.) major* foi estabelecido um paradigma vinculando a subpopulação de linfócitos T CD4⁺ produzindo principalmente IFN- γ (denominada T “helper” 1 = Th 1) à resistência e a de T CD4⁺ produzindo principalmente IL-4 e IL-10 (denominada T “helper” 2 cells =

Th2) à suscetibilidade. A importância de linfócitos T CD8⁺ na resistência somente foi definida recentemente. Quanto às citocinas envolvidas, a importância de interferon (IFN)- γ é reforçada no mecanismo de resistência, tanto como ativador eficiente de macrófagos, quanto como fator que direciona a diferenciação de linfócitos T “helper” não diferenciados (denominado T “helper” 0 = Th0) para Th1, com a participação de interleucina (IL)-12 e células “natural killer” (NK) e com a participação central de T-bet, um fator de transcrição T-box, neste processo. Por outro lado, IL-4, IL-13 e IL-10 estão ligadas à suscetibilidade ou persistência da infecção, esta última ligada à ativação de células T regulatórias CD4⁺CD25⁺ (informações detalhadas na revisão de Sacks e Noban-Trauth, 2002).

Nos outros modelos de leishmanioses, no entanto, tanto de cutânea utilizando outras espécies de *Leishmania* como de visceral, observam-se diferenças marcantes quanto à participação de diferentes populações de células e citocinas. Na leishmaniose cutânea por *L. amazonensis*, além da diferença nas linhagens que são suscetíveis ou resistentes à infecção, os linfócitos T CD4⁺ são os responsáveis pelo desenvolvimento da lesão (Soong et al., 1997, Terabe et al., 2000) e na interação de macrófagos com *L. amazonensis*, por exemplo, IFN- γ ativa estas células para matar promastigotas, mas, esta citocina promove crescimento de amastigotas no seu interior (Qi et al, 2004). No modelo de leishmaniose visceral, a resistência envolve tanto células T CD4⁺ quanto CD8⁺ T e IL-2, IFN- γ e IL-12, esta última num mecanismo independente de IFN- γ e ligada à produção de fator de crescimento de transformação (“transforming growth factor” =TGF) β . Suscetibilidade envolve IL-10 mas não IL-4. Em animais imunes, quando reinfectados, os elementos implicados na resistência são diferentes e são células T CD8⁺ e IL-2 (Anexo XI).

A participação de linfócitos B e de imunoglobulinas na suscetibilidade ou resistência nas leishmanioses nunca foi muito clara, mas, atualmente tem dados mais consistentes que indicam a sua participação como fator de suscetibilidade. Os animais depletados ou deficientes em linfócitos B são resistentes à leishmaniose (Hoerauf et al., 1994, Sacks et al., 1984, Smelt et al., 2000) e se tornam suscetíveis quando se transferem linfócitos B (Hoerauf et al., 1994) e há ainda estudos indicando que é a imunoglobulina, produto dos linfócitos B, o fator de suscetibilidade (Kima et al., 2000).

A5. Imunossupressão na leishmaniose visceral

Uma das conseqüências imunopatológicas mais importantes na leishmaniose visceral, principalmente na forma clássica, é a imunossupressão de células T, com teste de hipersensibilidade do tipo tardio negativo ao antígeno de leishmânia (teste de Montenegro ou leishmanina) e ausência de resposta proliferativa principalmente a antígeno de leishmânia e a mitógeno em alguns relatos, sendo observados no homem (Carvalho et al., 1981, Ghose et al, 1979, Hepner Levy e Mendes, 1981, Ho et al 1983, Manson-Bahr, 1961) e na leishmaniose visceral em hamster que desenvolve doença similar à humana, plenamente manifesta (Gifawesen e Farrell, 1989, Nickol e Bonventre, 1985, Rodrigues Jr et al., 1992, Vasconcellos et al., 1996). Em camundongo BALB/c, suscetível, infectado com *Leishmania (L.) donovani*, observa-se teste de hipersensibilidade do tipo tardio negativo aos antígenos de *Leishmania* na fase onde há aumento progressivo da carga parasitária (Basak et al., 1992).

Estudo dos mecanismos pelo qual ocorre imunossupressão na doença ativa indica que esta tem causa multifatorial. Inicialmente foram referidas redução do número de células T circulantes (Rezai et al., 1978) e depleção de linfócitos em áreas T

dependentes de linfonodos e de baço (Veress et al., 1977), mas, depois foram identificadas alterações comprometendo desde a apresentação de antígeno, supressão mediada por células, fatores séricos supressores e interação de citocinas.

Macrófagos como célula apresentadora de antígeno podem ter defeito na sua função e contribuir para a imunossupressão, fato observado em leishmaniose visceral em hamsteres (Rodrigues Jr et al., 1992) e em camundongos suscetíveis a infecção por *Leishmania* em que macrófagos apresentavam diminuição no número de moléculas do complexo principal de histocompatibilidade (“major histocompatibility complex” = MHC) de classes I e II na sua superfície (Nacy et al., 1983) ou de molécula coestimulatória B7-1 (Saha et al., 1995). Além disso, outros estudos mostram que há expressão da molécula CTLA-4 nos linfócitos T que inibe a sua ativação na leishmaniose visceral experimental em camundongo (Gomes et al., 1998, Murphy et al., 1998) e que resulta na produção de TGF- β (Gomes et al., 2000).

Células supressoras, caracterizadas ora como células aderentes (Koech et al., 1987), ora como células CD8⁺ (Holaday et al., 1993) ou sem nenhuma dessas características (Carvalho et al., 1989) foram, em diferentes estudos com pacientes, implicados também na supressão da resposta proliferativa. Na leishmaniose visceral em hamsteres e camundongos, observou-se participação de células não aderentes, possivelmente linfócitos T (Blackwell e Ulczak 1984, Nickol e Bonventre, 1985) e, em outro estudo, célula aderente e Th2 (Basak et al., 1992).

Fatores séricos foram também apontados na supressão da resposta linfoproliferativa a mitógenos na leishmaniose visceral ativa em pacientes (Barral et al., 1986) ou hamsteres (Evans et al 1990, Vasconcellos et al, 1996) bem como a resposta a antígeno de leishmânia (Wyler, 1982). Componente lipídico (Vasconcellos

et al, 1996) e receptor solúvel de IL-2 (Barral-Neto et al., 1991) foram apontados como possíveis fatores presentes no soro.

A produção de citocinas está alterada na fase ativa da doença. A grande maioria dos estudos é em amostras de pacientes e os dados aparentam ser controversos, pois são pesquisados em amostras diferentes e em número diferente de casos.

Quando citocinas do tipo Th1, pesquisadas em sobrenadantes de cultura de células mononucleares do sangue periférico estimuladas por antígeno de leishmânia, IL-12 estava ausente (Ghalib et al., 1995) e IFN- γ , negativo (Bacellar et al., 1996) ou em nível baixo (Cillari et al., 1995, Ghalib et al., 1995). No entanto, IFN- γ no soro estava positivo na frequência que variava de 38,5% (Babaloo et al., 2001) a cerca de 90% (de Medeiros et al., 1998, Zwingenberger et al., 1990), sendo encontrado até em nível alto no início (Cillari et al., 1995). Além disso, a expressão de mRNA de IFN-gama na medula óssea estava aumentada, sugerindo que os linfócitos nos órgãos onde ocorre a interação de linfócitos com o parasito ou antígeno da leishmânia, produz IFN- γ , refletindo no encontro no soro. Por outro lado, as células do sangue periférico, nas condições de cultura, sofreriam efeitos supressores diversos.

Quanto a citocinas do tipo Th2, IL-4 no soro foi positivo em um (Zwingenberger et al., 1990), mas negativo num outro estudo (Cillari et al, 1995) e detectado em nível elevado no sobrenadante quando a cultura foi estimulada com mitógeno (Cillari et al., 1995) e expresso como mRNA na medula óssea em cerca de metade dos casos (Kenney et al., 1998). IL-10, por outro lado, estava em nível baixo no sobrenadante de cultura (Cillari et al., 1995), mas, encontrada em nível elevado no soro (Cillari et al, 1995) e em 93,3% dos casos (de Medeiros et al., 1998) ou tinha sua expressão como mRNA em cerca de metade dos casos (Kenney et al., 1998) ou estava aumentada em linfonodo (Ghalib et al., 1993) ou medula óssea (Karp et al., 1993). O

papel de IL-10 na supressão parece mais consistente na leishmaniose visceral humana, esta sendo produzida pela célula CD8⁺ supressora (Holaday et al., 1993), contribuindo na recuperação da produção de IFN- γ com o uso de anticorpo anti-IL-10 (Ghalib et al., 1995).

Na leishmaniose visceral em hamsteres, por sua vez, mRNAs de citocinas tanto do tipo Th2 (IL-4 e IL-10) quanto do tipo Th1 (IFN- γ) (Melby et al., 1998) estão expressos.

Um outro fator que vem sendo estudado na imunossupressão na leishmaniose visceral é o TGF- β já revisto acima como um dos fatores de crescimento. Vimos que na doença em atividade há produção aumentada de TGF- β tanto em leishmaniose cutânea humana quanto experimental e pelo seu efeito inativador de macrófagos promove a proliferação de leishmânia intracelular.

Apesar da descrição de vários fatores na leishmaniose visceral acreditamos que o mecanismo de imunossupressão não esteja completamente esclarecido podendo haver participação de outros fatores ou fenômenos até agora pouco estudados. Nesta tese desenvolvemos estudos de apoptose como mecanismo de supressão e progressão da infecção.

A6. Apoptose na imunossupressão e na progressão da infecção

O fenômeno de apoptose está presente em várias infecções, participando de diversas maneiras influenciando no desenvolvimento da infecção.

Apoptose atinge células do sistema imune, participando do mecanismo de imunossupressão. Em pacientes com infecção pelo vírus da imunodeficiência humana a apoptose de células T CD4⁺ foi relacionada à destruição destas células ao longo da infecção (Ameisen et al., 1995 ; Groux et al., 1992). Descreve-se também apoptose em

células T do granuloma hepático em camundongos infectados com *Schistosoma mansoni* (Estaquier et al., 1997).

Em protozooses, aumento de apoptose em linfócitos de pacientes com malária por *Plasmodium falciparum* foi associado a baixa resposta linfoproliferativa de células mononucleares sugerindo sua participação no mecanismo de imunossupressão (Toure-Balde et al., 1996). Em toxoplasmose em camundongos com doença disseminada e ocular, ocorre apoptose de células T CD4⁺ e de células T CD8⁺ com ausência de resposta de células T (Hu et al., 1999, Khan et al., 1996).

Em doença de Chagas experimental, ocorre apoptose de células T esplênicas, por estimulação (Lopes et al., 1995) e, por efeito inativador de macrófago após sua fagocitose, favorece a progressão da doença (Freire-de-Lima et al., 2000).

Em leishmanioses, apoptose tem sido descrito como fator que pode participar da imunopatogenia da doença. Em camundongos com leishmaniose visceral há aumento de apoptose em células T CD4⁺ que poderia contribuir para imunossupressão e desenvolvimento da doença (Das et al., 1999). Em leishmaniose cutânea, camundongos deficientes de Fas e ligante de Fas não controlam a infecção por *Leishmania major* (Conceição-Silva et al., 1998, Huang et al., 1998) sugerindo participação de apoptose no mecanismo de proteção da infecção. Em leishmaniose cutânea humana, Bertho et al. (2000), analisando lesões de pacientes com doença progressiva e com cura espontânea, mostram que apoptose em células T CD4⁺ e CD8⁺ possam ter papéis diferentes no desenvolvimento ou cura da lesão.

Apoptose participa na infecção, com influência na sua evolução, também de outras maneiras. Observa-se, por exemplo, que alguns patógenos infecciosos protegem as células hospedeiras contra apoptose. Apoptose induzida em macrófagos é inibida por infecções diversas, por exemplo, *Candida albicans* (Heidenreich et al., 1996),

Mycobacterium bovis (BCG) (Kremer et al., 1997) e *Toxoplasma gondii* (Nash et al., 1998). Também Moore e Matlashewski (1994) observam que macrófagos infectados com *Leishmania donovani* ou tratados com lipofosfoglicano de *Leishmania* são protegidos da apoptose. A inibição da apoptose de células hospedeiras de microrganismos intracelulares contribuiria na progressão da infecção.

Alguns fatores foram relacionados à indução ou proteção da apoptose. Em células T, IL-10 induziu apoptose em células do granuloma hepático em camundongos infectados por *Schistosoma mansoni* (Estaquier et al., 1997), mas protegeu as células infectadas por *Salmonella choleraesuis* (Arai et al., 1995). Em monócitos/macrófagos, TNF- α induziu apoptose em células infectadas por *Candida albicans* (Heidenreich, 1996), mas protegeu da apoptose as células infectadas por *Mycobacterium bovis* (Kremer et al., 1997) ou por *Leishmania donovani* (Moore e Matlashewski, 1994).

O parasito, por seu lado, pode ser atingido por apoptose, mesmo em protozoários. A apoptose era até recentemente conhecida somente em organismos multicelulares como mecanismo de controle fisiológico de células, porém recentemente este processo vem sendo descrito também em organismos unicelulares. Em vários protozoários como *Tetrahymena* (Davis et al., 1992) *Dictyostelium* (Cornillon et al., 1994) e também em *Trypanosoma brucei* (Welburn et al., 1996), incluindo promastigotas de *Leishmania (L.) amazonensis* submetida a choque térmico (Moreira et al., 1996), promastigotas de *Leishmania (L.) donovani* submetidas a tratamento com diferentes doses de peróxido de hidrogênio (Das et al., 2001), assim como amastigotas axênicas em cultura (Serenio et al., 2001) a morte celular apresenta característica de apoptose. A apoptose tem sido considerado um mecanismo de controle populacional dos parasitos, sendo este mecanismo acionado quando há uma “superpopulação” de parasitos (Welburn et al., 1997).

Na leishmaniose visceral, as pesquisas voltam-se principalmente para o estudo da imunossupressão para explicar a evolução progressiva da infecção. No entanto, diversos estudos de apoptose mostram que este fenômeno possa atingir outras células, além de linfócitos. Na leishmaniose visceral a apoptose de linfócitos pode ser um dos mecanismos de imunossupressão, porém, há indicações de que possa ocorrer apoptose de outras células como macrófagos e do próprio parasito. Portanto, propomos neste estudo, investigar a apoptose em diferentes elementos celulares na leishmaniose visceral em hamsteres, o que implicaria na participação de outros mecanismos e interações no desenvolvimento da doença.

A7. Imunopatogenia das lesões na leishmaniose visceral

Na leishmaniose visceral no homem ocorrem alterações histopatológicas nos vários órgãos (Duarte, 2000). No baço observam-se hiperplasia e hipertrofia das células do sistema fagocítico mononuclear que são parasitadas por amastigotas, acompanhadas de depleção de linfócitos e infiltração de plasmócitos e ainda eventuais focos de amiloidose na polpa branca ou nos sinusóides. No fígado as alterações histopatológicas são variadas e classificadas em três padrões distintos: padrão típico ou clássico, onde se observam hiperplasia e hipertrofia de células de Kupffer que contêm amastigotas e infiltrado linfoplasmocitário focal intralobular e nos espaços porta; padrão nodular, com hipertrofia e hiperplasia de células de Kupffer e formação de agregados de células mononucleares (macrófagos, plasmócitos e linfócitos) com pequeno número de amastigotas fagocitadas; padrão fibrogênico, verificam-se discreto infiltrado inflamatório mononuclear portal e intralobular, focos múltiplos de fibrose intralobular, hipertrofia e hiperplasia de células de Kupffer com raras amastigotas. No pulmão observa-se pneumonia intersticial, multifocal, caracterizada por espessamento dos septos alveolares por macrófagos, linfócitos, plasmócitos e células intersticiais

com inclusões lipídicas e congestão de capilares septais e edema discreto. Dentro os órgãos afetados o rim é o mais estudado.

Há vários estudos e relatos de alterações renais na leishmaniose visceral no homem (Andrade e Iabuki, 1972, Brito et al., 1975, Caravaca et al., 1991, Duarte et al., 1983, Dutra et al., 1985, Weisinger et al., 1978) e no cão (Alencar, 1977/1978, Benderitter et al., 1988, Bray, 1982, Mancianti et al., 1989, Nieto et al., 1992, Poli et al., 1991). Para o estudo da patogenia, a definição inicial de padrões histopatológicos é fundamental. No entanto, até os dias atuais os estudos realizados ou são de descrição de um número pequeno de casos ou análises utilizando critérios não homogêneos. Na leishmaniose visceral naturalmente adquirida canina, no Mediterrâneo, os padrões histopatológicos observados foram de glomerulonefrite membranoproliferativa e glomerulonefrite mesangioproliferativa (Benderitter et al., 1988, Nieto et al., 1992, Poli et al., 1991). No homem, as alterações glomerulares mais frequentemente encontradas foram glomeruloesclerose e proliferação de células mesangiais (Andrade e Iabuki, 1972, Brito et al., 1975, Weisinger et al., 1978), mas, as alterações intersticiais foram consideradas predominantes (Duarte et al., 1983).

Na leishmaniose visceral há indícios de que as lesões são mediadas imunologicamente devido à escassez de parasitos em alguns órgãos onde há lesão. No pulmão, a lesão foi correlacionada com a presença de antígeno de *Leishmania* (Duarte et al. 1989). No rim, onde se concentra a maioria dos estudos, as lesões glomerulares tanto no homem quanto em modelos experimentais têm sido atribuídas à deposição de imunocomplexos (Dutra et al., 1985, Poli et al., 1991, Sartori et al., 1987, Tafuri et al., 1989, Weisinger et al., 1978), embora antígenos de leishmânia não tenham sido demonstrados no homem (Brito et al., 1975), sendo detectados somente em modelo em hamster, quando o parasito foi inoculado por via endovenosa (Sartori et al., 1987).

Deposição de imunocomplexo é também o mecanismo predominantemente proposto nas outras glomerulonefrites no homem e nos animais (Couser et al., 1998). Outros mecanismos, no entanto, vem sendo identificados no desenvolvimento das glomerulonefrites.

A participação de imunidade mediada por células começou a ser estudada na década de 70 quando se observaram a produção de fator de inibição de migração de macrófagos por linfócitos T a antígeno da membrana basal de glomérulo (Rocklin et al., 1970) e a inexistência de depósitos de imunoglobulinas na maioria dos casos de glomerulonefrite rapidamente progressiva (Stilmant et al., 1979). Surgiram outras evidências em glomerulonefrites experimentais (Bhan et al., 1978, Bolton et al., 1984, Penny et al., 1997.; Tipping et al., 1998) e em nefropatias humanas (Cunningham et al., 1999, Hooke et al., 1984, Hotta et al., 1998, Li et al., 1990, Markovic-Lipkovski et al., 1991, Saito et al., 1990, Stachura et al., 1984), sendo que em alguns casos a população implicada é de células T CD4⁺ (Bolton et al., 1987, Tipping et al., 1998), em outros, T CD8⁺ (Penny et al., 1998) e ainda ação concomitante de anticorpos e células T (Bhan et al., 1978, Tipping et al., 1985).

Um outro mecanismo patogênico com a participação de imunoglobulinas, diferente de deposição de imunocomplexo, vem sendo estudado em modelo murino de Lupus eritematoso sistêmico. Em camundongos com imunodeficiência combinada severa, lesões glomerulares proliferativas e em alça de arame com características semelhantes às que são encontradas na nefrite lúpica humana foram induzidas por anticorpos monoclonais (IgG3) produzidos a partir de camundongos MPR/gld, deficientes em ligante de Fas, que desenvolve espontaneamente manifestações de doenças autoimunes (Itoh et al., 1993, Nose et al., 1996). A análise da seqüência do gene dessas imunoglobulinas desencadeadoras de lesões lúpicas mostrou que há

poucas mutações somáticas na região variável da cadeia pesada (Ono et al., 1995), diferindo de outros autoanticorpos como anticorpos IgG anti-DNA e fatores reumatóides IgG observados nesses modelos (Shlomchik et al., 1987 e 1990) que se supõem causadores de lesões por imunocomplexos (Theofilopoulos et al., 1985). O mecanismo pelo qual essa imunoglobulina leva à lesão glomerular foi caracterizado como sendo pela internalização de imunoglobulinas por células endoteliais (Fujii et al., 2003).

Outros aspectos estudados na patogenia das glomerulonefrites são a expressão de moléculas de adesão e apoptose. Moléculas de adesão, ICAM-1, VCAM-1 e selectinas E e P têm sido observadas em diferentes tipos de glomerulonefrites (Adler e Brady, 1999). Na patogenia, apoptose é apontada como mecanismo responsável pelo fim da proliferação de células glomerulares em alguns estudos (Baker et al., 1994; Nitsch et al., 1997, Mooney et al., 1999, Shimizu et al., 1995 e 1996), sendo implicados como mediadores neste processo, TNF- α e IL-1 α (Liu et al., 1996).

B. Objetivos

Avaliar no contexto global da infecção/doença leishmaniose, as pesquisas realizadas e aquelas em andamento voltadas para o estudo da participação de seguintes elementos e/ou processos:

- elementos inespecíficos da resposta do hospedeiro: complemento, células “natural killer”, polimorfonucleares neutrófilos e fator de crescimento insulina-símile;
- imunossupressão e apoptose de macrófagos e amastigotas na evolução da forma visceral da doença;
- mecanismos imunopatogênicos das lesões teciduais na forma visceral da doença.

C. Resultados comentados

C1. Imunidade inata nas leishmanioses

Nas leishmanioses humanas e experimentais a grande maioria dos estudos de imunidade enfoca a resposta específica nas leishmanioses. No entanto, entendendo que os eventos iniciais ainda pouco elucidados são cruciais no estabelecimento da infecção, onde participam, entre outros elementos, complemento, polimorfonucleares neutrófilos (PMN), macrófagos, células “natural killer” (NK) e fatores de crescimento, desenvolvemos alguns estudos neste campo. A grande maioria dos dados na literatura de avaliação do papel de elementos inespecíficos, principalmente PMN e macrófagos, nas leishmanioses é proveniente de estudos in vitro. Como consideramos importante a sua avaliação in vivo, realizamos estudos do complemento, células NK e inflamação aguda na leishmaniose experimental. A participação do fator de crescimento insulina-símile, ainda não conhecida na leishmaniose na época, foi avaliada tanto em estudos in vitro como in vivo.

1.1. Papel do complemento na leishmaniose visceral em hamsteres

Na leishmaniose, o complemento exerce vários papéis como efeitos leishmanicida e quimiotático e de favorecimento da evasão do parasito no macrófago. Avaliamos alguns aspectos desses efeitos in vivo (Anexo I). O papel do complemento foi estudado em hamsteres infectados com *L. (L.) chagasi*, depletados ou não em complemento com a administração de 0,3 mg/Kg do fator de veneno de cobra, 12 e 24 horas antes da inoculação subcutânea de um milhão de promastigotas. Analisamos

vários aspectos na lesão incluindo a reação inflamatória no sítio de inoculação e os elementos celulares no período de duas a 72 horas de infecção.

Observou-se destruição de parasitos extracelulares em grande quantidade tanto nos animais depletados ou não em complemento, sugerindo que outros fatores, além do complemento, são responsáveis por essa destruição.

Um aspecto intrigante observado no estudo foi uma maior densidade de PMN na lesão com duas horas e, na época do estudo, especulamos sobre vários fatores principalmente ligados ao efeito do fator de veneno de cobra para explicar esse achado. No entanto, à luz dos conhecimentos mais recentes, a explicação pode ser um fator quimiotático de granulócitos liberado por promastigotas, descrito por van Zandbergen et al. (2002), que possivelmente estaria presente em quantidade maior nos animais decompementados, conseqüente a possível quantidade maior de parasitos na lesão pela depleção do complemento. Nos animais depletados de complemento, a migração maior de células PMN no sítio de inoculação do parasito pode levar a maior destruição de parasitos e isto condiz com o menor parasitismo encontrado nesses animais. No entanto, recentemente o seu papel na evasão está em discussão após a observação do retardo ou inibição da apoptose de PMN, possibilitando a manutenção e proteção da leishmânia no seu interior até a sua transmissão ao macrófago, com esta célula fagocitando PMN contendo parasitos (Aga et al., 2002). Este é um aspecto que seria interessante avaliar e que no nosso trabalho tampouco encontramos parâmetros para validação.

Avaliando o efeito da decompementação sobre macrófagos, observamos efeitos favoráveis ao hospedeiro. Observaram-se nos animais depletados uma migração menor de macrófagos com 72 horas de infecção e densidade menor de células contendo parasitos no seu interior, provavelmente conseqüente à diminuição da

opsonização pelo complemento. São efeitos que favorecem o hospedeiro, não disponibilizando na lesão células que são hospedeiras da leishmânia, além de albergar menor quantidade de parasitos no seu interior. Além disso, a depleção estaria interferindo num dos efeitos do complemento na evasão do parasito no interior do macrófago, de inibição da ativação eficiente do mecanismo leishmanicida do macrófago quando o parasito opsonizado é fagocitado ligando-se a um dos receptores do complemento, CR1 ou CR3 (Blackwell, 1985, Wright e Silverstein, 1983). O efeito nos tempos tardios desses eventos iniciais, desfavoráveis ao progresso da infecção, foi verificado examinando a infecção após 240 dias de infecção em fígado e baço. Observamos uma repercussão esperada de não detecção de parasitos íntegros nos animais deplecionados.

Nesse modelo de leishmaniose visceral em hamsteres, mostramos que o papel predominante do complemento é na evasão da *Leishmania*. Embora esse estudo tenha sido publicado em 1996, esse dado do papel do complemento in vivo continua inédito. Vale ressaltar, por outro lado, que no modelo de leishmaniose cutânea em camundongo infectado com *L. (L.) amazonensis*, outros dados sugerem que o efeito predominante do complemento é de destruição de parasitos pelo complemento na fase inicial e não tanto na evasão (Laurenti et al., 1999).

1.2. Papel das células “natural killer” (NK) na leishmaniose cutânea murina

Uma das células com possível papel no início da infecção nas leishmanioses é a célula NK. Nas leishmanioses, o papel das células NK é enfatizado no desenvolvimento da resposta imune específica, na diferenciação de células T CD4⁺,

Th0 para Th1 (Scharton e Scott, 1993), mas, existem outros estudos que apontam seu papel na fase inicial como elemento inespecífico, utilizando camundongos bege (Kirkpatrick et al, 1985) ou utilizando anticorpos para sua depleção (Laskay et al., 1993 e 1995). No entanto, esses modelos suscitam algumas dúvidas. O camundongo bege tem células NK reduzidas, mas, tem também alterações no compartimento lisossomal que afetam várias funções de macrófagos e PMN, não permitindo, portanto, atribuir os resultados obtidos nesse animal exclusivamente ao papel de células NK. O uso de anticorpos que depletam as células NK, por seu lado, leva à ativação maciça do complemento que sabe-se ter papel importante na fase inicial da infecção. Diante desses questionamentos, buscamos um modelo alternativo de depleção de células NK e decidimos por um método de utilização de ^{90}Sr . Este método tem a vantagem de promover depleção permanente de células NK, sem interferência nas células fagocíticas e complemento e ainda mantendo os compartimentos de células T e B intactos (Gidlund et al., 1990). Quando camundongos BALB/c foram depletados em células NK com a utilização do ^{90}Sr e infectados com *L. (L.) amazonensis* no subcutâneo, constatamos parasitismo maior no sétimo dia de infecção na lesão de pele em relação ao controle (Anexo II). Com a utilização de um modelo mais focalizado na célula NK, sem outras repercussões nas células fagocíticas ou complemento, naquele momento parecia claro o papel de células NK no controle da infecção por *Leishmania* na fase inicial. No entanto, dados surgidos na sequência desse trabalho, mostraram que existiam outros elementos como $\text{IFN}\gamma$ e complemento que eram afetados pela depleção das células NK, contribuindo no resultado do parasitismo na pele (Laurenti, 1998). Parte da alteração dos níveis de complemento seria por serem as células NK produtoras de $\text{IFN}\gamma$ (Scharton e Scott, 1993) que por sua vez tem efeito na transcrição do gene da fração do complemento C3 (Volanakis, 1995). Os dados mostraram também que o

tratamento com ^{90}Sr afetava uma população de células NK que não as células NK1.1 que estavam em quantidade aumentada no baço.

Se observamos a existência dessas interferências nos nossos experimentos, podemos dizer que a mesma restrição pode ser estendida a outros trabalhos que mostram o efeito das células NK onde a participação de outros elementos da resposta inata não é analisada.

1.3. Papel das células polimorfonucleares neutrófilos (PMN) na leishmaniose visceral murina

Outra célula importante da fase inespecífica são os polimorfonucleares neutrófilos. Estudos com PMN in vitro mostram seu efeito leishmanicida (Chang, 1981, Pearson e Steigbigel, 1981). Outros estudos in vivo, com depleção de PMN com anticorpos, também mostram seu papel no controle da infecção nas leishmanioses em camundongos (Lima et al, 1998, Rousseau et al, 2001). Mais uma vez este modelo traz no bojo o fato de levar a uma ativação maciça do complemento que se sabe ter papel importante na fase inicial da infecção. Além disso, há outros estudos controversos que indicam papel de PMN na indução da produção de IL-4, implicando-as na suscetibilidade à infecção na leishmaniose cutânea (Tacchini-Cottier et al., 2000), ou na secreção de IFN- γ levando à estimulação de macrófagos para uma resposta anti-leishmânia efetiva (Venuprasad et al. 2002).

Para contribuir na pesquisa neste campo, estamos estudando no momento o papel da inflamação aguda na evolução da leishmaniose visceral em camundongo. Desenvolvemos este estudo utilizando linhagens de camundongos com reatividade inflamatória aguda (“acute inflammatory reaction” = *AIR*) máxima (*AIRmax*) e

mínima (*AIRmin*) que foram selecionadas de acordo com a capacidade de recrutar leucócitos polimorfonucleares neutrófilos no exsudato inflamatório (Ibañez et al., 1992). Recorremos a essas linhagens porque camundongos isogênicos normalmente utilizados para o estudo da imunidade na leishmaniose, apesar de apresentarem diferenças na dinâmica de migração de células inflamatórias no início da infecção (Beil et al., 1992; Sunderkötter et al., 1993), também têm diferenças na resposta imune específica, o que inviabiliza o seu uso neste tipo de abordagem que propomos aqui e por isso descartamos. Além disso, com o uso das linhagens propostas, fugimos do uso de anticorpos para depletar PMN, com os inconvenientes referidos acima. Estudos iniciais de infecção dessas linhagens de camundongos por *L. (L.) chagasi* (Anexo III), após injeção intraperitoneal de 2×10^3 , 2×10^5 e 2×10^7 amastigotas, mostraram inicialmente ser 2×10^5 o melhor inóculo para evidenciar as diferenças entre as linhagens. Com este inóculo, a carga parasitária observada nos camundongos *AIRmin* tendia a ser maior após 14 e 28 dias de infecção e mostrou-se até mais suscetível que os camundongos BALB/c, sugerindo a existência de mecanismo compensatório, se admitimos esse papel leishmanicida dessas células na fase inicial da infecção. Esse mecanismo compensatório poderia ser a migração mais precoce de fagócitos mononucleares para o local da inflamação nessa linhagem de camundongo, observada anteriormente no rim (Yokoo, 2001).

Passamos à análise também do período mais precoce, aos 3 dias de infecção, que podemos considerar como fase final de interações quase que exclusivamente de elementos e fatores inespecíficos e aos 7 dias onde a resposta imune específica estaria sendo gradativamente ativada, além dos tempos de 14 e 28 dias, agora com outro método. Nesta análise, de fato, constatou-se quantidade maior de parasitos no líquido peritoneal no dia 3 nos camundongos *AIRmin*, o que podemos atribuir à menor

quantidade de PMN. No entanto, a carga parasitária nos dias 3 e 7 no linfonodo mesentérico, no fígado e no baço, é muito semelhante. Observa-se, por outro lado, alguma diferença entre as linhagens aos 14 dias de infecção no fígado. Este fato sugere que PMN é elemento que atua destruindo parasitos na fase inicial, mas, na evolução, um elemento que parece modular a resposta imune específica e este papel parece ser o mais importante. No prosseguimento, estão em estudo os tempos mais precoces e também as citocinas, quimiocinas e complemento.

1.4. Papel do fator de crescimento insulina-símile sobre leishmânia e na leishmaniose

Diversos fatores de crescimento presentes no meio têm efeito sobre leishmânia e nas leishmanioses: GM-CSF favorecendo o parasito na infecção (Barcinski et al., 1992, Greil et al., 1988, Scott et al., 2000) ou como efector leishmanicida (Ho et al., 1990, Murray et al., 1995) ou ainda TGF- β como inativador de macrófagos (Barral-Neto et al., 1992, Wilson et al., 1998) ou como indutor de expressão de CTLA-4 por linfócitos T (Gomes et al., 2000). Um fator de crescimento que vem despertando grande interesse em diversos campos, por sua ação diversificada e pleiotropismo, são os fatores de crescimento insulina-símile (“insulin-like growth factor” = IGF) (Kooijman et al., 1996, Jones e Clemmons, 1995). Como na leishmaniose, o IGF-I é supostamente um dos primeiros fatores encontrados pelas formas promastigotas de leishmânia na pele do hospedeiro e possivelmente após a internalização por macrófagos que expressam IGF-I (Arkins et al. 1993, Rom et al., 1988, Nagaoka et al., 1990, 1991), estudamos seu efeito sobre amastigotas e promastigotas de leishmânia in vitro e também in vivo na leishmaniose cutânea murina.

Os estudos nesta linha de pesquisa foram enfocados inicialmente no efeito dos IGF-I diretamente sobre a leishmânia, estenderam-se para avaliar o seu efeito na evolução da lesão no modelo experimental e atualmente estão centrados nos seus efeitos na interação macrófago/leishmânia.

Observamos inicialmente in vitro que IGF-I em concentrações fisiológicas induz a um aumento na proliferação de promastigotas de *Leishmania* de três espécies diferentes (Anexo IV) na presença de concentrações subótimas de soro fetal bovino, estudo este que foi estendido a amastigotas axênicas de *L. (L.) mexicana* com indução da sua proliferação (Anexo V). Estes efeitos não foram observados com IGF-II, tanto em promastigotas quanto amastigotas axênicas (Anexo V).

Atestando o seu efeito no parasito, com IGF-I observamos indução de fosforilação de resíduos de tirosina e de serina-treonina em diversas proteínas do parasito, mas de massas moleculares distintas em promastigotas e amastigotas de *L. (L.) amazonensis*. A fosforilação de resíduos de tirosina ocorreu em proteínas de 185 kDa em promastigotas e de 60 e 40 kDa de amastigotas. A fosforilação de outros resíduos, provavelmente serina/treonina, ocorreu em proteínas de 110 kDa em promastigotas e nas de 120 e 95 kDa de amastigotas (Anexos V e VI).

Na interação do IGF-I com o parasito, estudamos as características de ligação de IGF-I a promastigotas e amastigotas axênicas. Análises de ensaios de saturação e de deslocamento homólogo indicaram ligação de IGF-I ao parasito por receptor com um único sítio de ligação, de afinidade média a alta, sendo que tanto a afinidade quanto o número de supostos receptores em amastigotas foram superiores que em promastigotas (Anexos IV e V). O suposto receptor de IGF-I em *Leishmania* foi caracterizado como sendo constituído por uma glicoproteína monomérica de 65 kDa que tem antigenicidade cruzada com a cadeia alfa do receptor humano de IGF-I (Anexo VIII).

A comparação deste suposto receptor de IGF-I da *Leishmania* com o receptor presente na célula humana mostra uma grande diferença estrutural. Enquanto o receptor do parasito é constituído por uma molécula monomérica, a de célula humana é tetramérica, constituída por duas cadeias alfa e duas beta, com massas moleculares respectivamente de 135 e 93 kDa, perfazendo massa molecular total de 456 kDa (Rechsley e Nissley, 1990, Drakenberg et al., 1993). Como esta diferença estrutural vislumbra a possibilidade de existência de seqüências diferentes no receptor dessas duas espécies que possa ser atingida no parasito sem interferência no receptor da célula do hospedeiro, esforços estão sendo dirigidos atualmente para a clonagem do receptor de IGF-I de *L. (L.) chagasi* objetivando no futuro a análise de suas diferenças em relação ao receptor humano para gerar candidato a vacina contra leishmaniose.

Numa outra abordagem, em camundongos BALB/c infectados com *Leishmania* pré-ativada com IGF-I, observou-se tamanho maior da lesão a partir de 21 dias pós-infecção (PI), com número maior de parasitos viáveis no sítio da lesão a partir de 7 dias de infecção acompanhado de maior infiltrado inflamatório (Anexos V e VII). O maior parasitismo poderia ser devido a maior quantidade de células hospedeiras, macrófagos, na lesão, mas uma análise mais detalhada mostrou que esse aumento do parasitismo foi devido também ao aumento do número de parasitos por macrófago, mostrando que IGF-I na interação parasito/hospedeiro favorece o crescimento do parasito no interior da célula hospedeira (Anexo VII).

Se IGF-I favorece maior crescimento do parasito no macrófago, é provável que este fator exerça algum efeito sobre mecanismos microbicidas do macrófago. Os dois principais mecanismos efetores com geração de produtos lesivos a leishmânias em macrófagos de camundongos são a liberação de espécies de oxigênio reativo, principalmente peróxido de hidrogênio (H_2O_2) e de espécies de nitrogênio reativo

como o óxido nítrico (NO) (Green et al., 1990, Haidaris e Bonventre, 1982, Liew et al., 1990, Murray, 1981, Stenger et al., 1994). Desta forma, passamos a aprofundar o estudo do mecanismo de atuação de IGF-I na interação parasito/macrófago in vitro (Anexo IX). Observamos que a produção de H_2O_2 não é afetada na presença de IGF-I em nenhuma situação. Era um resultado de certa forma esperado pelas observações anteriores em outras condições que não a infecção por leishmânia onde outros fatores como hormônio de crescimento e prolactina, mas não o IGF-I, aumentavam a produção de H_2O_2 (Warwick-Davies et al., 1995). Por outro lado, a produção de NO por macrófagos infectados por promastigotas de *Leishmania* é diminuída quando macrófagos ou promastigotas são pré-incubados com IGF-I por cinco minutos ou o fator é mantido todo tempo em cultura. Concomitantemente observamos aumento do grau de infecção de macrófagos. Os dados até agora obtidos sugerem que o efeito do IGF-I sobre o aumento do parasitismo de macrófagos seja pelo seu efeito no metabolismo de L-arginina, o que está sendo aprofundado no momento, iniciando pela detecção da expressão de sintase do óxido nítrico. Baseado nas observações de Balanco et al. (2001) que observaram expressão de fosfatidil serina por leishmânia, simulando apoptose, levando a inativação de macrófagos, estamos propondo a avaliação da expressão de dessa molécula pelo parasito sob estímulo de IGF-I.

Iniciamos os nossos estudos e por muito tempo seguimos focalizando o IGF-I dentro do contexto da resposta inespecífica, mas, o seu efeito em macrófagos por nós observado e os dados de efeitos de citocinas na produção de IGF-I em macrófagos trazem uma nova dimensão ao estudo deste fator nas leishmanioses. O fato de citocinas $TNF-\alpha$ na ausência de $IFN-\gamma$ (Winston et al., 1999), IL-4 e IL-13 (Wynes e Riches, 2003) estimularem a produção de IGF-I em macrófagos e $IFN\gamma$, inibi-la (Arkins et al., 1995) e os nossos dados mostrando IGF-I como promotor de crescimento de parasito

fazem-nos especular sobre o papel de IGF-I como um dos elementos efetores do macrófago com ação sobre o crescimento de leishmânia, modulado por essas citocinas.

C.2. Imunossupressão e participação da apoptose na evolução da leishmaniose visceral em hamsteres

Os estudos da participação do sistema imune durante o desenvolvimento de uma infecção analisam as respostas imunes protetora ou indutora de suscetibilidade ou a supressão da resposta, do ponto de vista dos mecanismos envolvidos na ativação ou desativação de células, participação de citocinas diversas e interações de moléculas co-estimulatórias, etc. Pelo nosso interesse na imunopatogenia da leishmaniose visceral, realizamos uma revisão desses aspectos (Anexo XI) como ponto de partida para embasar o desenvolvimento de pesquisa em modelo experimental de leishmaniose visceral.

No nosso laboratório, iniciamos uma abordagem diferente dos mecanismos de suscetibilidade/imunossupressão na leishmaniose visceral, isto é, dentro da óptica de morte e sobrevivência de diferentes populações celulares, especificamente envolvendo o processo de apoptose, iniciando com a caracterização da imunossupressão no modelo de leishmaniose visceral em hamster infectado com *L. (L.) chagasi* (Anexo XII).

Inicialmente verificamos se os hamsteres infectados com *L. (L.) chagasi* desenvolviam imunossupressão como na leishmaniose visceral humana. A resposta proliferativa de células esplênicas a concanavalina A estava aumentada em relação ao controle, mas, frente a antígeno total de *Leishmania*, manteve-se nos níveis do controle sem estimulação a partir do dia 15 pós-infecção. Neste resultado, o que nos chamou a atenção foi que as células esplênicas de animais infectados já apresentavam um nível elevado de proliferação, mesmo sem nenhuma estimulação. Interpretamos

este dado como se as células estivessem previamente ativadas no animal pela presença da infecção no órgão e, conseqüentemente, sem possibilidade de reativação *in vitro*. De qualquer forma, essa ativação parece não ser eficiente para ativar o macrófago para matar o parasito.

Considerando que a ativação continuada de linfócitos possa levar à apoptose, estudamos este aspecto na leishmaniose visceral em hamster. Observamos apoptose de células inflamatórias nos tecidos hepático e esplênico no hamster infectado por *L. (L.) chagasi* utilizando o método de “TdT-mediated dUTP nick end labelling” (TUNEL). Avaliamos as células esplênicas em suspensão *ex-vivo* e após estímulo com Concanavalina A e antígeno total de *Leishmania* por 6 e 18 horas. Observamos marcação por TUNEL na suspensão celular *ex-vivo*, mas houve aumento de marcação nos linfócitos tanto nos controles não infectados mas mais nos animais infectados nas culturas de células de animais com 15 e 30 dias do experimento estimuladas com Concanavalina A e antígeno de *Leishmania*. Em suspensão celular, freqüentemente há marcação por TUNEL quando há proliferação, portanto, avaliamos a apoptose utilizando anexina V que se liga a fosfatidilserina externalizada no processo de apoptose. Não houve marcação, portanto, concluímos pela não ocorrência de apoptose na população de linfócitos; a marcação seria no caso pela proliferação.

Um outro aspecto estudado foi a expressão de citocinas, pois já se observou mudança no perfil de citocina durante a evolução da leishmaniose visceral em camundongo (Das et al., 1999), mas no nosso modelo o perfil de citocina, analisado por RT-PCR com RNA extraído de células esplênicas, era semelhante tanto nos animais infectados quanto não infectados e nos diferentes períodos de infecção. Baseados nesses resultados, acreditamos que a imunossupressão seja devida a pré-

estimulação de linfócitos especificamente a antígenos de leishmânia e de maneira ineficiente em relação à ativação de macrófagos.

Em certas infecções incluindo protozooses, apoptose é descrita também em outras células. Como não há nenhum dado na literatura sobre apoptose de macrófagos na leishmaniose visceral, pesquisamos a ocorrência de eventual apoptose e proteção da apoptose in vivo em hamsteres infectados intraperitonalmente com amastigotas de 2×10^7 *L. (L.) chagasi*. Inicialmente pesquisamos a presença de apoptose no fígado e observamos a sua ocorrência em células de Kupffer na fase inicial de infecção (15 a 30 dias). Foi um achado de certa forma inesperado pois ocorrendo apoptose de células que se constituem em habitat de *Leishmania*, esperaríamos a não progressão da infecção, mas o que ocorre é a evolução com aumento da carga parasitária, sugerindo a presença de outro mecanismo com efeito oposto. Prosseguimos o estudo da apoptose no fígado em períodos posteriores. Aos 90 dias de infecção a apoptose não é observada mais em células de Kupffer. Este achado pode ser resultado de ausência de estímulo para apoptose nessa fase ou a presença de mecanismo de proteção da apoptose. Como foi observado in vitro que a infecção de macrófagos por *Leishmania* protege-os da apoptose (Moore and Matlashewski 1994), os nossos achados sugerem que isto esteja ocorrendo também in vivo. Prosseguimos analisando a apoptose na população de células aderentes esplênicas. Detectamos apoptose na fase inicial da infecção que diminuiu nas fases posteriores frente a estímulo com antígeno total de *Leishmania*. Este resultado confirma o que observamos no fígado de hamster com leishmaniose visceral e mostra que macrófagos na fase inicial entram em apoptose sob estímulo específico e nas fases tardias parecem não ser sensíveis a estímulos que levem a apoptose ou possam ter mecanismos que levem à proteção da apoptose.

Células do hospedeiro são atingidas por processo de apoptose, mas, o parasito também pode sofrer esse processo. Na literatura há demonstração de apoptose ou fenômenos sugestivos de apoptose em organismos unicelulares incluindo várias espécies de *Leishmania* em estudos in vitro. No nosso laboratório realizamos estudos in vivo na leishmaniose visceral em hamsteres e constatamos que amastigotas de *L. (L.) chagasi* aparentemente entram em apoptose aos 60 e 90 dias pós-infecção (PI) tanto no baço como no fígado, o que não se observa na fase inicial aos 15 e 30 dias PI (Anexo X). Afastamos a ocorrência de necrose de tecido e apoptose de célula do hospedeiro na fase onde ocorre apoptose de amastigotas. Comprovamos apoptose analisando também a fragmentação de DNA de amastigotas purificadas de baço do período onde se observou reação ao TUNEL em tecido. Quanto ao mecanismo de indução de apoptose em amastigotas, embora não tenhamos comprovação, acreditamos que seja em parte por superpopulação ou presença de produtos oxidados de lipídios (baseado em dados preliminares não mostrados).

Neste estudo na leishmaniose visceral experimental observamos uma interação interessante entre hospedeiro e parasita. Infecção por *L. (L.) chagasi* induz imunossupressão por superativação prévia de linfócitos especificamente ao antígeno de *Leishmania* na fase inicial. Nesta fase induz por ativação, apoptose em macrófagos, sua célula hospedeira. Essas células, com a evolução da infecção, tornam-se resistentes à apoptose. A não morte de macrófagos passa a favorecer a sobrevivência do parasito. A imunossupressão aliada à proteção de macrófagos da apoptose proporcionaria uma evolução crescente da infecção, o que não ocorre, pois apoptose atinge também amastigotas na fase final, provavelmente desencadeada por superpopulação, levando ao controle em certa medida da progressão da infecção. O hamster com leishmaniose

visceral, no entanto, evolui para a morte, provavelmente por comprometimento de diversos órgãos e alterações metabólicas decorrentes da infecção crônica.

C.3. Imunopatogenia das lesões na leishmaniose visceral

Na leishmaniose visceral ocorrem alterações patológicas nos vários órgãos. Há indícios de que essas lesões são mediadas imunologicamente devido à escassez de parasitos em alguns órgãos com alterações. No rim, onde se concentra a maioria dos estudos, as lesões glomerulares têm sido atribuídas à deposição de imunocomplexos (Weisinger et al., 1978; Sartori et al., 1987; Poli et al., 1991, Tafuri et al., 1989; Dutra et al., 1985). Deposição de imunocomplexo é também o mecanismo predominantemente proposto nas outras glomerulonefrites no homem e nos animais (Couser et al., 1998), porém, outros mecanismos vêm sendo identificados no desenvolvimento das glomerulonefrites.

Para o estudo da patogenia, a definição de padrões histopatológicos é uma das questões fundamentais. No entanto, até recentemente os estudos realizados na glomerulonefrite na leishmaniose visceral ou eram de descrição de um número pequeno de casos ou eram análises utilizando critérios não homogêneos. Como contribuição nesse campo, realizamos estudos de padrão histopatológico da glomerulonefrite utilizando critério definido pela Organização Mundial da Saúde. Em 55 cães com leishmaniose visceral naturalmente adquirida da área endêmica, Teresina, Piauí, constatamos a presença de seis padrões histopatológicos: glomerulonefrite de alterações mínimas (14,5%), glomeruloesclerose segmentar focal (18,2%), glomerulonefrite proliferativa mesangial (32,7%), glomerulonefrite membranoproliferativa (30,9%), glomerulonefrite crescêntica (1,8%) e glomerulonefrite crônica (1,8%). (Anexo XV). A diversidade de padrões indica a

possível existência de patogenia diversa para a lesão glomerular na leishmaniose visceral, no entanto, não invalida a busca de mecanismos comuns como passo inicial.

Na busca de mecanismos patogênicos na leishmaniose visceral, particularmente da glomerulonefrite, analisamos alguns aspectos em hamsteres e outros em cães. Hamsteres infectados por *L. (L.) donovani* ou *L. (L.) chagasi* constituem-se num bom modelo de leishmaniose visceral ativa, plenamente manifesta, onde, por ser experimental, permite-se, por exemplo, o estudo da evolução da doença, mas, tem a desvantagem de não se dispor no mercado de reagentes para marcadores celulares e outros elementos do sistema imune. Desta forma, também pela oportunidade, estudamos alguns aspectos em leishmaniose visceral em cães naturalmente infectados, provenientes de área endêmica, Teresina, Piauí.

Como o mecanismo patogênico aceito de glomerulonefrite na leishmaniose visceral é de deposição de imunocomplexos, pesquisamos inicialmente a presença de imunoglobulina G (IgG) e fragmento do complemento C3b no rim de hamster com leishmaniose visceral. IgG estava presente, mas, a intensidade do depósito não era uniforme em todos os tempos e o depósito de C3b era de intensidade moderada (Anexo XIV). Subseqüentemente, analisamos os depósitos de imunoglobulinas e C3b em cães com leishmaniose visceral que refletiria o quadro numa fase crônica, pela evolução longa da doença nesses animais. Os depósitos de IgG, IgM, IgA e C3b estavam presentes, mas, com intensidade semelhante tanto em animais com leishmaniose visceral quanto em controles sem leishmaniose visceral, sugerindo ausência de papel patogênico de imunoglobulinas e complemento na fase de evolução da infecção em que estavam os cães (Anexo XVII).

Analisando conjuntamente os dados de depósitos de imunoglobulinas nesses modelos de leishmaniose visceral, não podemos descartar a participação de

imunoglobulinas na patogenia, pelo menos em alguma fase da infecção. Outras observações em paralelo, no entanto, trouxeram outros argumentos contra o mecanismo patogênico por imunocomplexos. Nos hamsteres com leishmaniose visceral, foram detectados depósitos de IgG no fígado e no pulmão, de maior intensidade no período de 30 a 45 dias pós-infecção, mas, ausência de depósito de C3b (Anexo XIV). Consideramos esses achados como fortes indícios contra o mecanismo patogênico de depósito de imunocomplexos, pois em doenças caracteristicamente por imunocomplexos não se observam depósitos de imunoglobulinas no fígado (Cochrane e Dixon, 1978), além da ausência de depósito de C3b. Acresce-se ainda que quando detectamos antígenos de *Leishmania* no glomérulo, estes estavam presentes, mas, em localização diferente da de imunoglobulinas e C3b: antígenos se localizavam nas células do mesângio enquanto que imunoglobulinas e C3b, detectados ao longo do contorno capilar (Anexo XVII). Imunocomplexos com outros antígenos podem estar implicados, mas, os nossos dados atestam que antígenos de *Leishmania* não formam complexos com imunoglobulinas depositadas no glomérulo.

Na literatura, mecanismos alternativos de lesão renal com a participação de imunoglobulinas são sugeridos em glomerulonefrite lúpica experimental e um destes envolve processo de internalização de imunoglobulinas por células endoteliais (Fujii et al., 2003). Como esse processo estava sendo estudado pelo Prof. Dr. Masato Nose do Japão quando iniciamos a nossa colaboração, desenvolvemos pesquisa desse mecanismo também na leishmaniose visceral. Inicialmente estudamos a interação de soros de pacientes com leishmanioses visceral e tegumentar e soros de hamsteres com leishmaniose visceral com células endoteliais da veia umbilical humana (“human umbilical vein endothelial cells” = HUVEC) em cultura (Anexo XVI). Observamos intensa internalização de IgG com soros de pacientes com leishmaniose visceral, mas,

discreta com soros de pacientes com leishmaniose tegumentar. Observamos também intensa internalização de IgG de hamsteres pelas células HUVEC quando soros de animais com 30 a 45 dias de infecção foram utilizados, justamente nos tempos onde foram detectados depósitos de IgG mais intensos nos órgãos, como vimos anteriormente (Anexo XIV). Prosseguimos a investigação deste mecanismo também no rim e fígado de hamsteres com leishmaniose visceral (Anexo XVI). Quando analisamos amostras à microscopia eletrônica de transmissão, submetidas a imunomarcação para imunoglobulina total e IgG, observamos depósito maior de imunoglobulina total aos 30 dias de infecção, sendo maior depósito de IgG observada aos 30 e 60 dias de infecção no fígado e aos 60 dias no rim, embora esta análise deva ser ampliada, principalmente no rim. Estes dados sugerem que imunoglobulinas estão implicadas na patogenia por um processo que não é de deposição de imunocomplexos. Por outro lado, na análise histopatológica observa-se hiper celularidade em hamsteres com leishmaniose visceral que é constituída transitoriamente de polimorfonucleares neutrófilos em intensidade moderada que é substituída gradativamente por células mononucleares. No rim, ainda nas fases tardias, ocorre em alguns casos a substituição do infiltrado inflamatório por substância eosinofílica amorfa. Embora os dados sugiram papel da internalização de imunoglobulinas na patogênese, a sua relação com o processo inflamatório ainda não está totalmente esclarecida.

Para certificarmos da implicação de imunoglobulinas no processo inflamatório, testamos uma abordagem fisiopatológica. Como em processos inflamatórios, há alteração na microcirculação e um dos fenômenos importantes é a ocorrência de extravasamento de macromoléculas, analisamos este fenômeno utilizando observação intravital em vasos da bolsa evertida da bochecha de hamster, tendo como marcador Dextran-fluoresceinado (Anexo XVI). Na abordagem inicial, hamsteres com

leishmaniose visceral foram examinados quanto à resposta à aplicação de histamina, vasodilatadora. Neste experimento, os animais com 30 dias de infecção tinham a resposta inibida à histamina, sugerindo uma vasodilatação prévia, antes da aplicação do agente. No prosseguimento do estudo, purificamos IgG de hamsteres controles e infectados de 30 e 60 dias e foram aplicadas em bochecha evertida de hamster normal. Observou-se extravasamento significativo quando IgG de animais com 60 dias de infecções foi aplicada à bochecha, revelando papel vasoativo em IgG deste período de infecção. O papel de IgG de animal tornou-se evidente no extravasamento e, no campo da especulação, podemos sugerir que aos 30 dias de infecção IgG seria internalizada por células endoteliais provocando vasodilatação e promovendo inflamação, razão pela qual o hamster aos 30 dias não responderia ao estímulo com histamina com extravasamento. Estando a imunoglobulina internalizada por células endoteliais, não estariam disponíveis na circulação, não apresentando nenhum efeito sobre vasos de hamster normal. Por outro lado, aos 60 dias a IgG, não estando internalizada por células endoteliais, ficaria disponível na circulação, exercendo efeito vascular.

Além de imunoglobulinas, outros elementos como linfócitos T e moléculas de adesão podem estar implicados no desenvolvimento das glomerulonefrites, o que analisamos em cães com leishmaniose visceral. Em 55 cães com leishmaniose visceral, naturalmente infectados, observamos a presença de glomerulonefrite em 100% dos animais e detectamos linfócitos T $CD4^+$ em 80% e, concomitantemente, células T $CD8^+$ em 25% no glomérulo (Anexo XVII). Em cinco animais provenientes da mesma área, não se detectou nenhuma célula T nos glomérulos. Quando a ocorrência de proliferação no glomérulo foi pesquisada com a marcação com KI-67, não houve reação na maioria dos casos (Anexo XVIII), dados que indicam que as células são

imigradas para o interior do glomérulo e não produtos de proliferação local. Se há migração de células, a presença de moléculas de adesão é esperada. Tanto em 25 cães com leishmaniose visceral como em cinco controles não infectados foram detectadas ICAM-1 e P-selectina, porém, em maior intensidade nos animais com leishmaniose visceral (Anexo XVII).

Quando há acúmulo de células por processo proliferativo ou inflamatório, ocorre geralmente a participação de apoptose para controle da população celular. Na evidência de infiltrado inflamatório na maioria das amostras (Anexo XV) analisamos na leishmaniose visceral canina também este aspecto. Em 36 cães com leishmaniose visceral naturalmente adquirida observamos menos células em apoptose em glomérulos, expressão não alterada de IL-1alfa mas menor de TNF-alfa do que no controle não infectado (Anexo XVIII).

Os dados em conjunto sugerem que os mecanismos patogênicos das lesões na leishmaniose visceral, com enfoque na glomerulonefrite, são complexos e envolvem imunoglobulinas, por processo diferente de deposição de imunocomplexos, inflamação com migração de linfócitos T, principalmente CD4⁺, provavelmente com a participação de moléculas de adesão ICAM-1 e P-selectina e inibição da apoptose.

D. Discussão geral

As discussões específicas estão em cada artigo e manuscrito e os comentários gerais dentro de cada área no capítulo anterior, portanto, nesta seção pretendo expor, mais do que fazer uma discussão formal, um pouco da minha visão e inquietação em relação aos estudos dos mecanismos imunes e imunopatogênicos nas leishmanioses a partir das nossas observações. Será também um exercício para delinear o prosseguimento das nossas linhas de pesquisa.

Na introdução das teses, freqüentemente lemos que a relação *Leishmania*-hospedeiros vertebrados, onde se inclui o homem, é bem longa e que persiste há muitos séculos. Esta afirmação, que é em geral meramente ilustrativa, convida a uma reflexão profunda e, ao mesmo tempo, nos alerta sobre o campo complexo em que estamos embrenhando. Faz-nos antever o emaranhado de interações e mecanismos desenvolvidos no decorrer desses séculos de convivência.

A grande maioria dos estudos volta-se para a resposta imune específica, supostamente em busca de um mecanismo ou imunógeno que leve à proteção dos indivíduos, que bloqueie a transmissão dos parasitos. A busca nessa área não é equivocada, mas, com o passar do tempo, percebe-se que é muito mais complexa do que aparentava há duas ou três décadas. Mesmo em estudos com camundongos isogênicos, onde as variações são menores ou supostamente previsíveis, as diferentes espécies de *Leishmania* não induzem o mesmo tipo de resposta e os elementos de proteção do hospedeiro diferem com as espécies do parasito. A dicotomia Th1 – Th2 definida por Mosmann e Coffman (1989) e por Locksley et al. (1987) na leishmaniose cutânea por *L. major*, não ocorre na leishmaniose visceral (Anexo XII) e nem na leishmaniose cutânea por *L. amazonensis* (Afonso e Scott, 1993, Lemos de Souza et al., 2000, Soong et al., 1997). A citocina IL-10, considerada fator de suscetibilidade,

foi recentemente vista como importante na persistência da resposta imune protetora nos camundongos resistentes (Belkaid et al., 2002). A citocina IFN- γ considerada fator protetor, foi recentemente relatada como fator de crescimento de amastigotas em macrófagos (Soong et al. 2004). Também na pesquisa direcionada à busca de candidato a vacina contra leishmanioses, fatos inquietantes são relatados. Vacina similar a Leishvacin induz piora da lesão em camundongos (Pinheiro, 2003) e a infecção de *L. braziliensis* exacerba em macacos *Cercopithecus* previamente infectados com *L. amazonensis* e curados espontaneamente (Abdalla e Shaw, comunicação pessoal).

Na pesquisa, é necessário limitar a área de pesquisa para chegar-se a alguma conclusão, mas, ao ampliar o campo de estudo, percebe-se facilmente a relatividade dos conhecimentos e seus condicionantes. Ao mesmo tempo, na leishmaniose, percebe-se quão imbricada é a interação do parasito com o hospedeiro, com o parasito utilizando-se de muitos ou quase todos os elementos ou mecanismos de proteção do hospedeiro a seu favor na evasão. No decorrer desses anos, nas pesquisas realizadas, constatamos esse fato em praticamente todos os passos. Por interesse e também por questão de oportunidade, lidamos com a resposta imune inata e adquirida e mecanismos imunes patogênicos. Observamos o papel fundamental do complemento no desenvolvimento da leishmaniose visceral (Anexo I), a aparente inocuidade do PMN no controle da infecção (Anexo III), a utilização do fator de crescimento do hospedeiro para a sua proliferação (Anexos IV e V) e no estabelecimento da infecção (Anexos V e VII), a infecção de macrófago por leishmânia salvando esta célula hospedeira da morte por apoptose para garantir a sua sobrevivência (Anexo XII) e elementos imunes como patogênicos nas lesões (Anexos XIII a XVII).

A peculiaridade da leishmaniose está na célula que alberga o parasito, o fagócito mononuclear que é um componente do sistema imune inato e também elemento desencadeante da resposta imune específica como célula apresentadora de antígeno e ao mesmo tempo seu efetor. Sendo o macrófago um elemento efetor, a sobrevivência deve ser garantida com mecanismos de evasão e estes devem ser dentro das potencialidades e abrangência funcional desta célula que são muitas. Desta forma os mecanismos de evasão passam por inibição da produção de reativos efetores gerados no macrófago que ocorrem pelo efeito de diversos fatores como TGF- β e provavelmente IGF-I (Anexo IX) e utilização de determinados receptores, p. ex. CR1 e CR3 (Blackwell, 1985, Wright e Silverstein, 1983). Passam por mecanismos que levam à diminuição da eficiência como célula apresentadora de antígeno, diminuindo a expressão de moléculas do complexo principal de histocompatibilidade (Nacy et al., 1983) ou levando à supressão da resposta específica de linfócitos T por diversos mecanismos (ver Anexo XI, XII), ou por expressão de molécula co-estimulatória CTLA-4 em linfócitos T (Gomes et al., 1998). Esta molécula cuja expressão é induzida em leishmaniose visceral, por sua vez, tem papel diversificado, resultando em indução da subpopulação Th1 se a célula for virgem, mas, levando à diferenciação para Th2 se a célula for de memória (Gomes e DosReis, 2001), o que nos leva a especular que na evolução da leishmaniose visceral, com o aparecimento das células de memória, a resposta inicialmente com perfil Th1 possa passar para resposta do tipo Th2 nos modelos em que isto ocorre.

Além dos diversos mecanismos de evasão utilizados pelo parasito para escapar dos elementos inespecíficos e específicos da resposta imune, cada um em seu setor, é importante atentar também para a interação e efeitos recíprocos que vem sendo descritos paulatinamente. Relatam-se PMN induzindo a produção de IL-4 (Tacchini-

Cottier et al., 2000) ou produzindo IFN- γ (Venuprasad et al., 2002), IFN- γ controlando a expressão de gene da fração C3 do complemento (Volanakis, 1995), IL-4 e IL-13 induzindo a produção de IGF-I no macrófago (Wynes e Riches, 2003) ou IFN- γ inibindo a sua produção (Arkins et al., 1995), CD4 solúvel influenciando a migração de PMN (Goto e Gidlund, 1996), atividade do complemento aumentada em camundongos com deleção de CD4 e CD8 (dados pessoais), etc. São aspectos que nem são cogitados na maioria dos estudos no campo da resposta específica. Além disso, relata-se a determinação da resistência por fatores independentes de T em linhagem de camundongo C57BL/6, resistente a *L. major* (Shankar e Titus, 1995).

No estudo da imunidade específica, um aspecto importante é a peculiaridade da resposta, diferença nos elementos efetivos a cada espécie de leishmânia, o que apontamos no nosso trabalho de revisão (Anexo 11) que, no nosso entender, deveria trazer preocupação pela sua implicação nas pesquisas de vacinas contra leishmanioses para uso no nosso meio em que freqüentemente co-existem várias espécies na mesma área.

Dos componentes inatos, destacam-se o complemento e o IGF-I, a nosso ver, o primeiro porque tem efeito patente tanto in vivo como in vitro e é influenciado por vários outros elementos como IFN- γ , moléculas CD4 e CD8. O segundo por ser um fator do hospedeiro que tem efeito direto sobre leishmânia, além de efeito na migração celular (Anexos IV, V, VII), a sua produção e efeito no macrófago e a sua produção modulada por citocinas.

Na área de imunopatologia também, uma atitude de não aceitação do que é tido como conhecimento definitivo pode levar a esclarecimento e descoberta de mecanismos novos como vimos em alguns dos nossos trabalhos (Anexos XIII a XVIII). Detectamos e invocamos a participação de linfócitos T CD4⁺ na patogenia da

glomerulonefrite na leishmaniose visceral canina (Anexos XIII e XVII) e recentemente relatamos dois casos de leishmaniose tegumentar em pacientes aidéticos, um que desenvolveu o quadro cutâneo e outro que exacerbou a lesão com o início do tratamento do HIV e com recuperação dos níveis de células T CD4⁺ (Posada-Vergara et al., 2004), achados que reforçam a convicção da participação dessas células na patogenia.

O parasito, por seu lado, precisa ser estudado com espírito desarmado. Para nós que transitamos na área biomédica, tendemos a observá-lo como um eucarioto como as células do nosso organismo, o que é um grande equívoco. Incursionando na protozoologia propriamente dita por causa do estudo do efeito do IGF-I sobre leishmânia e depois com a observação da apoptose de amastigotas, percebemos a necessidade de um olhar sem preconceito para poder encontrar respostas às nossas indagações. Se pretendemos estudar a resposta protetora do hospedeiro, é imprescindível entender até certa medida a biologia do parasito.

Acreditamos que com o prosseguimento dos nossos estudos possamos contribuir para o esclarecimento de questões pouco abordadas na pesquisa de imunidade inata e na imunopatogenia e entender melhor a interação dos mecanismos específicos e inespecíficos da resposta imune. Estudamos questões que desafiam a nossa curiosidade, mas, mais do que isso, elas irão contribuir para a aquisição de conhecimentos que possam orientar os procedimentos com os pacientes ou gerar produtos para o tratamento ou a prevenção de doenças. A vacina contra leishmanioses que atinja todas as espécies de *Leishmania* é um sonho, mas um sonho que não podemos nunca deixar de sonhar.

E. Conclusões

Avaliando as nossas pesquisas realizadas e aquelas em andamento voltadas para o estudo da participação de diversos elementos e/ou processos, no contexto global da infecção/doença leishmaniose, podemos dizer que obtivemos dados inéditos em vários campos e muito interessantes, principalmente *in vivo*, e podemos concluir que:

- o complemento *in vivo* constitui-se num fator de evasão dos parasitos que determinam a doença visceral;
- a avaliação do papel de células NK é difícil pela sua implicação estreita com o complemento;
- polimorfonucleares neutrófilos *in vivo* aparenta não ter papel decisivo na determinação da evolução da infecção;
- fator de crescimento insulina-símile é um fator importante na leishmaniose e que deva ser explorado na interação com o macrófago e com componentes da resposta imune específica.
- imunossupressão na leishmaniose visceral em hamster parece ser conseqüente a uma superativação prévia dos linfócitos e não por anergia ou apoptose de linfócitos. Por outro lado, macrófagos são protegidos de apoptose, mas, as amastigotas entram em apoptose nas fases finais da infecção.
- sugerimos mecanismos imunopatogênicos inéditos com a participação de imunoglobulinas, linfócitos T e moléculas de adesão na leishmaniose visceral.

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Anexos I, IV, VI, VII, VIII,X, XV

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- I. Laurenti, M.D., Corbett, C.E.P., Sotto, M.N., Sinhorini, I.L. & Goto, H. - The role of complement on the acute inflammatory process in the skin and on host parasite interaction in hamsters inoculated by *Leishmania (Leishmania) chagasi*. Int. J. Exp. Pathol. 77: 15-24, 1996.

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The role of natural killer cells in the early period of infection in murine cutaneous leishmaniasis

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Abstract

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In order to study the role of natural killer (NK) cells during the early period of *Leishmania* infection, BALB/c mice were selectively and permanently depleted of NK cells by injection with ⁹⁰Sr and subsequently infected with *Leishmania (Leishmania) amazonensis* (HSJD-1 strain). ⁹⁰Sr is known to selectively deplete NK cells, leaving an intact T- and B-cell compartment and preserving the ability to produce both interferon alpha and IL-2. This method of depletion has advantages when compared with depletion using anti-NK cell monoclonal antibodies because the effect is permanent and neither activates complement nor provokes massive cell death. In the present study, after one month of treatment with ⁹⁰Sr, the depletion of NK cells was shown by a more than ten-fold reduction in the cytotoxic activity of these cells: 2 x 10⁶ spleen cells from NK-depleted animals were required to reach the same specific lysis of target cells effected by 0.15 x 10⁶ spleen cells from normal control animals. The histopathology of the skin lesion at 7 days after *Leishmania* infection showed more parasites in the NK cell-depleted group. This observation further strengthens a direct role of NK cells during the early period of *Leishmania* infection.

Both innate and specific elements of the immune system contribute to the control or the progression of leishmaniasis. At the beginning of the infection, innate elements have been shown to have an important role in influencing the outcome of the disease. Among them, complement has been shown to contribute to the evasion of the parasite and for visceral dissemination in hamsters infected with *Leishmania (Leishmania) chagasi* (1). It has also been shown that nonimmune natural killer (NK) cells are im-

Key words

- Cutaneous leishmaniasis
- NK cells
- Strontium 90
- *Leishmania (Leishmania) amazonensis*

portant in *Leishmania* infection as a source of IFN γ with the potential to trigger the Th-1 type of immune response in cutaneous leishmaniasis (2,3). In addition, using the mutant beige mice with low NK activity, the direct importance of NK cells in the development of visceral leishmaniasis has been shown (4). Recently, in mice with an intermittent suppression or depletion of NK cells by anti-asialo GM1 or anti-NK1.1 monoclonal antibodies resulted in an increased susceptibility of mice to *Leishmania major* (5).

Figure 1 - In vitro spleen cell NK activity from normal (striped column) or NK-depleted (open column) mice. Spleen cells from control or ^{90}Sr -treated mice ($0.6 \mu\text{Ci/g}$ body weight intraperitoneal injection) were tested in a 4 H ^{51}Cr -release microcytotoxicity assay at the indicated effector to target ratio using YAC-1 as described in Ref. 6. Specific lysis = ((release (cpm) with effector cells - release in medium alone)/(release in distilled water - release in medium alone)) $\times 100$. Data are from two separate experiments yielding similar results. Data represent the mean of 5 animals in each group and the SD was less than 5% of the mean.

In the present study we have used ^{90}Sr to deplete NK cells. This treatment is well established and provides an intense local irradiation of the bone marrow leading to severe bone marrow aplasia with concomitant extramedullary myelopoiesis in the spleen (6,7). The treatment has been shown to lead to a severe and permanent depletion of NK cell activity in the spleen, in the lymph nodes and in the periphery without any noticeable alteration in the T- or B-cell compartment or in the capacity to rapidly produce IL-2 or interferon alpha upon stimulation (6). Here we studied the effect of NK cell depletion by ^{90}Sr on the course of *Leishmania* (*Leishmania*) *amazonensis* infection.

Ten newly weaned BALB/c mice were depleted of NK cells by intraperitoneal injection of ^{90}Sr ($0.6 \mu\text{Ci/g}$ body weight) as

previously described (6). After 30 days (i.e., after elimination of free ^{90}Sr) they were infected subcutaneously in the hind footpad with 5×10^7 stationary phase promastigotes of *Leishmania* (*L.*) *amazonensis* (HSJD-1 strain) characterized by Prof. J.J. Shaw (Instituto Evandro Chagas, Brazil) according to the reactivity to monoclonal antibodies specific for *L. (L.) amazonensis*, *L. (V.) panamensis* and for the subgenus *Viannia*, and also by Dr. S.R. Uliana (Department of Parasitology, ICB, University of São Paulo, Brazil) according to the reactivity to subunit ribosomal DNA probes for *L. amazonensis* and the subgenus *Viannia* (8). Samples were taken to evaluate NK activity of spleen cells at the time of inoculation by a ^{51}Cr release cytotoxic assay of YAC-1 target cells. The level of parasite growth was verified by histopathological analysis of the skin lesion at seven days of infection.

The severe depletion of NK activity was confirmed by the lytic activity against YAC-1 cells in ^{90}Sr -treated animals (Figure 1). The calculated number of cells required in control animals to obtain the same level of lysis of target cells as found in NK-depleted animals (i.e., 7% specific lysis) was shown to be 0.15×10^6 cells versus 2×10^6 spleen cells in NK-depleted animals. This demonstrated that the NK cell activity of ^{90}Sr -

Figure 2 - Histopathology of skin lesions from control or NK-depleted mice infected with *Leishmania* (*L.*) *amazonensis*. Hematoxylin and eosin. Magnification, 40X. A, Mixed inflammatory infiltrate with few parasites is shown in control BALB/c mice. B, Mixed inflammatory infiltrate with more parasites is shown in NK-depleted BALB/c mice.

treated mice was reduced more than ten times. As shown in Figure 2A and B, seven days after infection more parasites were observed in the skin lesion in ^{90}Sr -treated mice. The inflammatory infiltrate characterized mainly by mononuclear cells with few polymorphonuclear neutrophils was similar in both groups.

Previous studies have similarly indicated a role for NK cells in leishmaniasis; however, our study clarified several important points. Several color mutants, including the beige mutant, have been shown to have reduced NK cells compared with their wild type counterpart (9). These mice also have severe alterations in the lysosomal compartment which affect macrophage and neutrophil functions. Therefore, there is uncertainty about the data obtained in beige mice and how the defects in phagocytes could interfere with the susceptibility of these mutant mice to *Leishmania* infection. The use of anti-asialo GM1 or NK1.1 antibodies leads to an intermittent and short-lived depletion of NK cells (5,10) and furthermore, as is the case for any antibody used to deplete cell components *in vivo*, to a rapid activation of

the complement that is known to be important in the initial phase of infection (1). Finally, it is highly likely that the rapid elimination of a sizable portion of the lymphocyte pool by the antibody can cause secondary effects due to complement activity and massive cell death. The ^{90}Sr -treated mice have an advantage since they do not present any apparent change in monocyte function, as shown by the ability to mount a normal T- and macrophage-dependent response to Con A detected by IL-2 production (6).

In a system with a selective depletion of NK cells along with an intact T- and B-cell compartment and with preserved ability to produce both interferon alpha and IL-2, we have shown increased *Leishmania* growth in the skin lesion. We conclude that the present data further support a direct role of NK cells in the early period of *Leishmania* infection.

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Title: Influence of acute inflammatory response in the course of *Leishmania (L.) chagasi* infection in mice

Running Title: Influence of neutrophils on murine visceral leishmaniasis

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ABSTRACT

In visceral leishmaniasis specific and non specific immune responses are important. PMN in vitro are able to kill engulfed *Leishmania* and seemingly can serve as host cells in the early phase of infection. Since it is rare models for in vivo studies of PMN, two strains of mice selected for maximal (AIRmax) or minimal (AIRmin) acute inflammatory reactivity (AIR) according to the ability to recruit PMN during acute inflammatory process were used to analyze the role of PMN in the development of visceral leishmaniasis. When we initially inoculated intraperitoneally 2×10^7 purified amastigotes in BALB/c, AIRmax and AIRmin mice, the parasite burden evaluated at 28 and 56 days post-infection was significantly different among strains; bigger parasite burden was seen in the liver and spleen of AIRmin and AIRmax was similar to BALB/c (susceptible to visceral strain of *Leishmania*), and AIRmin more susceptible than the BALB/c. Different inoculum, 2×10^3 , 2×10^5 and 2×10^7 were tested in BALB/c, DBA/2, AIRmax and AIRmin, 2×10^5 was defined as the best amount to evidence the differences between the strains and significant between male and female AIRmax mice with 2 weeks of infection. With this inoculum parasite burden was determined in different localizations. The parasite in peritoneal liquid was still present at day 3, and the its amount was bigger in AIRmin. In mesenteric lymph node it was present at days 3 and 7 and in liver and spleen the parasite burden was similar in all four strains. Some differences among strains appears at 14 days post infection in the mesenteric lymph node with a peak in BALB/c, DBA/2 and AIRmin and a increase in the liver the organ where the infection mainly establishes in mice. In the spleen the parasite burden started to increase at 14 days and it decreased at 28 days post infection. Our present data suggest that PMN is element that acts destroying parasites

in the initial phase, but, in the evolution, an element that seems to modulate the specific immune response and then, this role seems to be more important in the evolution of leishmanial infection.

INTRODUCTION

Visceral leishmaniasis is caused by members of the *Leishmania donovani* complex (*L. donovani*, *L. infantum*, *L. chagasi*) that transmitted to human by the bite of an infected phlebotomine sand fly vector during a bloodmeal. In the mammalian host, the organism multiplies within mononuclear phagocytic cells resulting in infection/disease. In visceral leishmaniasis specific and inespecific immune response participate in the resolution or evolution of the disease. The role of lymphocytes in the specific defence against *Leishmania* has been well established, but the part played by the initial events of response, specially polymorphonuclear neutrophil (PMN) cells is much less studied even though that is crucial for the survival of the parasites (Solbach and Laskay, 2000, Rousseau et al., 2001). PMN are attracted to the site of infection within 24h (Sunderkotter et al., 1993), and this rapid attraction of PMN suggests that these cells may serve as host for *Leishmania* in the early phase (Laufs et al., 2002). Although the uptake of *Leishmania* by macrophage has been characterized in detail, no such comprehensive study with PMN has been carried out so far. Studies with PMN in vitro show its leishmanicidal effect (Chang, 1981, Pearson and Steigbigel, 1981). Moreover, it has other controversial studies that indicates a role of PMN in the induction of IL-4 production, implying them in the susceptibility to the infection in cutaneous leishmaniasis (Tacchini-Cottier et al., 2000). Other studies in vivo, with depletion of PMN with antibodies, also show its role in the control of the infection in murine leishmaniasis (Rasp et al., 1998, Rousseau et al., 2001). However, the depletion of PMN using antibodies lead to a great activation of the complement cascade that it is known to have important role in the initial phase of the infection. It is important to have an alternative model to study the role of PMN in vivo. It has been

reported that susceptible BALB/c and resistant C57BL/6 mice present different acute inflammatory migration dynamic, BALB/c having sustained level of neutrophils whereas in C57BL/6 is transitory (Beil, 1992), but to study the role of PMN is not adequate since they also present differences in specific immune parameters. It has been developed two strains of mice genetically selected for high or low acute inflammatory response through a selective breeding based on the capacity to mount strong (maximal) or weak (minimal) acute inflammatory responses to polyacrylamide beads (Biogel) whereby its intensity measured by the cell and protein concentration of the local exudates and BALB/c and DBA/2 mice, respectively, used as parameters of susceptibility and resistance to visceral leishmaniasis, and standard of response of strain unselected genetically for the acute inflammatory response. The selection of Acute Inflammatory maximal (AIRmax) and minimal (AIRmin) starting from a founding population resulting from the intercross of eight inbred mouse strain, which display a continuous range of inflammatory responses. Using these mouse strains, the purpose of this study is to search for the role of PMN in the development of visceral leishmaniasis.

MATERIAL AND METHODS

Animals:

Male, 45-60 days old outbred hamsters (*Mesocricetus auratus*) and 60-90 days old mice. BALB/c mice were obtained from the Animal Breeding Facility of Faculdade de Medicina da Universidade de São Paulo, DBA/2 mice from the Animal Facility for Isogenic Mice of Instituto de Biociências da Universidade de São Paulo and the AIRmax and AIRmin mice, from generation F₃₇ and F₃₈ of selection, were obtained from the Laboratório de Imunogenética of Instituto Butantan. All the animals had

been maintained Animal Facility of Tropical Medicine Institute of São Paulo of University of São Paulo during the experiments. Mice of both sex were used in experiments indistinctly.

Experimental Infections:

Leishmania (L.) chagasi amastigotes (MHOM/BR/73/strain 46) were obtained as according to Dweyer (1976). Briefly, the spleens were homogenized in cold RPMI 1640 medium (GIBCO, USA), the cellular suspension maintained for 10 minutes on ice, the amastigotes, filtered, processed 4 times through fine gauge needle (24G) and spun at 250g for 10 minutes. The supernatant was again spun at 250g for 10 minutes and then the resulting supernatant was spun at 2100g for 25 minutes. The pellet was resuspended in RPMI 1640 medium and the concentration of parasites adjusted to 2×10^8 /ml in RPMI 1640 medium. Depending on the experiments, mice were injected either with 2×10^3 , 2×10^5 or 2×10^7 purified amastigotes in RPMI 1640 medium by intraperitoneal route (100 μ L) and after different time periods they were euthanized by cervical dislocation under general anesthesia to collect the samples.

Parasite burden in the liver and spleen was determined according to Stauber (1958)

Quantification of the parasite burden by the Limiting Dilution Assay (LDA):

Parasite burden was quantified in peritoneal liquid, draining mesenteric lymph node, liver and spleen tissues by LDA, using a modified method of Lima et al. (1997). The total cell was counted in the peritoneal liquid and lymph nodes, and the weight was determined for the liver and spleen. Samples were processed as described above and suspended in complete Schneider's medium (Sigma, USA). Threefold serial dilutions of homogenized tissue suspension were then plated in ten replicate wells per dilution into 96-well tissue culture plates (TPP, Switzerland), sealed with Parafilm®, and incubated at 26 °C for 20 days. The experiments were followed observing the plates

under an inverted light microscope (Zeiss, German) and read at ELISA reader (Multiskan MCC/340, USA) at 450 nm, considering negative when OD was <0.05 (OD value of complete Schneider's medium incubated for 20 days at 26 °C). The assay was analyzed by scoring the number of positive wells for parasite growth, with both inverted microscope observation and OD value obtained and processed by ELIDA Program, utilizing a single-hit Poisson model equation and statistical method of chi-squared minimization (Taswell, 1986).

Statistical analysis: Results were expressed as the mean and standard error of the mean and analysed using Students *t*-test or Mann-Whitney test, and differences were considered significant when *p* was <0.05 .

RESULTS

When we initially inoculated 2×10^7 purified amastigotes in BALB/c, AIRmax and AIRmin mice, parasite burden was evaluated at 28 and 56 days post-infection in the splenic and hepatic tissue imprints. Significant difference between AIRmax and AIRmin mice was observed with bigger parasite burden in the liver and spleen of AIRmin mice. It was bigger in the liver when compared with that of spleen. The parasite burden of the AIRmax mice was similar to the BALB/c that is susceptible to visceral strain of *Leishmania*, and AIRmin more susceptible than the BALB/c (data not shown). We proceeded the study carrying out protocols to evaluate the infection at 14 and 28 days post infection using different inoculum.

Determination of the amount of *L.(L.) chagasi* amastigotes for infection of mice.

BALB/c, DBA/2, AIRmax and AIRmin male and female mice were inoculated intraperitoneally with 2×10^3 , 2×10^5 and 2×10^7 purified amastigotes and parasite

burdens of the infected liver and spleen were determined at 14 (Fig.1A, 1C) and 28 days (Fig.1B, 1D) post infection in hepatic (Fig.1A, 1B) and splenic (Fig. 1C, 1D) imprints with counting by Stauber's method. BALB/c and DBA/2 mice were used, respectively, as parameters of susceptibility and resistance to visceral leishmaniasis. In this experiment, significant differences were observed in the liver between AIRmax and AIRmin at 14 days post infection with inoculation of 2×10^7 amastigotes ($p < 0,001$) (Fig. 1A) and at 28 days post infection with inoculation of 2×10^3 and 2×10^5 amastigotes in the same mice ($p < 0,01$) (Fig. 1B). However, in the spleen, it was observed significant difference between AIRmax and AIRmin ($p < 0,001$) with inoculation of 2×10^5 amastigotes at 14 days post infection (Fig. 1C). The inoculum of 2×10^5 amastigotes was defined as the best amount to evidence the differences among mouse strains. It was also observed significant difference between male and female AIRmax mice with inoculation of 2×10^5 amastigotes at 14 days post infection ($p < 0.05$) (data not shown).

Determination of the parasite burden by the Limiting Dilution Assay.

BALB/c, DBA/2, AIRmax and AIRmin mice were inoculated intraperitoneally with 2×10^5 purified amastigotes and parasite burdens of the in the peritoneal liquid, draining mesenteric lymph node, liver and spleen of *Leishmania(L.) chagasi*-infected BALB/c, DBA/2, AIRmax and AIRmin mice at 3, 7, 14 and 28 days of infection.

Stauber's method is not sensitive enough to evaluate parasite burden in early times of the infection, therefore we proceed using Limiting Dilution Assay (LDA).

Results presented in Fig. 2A and 2B show that the parasite burden in peritoneal liquid was still present at day 3, but it started to decline sharply after that in all four studied groups. Bigger amount of parasites was detected in AIRmin (Fig. 2B) and BALB/c strains (Fig. 2A). The parasite burden in AIRmax (Fig. 2B) was smaller

which we can attribute to the role of polymorphonuclear leukocytes. Surprisingly, the parasite burden at days 3 and 7 in mesenteric lymph node, the liver and spleen was similar in all four strains. In the inbred BALB/c and DBA/2 mice and AIRmin strain draining lymph node started rising moderately thereafter, the increase being more marked at 14 days post infection and started declining retaining basal amount of parasite even at post infection day 28 day, very different of that was observed in AIRmax mice. It is observed, on the other hand, some differences among lineages at 14 days post infection in the liver (Fig. 2E, 2F), the organ where the infection mainly establishes in mice. In the spleen the parasite burden started to increase at 14 days post infection and decreased at 28 days post infection (Fig. 2G, 2H).

DISCUSSION

Leishmania spp. are inoculated into the skin of the mammalian host during the bite of an infected sand fly. Infection may be controlled by the host without the development of overt clinical symptoms or active disease. A crucial step in the initial phase of *Leishmania* infection is evasion from the innate response of the host and establishment in macrophages. PMN is one of first elements that take part in the initial barrier to avoid the progression of the infection, being recruited to the site of inoculation in few minutes. The depletion of PMN using antibodies with consequent activation of complement cascade is an obstacle to use animals with this treatment, therefore the choice of an animal model without this interference is crucial for the development of the study. For this reason strains of genetically selected mice for Acute Inflammatory Response, AIRmax and AIRmin turn to be an interesting option to understand the non especific immune mechanisms of modulation of the susceptibility and resistance to

infections. These strains during the selection, had been bred with similar extreme phenotypes, of high reactivity and low response (Ibañez et al., 1992) and the evaluation of the reaction in the inflammatory exsudate along of the generations was determined by the number of PMN and concentration of proteins after Biogel particle inoculation in the subcutaneous tissue (Stiffel et al., 1987, Ibañez et al., 1992). To better understand the initial events of response and the involvement of PMN in the control and evolution of murine visceral leishmaniasis we quantified the parasite burden in the peritoneal liquid, draining mesenteric lymph node, liver and spleen in model inbred mice strains (BALB/c and DBA/2) and mice strains genetically selected for high or low acute inflammatory response (AIRmax and AIRmin).

Previous studies had been carried out to evaluate the evolution of *Leishmania (L). chagasi* infection in different mouse strains, with 2×10^7 purified amastigotes inoculated intraperitoneally in BALB/c, AIRmax and AIRmin mice and the parasite burden evaluated at 28 and 56 days post-infection, and the spleen and the liver were analyzed. Significant difference between AIRmax and AIRmin mice was observed with bigger parasite burden in the liver and spleen of AIRmin mice. It was bigger in the liver when compared with that of spleen. The parasite burden of the AIRmax mice was similar to the BALB/c that is susceptible to visceral strain of *Leishmania*, and AIRmin more susceptible than the BALB/c (data not shown). To proceed carrying out protocols, we evaluated the infection at 14 and 28 days post infection using different inoculum, 2×10^3 , 2×10^5 and 2×10^7 purified amastigotes. Initially it was shown to be 2×10^5 the best amount to evidence the differences between the lines and sex in AIRmax mice. With this inoculum the parasite burden increased in the BALB/c, AIRmax and AIRmin in both the liver and the spleen between 14 and 28 days post infection. The parasite burden in the AIRmin mice tended to be bigger after

14 and 28 days of infection and revealed to be even more susceptible than the BALB/c mice, suggesting the existence of compensatory mechanism, if we admit this leishmanicidal role of these cells in the initial phase of the infection. This compensatory mechanism could previously be the early migration of mononuclear for the site of the inflammation in this strain of mouse. And the lower-dose model is thus representative (at least at the visceral level) of most human infections, in which the early parasite replication is controlled by the induction of a protective immune response, and the infection remains subclinical (Badaro, et al. 1986, b). Using the dose inoculum of 2×10^5 purified amastigotes, we studied the peritoneal liquid, draining mesenteric lymph node, liver and spleen at 3, 7, 14, 28 days post infection. At 3 days post infection, that we can be almost considered as final phase of interactions exclusively of inespecíficos elements and at 7 days where the specific immune response would gradually be activated, beyond the times of 14 and 28 days post infection. In this analysis, in fact, greater amount of parasites in the peritoneal liquid in day 3 in the AIRmin mice was evidenced, what we can attribute to the lesser amount of PMN. However, the parasite burden in days 3 and 7 in draining mesenteric lymph node, liver and spleen, is very similar in all four strain. In the inbred strain and AIRmin mice, parasite burden in lymph node started increase moderately thereafter, and the increase being more marked at 14 days post infection what start to decline retaining basal amount of parasite even at post infection at day 28. This profile was very different from that it observed in the AIRmax mice. It is observed, on the other hand, some difference between the strain at 14 days of infection in the liver, the organ where the infection mainly establishes in mice. In the spleen the parasite burden started to increase at 14 days post infection and decrease at 28 days post infection .

The early influx of neutrophils into the infected tissue has been reported to be associated with the development of disease after *L. major* infection in mice (Tacchini-Cottier et al. 2000). However, the mechanism by which PMN promote disease development has remained unclear. Laufs et al.(2002) demonstrated the intracellular survival of *L. major* in PMN, therefore, can serve as host cells for leishmania in the early phase of infection. according to these authors, rapid recruitment of PMN is beneficial for the survival of parasites.

Our present data suggest that PMN is an element that acts destroying parasites in the initial phase, but, in the evolution, an element that seems to modulate is the specific immune response and this role seems to be more predominant during infection.

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Figure Legends

Figure 1. Parasite burden of *L.(L.) chagasi*-infected BALB/c, DBA/2, AIRmax and AIRmin mice in the liver (A) at 14 day and (B) at 28 day post infection, and in the spleen (C) at 14 day and (D) at 28 day post infection. Mice were infected intraperitoneally with purified suspension of 2×10^3 , 2×10^5 and 2×10^7 *L.(L.) chagasi* amastigotes. Results were expressed in mean $\pm$ standard error of the mean of seven mice per group and comparisons by Student's *t* test between AIRmax and AIRmin mice resulted in significant difference in the suspension 2×10^7 (** $p < 0,001$) (A) and 2×10^3 and 2×10^5 (** $p < 0,01$) (B) and 2×10^5 *L.(L.) chagasi* amastigotes (** $p < 0,001$)(C).

Figure 2. Kinetics of visceral parasite burden in *L.(L.) chagasi*-infected BALB/c, DBA/2, AIRmax and AIRmin mice. Mice were infected intraperitoneally with purified suspension 2×10^5 *L.(L.) chagasi* amastigotes. The parasite burden in the (A and B) peritoneal liquid, (C and D) draining mesenteric lymph node, (E and F) liver and (G and H) spleen was determined by quantitative limiting diluting assay and analyzed by the ELIDA Program. Results were expressed in mean $\pm$ standard error of the mean of three mice per group and comparisons between AIRmax and AIRmin mice by Mann-Whitney test resulted no differences significances between mice.

Figure 1

Figure 2

Insulin-like growth factor I is a growth-promoting factor for *Leishmania* promastigotes and amastigotes

(tyrosine phosphorylation/protozoa/binding/leishmaniasis)

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ABSTRACT Leishmaniasis are diseases caused by protozoa of the genus *Leishmania* that affect more than 20 million people in the world. The initial phase of the infection is fundamental for either the progression or control of the disease. The *Leishmania* parasites are injected in the skin as promastigotes and then, after been phagocytized by the host macrophages, rapidly transform into amastigotes. In this phase different nonspecific cellular and humoral elements participate. We have shown previously that insulin-like growth factor (IGF)-I that is constitutively present in the skin induces growth of *Leishmania* promastigotes. In the present paper we show further evidence for the importance of this factor: (i) IGF-I also can induce a growth response in *Leishmania* (*Leishmania*) *mexicana* amastigotes; (ii) IGF-I binds specifically to a putative single-site receptor on both promastigotes and amastigotes; (iii) IGF-I induces a rapid tyrosine phosphorylation of parasite proteins with different molecular mass in promastigotes and amastigotes of *L. (L.) mexicana*; and, finally, (iv) the cutaneous lesion in the mice when challenged by IGF-I-preactivated *Leishmania* (*Viannia*) *panamensis* is increased significantly because of inflammatory process and growth of parasites. We thus suggest that IGF-I is another important host factor participating in the *Leishmania*–host interplay in the early stage during the establishment of the infection and presumably also in the later stages.

Leishmaniasis are diseases caused by protozoa of the genus *Leishmania* that affect more than 20 million people in the world (1). The infectious cycle in the vertebrate host is initiated when *Leishmania* promastigotes are injected into the skin by the insect vector. In the host the promastigotes transform into amastigotes inside the macrophages where they continue proliferating. Immunity in leishmaniasis is considered mainly to be T cell-mediated (2, 3), but more and more nonspecific factors acting in the early stage of the infection have been considered as important for the outcome of the infection (3, 4). Besides complement, polymorphonuclear neutrophils, and eosinophils (2, 5), other growth factors or cytokines such as granulocyte-macrophage colony-stimulating factor, transforming growth factor β , and tumor necrosis factor (6–9) have been reported to participate in this phase. Macrophages and natural killer cells also have been considered important in this phase as source of interleukin-12 and interferon γ , respectively, and thus guiding the preferential activation of T helper 1 or T helper 2 subpopulation of CD4+ T cells (10, 11).

Insulin-like growth factors (IGFs) are evolutionary well conserved polypeptides with an approximate molecular mass of 7.5 kDa. The most abundant forms are IGF-I and -II. They are detectable both in circulation and in tissues, mainly

associated to IGF-binding proteins. Most cell types have the ability to produce IGFs although the main site of production is the liver. Upon stimulation several cell types, including monocytes and mitogen-stimulated T cells, display an enhanced production of IGF-I (for excellent reviews see refs. 12–14). Functionally, IGFs have been shown to affect cell metabolism (e.g., glucose) and to be an important endocrine growth and differentiation factor in inflammation, immune activation, and wound healing (14–16). In the skin it is possible to detect a constitutive production of IGF-I, and an increase in the mRNA level of IGF-I at the site of the lesion has been reported when a wound is inflicted (17). In the macrophages, habitat of *Leishmania* amastigotes, immunoreactive IGF-I has been detected (18). Therefore, it is likely that IGF-I is one of the first growth/growth-inducing factors that the *Leishmania* promastigotes encounter in the skin soon after their transmission and that amastigotes interact with within the macrophages. We have shown previously that IGF-I but not IGF-II induces a growth response of promastigotes of several species of *Leishmania* (19). In the present report we have extended these studies and show that IGF-I also induces a growth response in *Leishmania* amastigote-like forms. IGF-I has a relatively high specific binding to both forms and induces a stage-specific phosphorylation of tyrosine residues of respective parasite proteins. Finally, we show that *Leishmania* promastigotes preactivated with IGF-I greatly enhance the size of the lesion and the number of intact parasites when injected *in vivo*.

MATERIALS AND METHODS

Mice. Six- to 10-week-old inbred BALB/c strain mice of both sexes were obtained from our own breeding facility at the Medical School at the University of São Paulo, Brazil.

Parasites. *Leishmania* (*Viannia*) *panamensis* (HSJD-1 strain) (20) and *Leishmania* (*Leishmania*) *amazonensis* (IOC-L 185 strain, WHOM/BR/OO/LTBOO16) were used. The established axenic culture of amastigote-like forms of *Leishmania* (*Leishmania*) *mexicana* (MNYC/BZ/62/M379), originally from D. G. Russel's laboratory (Washington University School of Medicine), was kindly provided by S. C. Alfieri (University of São Paulo) (21). *Leishmania* (*Leishmania*) *mexicana* promastigotes were derived from this strain. The promastigotes were maintained in NNN medium overlaid by RPMI 1640 medium (GIBCO) with 10% heat-inactivated fetal calf serum (FCS) (WL. Imunoquímica, Rio de Janeiro, Brazil) at 25°C, with periodical passage in BALB/c mice. The parasite culture was expanded in RPMI 1640 medium with 10% FCS and grown until stationary phase for experiments. After washing, for injection in mice the concentration was adjusted to 2×10^8

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Abbreviation: IGF, insulin-like growth factor.

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parasites per ml in RPMI 1640 medium. The amastigote-like forms were maintained at 33°C in M-54 medium, pH 6.0, consisting in medium 199 with Hanks' salts (GIBCO/BRL) supplemented with 2.5 mg/ml glucose/5 mg/ml trypticase/750 µg/ml L-glutamine/20 µg/ml hemin/5 µg/ml gentamicin/20% of heat-inactivated FCS and 25 mM Hepes (21).

Reagents. Recombinant human IGF-I and -II, produced in *Escherichia coli*, was a kind gift from M. Lake, Kabi-Pharmacia, Sweden (for details on purification see ref. 15) or purchased from R & D Systems. Monoclonal anti-phosphotyrosine antibody (22) was kindly provided by R. I. Schumacker (University of Sao Paulo, Brazil). ¹²⁵I (100 mCi/ml), ¹²⁵I-IGF (6.6 µCi/µg), ¹²⁵I-protein A (35 µCi/µg), and [³H]thymidine (2 Ci/mmol), and ¹⁴C-Rainbow molecular weight markers were purchased from Amersham. Phenylmethylsulfonyl fluoride, aprotinin, leupeptin, sodium pyrophosphate, sodium fluoride, sodium orthovanadate, tris(hydroxymethyl)amino-methane (Tris), Triton X-100, and BSA RIA grade (with no detectable amounts of IGF contamination as determined by a radioreceptor assay; Daniel Gianella Neto, personal communication) were from Sigma. Enzymobeads for lactoperoxidase labeling were purchased from Bio-Rad. Mouse monoclonal anti-phosphotyrosine IgG3 antibody secreted by the hybridoma cell line FB2 and purified by affinity chromatography (22, 23) was kindly provided by R. I. Schumacher (University of Sao Paulo, Brazil).

Measurement of Proliferative Responses to IGF-I and -II. Stationary-phase *L. (L.) amazonensis* and *L. (L.) mexicana* promastigotes and *L. (L.) mexicana* amastigote-like forms after washing in 0.01 M PBS, pH 7.2, had the concentration adjusted to 10⁷ parasites/ml in RPMI 1640 medium without FCS. Parasites (2 × 10⁵/well) in 96-well flat-bottomed microculture plates (Costar) were cultured in the presence of recombinant IGF-I or IGF-II (1–50 ng/ml) and in the presence of FCS (4–8%) as indicated in the figures. The cultures were pulsed with [³H]thymidine (1 µCi/well) either at time 0 (for 24-hr culture) or after 24 hr (for 48-hr culture) and incubated either at 25°C (promastigotes) or 33°C (amastigote-like forms) for an additional 24 hr. The parasite cultures were then harvested onto glass-fiber filter paper (Flow Laboratories) by using a cell harvester (Titertek, Huntsville, AL). The incorporated radioactivity was assessed by liquid scintillation in a beta counter (Beckman). Besides [³H]thymidine incorporation, the proliferation of *L. (L.) mexicana* amastigote-like forms grown in the presence of 0, 10, or 50 ng/ml of IGF-I in RPMI medium with 4% FCS also was evaluated after 24 hr, counting the actual number of amastigotes under light microscope. The results are expressed either in cpm or as parasite index = number of parasites in cultures with IGF-I/number of parasites in cultures without IGF-I.

Radioiodination. When commercial ¹²⁵I-IGF-I was not available, 0.5 µg of recombinant IGF-I was labeled with 0.5 mCi ¹²⁵I by lactoperoxidase using Enzymobeads according to the protocol supplied by the manufacturer (Bio-Rad). The labeling resulted in a specific activity of 30–40 µCi/µg, and the free ¹²⁵I represented less than 1% as determined by trichloroacetic acid precipitation.

Determination of IGF-I Binding to Parasites. Homologous displacement was performed essentially as described in ref. 25. To each tube containing 1 ml of 6 × 10⁶ *L. (L.) mexicana* promastigotes or amastigote-like forms in ice-cold buffer (100 mM Hepes/120 mM NaCl/5 mM KCl/1.2 mM MgCl₂/1 mM EDTA/0.2% BSA, pH 7.4) 25 µl of 0.6 nM ¹²⁵I-IGF-I was added in the absence or in the presence of increasing amounts of unlabeled IGF-I ligand and left overnight at 4°C. After incubation the samples were immediately centrifuged at 10,000 × g for 3 min, the supernatants were collected, and the pellet was washed twice with ice-cold PBS, resuspended in 0.2 M NaOH, and incubated for 1 hr at 37°C with mild agitation. Both the supernatants and the lysates were measured for

radioactivity in a gamma counter (Beckman). The data were analyzed by the LIGAND computer program (24).

Analysis of Tyrosine Phosphorylation. Detection of tyrosine phosphorylation was done essentially as described in refs. 22 and 24. *L. (L.) mexicana* promastigotes or amastigote-like forms (2 × 10⁸ parasites/ml) were stimulated with IGF-I (50 ng/ml) for 1, 5, and 25 min in RPMI 1640 medium in the presence of 4% FCS. The parasites then were lysed in 1 ml of lysis buffer (20 mM Tris-HCl, pH 7.4/10 mM sodium pyrophosphate/50 mM sodium fluoride/2 mM sodium orthovanadate/1 µg/ml leupeptin/1 µg/ml aprotinin/1 µM phenylmethylsulfonyl fluoride/1% Triton X-100) for 30 min on ice. Thereafter, the solubilises were centrifuged at 12,000 × g for 10 min, and an aliquot of the supernatants was adjusted to the same protein concentration and separated on a 7% SDS/PAGE gel under reducing conditions. Then the proteins were electrotransferred by using a semidry blotter (Bio-Rad) onto nitrocellulose membranes (GIBCO), blocked for 2 hr with 5% BSA in 20 mM Tris-buffered saline (TBS), pH 7.4, and thereafter incubated with 5 µg/ml of a monoclonal anti-phosphotyrosine antibody for 18 hr. After extensive washing with TBS/0.05% Triton X-100, 4 µCi of ¹²⁵I-protein A was added and allowed to react for 1 hr. The membranes again were washed extensively, and the bands representing tyrosine-phosphorylated proteins were detected by autoradiography by using X-Omat-AR film (Kodak) and cassette with enhancing screen (Amersham). Laser densitometric analysis was done by using an Ultrascan XL (LKB).

Analysis of the *in Vivo* Effect of IGF-I. *L. (V.) panamensis* promastigotes were preincubated with or without 50 ng/ml of IGF-I in PBS for 5 min. Thereafter, 10⁷ stationary phase promastigotes in 50 µl were injected in the right hind-footpad of age- and sex-matched BALB/c mice (five in each group). The growth of the lesion was monitored at indicated time points by measuring the thickness of the footpad using a dial caliper (Mitsutoyo, Tokyo, Japan). The contralateral footpad of each animal injected with PBS represented the control value and the swelling calculated as: thickness of the right footpad – thickness of the left footpad. The data were analyzed by Mann-Whitney test.

Quantitative Morphometric Analysis. At 24 and 48 hr and 7 and 30 days postinfection (PI), five animals from each group were killed and the specimens from the inoculation site were taken for quantitative morphometric analysis. The skin specimens were fixed in 0.01 M phosphate-buffered 4% paraformaldehyde and embedded in plastic resin (Technovit 7100). Semithin sections (1 µm) were obtained in 11800 Pyramitone (LKB) and stained with hematoxylin-eosin. The morphometric analysis was made on three different levels of semithin section per animal by using a graticule eyepiece in an area of 0.01 mm² with an Olympus planapochromatic immersion objective lens (×100). In these levels 900 cells were counted, where each cell type constituted at least 10% of the total to keep the relative SE below 0.1% as detailed in ref. 25. Samples from five experimental animals were analyzed in each of these points, and the data were submitted to the Mann-Whitney test.

RESULTS

***In Vitro* Growth Response of *Leishmania* Amastigotes to IGFs.** We have shown previously the growth-inducing effect of recombinant IGF-I (but not IGF-II) on various *Leishmania* promastigotes (19). To analyze whether amastigotes could respond to IGF-I or -II we used cell-free amastigotes or so-called amastigote-like forms derived from the promastigotes of *L. (L.) mexicana* and adapted to axenic culture conditions (21). These amastigotes showed a growth response similar to the promastigotes as shown both by [³H]thymidine incorporation and actual increase in the parasite number (Fig. 1A–C). As for promastigotes, this growth-inducing effect was

observed under suboptimal concentration of fetal calf serum, i.e., concentration where no spontaneous proliferative response occurs. The growth response occurred during the first 24 hr in both promastigotes and amastigote-like forms, and no further growth was detected in the following 24 hr (data not shown).

FIG. 1. (A) *In vitro* proliferative response of *Leishmania* (*L.*) *amazonensis* and *Leishmania* (*L.*) *mexicana* promastigotes to IGF-I. Stationary-phase *Leishmania* (*L.*) *amazonensis* and *Leishmania* (*L.*) *mexicana* promastigotes were tested for proliferative response ($[^3\text{H}]$ thymidine incorporation) with IGF-I in the presence of 4% FCS: control (open bar); with 50 ng/ml IGF-I (hatched bar). Each point represents data from triplicate determinations with less than 5% SD of the mean. (B) *In vitro* proliferative response of *Leishmania* (*L.*) *mexicana* amastigote-like forms to IGF-I ($[^3\text{H}]$ thymidine incorporation). Stationary-phase *Leishmania* (*L.*) *mexicana* amastigote-like forms were tested for proliferative response with IGF-I in the presence of 0% FCS (circle), 4% FCS (square), or 8% FCS (triangle). (C) *In vitro* proliferative response of *Leishmania* (*L.*) *mexicana* amastigote-like forms to IGF-I (parasite counting). Stationary-phase *Leishmania* (*L.*) *mexicana* amastigote-like forms were tested for proliferative response to IGF-I in the presence of 4% FCS. Parasite index = number of parasites in cultures with IGF-I/number of parasites in cultures without IGF-I. For further details see *Materials and Methods*.

Analysis of Specific Binding of IGF-I to *Leishmania* Promastigotes and Amastigote-Like Forms. To analyze the specific binding and the characteristics of interaction between IGF-I and *Leishmania*, we performed homologous displacement analysis by using ^{125}I -labeled and unlabeled IGF-I (cold). Fig. 2 shows one of two experiments that yielded similar curves for the analysis of the binding of IGF-I to *L. (L.) mexicana* promastigotes and amastigote-like forms. IGF-I binding to amastigote-like forms consistently was higher than to promastigotes, although both forms presented a high to medium affinity binding. Other calculated values are shown in Table 1 and show a remarkably high number of specific receptors for IGF-I. The analysis showed a single-site binding for IGF-I on both promastigotes and amastigote-like forms.

IGF-I-Mediated Tyrosine Phosphorylation in *Leishmania* Promastigotes and Amastigote-Like Forms. To further strengthen the link between IGF-I and the induced proliferative response in *Leishmania* parasites we studied the time-dependent induction of protein phosphorylation occurring at the tyrosine residues upon stimulation with IGF-I for 1–25 min. This was done by immunoblotting parasite proteins with a mAb recognizing phosphotyrosine. IGF-I induced tyrosine phosphorylation of proteins both in promastigotes and amastigote-like forms of *L. (L.) mexicana* from 1-min stimulation. Promastigotes showed strong tyrosine phosphorylation of a 185-kDa protein and some less-pronounced lower-molecular-mass bands while amastigote-like forms showed tyrosine phosphorylation of 60- and 40-kDa proteins (Fig. 3 A and B).

FIG. 2. Determination of the specific binding of IGF-I to *Leishmania* (*L.*) *mexicana* promastigotes and amastigote-like forms. The binding of IGF-I to *Leishmania* (*L.*) *mexicana* promastigotes (A) and amastigote-like forms (B) were analyzed by homologous displacement as described in *Materials and Methods*.

Table 1. Physical constants and the number of receptors for the binding of IGF-I to *Leishmania (L.) mexicana* promastigotes and amastigote-like forms

Parameter	Promastigotes	Amastigotes
K_a , liter/mol	3×10^7	1×10^7
K_d , mol/liter	4×10^{-8}	10×10^{-8}
Receptor number/parasite	2×10^6	6×10^6

The values were obtained from the computer program LIGAND as described in *Materials and Methods*.

In Vivo Effect of IGF-I on *Leishmania* Infection. Finally, to evaluate the importance of IGF-I for the establishment of the *Leishmania* infection *in vivo* we used a *L. (V.) panamensis*-induced leishmaniasis model that we characterized recently (21, 26). We infected susceptible BALB/c mice with *L. (V.) panamensis* with or without IGF-I. This was done by preincubating and preactivating the parasite with IGF-I (50 ng/ml) for 5 min before injection in the hind footpad. This procedure resulted in significantly increased foot swelling from 21 days and onward postinfection. One of two independent experiments showing a significant difference between the IGF-I-activated *Leishmania*-injected and control groups is shown in Fig. 4. We also made morphometric analysis in the skin lesions in the acute phase at 24 and 48 hr PI and later at 7 and 30 days PI. Counting the number of intact parasites/0.01 mm², the density of parasites was higher in mice injected with IGF-I-activated parasites (Fig. 5).

FIG. 3. (A) IGF-I-induced tyrosine phosphorylation. Analysis of IGF-I-induced tyrosine phosphorylation in *Leishmania (L.) mexicana* promastigotes (lanes a–d) and amastigote-like forms (lanes e–h) was done by immunoblotting with a monoclonal antiphosphotyrosine antibody. Stimulation time: 0, lanes a and e; 1 min, lanes b and f; 5 min, lanes c and g; and 25 min, lanes d and h. (B) Quantitative analysis of the IGF-I-induced tyrosine phosphorylation in *Leishmania (L.) mexicana* amastigote-like forms. Evaluation of the phosphorylation in *Leishmania (L.) mexicana* promastigotes at 0 (broken line) and 5 min (solid line) of IGF-I induction was performed by using laser densitometry.

FIG. 4. *In vivo* development of the cutaneous lesions after challenge with *Leishmania (V.) panamensis* promastigotes preincubated with IGF-I. *Leishmania (V.) panamensis* promastigotes preincubated with 50 ng/ml of IGF-I (triangles) or without IGF-I (circles) were injected in the hind footpad, and the lesion size (mean $\pm$ SEM) was determined as stated in *Materials and Methods*.

DISCUSSION

Directly after transmission into the vertebrate host, the *Leishmania* promastigotes encounter drastic changes of physiological nature, such as changes in temperature and pH, and a highly oxidative milieu (ref. 27; R. Chebabo, unpublished data). The parasite also will encounter host-derived humoral factors, including growth factors (5, 7), that can be beneficial for the host leading to the elimination of the parasites, but it is also possible that they can directly or indirectly favor the parasite survival in the initially hostile host environment. IGFs, particularly IGF-I, could belong to this category of factors since IGF-I is an evolutionary well conserved polypeptide, constitutively present in the skin, and known to be associated with inflammation and wound healing (12–17). Moreover, the effect of IGFs has been reported on the protozoan *Giardia lamblia* (28) and on cells from primitive vertebrates such as *Cottus scorpius* (sea scorpion), *Raja clavata* (ray), and *Myxine glutinosa* (Atlantic hagfish) (29). Further-

FIG. 5. Density of intact parasites (mean $\pm$ SEM) in the skin lesion of BALB/c mice infected with *Leishmania (V.) panamensis* promastigotes with and without preincubation with IGF-I. *Leishmania (V.) panamensis* promastigotes preincubated with 50 ng/ml of IGF-I (open column) or without IGF-I (solid column) were injected in the hind footpad, and the skin samples taken from five animals from each group at different time points were submitted to morphometric analysis as stated in *Materials and Methods*.

more, we previously have analyzed and shown the growth-inducing effect of IGF-I on promastigotes of different species of *Leishmania* (19).

In this study we extended our previous observation analyzing the effect of IGF-I on amastigotes and also how parasites already stimulated by IGF-I would behave during an experimental infection *in vivo*.

In the presence of suboptimal concentrations of fetal calf serum, IGF-I but not IGF-II could induce a rapid growth response of *Leishmania* amastigote-like forms *in vitro* as it could of promastigotes. The amastigote-like forms used here are very similar to amastigotes directly taken from the lesion, and it has been shown that they induce cutaneous lesions in mice (30). In *G. lamblia* it was shown that IGF-II induced a growth response while IGF-I was inert; however, both showed the capacity to bind to the parasite (28). We cannot completely rule out the effect of IGF-II on *Leishmania* under circumstances not tested here, but the difference may reflect species or phylogenetic differences.

Both amastigotes and promastigotes showed a strong specific binding of IGF-I through a single-site putative receptor of IGF-I. The strength of their binding is comparable to that of IGF-I to erythrocytes (31), fibroblasts, and smooth muscle cells (D. Gianella Neto, unpublished data).

IGF-I could induce a rapid tyrosine phosphorylation of a number of proteins in *L. (L.) mexicana* promastigotes and amastigotes. Reports on phosphorylation-dependent signaling in protozoas and particularly in *Leishmania* are still sparse and mainly show differences in the total phosphorylation pattern in various species of *Leishmania* promastigotes depending on whether the growth phase is stationary or exponential (32). In the study presented here and also by us in ref. 23, a single specific ligand (IGF-I) is used to study the tyrosine phosphorylation in *Leishmania*. One of the more prominent phosphorylation occurred at the protein of 185 kDa in promastigotes, and it represents a hitherto nonreported tyrosine-phosphorylated molecule in *Leishmania*. This is interesting because it corresponds in molecular mass to the molecule described in mammalian cells as an endogenous substrate for the IGF-I and insulin receptor, the insulin receptor substrate-1 (33, 34). The tyrosine phosphorylation of proteins in the amastigote-like forms upon IGF-I stimulation was shown to be different and occurred at 60- and 40-kDa proteins. The 60- and 40-kDa proteins also were present in the control sample from non-stimulated amastigotes and could represent autophosphorylating molecules or could reflect the experimental difficulties in achieving completely synchronized and quiescent parasites. However, stimulation with the IGF-I led to a significantly increased phosphorylation as shown by laser densitometry. Interestingly, an autophosphorylating protein kinase of 60 kDa has been described in *Trypanosoma brucei* (35). The difference in phosphorylation pattern found between promastigotes and amastigote-like forms could reflect evolutionary adaptation to different environments of the parasites, i.e., promastigotes for the extracellular and the amastigotes for the intracellular milieu. The use of the same strain of *Leishmania* for the study on promastigote and amastigote-like forms strengthens the significance of this difference in pattern. Similar cell cycle-dependent differences in the tyrosine-phosphorylation pattern in protozoa has been described in *T. brucei* (36, 37). A more detailed study and discussion on the IGF-I-mediated phosphorylation of *Leishmania* parasites is published in ref. 23.

The exact mode of action of IGF-I to induce proliferation of *Leishmania* promastigotes and amastigotes *in vitro* has to be elucidated. Here we present evidence for an IGF-I-induced sequential event starting with an initial binding, induction of intracellular signaling through tyrosine phosphorylation, and increase in both DNA synthesis rate and number of parasites. The requirement for suboptimal levels of FCS points to the previously suggested endocrine role of IGF-I, in which IGF-I

induces expression of receptors for other growth or activation factors (12–15).

To test the hypothesis that IGF-I could activate the parasites directly when entering in the host at the site of infection and thus influence the outcome of the infection, the *in vivo* experiment was designed to make the challenge with parasites already activated with a physiological dose of IGF-I. Two independent experiments showed that *Leishmania* promastigotes preactivated with IGF-I induced a significantly bigger lesion. The actual increase in the number of intact parasites also observed suggests that IGF-I may have induced a rapid proliferative response of the parasites and thus would have increased the number of available and infectious parasites. An alternative mechanism could be an increase of the virulence of the parasites by altering or inducing surface molecules (e.g., lectin-like) or the induction of mechanisms that prevent parasite death, as in the case of granulocyte–macrophage colony-stimulating factor (6).

The *in vitro* data showing a specific response of the *Leishmania* amastigotes indicate that IGF-I also may affect later stages of the infection. The operational mechanisms for such an intracellular effect remain unclear. Intracellular IGF-I in macrophages detected by immunohistochemistry have been reported (18). Whether extracellular IGF-I also can reach the parasitophorous vacuole, however, remains speculative. A similar *in vitro* effect on facultative intracellular *Mycobacteria* by epidermal growth factor has been described (38).

Thus, we conclude that IGF-I with a growth-inducing effect on *Leishmania* parasites *in vitro* and effect on the course of infection *in vivo* may constitute another important pathogenic factor in leishmaniasis.

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Title: Insulin-like growth factor (IGF)-I affects nitric oxide production and *Leishmania* growth in macrophages in vitro

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Running title: IGF-I affects NO production and *Leishmania* growth in macrophages

Key words: *Leishmania amazonensis*, macrophage, nitric oxide, hydrogen peroxide, flow cytometry.

Abstract

Insulin-like growth factor (IGF) – I affects parasite growth in vitro and increases the lesion size in cutaneous leishmaniasis in mice. Here we studied the effect of IGF-I on *Leishmania*-macrophage interaction in vitro, evaluating hydrogen peroxide (H₂O₂), nitric oxide (NO) production and parasitism, the latter using two distinct methods, counting under light microscope and flow cytometry. In the presence of IGF-I, we have observed lower level of NO in the supernatant of culture, whereas no effect was observed in the hydrogen peroxide level. In parallel, we have observed an increase in the parasitism that was evident when flow cytometer was used. Based on our results we suggest that IGF-I inducing decreased NO level possibly allowed increased parasite growth within macrophages.

Introduction

Leishmaniasis are parasitic diseases caused by protozoan parasites of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae) that affects more than 15 million people, mainly in tropical and subtropical areas around the world. In humans, *Leishmania* infection results in cutaneous or visceral diseases (1). Control or progression of leishmaniasis in the vertebrate host involves both non-specific and specific factors of the host immune response, and in certain circumstances the parasite survival depends on the ability of promastigotes and amastigotes to evade from the microbicidal mechanism of macrophage (2).

Insulin-like growth factor (IGF)-I is a pleiotropic growth factor detectable in circulation and in tissues (3,4), particularly in macrophages (5) and its secretion increases during inflammatory process (6). Probably it is one of the first growth-inducing factor that *Leishmania* encounter in the skin as soon as they are injected and subsequently after internalization by macrophages. Biological importance of IGF-I on leishmaniasis was first observed by our group showing that IGF-I in physiological concentration induces a rapid growth of *Leishmania* promastigotes and cell-free amastigotes and induces phosphorylation of intracellular molecules up on stimulation in vitro (7-9). In in vivo study, a significant increase in lesion size and in number of viable parasites in skin sections were observed in mice infected with *Leishmania* promastigotes pre-incubated with IGF-I (9,10). In the more detailed study it was shown an increased intracellular parasite/cell ratio upon IGF-I activation suggesting that the effect of IGF-I in leishmaniasis was due not only to the availability of more macrophages in the lesion but also to the increased growth of the parasites within macrophages (10).

Macrophages play a central role in the parasite/host interaction and particularly exerting leishmanicidal function when activated (11). Two macrophage-derived products have been identified as critical in the control of *Leishmania* infection: the reactive oxygen intermediates, mainly hydrogen peroxide (H₂O₂), and the nitrogen intermediates, specially nitric oxide (NO) (12,13). Nitric oxide has been identified as a major effector molecule in the destruction of intracellular *Leishmania* spp by murine macrophage (14-16).

Since IGF-I induces increased *Leishmania* growth within macrophages, and it may be due to the effect of IGF-I on leishmanicidal mechanism of macrophages, we studied in vitro the role of IGF-I on hydrogen peroxide and nitric oxide production and on the growth of *Leishmania* within macrophages.

For evaluation of parasite growth within macrophages, the usual method is the parasite and macrophage counting under light microscope that is tedious and subjective. In this study, besides evaluation of the in vitro effect of IGF-I on *Leishmania*/macrophage interaction using microscopic counting, we used another method for evaluation of parasitism using flow cytometer.

Materials and Methods

Parasite

Promastigotes of *Leishmania* (*Leishmania*) *amazonensis* (WHOM/BR/00-LTB-0016) derived from amastigotes purified from the footpad lesions of hamster were expanded and maintained in 199 medium (Gibco, USA) with 10% heat-inactivated fetal calf serum (FCS) and 0.25% hemin. Promastigotes in the stationary-phase and only until the fourth passage were used in the experiments.

Leishmania infection of murine macrophages

Peritoneal macrophages obtained from six to 10-week-old inbred BALB/c mice were suspended in nitrite- and phenol red-free Eagles' minimum essential medium (MEM) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 2 % heat inactivated mice serum.

For the study of the effect of IGF-I, either *Leishmania* promastigotes or macrophages were pre-incubated during 5 minutes with 50 ng/mL recombinant human IGF-I (R & D Systems, GB) or this factor was maintained throughout the culture period. Control was maintained without IGF-I. Tests were run in sextuplicates. 5×10^5 cells/well were allowed to adhere on glass coverslips placed in the wells of 24-well plates (Costar, USA), non adherent macrophages were washed out, and the parasite suspension (two parasites/macrophage) was dispensed in the wells, allowed to infect for one hour at 33 °C in humid atmosphere with 5 % CO₂, and then non-internalized parasites were washed out. Then part of samples was processed for evaluation of the initial parasitism in cells on coverslips or in suspension. Part of cultures was prepared for H₂O₂ dosage, and in some experiments, culture supernatants were harvested for NO dosage.

Detection of NO

Nitrite level was determined as an indicator of NO production, having sodium nitrite as standard as previously described (17), and read at 540 nm in a spectrophotometer Multiskan MCC/340 P version 2.20 (Labsystems, Finland).

Detection of H₂O₂

It was determined by a microassay described by Pick & Keisari (18). 5×10^5 monocytes were cultured in 96-well flat-bottomed tissue plates and with parasites and IGF-I in similar conditions as above. After the culture period, the phenol red solution was added and it was maintained at 33°C in humid atmosphere with 5 % CO₂ for 3

hours stopped by the addition of 1M NaOH, and read at 620nm in a spectrophotometer. Standard curve was established using H₂O₂ solution of known concentration.

Results and Discussion

Activated macrophage-derived products with leishmanicidal effect were searched during *Leishmania*-macrophage interaction in the presence of IGF-I. The hydrogen peroxide level (Fig 1) did not show any alteration when stimulated with IGF-I. Our data are in agreement with Warwick-Davies et al that have shown no effect of IGF-I on human macrophage H₂O₂ production in contrast to other growth factors such as growth hormone and prolactin (21). When supernatants of cultures with promastigotes were analyzed, we observed diminished levels of nitric oxide in every situation when IGF-I was used (Fig. 2). Different effects on distinct leishmanicidal mechanisms show that the effect of IGF-I is not a general downregulation of macrophage activity.

IGF-I leading to lower NO level has not been observed before. A toxic effect of IGF-I that could determine decrease in cell population and lower NO level was discarded since the viability of macrophages was maintained at 96 – 98% after one and three hour incubation with IGF-I, using Trypan blue staining. It is also known that IGF-I induces macrophage proliferation (18), and it induces production of some cytokines as tumor necrosis factor alpha (19). From our previous data, a decrease in the nitric oxide level was somehow expected when macrophage was pre-incubated with IGF-I or when the growth factor was maintained throughout the experimental period, since in this situation effector cell was targeted directly by IGF-I. However, decreased NO level when promastigotes were pre-incubated with IGF-I was surprising since

most of IGF-I is supposed to be removed with washing after incubation. To explain the observed effect, we speculate on either an unknown effect of IGF-I activated promastigotes or an effect exerted by a tiny amount of remained IGF-I on downregulation of NO synthesis.

To relate the nitric oxide levels with the infection ratio in the same experiment, we evaluated the parasitism by two distinct methods, a traditional optical microscopy method and by flow cytometry: a) For analysis with light microscope, the coverslips with infected macrophage cultures were stained with Giemsa, and phagocytosis of parasites examined under light microscope (Carl Zeiss, Germany) counting a total of 100 cells. Then the infection index was calculated following the formula [(number of infected macrophages x number of parasites per number of macrophages):100]. Then infection index ratio was calculated dividing the value obtained in culture with IGF-I by the value obtained in control culture without IGF-I. b) For flow cytometry, staining of intracellular parasites was done on macrophages detached from the plate with 0.02 % EDTA in RPMI 1640 medium, permeabilized with 0.5% saponin in Hank's balanced salt solution (HBSS) containing 2% FCS, and sequentially incubated for 30min at 37 °C with polyclonal anti-*Leishmania* antibody from *L. (L.) amazonensis*-infected hamster, produced in our laboratory, and then with fluorescein-conjugated goat anti-hamster IgG (Rockland, USA). The cell suspensions were run on a Facsort analytical flow cytometer (Becton Dickson, USA). Analysis were performed on 10,000 gated events, and the data were processed with Cell Quest software (Becton Dickson, USA), The anti-*Leishmania* antibody stained the intracellular amastigotes, and we did not observe any significant binding of goat anti-hamster IgG antibody on macrophage surface, when tested alone as control.

Using the traditional method, when the parasite number was counted after 48 hours of culture, we did not observe evident difference in the infection index ratios: 1.1 (macrophage + IGF-I), 0.9 (promastigote + IGF-I) and 1.2 (IGF-I maintained throughout the culture period). However, we observed important increase in the intracellular parasitism with IGF-I, accompanying nitric oxide level reduction, when analyzed by flow cytometry (Figure 3). This increase in parasitism was not determined by higher parasite uptake by macrophages in the presence of IGF-I, since after one hour of *Leishmania*-macrophage interaction, the parasite count was similar in the cultures with and without IGF-I using flow cytometer (Figure 3) and light microscope (data not shown).

Our results showed in vitro that IGF-I determined decrease of NO level that possibly allowed increased parasite growth within macrophages. Use of flow cytometer as an alternative method for quantifying the parasite within macrophages turned up valuable. Studies are in progress with amastigotes and analyzing further the nitric oxide metabolism under IGF-I effect..

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Figure legends

Fig. 1. Hydrogen peroxide (nmol/mL) in the culture supernatant of macrophages infected with *L. (L.) amazonensis* promastigotes either pre-incubated for 5 minutes with IGF-I (50 ng/ml) or maintained in the culture for 3 hours, or without IGF-I (control).

Fig. 2. Nitric oxide levels (μM) in the culture supernatant of macrophages infected with *L. (L.) amazonensis* promastigotes either pre-incubated for 5 minutes with IGF-I (50 ng/ml) or maintained in the culture for 48 hours, or without IGF-I (control). Data representative of 6 similar experiments.

* $p \leq 0.05$ in relation to control (ANOVA and Student Newman-Keuls tests) .

Fig. 3. Detection of intracellular *Leishmania* by flow cytometry after one and 48 hours of parasite-macrophage interaction. BALB/c mouse peritoneal macrophages were infected with *L. (L.) amazonensis* promastigotes (2 parasites/macrophage). Either macrophages or promastigotes were pre-incubated with IGF-I (50 ng/ml) for 5 minutes, or IGF-I was maintained throughout the culture period. Macrophages were permeabilized with 0.5% saponin in HBSS, intracellular *Leishmania* was stained with polyclonal anti-*Leishmania* antibody from *L. (L.) amazonensis*-infected hamster and sequentially by fluorescein-conjugated goat anti-hamster IgG, and the cell suspensions were run on a flow cytometer. The result is presented as histogram where slim line corresponds to data of *Leishmania* infected-macrophage (control), and the bold line to data where IGF-I was incubated with different elements of parasite-macrophage interaction.

Figure 1

Figure 2

Figure 3

Immunity and immunosuppression in experimental visceral leishmaniasis

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Abstract

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Leishmaniasis is a disease caused by protozoa of the genus *Leishmania*, and visceral leishmaniasis is a form in which the inner organs are affected. Since knowledge about immunity in experimental visceral leishmaniasis is poor, we present here a review on immunity and immunosuppression in experimental visceral leishmaniasis in mouse and hamster models. We show the complexity of the mechanisms involved and differences when compared with the cutaneous form of leishmaniasis. Resistance in visceral leishmaniasis involves both CD4⁺ and CD8⁺ T cells, and interleukin (IL)-2, interferon (IFN)- γ , and IL-12, the latter in a mechanism independent of IFN- γ and linked to transforming growth factor (TGF)- β production. Susceptibility involves IL-10 but not IL-4, and B cells. In immune animals, upon re-infection, the elements involved in resistance are different, i.e., CD8⁺ T cells and IL-2. Since one of the immunopathological consequences of active visceral leishmaniasis in humans is suppression of T-cell responses, many studies have been conducted using experimental models. Immunosuppression is mainly *Leishmania* antigen specific, and T cells, Th2 cells and adherent antigen-presenting cells have been shown to be involved. Interactions of the co-stimulatory molecule family B7-CTLA-4 leading to increased level of TGF- β as well as apoptosis of CD4⁺ T cells and inhibition of macrophage apoptosis by *Leishmania* infection are other components participating in immunosuppression. A better understanding of this complex immune response and the mechanisms of immunosuppression in experimental visceral leishmaniasis will contribute to the study of human disease and to vaccine development.

Key words

- Visceral leishmaniasis
- Immunosuppression
- T cell
- Cytokines
- Mice
- Hamster

Introduction

Protozoa of the genus *Leishmania* cause cutaneous, mucocutaneous or visceral diseases in man depending on the species of the parasite and the host immune response. There

are many different species of *Leishmania* causing different clinical manifestations, mainly in the New World. Visceral leishmaniasis is caused by *Leishmania* (*Leishmania*) *donovani* in the Old World, by *L. (L.) infantum* in the Southeast of Europe and

Mediterranean area and by *L. (L.) chagasi* in the New World, including Brazil. A spectrum of clinical manifestations occurs in visceral leishmaniasis, from asymptomatic or oligosymptomatic disease to progressive disease with severe manifestations such as hepatosplenomegaly, fever, pancytopenia, and hypergammaglobulinemia (1).

While extensive information is available about the immune response in experimental cutaneous leishmaniasis, the nature of immunity in experimental visceral leishmaniasis, which is different in many aspects, is poorly understood. In order to develop vaccines for different forms of leishmaniasis, and since there are many areas where different species and different forms of the disease overlap, a detailed knowledge of the particularity of the immune response and pathogenesis is extremely important. In the present review on immunity and immunosuppression in experimental visceral leishmaniasis we present the many important advances made in the last three decades and the complexity of the mechanisms involved. We show that there are marked differences in immunity between experimental visceral leishmaniasis and cutaneous leishmaniasis. Since updated and comprehensive reviews on immunity in cutaneous leishmaniasis are available (2), here we will mention this form of the disease only when pertinent for comparison.

Since one of the immunopathological consequences of active visceral leishmaniasis in humans is suppression of the T-cell response, many studies on experimental models are available in this field, which will be covered in the last part of this review.

Resistance and susceptibility in experimental visceral leishmaniasis

Throughout the review, when we compare data on experimental cutaneous leishmaniasis that we briefly summarize here, we do it mainly referring to the most extensively studied models of cutaneous leishmaniasis

which involve certain isogenic mouse strains infected with *L. (L.) major*. In this model a paradigm was established linking a subpopulation of CD4⁺ T cells producing mainly interferon (IFN)- γ (T helper 1 cells = Th1 cells) to resistance, and CD4⁺ T cells producing mainly interleukin (IL)-4 and IL-10 (T helper 2 cells = Th2 cells) to susceptibility. The importance of CD8⁺ T cells has only recently been appreciated. The importance of IFN- γ is stressed in the mechanism of resistance as both an efficient activator of macrophages and as a factor directing the differentiation of non-differentiated T helper cells to Th1 cells with participation of IL-12 and natural killer (NK) cells, and a T-box transcription factor T-bet playing a central role in this process. Conversely, IL-4, IL-13 and IL-10 are linked to susceptibility or persistence of infection, with the latter cytokine being linked to the CD4⁺CD25⁺ regulatory T-cell activation (see Ref. 2 for detailed information).

The data reviewed here are based on two models. The most widely studied model of visceral leishmaniasis is the BALB/c strain of mice infected with *L. (L.) donovani* or *L. (L.) chagasi*. Although this strain is considered to be susceptible and the infection progresses during the first two weeks, the infection is then controlled by the host immune response (3). As mentioned above, human visceral leishmaniasis presents a spectrum of clinical manifestations from a self-controlled infection to a progressive disease. The mouse model is comparable to self-controlled oligosymptomatic cases and therefore is useful for the study of the protective immune response. Alternatively, the better model to study the progressive disease is hamsters infected with *L. (L.) donovani* or *L. (L.) chagasi* that develop a disease similar to human progressive visceral leishmaniasis with hepatosplenomegaly, hypoalbuminemia, hypergammaglobulinemia, and pancytopenia (4). Therefore, this model is mainly used to study the mechanisms of immuno-

suppression that we will present in the final section.

Role of T lymphocyte populations

In human and experimental leishmaniasis immunity is predominantly mediated by T lymphocytes (2). Initial studies on mice using T cell-depleted mice (5) and nude BALB/c mice (3) have shown the importance of T lymphocytes for protection against *L. donovani* infection. Adoptive transfer of T cells, immune to *Leishmania* antigen, conferred resistance against *L. donovani* infection (6). Reconstitution experiments using nude BALB/c mice, and cell depletion experiments in euthymic mice using monoclonal anti-CD4 or anti-CD8 antibodies showed the necessity of both CD4⁺ and CD8⁺ T cells in the protection against *L. donovani* infection (7). In *L. donovani*-infected BALB/c mice, there was a time course-related participation of different cell populations: L3T4⁺ (CD4⁺) cells are important in the initial two weeks of infection, when the parasite replication is still occurring, mainly in the formation of hepatic granulomas, but later this cell population decreases and is replaced by Lyt 2⁺ (CD8⁺) cells when the progress of the infection is controlled (8). However, other studies point to the differences in the participation of immune elements in the protection observed in immune animals upon re-infection compared with those in naive animals with a primary infection. In immune BALB/c mice, depletion of Lyt 2⁺ but not L3T4⁺ abolishes resistance. Furthermore, granuloma formation is not affected by depletion of Lyt 2⁺ or L3T4⁺. Conversely, cyclophosphamide A treatment abolishes granuloma formation but does not interfere with parasite replication (9).

Role of cytokines

T lymphocytes participate in the immune response to *L. donovani* infection by produc-

ing different cytokines. While euthymic *L. donovani*-infected BALB/c mice are able to control infection with granuloma formation and IFN- γ and IL-2 production, nude BALB/c mice neither form granulomas nor produce IFN- γ (3). Human recombinant IFN- γ restores the ability of nude BALB/c mice to control *L. donovani* infection. Furthermore, anti-IFN- γ antibody abolishes granuloma formation (10), confirming the importance of this cytokine in protection. Moreover, depletion experiments using anti-IL-2 monoclonal antibodies and reconstitution using recombinant IL-2 showed a role for IL-2 in leishmanicidal activity apparently through the induction of IFN- γ , and in granuloma formation with involvement of L3T4⁺ and Lyt 2⁺ T cells (11). Results in immune mice differ from those described for naive animals. Neutralization of IL-2 but not IFN- γ abolishes resistance, and granuloma formation is not affected by neutralization of IL-2 or IFN- γ (9).

The role of IL-4 as a cytokine related to susceptibility has been recently questioned in experimental cutaneous leishmaniasis (2), but most studies on experimental visceral leishmaniasis had raised this question about the role of IL-4 in susceptibility from the beginning. In one study, besides predominant IFN- γ production in the initial and late phase of infection, IL-4 production was detected in the intermediary phase coinciding with peak of parasite burden in the susceptible strain, and no IL-4 production in the resistant mouse strain (12). Nevertheless other studies contradicted these findings. No IL-4 or IL-5 production was observed in three different strains of mice infected with *L. donovani* (13), and in the liver, only IFN- γ RNA was detected by Northern blot, and both Th1 and Th2 cytokine mRNAs, IL-4, IL-10, IFN- γ and IL-2 mRNA were detected by PCR (14). Furthermore, mice treated with anti-IL-4 monoclonal antibodies (14) and mice with IL-4 gene disruption (15) did not show better control of the infection. IL-10,

another Th2 cytokine, however, was related to progressive disease in human visceral leishmaniasis (16) and was shown to have a role in susceptibility in experimental visceral leishmaniasis. A progressive increase in IL-10 mRNA level in tissues during infection suggested a role in susceptibility (17). In addition, the control of parasite growth in inner organs in BALB/c IL-10^{-/-} mice and in normal mice with IL-10 receptor blockade by antibodies confirmed the role of IL-10 in susceptibility (18,19). Since IL-10 receptor blockade increased serum IFN- γ levels, a protective effect was initially attributed to the non-suppressed leishmanicidal effect of IFN- γ . However, suppression of parasite growth with IL-10 receptor blockade even in IFN- γ gene-disrupted mice suggested a broader effect of IL-10 on the suppression of multiple leishmanicidal mechanisms (20). We should emphasize, however, that visceral leishmaniasis in mice is a self-controlled infection; therefore IL-10 is probably not responsible for uncontrolled progressive increase in the parasite burden, which does not occur in this model. Its role should rather be considered similar to that in cutaneous leishmaniasis in resistant strain of mice, in which important findings have been recently obtained. In *L. major*-infected resistant C57BL/6 mice, IL-10 was shown to be important for the persistence of the parasite in the lesion, preventing its complete clearance from the lesion despite the presence of a protective immune response (21). Furthermore, this apparently undesirable persistence of the parasite was shown to be of the utmost importance for the maintenance of protective immunity against re-infection, with CD4⁺CD25⁺ regulatory T cells with IL-10-dependent and IL-10-independent mechanisms probably involving transforming growth factor (TGF)- β being involved in the suppression of IFN- γ production (22,23).

In contrast, IL-12 was shown to be linked to protection against the infection. IL-12 treatment of *L. donovani*-infected BALB/c

mice significantly reduced the parasite burden with the participation of CD4⁺ and CD8⁺ T cells, NK cells and IFN- γ , IL-2 and tumor necrosis factor (TNF)- α (24). But a distinct antimicrobial effect of IL-12, independent of IFN- γ , was also demonstrated in experiments using IFN- γ gene-disrupted mice. These mice, as expected, show a progressive infection for the first eight weeks but in the late phase develop a capacity to reduce the parasite burden with the participation of TNF- α induced by IL-12 (25). Neutralization of IL-12 with anti-IL-12 monoclonal antibody (26) or IL-12 deficiency (IL-12^{-/-}) (27) shows the fundamental role of this cytokine in the control of infection in susceptible mice. Furthermore, increased levels of TGF- β were observed in IL-12^{-/-} mice and were further increased in IL-12/IFN- γ double knock-out mice, without expansion of the Th2 response (28).

Role of B cells and immunoglobulins

Polyclonal B cell activation is present both in human and experimental visceral leishmaniasis (29,30), but the actual role of B cells or immunoglobulins in the immunity in visceral leishmaniasis has been poorly evaluated. Most of the data in this area have been obtained with cutaneous leishmaniasis models, where resistance was observed with depletion of B cells using anti-IgM antibody (31) or in BALB xid mice, lacking B-1 B cells (32), and susceptibility was increased by transfer of B cells (33) or administration of B-cell hematopoietic factor, IL-7 (34). In visceral leishmaniasis, enhanced resistance was recently shown in mutant mice that lack mature B cells (35).

To distinguish the effect of B cells from that of immunoglobulins on susceptibility, experiments were performed using mice genetically altered to contain no circulating antibody, with or without functional B cells, and mice defective in Fc receptor. These studies showed that the circulating antibody is crucial for susceptibility to the develop-

ment of cutaneous leishmaniasis (36). Furthermore, amastigotes from the lesion of cutaneous leishmaniasis were shown to be coated by IgG, and internalization of immunoglobulin-coated amastigotes by macrophages was shown to lead to IL-10 production and consequent enhancement of intracellular parasite growth *in vitro* (37). Similar mechanisms might be acting in visceral leishmaniasis.

Immunosuppression in experimental visceral leishmaniasis

One of the immunopathological consequences of active visceral leishmaniasis in humans is suppression of the T-cell responses mainly to *Leishmania* antigen (38). Although the *L. donovani*-infected mouse is not a good model for the study of immune suppression, negative *Leishmania* antigen-induced delayed-type hypersensitivity can be observed coinciding with the peak of parasite burden in the susceptible mouse strain (39) and therefore some studies have been conducted using this model. However, the better model to study this aspect is hamsters infected with *L. (L.) donovani* or *L. (L.) chagasi* that develop progressive visceral leishmaniasis. We have studied immunosuppression in *L. (L.) chagasi*-infected hamsters and have observed a concanavalin A-induced lymphoproliferative response in all experimental periods but the total absence of a *Leishmania* antigen-induced response (40). In the literature, the *Leishmania* antigen-induced response was found to be suppressed in all studies (4,41), but there was disagreement about the concanavalin A-induced response. Some studies showed that the response was preserved during the experiment (4,29) while others did not observe a response after 42 days of infection (41). Antigen-specific T-cell anergy present during active disease recovers after treatment and cure (42).

Various factors have been reported to cause immunosuppression in studies using

either mouse or hamster models. Studies on mice have indicated T cells (43) and others Th2 cells and adherent cells (39) as being responsible for suppression. Macrophage-mediated suppression is reported to lead to increased parasite growth and to be linked to either defective antigen presentation, suppression of class I and class II major histocompatibility complex molecule expression or mediation by prostaglandin-like substances (44-46). In *L. (L.) donovani*-infected hamsters, adherent splenic cells have been shown to be important in the suppression of lymphoproliferation and in defective antigen presentation (4). TGF- β produced by adherent antigen-presenting cells from infected hamsters was implicated in immunosuppression since a high level of TGF- β was observed in the cell culture supernatant when the *Leishmania* antigen-induced lymphoproliferative response was inhibited (47). We have studied the effect of another growth factor, insulin-like growth factor-I, and we have shown its effect on *in vitro Leishmania* growth but also on enhancement of the lesion in cutaneous leishmaniasis (48). More recent data suggest that it is a suppressor factor of macrophages leading to the decreased production of nitric oxide in *Leishmania*-infected macrophages *in vitro* (49). As mentioned in a previous section, cytokine IL-10 has been studied as a susceptibility factor in cutaneous leishmaniasis, but in our opinion it should also be considered within the context of immunosuppression. The absence of data in this field may be due to the difficulty to study hamsters, which is the most appropriate model, for which no reagents for cytokines are available. Only recently RT-PCR primers for some cytokines have been developed (50), and using these primers, no qualitative change in the expression of different cytokine RNA was observed during visceral leishmaniasis in hamsters (50), suggesting the necessity to develop a more sensitive method for evaluation such as quantitative PCR.

On the basis of elements shown to participate in suppression, such as T cells and TGF- β and possibly IL-10, it becomes attractive to speculate on the possible participation of CD4⁺CD25⁺ regulatory cells in immunosuppression during visceral leishmaniasis.

Another aspect addressed in a number of studies of immunosuppression is the initial interaction between antigen-presenting cells and T cells. Decreased expression of costimulatory molecules B7-1 (46) and Th1-specific M150 protein (51) in antigen-presenting cells has been associated with immunosuppression. However, apparently paradoxical were the data observed with the blockade of B7-1 or B7-2 molecules that led to restoration of T-cell response and to increased IFN- γ and IL-4 production and parasite clearance, respectively, in *L. chagasi*- and *L. donovani*-infected mice (52,53). The use of different ligands for B7 molecules, searched in sequence, explained this contradiction since there are two receptors for the B7 molecules, CD28 for T-cell activation and CTLA-4 for termination of T-cell activation. Indeed, blockade of CTLA-4 has led to the recovery of resistance against infection, suggesting expression of the CTLA-4 molecule during visceral leishmaniasis (53,54). Furthermore, it has been shown that the effect of CTLA-4 linkage resulted in the production of TGF- β , a factor that favors parasite growth within macrophages (55). All of these data demonstrate a role of CTLA-4 in immunosuppression, favoring parasite growth, but there are other reports showing its role in the development of a Th1 response in *Leishmania major* infection in mice transfected with the CTLA-4 gene (56). These paradoxical findings were elucidated in a review showing a dual role of the CTLA-4 molecule with activation of Th1 cells when T cells involved were naive, but with activation of Th2 cells when memory cells were involved (57). This dual role of the CTLA-4 molecule is a crucial point to be further analyzed in an eventual study aiming at vac-

cine development. We should also emphasize the importance of TGF- β in susceptibility and immunosuppression since recent data have indicated it as one of the most important factors, maybe a determinant factor, leading to Th2 development through inhibition of T-bet in leishmaniasis (23).

Apoptosis of T cells has been reported in experimental visceral leishmaniasis. More than 40% of CD4⁺ T cells from susceptible but not from resistant mice undergo apoptosis, accompanied by a significant decrease in IL-2 and IFN- γ secretion, and unaltered IL-4 secretion during *L. donovani* infection, findings that were also related to immunosuppression (58). In addition, apoptosis was detected in inflammatory cells in the liver and spleen during *L. donovani* infection, but when the role of CD95-CD95 L was assessed using CD95 ligand-deficient mice, increased parasite growth, but no effect on apoptosis, was observed in the CD95 ligand-deficient mice, suggesting a role of CD95 L in the control of parasite growth that is independent of host cell apoptosis (59). Since apoptosis of host lymphocytes may have a role in immunosuppression leading to parasite growth, we addressed this question in the hamster model. We observed apoptosis of inflammatory cells in the liver and spleen of *L. chagasi*-infected hamsters that was induced by *Leishmania* antigen stimulation in the early period of infection. We have not seen so far a direct time-related correlation with the *Leishmania* antigen-induced suppression of the lymphoproliferative response since apoptosis was present in the initial phase of the infection and the suppression of the lymphoproliferative response throughout the experimental period (40). Based on these observations, we can speculate on the occurrence of selection of the lymphocyte populations, with apoptosis of *Leishmania* antigen-specific lymphocytes in the initial phase and survival of nonspecific ones in the later phase. This may explain the absence of a *Leishmania* antigen-specific lymphopro-

liferative response throughout the study period but the presence of apoptosis only in the initial phase. These are some of the scattered lines of evidence about the role of apoptosis in immunosuppression that are still indirect and incomplete and that should be explored in the future.

Macrophages are not only the habitat of *Leishmania* but also the main cells involved in the leishmanicidal process. Survival of *Leishmania* depends on the integrity and supply or proliferation of macrophages in the lesion, besides a suppression of the leishmanicidal machinery. It was shown that *in vitro* infection of macrophages by *Leishmania* renders them resistant to apoptosis (60). We studied this phenomenon *in vivo* in hamsters with visceral leishmaniasis and observed that apoptosis is induced in macrophages by *L. (L.) chagasi* infection in the initial phase. However, as the infection progresses, apoptosis of macrophages disappears from both the liver and spleen, suggesting protection of macrophages by *Leishmania* infection (40).

Soluble factors other than immunoglobulin have been involved in immunosuppression. Inhibition of concanavalin A-induced lymphoproliferative responses of splenic cells was seen in the presence of serum obtained during the acute phase of visceral leishmaniasis infection. Increased serum triglyceride levels were detected 60 days after infection and the suppression was abolished when serum was delipidated (41).

Concluding comments

The immunological responses induced during experimental visceral leishmaniasis are markedly different from those induced in cutaneous leishmaniasis. Although the amount of data is still modest when compared with that for cutaneous leishmaniasis, the studies have disclosed interesting facets of the immune response, even contradicting some dogmas such as the role of IFN- γ in

protection. These particularities should be studied in order to understand how different species of *Leishmania* by determining different forms of disease can generate such different immunological responses. Furthermore, detailed analysis of the differences is very important in view of the fact that the ultimate goal in the fight against any endemic parasitic disease including leishmaniasis, is the development of an efficient vaccine that is effective in leishmaniasis caused by different species of *Leishmania*.

Identification of the differences is the initial step directed at the development of an efficient vaccine for leishmaniasis, and further extensive studies are needed to identify parasite- or host-related factors leading to these differences. Parallel studies on human visceral leishmaniasis become imperative also to compare and identify differences related to the host. In this context, studies of the mechanisms of immunosuppression can also contribute to a better understanding of this undesirable consequence of *Leishmania* infection, and indicate a type of immune response that should be avoided when induced by a candidate vaccine, for example directing the enrollment of CTLA-4 molecule in the activation of Th1 cells, and avoiding responses leading to IL-10 and TGF- β production. Furthermore, the understanding of the mechanisms of immunosuppression, if proven similar in patients, can also contribute to the planning of their treatment. In patients resistant to conventional treatment or in those who present recurrences, like HIV/*Leishmania* co-infected subjects, eventual interference blocking the effects of IL-10 and TGF- β may be considered in the future.

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Title:

Immunosuppression and inhibition of macrophage apoptosis in visceral leishmaniasis in hamsters

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Key words: Apoptosis, Macrophages, Lymphocytes, Visceral Leishmaniasis, Hamster

Abstract

Visceral leishmaniasis causes immunosuppression, but the mechanism is not been clear. We evaluated evolution of experimental visceral leishmaniasis in hamster and we searched immunosuppression using spleen cell proliferative response. Hamsters were infected intraperitoneally with 2×10^7 amastigotes, and developed similar disease to human. The spleen cell proliferative response showed normal response to Concanavalin A, but a response to *Leishmania* total antigen in the similar level of control that presented high proliferation rate from 15 days trough 90 days post-infection and we observed a high basal proliferation of cells from infected hamsters and absence of increase in the response to *Leishmania* antigen can be attributed to pre-engagement and pre-activation of specific cells. We proceeded the study to analyze whether apoptosis would appear as a consequence of activation, but did not detect any apoptosis of lymphocytes. We searched cytokines in spleen cells using RT-PCR and we observed presence of mRNA of cytokines of both TH1 and TH2 profile. In macrophages we observed presence of TUNEL staining both in Kupffer cells and in spleen adherent cells in hamsters with visceral leishmaniasis in the initial phase of infection and decreased in later phases. *Leishmania* apparently induces apoptosis in macrophages in the initial phase of infection. With evolution of the infection *Leishmania* infection protects macrophages from the death by apoptosis.

Introduction

Visceral leishmaniasis in Brazil is caused by *Leishmania (Leishmania) chagasi* that proliferates within mammalian host macrophages in the liver, spleen, bone marrow and lymph nodes (1). In visceral leishmaniasis in mice both CD4⁺ and CD8⁺ T cells play an important role in the control of infection with participation in different phases of infection (2,3). However, during active disease, suppression of *Leishmania* antigen-induced T cell-mediated immune responses are observed both in humans and in experimental animals (4,5). Although many factors have been identified in various studies, from defect in antigen presentation, *Leishmania* antigen-specific lymphocyte unresponsiveness, absence of effector cytokine production through presence of serum factors and suppressor cells (6, 7, 8, 9,10), the mechanisms are not completely clarified yet.

Focusing the parasite, since macrophages are the main host cells for *Leishmania*, its survival depends on the integrity and supply or proliferation of macrophages in the lesion and, since these cells are the effector cells of the activated T lymphocytes, leishmanicidal immune response has to be suppressed and inhibit macrophage activation.

In certain infections, apoptosis was implied in the suppression of T cell response. In other situations, on the contrary, macrophages are protected from apoptosis. In HIV-infected patients lymphocyte apoptosis has been observed and related to lymphopenia and destruction of CD4⁺ T cells during progression of the infection (11, 12, 13). In parasite infection, T cell apoptosis has been observed in hepatic granuloma of *Schistosoma mansoni*-infected mice (14), and activation-induced CD4⁺ T cell death in experimental Chagas's disease (15) and malaria caused by *Plasmodium falciparum* (16). In *Leishmania donovani*-infected mice, more

apoptotic CD4⁺ T cells in the spleen was observed in susceptible than in resistant mice (17).

Protection of macrophages from apoptosis has been seen in human monocytes and U 937 cell line infected with *Candida albicans* (18), in human monocytes infected with *Mycobacterium bovis* (19), and in mouse BALB/c bone marrow derived-macrophages infected with *Leishmania donovani* or incubated with lipophosphoglycan from *Leishmania donovani* promastigotes (20).

Some factors are related to the induction of or protection from apoptosis. In T cells, IL-10 induced apoptosis in the hepatic granuloma in *Schistosoma mansoni*-infected mice (14), but protected in Epstein-Barr virus infected animals (21). In monocyte/macrophage, TNF- α induced apoptosis with *Candida albicans* infection (18), but protected from apoptosis in *Mycobacterium bovis* (19) or with *Leishmania donovani* infection (20).

In this study we evaluated immunosuppression during visceral leishmaniasis in hamsters and we observed *Leishmania* antigen-specific immunosuppression related to over-stimulation of lymphocytes but no lymphocyte apoptosis. Further, we observed protection of macrophages from apoptosis during infection, both phenomena contributing to the progression of the infection.

Material and methods

Animals - Outbred 45 - 60 days old male hamsters (*Mesocricetus auratus*) from Animal Breeding Facility of Medical School of University of Sao Paulo were maintained in the Animal Facility of Tropical Medicine Institute of Sao Paulo of University of Sao Paulo and were allowed free access to water and food during experiment.

Parasites - *Leishmania (Leishmania) chagasi* (MHOM/BR/72/cepa 46) were maintained in hamsters with successive inoculations with infected spleen homogenate every three months. For experiments, hamsters with visceral leishmaniasis with 2-3 months of infection were sacrificed under anesthesia and the spleen removed aseptically and the amastigotes purified according to Dwyer (22) with some modifications. Briefly, the spleens were homogenized in cold RPMI 1640 medium (GIBCO, USA), the cellular suspension maintained for 10 minutes on ice, the amastigotes, filtered, processed 4 times through fine gauge needle (24G) and spun at 250g for 10 minutes. The supernatant was again spun at 250g for 10 minutes and then the supernatant was again spun at 2100g for 15 minutes. The pellet was resuspended in RPMI 1640 medium and the concentration of parasites adjusted to 2×10^7 /ml in RPMI 1640 medium.

Experimental protocol - Hamsters were inoculated intraperitoneally with 2×10^7 purified amastigotes in 1 ml of RPMI 1640 medium. Control animals were injected with one ml of RPMI 1640 medium. The animals were sacrificed from 15 through 90 days of infection. The liver samples from some animals were taken and fixed in 0.01M, pH 7.4 phosphate-buffered 10% formalin to prepare histopathological section to submit to TUNEL method. Spleen samples from some animals were taken for in vitro cell culture and analysis under flow cytometry.

In vitro spleen cell culture - Spleen homogenate was spun at 250 g for 10 minutes at 4 °C, the erythrocytes in the pellet lysed with 2% ammonium chloride in distilled water for 2 minutes, the osmolarity immediately adjusted with 10x concentrated PBS and washed twice in RPMI 1640 medium. The cell concentration was adjusted to 2×10^6 cells/ml in RPMI 1640 medium supplemented with 10mM HEPES, 100 UI/ml Penicillin, 10 µg/ml gentamicine, 2 mM L-glutamine, 10 µM 2-mercaptoethanol

(Sigma, USA) and 1 % heat inactivated normal hamster serum. Cell suspension was dispensed in 24 or 96 well flat-bottom plates (Costar, USA), and either RPMI 1640 medium or 2 µg/ml Concanavalin A (Amersham Pharmacia, Sweden) or sonicated *L. (L.) chagasi* antigen (10^6 promastigotes/ml) were added, set in triplicate, and incubated at 37°C in humid atmosphere with 5% CO₂ either for 18 or 72 hours. For the lymphoproliferative assay, cell culture in 96 well plates was pulsed with 1 µCi/well of ³H-thymidine (specific activity = 5 Ci/mmol; Amersham Life Science, England), six hours before the end of culture period, and then the cells were harvested into the glass fiber filters (Millipore, USA) and counted in a β counter (Packard, USA). The results were expressed in mean cpm of triplicates. The cells cultured in 24 well plates were processed for detection of apoptosis by flow cytometry.

Spleen adherent and non adherent cells - Spleen cell suspension in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS-RPMI) was dispensed in Petri dish (Costar, USA), and incubated at 37°C in humid atmosphere with 5% CO₂ for two hours. Non adherent cells were then removed by three consecutive washes with FCS-RPMI. Adherent cells were obtained by scrapping the plate and washed with FCS-RPMI. Adherent and non-adherent cells were analyzed under flow cytometer (Beckton Dickson, EUA) and the gates were defined for each cell population based in FSC/SSC plots.

TdT-mediated dUTP nick end labeling (TUNEL) method, according to Gavrieli et al (23). Specific kits from BOEHRINGER MANNHEIM (German) were used following the protocols provided by the manufacturer on tissue sections and with cell suspensions to analyze, respectively, under light microscope and flow cytometer.

Anexin V staining - 2×10^6 spleen cells from *Leishmania (L.) chagasi* infected-hamsters and non-infected control stimulated in vitro with concanavalin A or

Leishmania antigen were washed with PBS and suspended in linkage buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl and 2.5 mM CaCl₂). 5 µL of 1.5 mg/ml fluorescein conjugated-anexin V (Pharmigen, USA) followed by 5 µL of 2 µg/ml propidium iodide (Pharmigen, USA) were added to the pellet and incubated for 20 minutes at 4 °C, in dark. After incubation, 700µl from linkage buffer were added and analyzed under flow cytometer.

Extraction of total RNA. Spleen cells were suspended in TRIZOL and incubated at room temperature for 10 minutes. To each mL of Trizol (GIBCO, EUA) 200µL chloroform were added, vigorously homogenized then spun at 13000g at 4°C for 15 minutes. Aqueous phase was then separated and incubated with isopropanol (1:1 vol.: vol.) for 15 minutes at room temperature then spun. 500µL absolute ethanol was added to the pellet and spun again at 13000g for 15 minutes at 4°C. Ethanol was discarded and the pellet was dried for 10 minutes and suspended in 50µL RNase-free distilled water.

Obtention of cDNA. Ten microliters of extracted RNA were mixed with 10µL of solution constituted by BRL buffer 5x (GIBCO, EUA), 5mM dNTP (GIBCO, EUA), 100 pmol/µL random primers (GIBCO, EUA), 100mM DTT (GIBCO, EUA), 10U/µL cloned RNase inhibitor (GIBCO, EUA), 200U/µL super script II (GIBCO, EUA) in autoclaved bidistilled water with diethylpirocarbonate (DEPC), incubated for 60 minutes at 37°C and 15 minutes at 90°C.

Polimerase chain reaction (PCR). The oligonucleotide sequences used to amplify 100 pmol/µL cytokines or HPRT cDNAs from hamster (24) are as follows: HPRT: forward, CCA CGC CTA CTA TAG AGT TTG ; reverse, CTT GCG ATG TCA TGG TAG AG; TNF-α: forward, CAC AAT CCT CTT CTG CCT GC; reverse, TGT CTT

TGA GAG ACA TCC CG; TGF- β : forward, GAG AAG AAC TGC TGT GTG CG; reverse, ACC CAC GTA GTA CAC GAT GG; IL-2: forward, AAC CCA GCA GCA CCT CGA GC; reverse, CAG TTA CTG TCT CAT CAT CG; IFN: forward, TCA TTG AGA GCC AGA TCG TC; reverse, GGC TAA GTT TTC GTG ACA GG; IL-10: forward, GGA CAA CAT ACT ACT CAC TG; reverse, ACA GGG GAG AAA TCG ATG AC and IL-4: forward, TCC TAT CAC TGA CGG TAG AG; reverse, TGC AAA TGA GGT CTT TCT CC. They were obtained from the Gene Bank and synthesized by GIBCO (BRAZIL). Three μ L of cDNA were mixed with 47 μ L of PCR mix constituted with 10x PCR buffer (GIBCO, EUA), 1.25mM dNTP (GIBCO, EUA), 10mM specific primers, 50mM magnesium chloride (GIBCO, EUA), 5U/ μ L Taq DNA polimerase (GIBCO, EUA) in autoclaved MilliQ water. The samples were submitted to amplification cycles in a DNA termocicler (MJ.RESERACH, USA) for 36 cycles of 2 minutes at 95°C, 2 minutes at 50°C and 2 minutes at 70°C. The amplified product was analyzed in 1.5 % agarose gel and visualized using ethidium bromide.

Results

1. Spleen cell proliferative response.

Hamsters infected with *Leishmania chagasi* presented specific immunosuppression. The spleen cell proliferative response showed normal response to Concanavalin A (figure 1A), but a response to *Leishmania* total antigen in the similar level of control that presented high proliferation rate from 15 days trough 90 days post-infection (figure 1B).

2. Dectetion of apoptosis in inflammatory cells from liver and spleen in situ.

Using TUNEL method we evaluated the presence of apoptosis in tissue section of liver and spleen. In liver we observed presence of TUNEL staining of cells from periportal inflammatory infiltrate and in inflammatory foci in parenchyma in the initial phase (15 and 30 days) of infection (figure 2B) and absence at the late phase (60 and 90 days) of infection (figure 2C). Non-infected control hamsters did not present TUNEL staining of any cell in the liver (figure 2A). When we analyzed spleen from infected hamsters and non-infected controls we observed TUNEL staining of cells from both groups in non-infected control and at initial phase (15 and 30 days) (figures 2D, 2E), however TUNEL staining decreased at late phase (60 and 90 days) of infection (figure 2F).

Interestingly, we observed TUNEL staining of Kupffer cells from infected hamster at the initial phase (15 and 30 days) (figure 3B) and absence at late phase (60 and 90 days) of infection (figure 3C) and absence in non-infected control animal (figure 3A).

3. Detection of apoptosis in spleen cell suspension.

Since it was difficult to quantify the apoptosis and to differentiate the cell populations in the spleen, we analyzed the parameters by flow cytometry. We analyzed TUNEL staining of spleen cell suspension in ex-vivo and in 18 hours Concanavalin A- and *Leishmania* total antigen-stimulated samples. We analyzed the gated populations of adherent (macrophages) and non-adherent (lymphocytes) cells, separately. In control non-infected and infected animals there was an increase of TUNEL staining of spleen non-adherent cells from animals at 15 and 30 days post-infection when stimulated with concanavalin A and *Leishmania* antigen. In cells from 60 and 90 days post-infection, no increase of TUNEL staining was observed under

stimuli in infected animals (figure 4). However, since it is known that this method can stain cell proliferation (25), and we have seen increased proliferation even in basal conditions, we used an alternative method to detect apoptosis. Using annexin V we observed no apoptosis in non-adherent cells (data not shown).

With adherent cells, in control non-infected and infected animals there was also an increase of TUNEL staining of spleen non-adherent cells from animals at 15 and 30 days post-infection when stimulated with concanavalin A and *Leishmania* antigen. In adherent cells from 60 and 90 days post-infection, no increase of TUNEL staining was observed under stimuli in infected animals (figure 5).

Cytokine detection

We evaluated the pattern of cytokines using RT-PCR in spleen cell RNA and we observed similar cytokine profile in non-infected and *Leishmania*-infected hamsters at different time periods studied (figure 6).

Discussion

Leishmania (Leishmania) chagasi infected -hamsters are excellent model to study immunosuppression in visceral leishmaniasis because it develops a similar disease to humans. When we searched concanavalin A- and *Leishmania* antigen-induced spleen cell proliferative response, apparently a *Leishmania* antigen specific immunosuppression was present. However, since we observed a high basal proliferation of cells from infected hamsters, we attributed the absence of increase in the response to the pre-engagement and pre-activation of specific cells. There is apparent antigen specific activation of lymphocytes but, seemingly, in some way it is not efficient to activate the macrophages for *Leishmania* killing. We proceeded the study to analyze whether apoptosis would appear as a consequence of activation and

whether it would be an additional mechanism of immunosuppression in *Leishmania* infection. Using TUNEL method we observed staining of non-adherent spleen cells (lymphocytes) at the initial phase of infection and non-infected control and decreased at final phase. However using annexin V we could not confirm the presence of apoptosis in non-adherent cells. Then, another mechanism of immunosuppression may be involved, so we searched cytokines in spleen cells using RT-PCR and we observed presence of mRNA of cytokines of both TH1 and TH2 profile. Our data suggest that the cytokines are not related to immunosuppression in experimental visceral leishmaniasis in hamsters. Similar results were shown by Melby et al (24) in hamsters infected with *Leishmania donovani* although the studied period was shorter in their study than in ours.

Macrophages are important cells involved in the interaction with *Leishmania* and we searched for apoptosis in these cells using TUNEL method in situ and in culture. Initially, we observed TUNEL staining of Kupffer cells in the liver at the initial phase of infection and disappearance at the final phase that was similarly detected in macrophages in spleen cell culture. In macrophages, we are confident that TUNEL staining is revealing apoptosis since we suppose no high proliferation is present in these cells in vitro, and further, TUNEL method is known rarely to stain proliferative cells in tissue, thus being more specific for apoptosis (26, 27).

Observation of apoptosis of macrophages that are habitat of *Leishmania* undergo apoptosis, we would expect no progression of the infection in the liver, but what we observe is an increase in the parasite burden (data not shown), that is likely to be consequent of absence of apoptosis in later phases of infection. Since it was shown that in vitro infection of macrophages by *Leishmania* renders them resistant for apoptosis (20) as with other microorganisms such as *Theileria parvum*, *Toxoplasma*

gondii, *Cryptosporidium parvum* (28), it is likely that it is occurring also in vivo in our model. Our data suggest this protection of macrophages from apoptosis in late phase of infection, and in fact most of the macrophages in the later phases were observed parasitized by *Leishmania* (data not shown). We know that different stimuli can be involved in the protection or apoptosis of macrophages, but so far we detected no cytokine profile changes in this study.

In this study, in visceral leishmaniasis in hamsters we observed an interesting interplay between host and parasite. *Leishmania* infection induces intense activation of specific immune response. However the parasite continues proliferation in host macrophages its host cell. *Leishmania* apparently induces apoptosis in macrophages in the initial phase of infection, but with evolution the situation changes with protection of macrophages from apoptosis favoring parasite survival and progression of infection.

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Figure legends

Figure 1. Box-plot representation of the spleen cell proliferative response (mean cpm of triplicate) to concanavalin A (A) and *Leishmania* antigen (B) from *L.eishmania* (*L.*) *chagasi*-infected hamsters at 15 - 30 days and 60 - 90 days post-infection and non-infected control. Median and 25 – 75 percentile interval are presented. * $p \leq 0.05$ in relation to non stimulated control .

Figure 2. TUNEL staining of cells from *Leishmania* (*L.*) *chagasi*-infected hamsters and non-infected control. A) Liver. Absence of staining of cells of non-infected control hamster. B) Liver. Presence of staining in cells from periportal inflammatory and infiltrating foci in parenchyma in the initial phase of infection (15 days). C) Liver. Absence of staining of cells from periportal inflammatory and infiltrate foci in parenchyma in the late phase of infection (60 days). D) Spleen. Presence of staining of spleen cells from non-infected control hamsters. E) Spleen. Presence of staining of spleen cells from hamsters at the initial phase (15 days) of infection. F) Spleen. Presence of staining of spleen cells from hamsters at the late phase of infection (60 days). A,B,C = x 40. D,E,F= x 20.

Figure 3. TUNEL method staining liver cells from *L. (L.) chagasi*-infected hamsters and non-infected control. A) Absence of staining of Kupffer cells in non-infected control hamsters. B) Presence of staining of Kupffer cells of hamsters at the initial phase (15 days) of infection. C) Absence of staining of Kupffer cells of hamsters at the late phase (60 days) of infection. x 40.

Figure 4. TUNEL staining of non-adherent spleen cells from *Leishmania (L.) chagasi*-infected hamsters and non-infected control. Spleen cells in culture were concanavalin A-stimulated (con A) or *Leishmania* antigen-stimulated (Leish). Cells were gated on non-adherent cells (lymphocytes) and analyzed under flow cytometer.

Figure 5. Figure 4. TUNEL staining of adherent spleen cells from *Leishmania (L.) chagasi*-infected hamsters and non-infected control. Spleen cells in culture were concanavalin A-stimulated (con A) or *Leishmania* antigen-stimulated (Leish). Cells were gated on adherent cells (macrophages) and analyzed under flow cytometer.

Figure 6. Cytokine mRNA expression in spleen cells from non-infected control hamsters and *Leishmania (L.) chagasi*-infected hamsters. Spleen cells total RNA was separated by TRIZOL reagent and cDNA was obtained. The DNA was amplified by RT-PCR using sequences HPRT, IL-2, IL-4, IL-10, TNF- α and TGF- β cDNA probes. The products were electrophoresed through a 1 % agarose gel containing ethidium bromide. A) Non-infected control hamster. B) Hamsters at the initial phase (30 days) of infection. C) Hamsters at the final phase (60 days) of infection.

Lanes: **1** – molecular weight markers, **2** – HPRT, **3** – TNF- α , **4** – TGF- β , **5** – IL-4, **6** – IL-2, **7** – IL-10, **8** – IFN- γ .

A

Figure 1

Time post-infection and stimuli

B

Time post-infection and stimuli

Figure 2.

Figure 3

Figure 4

Figure 5

Figure 6

CD4⁺ T cells participate in the nephropathy of canine visceral leishmaniasis

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Abstract

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Renal involvement in visceral leishmaniasis (VL) is very frequent. The renal lesions of humans and dogs are similar but their pathogenesis has not been clearly elucidated. There is growing evidence that the cellular immune response is involved in the pathogenesis of immunologically mediated glomerulonephritis. Since T cells could participate in the pathogenesis of nephropathy, in the present study we investigated the possible involvement of CD4⁺ and CD8⁺ T cells in the nephropathy of canine VL. Six dogs naturally infected with *Leishmania (Leishmania) chagasi* from the endemic area in the Northeast of Brazil, the town of Teresina in the State of Piauí, were studied. An expressive inflammatory infiltrate of CD4⁺ T cells both in glomeruli and in interstitium was present in 4 animals and absent in 2. CD8⁺ T cells were detected only in one animal. CD4⁺ T cells alone were observed in 3 animals; when CD8⁺ T cells were present CD4⁺ T cells were also present. CD4⁺ T cells were observed in cases of focal segmental glomerulosclerosis, diffuse membranoproliferative glomerulonephritis, diffuse mesangial proliferative glomerulonephritis and crescentic glomerulonephritis. CD8⁺ T cells were present only in a case of crescentic glomerulonephritis. *Leishmania* antigen was detected in glomeruli and in interstitial inflammatory infiltrate in 4 animals and immunoglobulins were observed in 4 dogs. In this study we observed that T cells, in addition to immunoglobulins, are present in the renal lesion of canine VL. Further studies are in progress addressing the immunopathogenic mechanisms involving the participation of immunoglobulins and T cells in canine VL nephropathy.

Key words

- Visceral leishmaniasis
- Dog
- *Leishmania (Leishmania) chagasi*
- Nephropathy
- Immunoglobulin
- T cells

In Brazil visceral leishmaniasis (VL) is caused by *Leishmania (Leishmania) chagasi* (1) and the dog is the most important reservoir of this parasite. Northeastern Brazil is an endemic area for VL. About 1600 dogs naturally infected with *Leishmania (L.) chagasi* are estimated to be present in

Teresina, State of Piauí. The dogs present lesions that are similar to those seen in human disease (2,3). The disease causes alterations in several organs and renal involvement is very frequent both in humans and in dogs (4-9). However, the pathogenesis of this nephropathy has not been clearly eluci-

dated. Until recently, the pathogenesis of renal lesion in both human and canine VL was mainly attributed to immune complex deposition as a probable mechanism of glomerular injury (10-12), but the scarcity of the antigen and the type of cell infiltrate are not fully compatible with this hypothesis. On the other hand, growing evidence that the cellular immune response is involved in the pathogenesis of some types of immunologically mediated glomerulonephritis has been recently obtained (13). The presence of T cells has been detected in cases of renal disease in humans and in experimental models (14-16). Since the nephropathy of canine VL has not been fully elucidated and T cells may participate in its pathogenesis, in the present study we investigated the possible involvement of CD4⁺ and CD8⁺ T cells in the nephropathy of dogs.

Six naturally infected dogs from the town of Teresina with positive sorology for VL and positive culture of *Leishmania* from bone marrow, spleen and/or popliteal lymph nodes and 2 non-infected controls were studied. Formalin-fixed and paraffin-embedded kidney samples were processed for histopathological and immunohistochemical studies. CD4⁺ and CD8⁺ T cells were detected using mouse monoclonal anti-canine CD4⁺ (VMRD, Pullman, WA, USA) and CD8⁺ (VMRD) antibodies diluted 1:500 in 0.01 M phosphate-buffered saline (PBS), pH 7.2, respectively, and a sensitive immunohistochemistry-catalyzed signal amplification (CSA) system (Dako Corporation, Carpinteria, CA, USA).

Of 6 naturally infected animals only 1 did not show any alteration. In 5 animals glomerular, interstitial and tubular changes were observed. Two control animals did not show any significant renal histopathological changes, and no CD4⁺ or CD8⁺ cells were observed. An inflammatory infiltrate of CD4⁺ T cells both in glomeruli (Figure 1A) and in interstitium was expressive in 4 infected animals and absent in 2. CD8⁺ T cells were only

detected in one animal both in glomeruli (Figure 1B) and in the interstitial infiltrate. CD4⁺ T cells alone were observed in 3 animals and when CD8⁺ T cells were present CD4⁺ T cells were also present. CD4⁺ T cells were observed in cases of focal segmental glomerulosclerosis, diffuse membranoproliferative glomerulonephritis, diffuse mesangial proliferative glomerulonephritis and crescentic glomerulonephritis. CD8⁺ T cells were present only in a case of crescentic glomerulonephritis. Of 2 cases in which CD4⁺ and CD8⁺ T cells were not observed, one did not present any renal lesion and the other presented chronic glomerulonephritis. To confirm that the renal changes were actually related to leishmaniasis, the presence of *Leishmania* antigen was determined using mouse polyclonal anti-*Leishmania* (*L.*) *amazonensis* antibody produced in our laboratory, diluted 1:1600 in 0.01 M PBS, pH 7.2, and the CSA system. *Leishmania* antigen was observed in the glomeruli (Figure 1C) and in the interstitial inflammatory infiltrate in 5 infected animals. The antigen was observed as characteristic diffuse dark brown peroxidase staining almost exclusively in phagocytic cells of glomeruli and in the interstitial mononuclear cell infiltrate, but whole parasites were not detected in any case. In 2 control animals *Leishmania* antigen was not detected in renal tissue.

Since in other studies on VL immunoglobulins have been always detected in renal lesions we also searched for the presence of IgG in our samples. IgG deposits were detected by streptavidin-peroxidase techniques in 4 dogs using commercially available goat monoclonal anti-dog IgG (Bethil Laboratories, Montgomery, TX, USA) antibody at the concentration of 10 µg/ml. Granular IgG deposits especially along the capillary walls (Figure 1D) were observed in 4 animals studied. Immunoglobulin deposits have also been observed in other studies on renal tissue of dogs with VL (8,17).

In the present study we used naturally

infected dogs as a model to study VL. Even though the parasite inoculation site has not been fully elucidated, we assumed that the disease of these naturally infected dogs in a way resembles more closely what occurs during the infection in man, with a spectrum of various organ specific lesion. In addition, the dog model has a great advantage in comparison to the well-known hamster model (18,19) since numerous reagents for cell markers and known parameters for an extensive immunological and pathological evaluation are available for this species.

The detection of CD4⁺ T cells both in glomeruli and in interstitium suggests the participation of these cells in the pathogenesis of the nephropathy in canine VL. Since CD8⁺ T cells were detected in only one case their participation in the pathogenesis of glomerulonephritis in canine VL is uncertain. These findings are similar to the requirement for CD4⁺ but not CD8⁺ T cells that has been shown in experimental crescentic glomerulonephritis in CD4⁻ and CD8⁻ mice (16). The predominance of CD4⁺ T cells over CD8⁺ T cells has also been ob-

served in different forms of glomerulonephritis (20), suggesting that activation of these cells leading to delayed-type hypersensitivity, cytolytic reactions, abnormal expression of major histocompatibility complex molecules, or B cell activation can result in renal injury (13).

To the best of our knowledge, this is the first report on the presence of T cells in kidney in cases of VL. Recent studies have shown the presence of T cells in glomeruli, especially in cases of crescentic glomerulonephritis (16), membranoproliferative glomerulonephritis (15), and anti-glomerular basement membrane glomerulonephritis in rats (14). In the present study we detected the presence of T cells in several patterns of glomerular lesion but not in chronic glomerulonephritis.

The detection of *Leishmania* antigen in glomeruli and in renal interstitium in the animals with infection was also an important finding since we used naturally infected dogs. In these animals the *Leishmania* infection was ascertained by parasitological and serological tests but other subclinical infections

Figure 1 - Detection of T cells, *Leishmania* antigen and immunoglobulin in glomerular lesions in canine visceral leishmaniasis by immunoperoxidase staining. A, Presence of CD4⁺ T cells of severe intensity in focal segmental glomerulosclerosis. Bar: 25 µm. B, Presence of CD8⁺ T cells of moderate intensity in crescentic glomerulonephritis. Bar: 25 µm. C, Presence of *Leishmania* antigen of moderately severe intensity in focal segmental glomerulosclerosis. Bar: 25 µm. D, Presence of an immunoglobulin G deposit of moderate intensity in diffuse mesangial proliferative glomerulonephritis. Bar: 25 µm.

or diseases could be present inducing a T cell inflammatory infiltrate in the kidney. However, the fact that the control non-infected dogs from the same endemic area did not present any lesion and the presence of *Leishmania* antigen in the studied animals strongly indicate that the presence of T cells is related to the presence of *Leishmania* infection. Furthermore, in the single case in which *Leishmania* antigen was not detected T cells were not present.

We observed that T cells besides immunoglobulins are present in the renal lesion of canine VL. Further studies are in progress

addressing the immunopathogenic mechanisms involving the participation of immunoglobulins and T cells in canine VL nephropathy.

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Detection of immunoglobulin G in the lung and liver of hamsters with visceral leishmaniasis

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Abstract

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Several organs are affected in visceral leishmaniasis, not only those rich in mononuclear phagocytes. Hypergammaglobulinemia occurs during visceral leishmaniasis; anti-*Leishmania* antibodies are not primarily important for protection but might be involved in the pathogenesis of tissue lesions. The glomerulonephritis occurring in visceral leishmaniasis has been attributed to immune complex deposition but in other organs the mechanism has not been studied. In the current study we demonstrated the presence of IgG in the lung and liver of hamsters with visceral leishmaniasis. Hamsters were injected intraperitoneally with 2×10^7 amastigotes of *Leishmania (Leishmania) chagasi* and the presence of IgG in the liver and lung was evaluated at 7, 15, 30, 45, 80 and 102 days postinfection (PI) by immunohistochemistry. The parasite burden in the spleen and liver increased progressively during infection. We observed a deposit of IgG from day 7 PI that increased progressively until it reached highest intensity around 30 and 45 days PI, declining at later times. The IgG deposits outlined the sinusoids. In the lung a deposit of IgG was observed in the capillary walls that was moderate at day 7 PI, but the intensity increased remarkably at day 30 PI and declined at later times of infection. No significant C3 deposits were observed in the lung or in the liver. We conclude that IgG may participate in the pathogenesis of the inflammatory process of the lung and liver occurring in experimental visceral leishmaniasis and we discuss an alternative mechanism other than immune complex deposition.

Key words

- *Leishmania (Leishmania) chagasi*
- Visceral leishmaniasis
- Immunopathology
- Immunoglobulin G
- Liver
- Lung

Leishmaniasis is a disease caused by a protozoan of the genus *Leishmania*. Visceral leishmaniasis affects mononuclear phagocytes mainly in the spleen and in the liver where hyperplasia and hypertrophy of these cells occur, but other organs such as lung and kidney are affected during progression of the disease (1). Interstitial inflammation is ob-

served in the lung, kidney and liver (1) but its pathogenesis has not been fully elucidated. Hypergammaglobulinemia occurs in humans and in hamsters with visceral leishmaniasis due to polyclonal activation of B lymphocytes (2,3). Antibodies have no evident protective effect in leishmaniasis (4) but may be involved in the pathogenesis of the lesions in

visceral leishmaniasis. In the kidney, immune complex deposition has been claimed as the mechanism of lesion (5-7). To study the immunopathogenesis of the lesions in the liver and lung, in the current study we evaluated the presence of IgG in these organs in hamsters with visceral leishmaniasis.

Twenty-eight outbred 45-60-day-old male hamsters (*Mesocricetus auratus*) from the Animal Breeding Facility of the Medical School of the University of São Paulo were maintained in the Animal Facilities of the Institute of Tropical Medicine of São Paulo during the experiment. Seventeen hamsters were inoculated intraperitoneally with purified 2×10^7 amastigotes of *Leishmania* (*Leishmania*) *chagasi* (MHOM/BR/72/strain 46) in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD, USA), and sacrificed at 7, 15, 30, 45, 80 and 102 days postinfection (PI). Eleven control animals were injected with RPMI 1640 medium and sacrificed at the same times. At the time of sacrifice, spleen, liver, kidney and lung were obtained and the presence of IgG in the liver, kidney and lung was evaluated by immunohistochemistry using biotinylated goat anti-hamster IgG antibody (20 µg/ml) (Rockland, Gilbertsville, PA, USA) in formalin-fixed and paraffin-embedded tissue sections. Complement fraction C3 was detected in

cryosections of the organs using a polyclonal rabbit anti-hamster C3 antibody produced by us (8). Anti-*Leishmania* antibody titer was determined by immunofluorescence using *Leishmania* (*L.*) *amazonensis* antigen. The parasite burden in the spleen and liver was calculated by counting the number of cells and parasites in each microscopic field of organ imprints up to 1000 cells or parasites. Parasite burden was calculated by the formula: (number of parasites/number of cells) $\times$ weight of the organ (mg) $\times 2 \times 10^4$.

Parasite burden in the spleen and liver and anti-*Leishmania* antibody titer in infected hamsters increased progressively during infection. Using immunohistochemistry we observed some background staining in control animals but in the liver of infected animals we clearly observed a weak deposit of IgG at day 7 PI that increased progressively until it reached highest intensity around 30 and 45 days PI, declining at later times (Table 1). IgG deposits outlined the sinusoids (Figure 1A). In the lung a deposit of IgG was observed in the capillary walls (Figure 1B) which was of moderate intensity at day 7 PI, but increased remarkably at day 30 PI and declined at later times of infection (Table 1). No significant C3 deposits were observed either in the lung or in the liver. In two preliminary experiments with 3-4 infected

Table 1 - Intensity of IgG deposits detected by immunoperoxidase staining using biotinylated goat anti-hamster IgG antibody in the liver, lung and kidney of hamsters infected with 2×10^7 amastigotes of *Leishmania* (*L.*) *chagasi* and non-infected control animals at different times of infection.

- = Negative. Intensity of positive reactions graded from + through +++++. Data are reported as the mean of 2-4 animals/group.

Days post-infection	Liver		Lung		Kidney	
	Control	Infected	Control	Infected	Control	Infected
7	-	+	+	++	-	+
15	+	++	+	+++	-	++
30	+	+++	+	++++	++	+++
45	+	+++	+	+++	+	++
80	++	++	+	+++	+	++
102	+	++	++	+++	-	+++

and control animals sacrificed at the same times, we observed similar results by immunofluorescence using polyclonal rabbit anti-hamster total immunoglobulin serum.

Since the absence of C3 deposits is not compatible with immune complex deposition in the pathogenesis of the disease and since an immune complex-mediated mechanism has been shown in the kidney in visceral leishmaniasis (5-7), as a control we examined kidney tissue from the same animals. In the kidney the deposit of IgG was observed outlining the capillary walls (Figure 1C). The intensity of the deposit was highest at 30 and at 102 days PI and slightly lower at other time points (Table 1). C3 deposits of moderate intensity were detected in the kidney throughout the experiment, with a slight increase in intensity at day 102. Thus, we conclude that the conditions for the presumed formation of an immune complex were present in the experimental animals. Furthermore, one of the factors for immune complex deposition is the incapacity to clear immune complexes due to dysfunction of the reticuloendothelial system (9), which is likely to occur in visceral leishmaniasis due to *Leishmania* parasite proliferation in mononuclear phagocytes.

The absence of C3 deposits in the liver and lung still contradicts the mechanism of immune complex deposition. However, the presence of IgG deposits in the liver and lung might indicate its role in the pathogenesis. IgG deposits in the lung have been reported in some situations such as the presence of anti-basement membrane antibody in the sep-

Figure 1 - Detection of IgG deposits in the liver, lung and kidney of hamsters infected with 2×10^7 amastigotes of *Leishmania* (*L.*) *chagasi* by immunoperoxidase staining using biotinylated goat anti-hamster IgG antibody. A, IgG deposits along the sinusoid are seen in the liver of infected hamsters at 45 days of infection. B, IgG deposits in the capillary wall of the lung septum from infected animals at 30 days of infection. C, IgG deposits outlining the capillary wall in the glomeruli of infected hamsters at 45 days of infection. Bar: 25 μ m.

tum in Goodpasture syndrome (10) and in acute and chronic immune complex-mediated disease models (11). In the present study, we observed the presence of IgG deposits in the lung mainly in the vessel wall, in agreement with findings for rabbits with chronic serum sickness, in which interstitial pneumonitis develops (12). In addition, in visceral leishmaniasis the presence of *Leishmania* antigen has been observed in inflammatory foci in the lung (13). In immune complex-mediated diseases and in animal models of acute and chronic serum sickness, a known model of immune complex-mediated disease (11), no liver lesion has been described. Furthermore, in diseases involving an immune mechanism such as viral hepatitis and primary biliary cirrhosis, the antibodies are directed at the hepatocytes and bile ducts, respectively (14,15). Therefore, to our knowledge, this is the first observation of IgG along the sinusoids.

To correlate the lesion with the IgG deposits histopathological analysis was performed. In the liver, a progressive increase of diffuse hyperplasia and hypertrophy of Kupffer cells were observed from 7 to 80 days PI, when hyperplasia became less pronounced. Foci of mononuclear cells were observed from 7 days PI, initially in periportal and centrilobular spaces, but later more disseminated with no preference for any particular zone. In the lung, foci of septal thickening due to congestion, edema and mixed infiltrate of polymorphonuclear neutrophils and mononuclear cells were observed at 7 days PI. Inflammation increased progressively and the polymorphonuclear neutrophils were gradually replaced by mononuclear cells in the infiltrate, and at 30 days PI only mononuclear cells were practically seen. In the kidney, hypercellularity and an increase in mesangial matrix were observed at 30-45 days PI followed by a decrease at 45 days, and at 102 days PI a deposit of an amorphous eosinophilic substance was seen. The lesions in different organs were progres-

sive, with noticeable changes in their features during evolution. In the liver, the mononuclear cell inflammatory infiltrate became more prominent than hyperplasia of Kupffer cells at later times and in the lung the cell population in the interstitial infiltrate changed during the course of infection. The changes in the characteristics of the lesion and the occurrence of more intense IgG deposits around 30-45 days PI in the lesion might suggest that participation of IgG in the pathogenesis occurs at a certain time during the evolution of the infection.

Concerning the mechanism of lesion, the presence of IgG deposits in the liver which has not been shown in immune complex-mediated diseases and the absence of C3 deposits both in the lung and in the liver led us to consider alternative mechanisms. We have some evidence that pathogenic mechanisms other than immune complex-mediated ones participate in the lesions of visceral leishmaniasis. We recently observed the presence of T cells in the kidney in canine visceral leishmaniasis (16). In addition, in systemic lupus erythematosus it has been shown that immunoglobulins might participate in the pathogenesis of glomerular lesions by a mechanism distinct from immune complex deposition (17,18). We have been working with the hypothesis that internalization of immunoglobulins by endothelial cells might have some role in the pathogenesis of visceral leishmaniasis. We observed *in vitro* internalization by endothelial cells of serum IgG from visceral leishmaniasis patients (19) and we preliminarily detected by transmission electron microscopy the presence of IgG in endothelial cells in the liver of hamsters with visceral leishmaniasis processed for immunodetection using anti-hamster immunoglobulin antibody and protein A-gold (20). If this alternative mechanism is present in visceral leishmaniasis, it might play a role in the early phase of infection when IgG deposition is more evident. This mechanism then might trigger processes leading to mi-

gration of mononuclear cells including T cells to the lesion, as observed in the nephropathy of canine visceral leishmaniasis (16). Our findings in the liver and lung are compatible with this hypothesis and studies are in progress for a better understanding of the immunopathogenesis of visceral leishmaniasis.

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Title: Internalization of immunoglobulins by endothelial cells in visceral leishmaniasis

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Running title: Internalization of IgG by endothelial cells in VL

Key words: Internalization, IgG, Endothelial cell, Visceral Leishmaniasis

ABSTRACT

In visceral leishmaniasis (VL) the immune complex deposition has been claimed as the mechanism of tissue lesions. However other mechanisms involving IgG have been described and in this work, we studied internalization of IgG by endothelial cells as an alternative mechanism of lesion. When human umbilical vein endothelial cells (HUVEC) were incubated in vitro with VL sera of patients and hamsters, high intensity of IgG staining was observed in endothelial cells whilst with tegumentary leishmaniasis sera only low intensity IgG staining in fewer endothelial cells was observed. Liver and kidney taken from hamsters with VL at different time periods were analyzed under transmission electron microscopy for internalization of immunoglobulin or IgG by endothelial cells. We observed greater amount of total immunoglobulin at 30 days PI in the endothelial cells from liver and kidney of infected hamsters when compared with non-infected controls. When IgG was searched, we observed more particles at 30 and 60 days PI in endothelial cells in the liver, and at 60 days in the kidney when compared with non-infected control. Vascular permeability changes were studied in cheek pouch of hamsters by intravital fluorescent microscopy, and using stimulation with histamine, plasma leakage was seen increased in non-infected animal and in infected animals at 60 days, but not in infected animals at 30 days of infections.. Increased leakage was detected when purified IgG from hamsters with 60 days PI was applied. These data suggest that internalization of IgG occurring in endothelial cells probably may be one of the mechanisms involved in the pathogenesis in the VL.

INTRODUCTION

Visceral leishmaniasis in Brazil is caused by the protozoa *Leishmania (Leishmania) chagasi* that proliferates within mononuclear phagocytes mainly in the spleen and in the liver. However, other organs are affected and inflammatory process is seen in the lung, kidney and liver (Duarte, 2000) which pathogenesis is not fully elucidated. Hypergammaglobulinemia occurs in visceral leishmaniasis due to polyclonal activation of B lymphocytes (Bunn-Moreno et al., 1985; Campos-Neto et al., 1982) and it might be involved in the pathogenesis of the lesions in mesenchimal organs.

In the kidney immune complex deposition has been claimed as the mechanism of glomerular lesion (Weisinger et al., 1978; Sartori et al., 1987; Poli et al., 1991), but we have some evidences on participation of other pathogenic mechanisms in the lesions in visceral leishmaniasis. We recently observed IgG deposits in the liver outlining the sinusoids in experimental visceral leishmaniasis (Mathias et al., 2001) what is noteworthy since in immune complex-mediated diseases and in animal models of acute and chronic serum sickness, a well known model of immune complex-mediated disease (Cochrane & Dixon, 1978), no lesion in the liver is known. We also observed a presence of T cells in the kidney in canine visceral leishmaniasis (Costa et al., 2000). Moreover, in systemic lupus erythematosus it was suggested that immunoglobulins may participate in the pathogenesis of glomerular lesions by a mechanism distinct from immune complex deposition (Itoh et al., 1993; Ono et al., 1995). Recently, Fuji et al. (2003) showed the interaction between particular antibodies and endothelial cell surface integrins via fibronectin and their internalization by endothelial cells leading to antibody deposition in glomeruli, and suggested this process to be one of the mechanisms of glomerular injury in lupus

nephritis. We present here, in vitro and in vivo evidences showing in visceral leishmaniasis internalization of immunoglobulins by endothelial cells and pathophysiological effect of IgG on microcirculation.

MATERIAL AND METHODS

Serum samples

Sera were obtained from 13 visceral leishmaniasis patients with diagnosis confirmed by finding of *Leishmania* in bone marrow aspirate, 15 patients with tegumentary leishmaniasis with diagnosis confirmed by Leishmanin skin test and analysis of histopathology of the skin lesion, and six normal controls with negative anti-*Leishmania* antibody. All VL patients were from the Northeast, tegumentary leishmaniasis patients were from either Northeast or Southeast of Brazil, and normal controls were laboratory personnel. All procedures with patients were complied with institutional ethical guidelines.

Parasites

Leishmania (Leishmania) chagasi (MHOM/BR/72/cepa 46) were maintained in hamsters with successive inoculations with infected spleen homogenate every three months. For experiments, hamsters with visceral leishmaniasis with 2-3 months of infection were sacrificed under anesthesia and the spleen removed aseptically and the amastigotes purified according to Dwyer (1976) with some modifications. Briefly, the spleens were homogenized in cold RPMI 1640 medium (GIBCO, USA), the cellular suspension maintained for 10 minutes on ice, the amastigotes, filtered, processed 4 times through fine gauge needle (24G) and spun at 250g for 10 minutes. The supernatant was again spun at 250g for 10 minutes and then the resulting supernatant was spun at 2100g for 15 minutes. The pellet was resuspended in RPMI 1640

medium and the concentration of parasites adjusted to 2×10^7 /ml in RPMI 1640 medium.

Animals and experimental protocol

Twenty-eight outbred 45-60 days old male hamsters (*Mesocricetus auratus*) from the Animal Breeding Facility of the Medical School of the University of São Paulo were maintained in the Animal Facilities of the Institute of Tropical Medicine of São Paulo during the experiment. The animals were handled and sacrificed under general anesthesia using sodium pentobarbital (0.1 ml of 60 mg/ml). Seventeen hamsters were inoculated intraperitoneally with purified 2×10^7 amastigotes of *Leishmania* (*Leishmania*) *chagasi* in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD, USA), and sacrificed at 15, 30, 45 and 60 days post-infection (PI). Eleven control animals were injected with RPMI 1640 medium and sacrificed at same time periods (Mathias et al., 2001). At the time of sacrifice, sera, liver and kidney were obtained. Formalin-fixed and paraffin-embedded tissue sections were submitted to immunohistochemistry.

Purification of IgG by affinity chromatography with protein G-Sepharose

Gamma-globulin precipitated from sera with $(\text{NH}_4)_2\text{SO}_4$ -40% was filtrated and purified by affinity chromatography with protein G-Sepharose (Pharmacia, USA), the protein concentration determined at 280 nm at spectrophotometer (Pharmacia, USA), aliquoted and maintained at 4 °C until use.

Internalization of IgG by human umbelical vein endothelial cell (HUVEC)

HUVEC obtained according to Jaffe et al. (1973) were cultured in E300 medium for endothelial cells (Kyokutou, Ibaraki, Japan) supplemented with 5% heat inactivated fetal calf serum (Gibco, EUA) on plastic dishes pre-coated with 10 µg/ml fibrinogen from bovine plasma (Sigma, St. Louis, MO) in Hanks' balanced salt solution (Gibco,

EUA). The cells were maintained at 37 °C, in 5% CO₂ and humid atmosphere until confluent stage and cells from 3rd through 10th passage were used in experiments. To study the internalization of serum IgG by HUVEC, cells were cultured in either Lab-Tek Chamber slide (NUNC, Denmark) or 6 well-cluster plates pre-coated with fibrinogen (SIGMA) until they become confluent, then incubated for 2 hours with E300 medium containing 30% of different serum samples. Cells cultured in 6 well-cluster plates were processed to quantify internalized IgG by capture ELISA as described bellow. Cultures in Lab-Tek Chamber were processed for fluorescent microscopic observation. Glass slides were removed from Lab-Tek chamber, slides were thoroughly washed 3 times with 0.01 M, pH 7.2 Dulbecco's phosphate-buffered saline (D-PBS) with 1% bovine serum albumin (BSA – Sigma, St. Louis, USA) and 0.02% sodium azide (Sigma, USA), fixed with 2% paraphormaldehyde (Sigma, EUA), 0.1M L-lysine hydrochloride, 0.01M sodium periodate in 0.05M, pH 7.4 phosphate buffer at 4 °C for 30 minutes, washed 3 times as above, then incubated with FITC-conjugated rabbit anti-human IgG (Dako) antibody for one hour at 4 °C, washed 3 times as above, incubated with 4 µg/ml propidium iodide (Sigma, USA) in PBS, washed 3 times as above and mounted with glycerol:PBS (v:v 9:1) and examined under fluorescent microscope (Zeiss, Germany).

Determination of internalized IgG by capture ELISA

HUVEC cultured in 6 well culture plates and incubated with test sera were ruptured with 0.1 % Triton X 100 in Na₃C₆H₅O₇2H₂O, pH 7.4, and the samples assayed for IgG determination. Biotin labeled goat anti-human IgG diluted 1:50 in DPBS (50 µL/well) was dispensed on a 96 well ELISA plate (MicrotiterR MIC-2000T.M. Dynatech Laboratories, Inc.), incubated overnight at 4 °C, washed with PBS three times, blocked with 10 % skimmed milk in DPBS (wt/vol) (100 µL/well) for 60 minutes at

room temperature, washed with DPBS 0.1 % Tween 20 (PBS/T) three times. Then it was incubated with test samples prepared in the flat plate or standard samples (human IgG) serially diluted in PBS/T/1 % BSA (Sigma, Fraction V) (PBS/T/BSA) (50 μ L/well) for 60 minutes at 37 °C, then washed with DPBS/T three times. It was incubated with alkaline phosphatase-conjugated goat F(ab')₂ anti-human IgG (Protos Immunoresearch) diluted 1:1000 in PBS/T/BSA (50 μ L/well) for 60 minutes at 37 °C. Before incubating with Sigma 104R in substrate buffer (1 mg/ml, 1 tab./5 mL) (100 μ L/well) for 15 to 60 minutes at 37 °C, in 9.7 % diethanolamine; 3.07 mM N_aN₃, 0.49 mM MgCL₂6H₂O solution, pH 9.8, the plate was washed with PBS/T four times.

Detection of IgG in the tissues for transmission electron microscope analysis

Fragments of tissues of around 0.3 mm³ were fixed for two hours in 4% paraphormaldeyde and 0.1% glutaraldeyde in PBS, washed 3 x 15 minutes with PBS, and incubated with 50 mM ammonium chloride in phosphate buffer 0.1 M pH 7.4 for 15 minutes. Then, they were dehydrated in increasing concentrations of ethanol, included in LR White hard grade resin (Sigma, U.S.A.)/ethanol 1:1 (vol:vol) for 30 minutes, LR White:ethanol 3:1 (vol:vol) twice for 30 minutes, LR White:ethanol 3:1 (vol:vol) overnight at 4 °C, pure LR White twice for 30 minutes, pure LR White overnight at 4 °C, once again in pure resin for one hour, and then placed in gelatin capsules that had been filled until the edge with pure LR White that was maintained at 52 °C for three to five days, for polymerization. Ultrathin sections on formwar-covered nickel mesh (Sigma, EUA) were washed once in PBS for 5 minutes. After hydration with PBS plus 0.1% bovine serum albumin (BSA buffer) pH 7.4 for five minutes, they were incubated with polyclonal rabbit anti-hamster immunoglobulin antibody (produced in our laboratory) and then with biotinylated goat anti-hamster

IgG antibody (20 µg/ml in BSA buffer) (Rockland, Gilbertsville, PA, USA) for two hours in humid chamber at room temperature. Before the incubation with protein A-gold 10 nm (Sigma, U.S.A.) diluted 1:10 in PBS pH 7.4 - 1% BSA for two hours in humid chamber at room temperature, they were washed twice for five minutes each, followed by four washes of four minutes in PBS pH 7.4, 0.05% Tween 20, 1% of BSA and for one minute in distilled water. Then they were fixed in 2% aqueous glutaraldehyde for 15 minutes, washed again three times in distilled water, incubated for five minutes with 1% aqueous osmium tetroxide, three times in distilled water for 15 minutes, then dried at room temperature, incubated with uranyl ultrafiltered for 10 minutes, washed some times quickly in distilled water and for three minutes with lead, and washed in distilled water. The processed ultrafin sections were observed at transmission electron microscope JEOL. Electromicrographs from the fields of interest were taken, analyzed and submitted the morphometric analysis.

Morphometry

The electromicrographs were analyzed, quantifying the deposits of protein-A gold-labeled immunoglobulin and IgG, using a 1 cm² square-lined transparent sheet on the electromicrographs. The endothelial cell area was delimited and gold particles were counted in whole area. The value obtained counting gold particles found on the cell nucleus was considered non specific and used to subtract from the counting obtained on endothelial cells. Tissue sections of liver and kidney from 5 infected animals with 30 and 60 days and of 3 non-infected control animals were used. The morphometry was carried out in a total of 43 electromicrographs of liver and in 79 of kidney.

Intravital fluorescence microscopy

Hamsters were anesthetized intraperitoneally with sodium pentobarbital (0.1 ml of 60 mg/ml) supplemented with i.v. chloralose (2.5 % solution, ~0.1 ml/hour) were injected through right femoral vein catheter. Another catheter (PE 10) in the left femoral vein was used for FITC-dextran infusion. A tracheal cannula (PE 190) was inserted to facilitate spontaneous breathing and the body temperature was maintained at 37 °C by a heating pad and a rectal thermistor. The hamster cheek pouch was prepared for intravital microscopy according to Duling (1973) as modified by Svensjö (1978 and 1990). Briefly, the cheek pouch was everted and mounted on a microscope stage and an area of about 1 cm² was prepared for intravital fluorescence microscopy. The cheek pouch was superfused with a warm (35 °C) bicarbonate buffered salt solution which was continuously bubbled with 95 % N₂ and 5 % CO₂ to maintain a low oxygen tension (~4kPa) and a pH of 7.35. Thirty minutes after complete preparation, fluorescein labelled dextran (FITC-dextran, MW= 150000, TdB Consultancy, Uppsala, Sweden) was injected intravenously (25 mg/100 gb.w) as a macromolecular tracer. The microvascular permeability increase for large molecules was quantified by counting fluorescent spots (leaky sites=leaks) at postcapillary venules (Svensjö, 1978 and 1990). Number of leaks were counted before and at 2, 5, 7, 15 and 30 minutes after topical application of histamine 5.10⁻⁶ M for 5 min. The vascular permeability response to histamine was studied in hamsters which had been injected intraperitoneally either with RPMI 1640 medium (control) or with purified *Leishmania (L.) chagasi* amastigotes and the microcirculation of the cheek pouch was studied at 15, 30 and 60 days post-infection by intravital fluorescent microscopy. For each point in time a number of 4-6 control hamsters and 6-7 *Leishmania*-infected hamsters were studied. In another approach, normal hamsters were prepared and

purified IgG (290 µg/mL) from *Leishmania*-infected and non-infected hamsters were topically applied on the cheek pouch.

RESULTS

We have studied internalization of immunoglobulins by endothelial cells in vitro and in vivo in visceral leishmaniasis in hamsters. Further we have searched the pathophysiological effect of IgG from visceral leishmaniasis hamster on microcirculation.

Internalization of IgG by HUVEC

When HUVEC were incubated with visceral leishmaniasis patient sera, high intensity of IgG staining was observed in endothelial cells with 11 from 13 sera (Figure 1A) whilst in tegumentary leishmaniasis only low intensity IgG staining in fewer endothelial cells was observed with 8 from 15 sera (Figure 1B). Very discrete IgG staining was observed with normal sera (data not shown).

VL patients have hypergammaglobulinemia, therefore internalization of IgG by endothelial cells were quantitatively evaluated after incubating HUVEC with the same concentration of IgG from different sera in E300 medium. Capture ELISA confirmed higher ingestion of IgG from visceral leishmaniasis patients (N= 10; median = 230, range - 60 to 2,610 pg/1000 cells) compared with those from tegumentary leishmaniasis (N= 8; median = 30, range – 30 - 260 pg/1000 cells).

Sera from hamsters with visceral leishmaniasis were also assayed with HUVEC, and we observed intense internalization of IgG with samples from 30 and 45 days post infection (PI) (Figures 1C and 1D, respectively). Internalization of IgG with sera from non-infected control and from infected animals of other experimental periods was very discrete (data not shown).

Detection of total immunoglobulin and IgG in the tissues of hamster with visceral leishmaniasis by transmission electron microscopy

As we verified in vitro the internalization of IgG of the serum of patients and hamster with visceral leishmaniasis by endothelial cells (HUVEC), we proceeded with in vivo study in visceral leishmaniasis in hamsters, detecting total immunoglobulin and IgG in endothelial cells of liver and kidney, at 30 and 60 days PI, by transmission electron microscopy. When we analyzed qualitatively the presence of total immunoglobulins in the liver, we observed greater amount of gold particles in the samples of infected animal than in non-infected control (Figures 2A and 2B). In quantitative analysis, we observed greater amount of immunoglobulin at 30 days PI in the endothelial cells when compared with non-infected controls (Table 1). In the kidney, qualitatively the amount of gold particles was similar in infected and in non-infected control animals, however, when quantified, greater amount of immunoglobulins was observed at 30 days PI in glomerular endothelial cells when compared with the non-infected controls (Table 1). When IgG was searched, we observed more particles at 30 and 60 days PI in endothelial cells in the liver when compared with non-infected control (Table 2). In the kidney, we observed more particles in endothelial cells at 60 days PI when compared with non-infected controls (Table 2).

Intravital microscopy study of microcirculation

Considering that endothelial cells are involved in any inflammatory process and that the change in vascular permeability is one of the main features in inflammation, this aspect was studied in hamster cheek pouch microcirculation. When we stimulated the cheek pouch with histamine, all hamsters responded to histamine

with reversible increase in the number of postcapillary leaks. In the control group there were no significant differences in the response to histamine at 15, 30 and 60 days. In the infected animals the response to histamine was less at 30 days ($P < 0.05$) than in the control animals (Figure 3). When we carry out the experiment using IgG purified from the sera of infected and non-infected hamsters, we observed a significant increase in the number of postcapillary leaks with sample from 60 days PI when compared with the control and 30 days PI samples (Figure 4).

DISCUSSION

The accepted pathogenic mechanism, mainly in the kidney, in visceral leishmaniasis is immunocomplex deposition. Internalization of antibodies was described recently as a novel mechanism of lupus nephritis in mice (Fujii et al., 2003). In visceral leishmaniasis may occur a similar mechanism, since deposition of immunoglobulin was seen in the liver, lung and kidney, but did not have typical components of immunocomplex-mediated lesion (Mathias et al., 2001). Initially in this work, we observed the interaction of immunoglobulin from sera from patients with visceral or tegumentary leishmaniasis and *Leishmania (L.) chagasi* infected hamsters with endothelial cells, in vitro. IgG of both human and hamster visceral leishmaniasis serum was intensively internalized by HUVEC cells. Intense internalization of IgG from hamster occurred when sera from 30-45 days of infection were used. In addition, in vivo, we evaluated the internalization of total immunoglobulin and IgG from serum from *Leishmania (L.) chagasi*-infected hamsters by endothelial cells both in the liver and kidney. We observed by transmission electron microscopy similar results obtained in vitro as the presence of internalized immunoglobulin and specifically IgG. The internalization of immunoglobulins by

endothelial cells was increased in the liver and kidney from infected hamster at 30 days of infection. When IgG was analyzed, we observed the internalization at 30 and 60 days PI in the liver, while in the kidney, these were verified at 60 days PI. These data indicates the existence of internalization immunoglobulins by endothelial cells both in the human and hamster visceral leishmaniasis that may be related to the pathogeny of the lesion. It is known that endothelial cells have main role in inflammation, and we searched the pathophysiological effect of IgG from visceral leishmaniasis. We studied vascular macromolecular permeability as parameter in hamster cheek pouch by intravital microscopy. Initially, when we stimulated the cheek pouch of control and visceral leishmaniasis hamsters with histamine, all responded to histamine (vasodilator) with reversible increase in the number of postcapillary leaks, except the group of 30 days PI. To test directly the effect of IgG from visceral leishmaniasis hamsters, it was purified and then applied on cheek pouch of normal hamster. When IgG of hamsters at 60 days PI, was applied, we observed a significant leakage that revealed a vasoactive property in the IgG obtained at this period of infection. We do not have enough data to connect the internalization of IgG with its vasoactive effect since it has occurred in different time periods. However, it strongly indicates a potential role in the pathogenesis of the lesion. Further studies are planned to study adhesion molecule expression and characteristics of IgG.

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Figure Legends

Figure 1. Internalization of IgG by human umbelical vein endothelial cells (HUVEC).

A) High intensity staining of IgG in HUVEC incubated with serum of patients with visceral leishmaniasis. B) Low intensity staining of IgG in HUVEC incubated with serum of tegumentary leishmaniasis patients. C and D) High intensity staining of IgG in HUVEC incubated with serum samples, respectively, from hamsters with visceral leishmaniasis with 30 and 45 days PI. Fluorescent microscopy. 400X.

Figure 2. Detection of immunoglobulin in the endothelial cells of infected and non-infected hamsters, using polyclonal rabbit anti-hamster immunoglobulin antibody and protein A-gold (10 nm). A) Liver of non-infected control hamster at 30 days. B) Liver of infected hamster at 30 days post-infection. C) Kidney of non-infected control hamster at 30 days. D) Kidney of infected hamster at 30 days post-infection. Presence of protein A-gold particles ($\uparrow$) in the endothelial cell (E). Eosinophil (Eo). Erythrocyte (Er). Hepatocyte (H). Disse space (D). Sinusoid (S). Basal membrane (M). Lume (L). Foot process of podocytes (Pp). TEM. 50.000X.

Figure 3. Maximal number of leaks observed after topical application of histamine in the hamster cheek pouch of *Leishmania* (L.) *chagasi*-infected and non-infected hamsters, at 15, 30 and 60 days of experiment. Results expressed as mean $\pm$ standard deviations. Control ($\square$) and infected animals ($\equiv$).

Figure 4. Maximal number of leaks observed in the hamster cheek pouch after application of IgG purified from the serum of *Leishmania* (L.) *chagasi*-infected and non-infected hamsters, at 15, 30 and 60 days of experiment. * = median. Control ($\square$) and infected animals ($\equiv$). PI= postinfection.

Figure 1.

Figure 2A

Figure 2B

Figure 2C

Figure 2D

Figure 3

Figure 4

Organ	Contro	30 days	60 days
Live	0.05 (n=4)	0.56*	0.34
Kidney (glomerulus)	0.21±0.25 (n=6)	1.35±0.02 (n=2)	0.18±0.22 (n=5)

Table 1. Detection of immunoglobulins using polyclonal rabbit anti-hamster immunoglobulin antibody and protein A-gold (10 nm) particle in the liver and kidney. Number of protein A-gold particles/cm² (mean± standard deviation) in the endothelial cells of infected and non-infected control hamster: morphometry. Liver. *Background* = 0.26/cm². * P< 0.05 in relation to non-infected animals (Kruskal Wallis and Dunn tests). Kidney. *Background* = 0.40/cm². *P< 0,05 in relation to non-infected animals (ANOVA and Student-Newman-Keuls). N= total number of electronmicrographs analyzed. PI= postinfection.

Organ	Contro	30 days	60 days
Live	0.03±0.05 (n=4)	0.11±0.0 4	0.13±0.06 (n=9)
Kidney (glomerulus	0.07±0.10 (n=2)	0.05±0.01 (n=2)	0.13±0.08 (n=2)

Table 2. Detection of IgG using goat anti-hamster IgG antibody (20 µg/ml) in the liver and kidney. Number of protein A-gold particles/cm² (mean ± standard deviations) in the endothelial cells of infected and non-infected hamsters: morphometry. N= number of electronmicrographs analyzed. PI= postinfection.

**T cells and adhesion molecules participate in the pathogenesis of
glomerulonephritis in canine visceral leishmaniasis**

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Abstract

Until recently, studies on glomerular changes in VL pointed the immune complex deposition as the mechanism of lesion. However, some recent researches have shown that the mechanisms of glomerular injury are multiple. Examining renal alterations in 55 dogs with naturally acquired visceral leishmaniasis (VL) we have shown that immunoglobulins and complement deposits were not present in greater quantity in glomeruli in infected dogs when compared with the non-infected control dogs. However, we observed an expressive presence of T cells in glomeruli in 46 (83.6%) infected dogs, mainly CD4⁺ T cells, but absent in non-infected control dogs. In addition correlation between the presence of *Leishmania* antigen and CD4⁺ T cells was observed. Adhesion molecules were also analyzed. The expression of ICAM-1 and P-selectin was significantly greater in glomeruli in infected dogs than in the non-infected control dogs and were correlated with the presence of *Leishmania* antigen. Considering that the pathogenesis of glomerulonephritis has not been completely elucidated, we discuss in this study some important aspects that may play a major role in glomerular injury in canine VL.

Introduction

Visceral leishmaniasis (VL) affect mainly organs of the mononuclear phagocyte system but renal changes in canine VL are frequent, and their pathogenesis has not been totally elucidated. Until recently, studies on glomerular changes in VL pointed the immune complex deposition as the mechanism of lesion (Weissinger et al., 1978; Tafuri et al., Ferrer, 1999). However, studies on the pathogenesis of glomerulonephritis of other etiologies has shown involvement also of T cells (van Alderwegen et al., 1997; Penny et al., 1997; Tipping et al., 1998) and adhesion molecules (Bonventre e Colvin, 1996) in some cases. In our previous study we have shown glomerulonephritis in all 55 dogs with VL, naturally-infected animals from endemic area. The glomerular alterations were characterized histopathologically, and classified in six different patterns: minor glomerular abnormalities, focal segmental glomerulosclerosis, mesangial proliferative glomerulonephritis, membranoproliferative glomerulonephritis, crescentic glomerulonephritis, and chronic glomerulonephritis (Costa et al., 2003). Although different patterns indicate possible involvement of different pathogenic mechanisms, common features may be present. In a preliminary study we have already detected CD4⁺ T cells in glomeruli in five dogs with VL (Costa, et al., 2000).

To better understand the immunopathogenesis of glomerulonephritis in VL, in this work we have extended the study evaluating the presence of immunoglobulins, CD4⁺ and CD8⁺ T cells and adhesion molecules in glomerulonephritis in dogs with visceral leishmaniasis.

Materials and Methods

Animals and diagnosis of VL

55 male and female adult dogs with different patterns of renal lesions and positive for leishmaniasis, as previously described (Costa et al., 2003), were selected for this study.

Detection of *Leishmania* antigen, immunoglobulins, CD4⁺ and CD8⁺ T cells and adhesion molecule in renal tissue

Formalin-fixed and paraffin-embedded kidney sections were deparaffinized in xylene, rehydrated in decreasing alcohol concentrations, then incubated with 0.03% hydrogen peroxide in methanol solution for 30 minutes in the dark to block endogenous peroxide activity. Antigen retrieval was performed using Tris-HCl (1.2 mg/ml), pH 1.0, in a microwave oven (Sanyo, Brazil), under maximum potency in consecutive cycles of 10 and 5 minutes. After washing in 0.01 M phosphate-buffered saline, pH 7.2 (PBS), the sections were treated using a 'Blocking Kit' (Vector Laboratories, Inc., Burlingame, USA), and a "protein block" (Dako Corporation). The tissues were then incubated overnight at 4°C in a humid atmosphere with different antibodies: mouse polyclonal anti-*Leishmania amazonensis* antibody diluted 1:1600 (vol:vol), goat polyclonal anti-canine IgG, IgM, IgA and C₃ antibody in concentration of 10 µg/ml, mouse monoclonal anti-canine CD4⁺ and CD8⁺ T cells antibody diluted 1:500 (vol:vol) in PBS and mouse monoclonal anti-canine ICAM-1 and P-Selectin antibody, gently provided by professor C. Wayne Smith, Baylor College of Medicine, Houston, Texas, E.U.A., in concentration of 10 µg/ml in PBS. The reaction proceeded using a catalyzed signal amplification (CSA) system- peroxidase (Dako Corporation) following protocols provided by the manufacturer, and the reaction developed using 0.06% hydrogen peroxide and 0.3 mg/ml 3,3'-diaminobenzidine (Sigma Chemical,

USA) in PBS. Counterstaining was performed using Harry's hematoxylin (Sigma Chemical, USA).

Morphometry

Morphometric analyses were performed on some sections from samples stained with H-E, and on those stained for *Leishmania* antigen and CD4⁺ and CD8⁺ T cells by immunohistochemistry using an automatic image analyzer employing the Bioscan Optimas software (Optimas, Edmonds, CA, US—Version—4.10). The following parameters were measured: glomerular area, total number of cells per glomerulus, total number of cells positive for *Leishmania* antigen, CD4⁺ and CD8⁺ T cells per glomerulus and total number of cells per glomerular unit in a total of 50 glomeruli per animal.

Statistical analyses

The morphometric parameters were analyzed using the Kruskal-Wallis or Dunn tests to compare multiple groups, and the Mann-Whitney or Student t-test to compare two groups, employing Sigma Stat software (Jandel Corporation, USA).

Lesion intensity was classified semi-quantitatively on a scale from 0 to 4⁺ where 0 = normal, 1 = minimal or doubtful; 1⁺ = medium; 2⁺ = moderate; 3⁺ = moderately severe; and 4⁺ = severe, according to Tisher and Brenner (1994).

Results

Detection of immunoglobulins and C₃b in glomeruli

Immunoglobulins IgG, IgM and IgA and C₃b were searched in 26 infected and in 5 non-infected control dogs. They were present in all patterns of glomerulonephritis and also in non-infected control dogs. Semi-quantitative analysis of immunoglobulins and C₃b deposits in the glomerular capillary wall did not reveal any significant

difference when infected dogs as a whole were compared with non-infected control dogs (Fig. 1).

Immunoglobulins and C₃b were localized outlining the glomerular capillary wall whereas the *Leishmania* antigen was localized in the mesangium as granulous deposit (Fig. 2 a,b e c).

Detection of CD4⁺ and CD8⁺ T cells in glomeruli

CD4⁺ and CD8⁺ T cells were not detected in any non-infected control dogs. However, in infected dogs, CD4⁺ T cells (Fig. 3a) were observed in glomeruli in 44 cases (80%), and CD8⁺ T cells (Fig. 3b) in 14 cases (25.4%). In all cases with CD8⁺ T cells, CD4⁺ T cells were also present. T cells, mainly CD4⁺ T cells, were present in almost all patterns of glomerulonephritis but not in chronic glomerulonephritis, probably because almost no cells were present in glomeruli.

Morphometric analysis showed more CD4⁺ T cells per glomerulus ($p = 0.007$, Mann-Whitney) and per glomerular area ($p < 0.0001$, Mann-Whitney test) in infected dogs compared with the non-infected control dogs, but there was no difference with CD8⁺ T cells. More CD4⁺ T cells in mesangial proliferative glomerulonephritis and membranoproliferative glomerulonephritis compared with the non-infected control dogs ($p < 0.05$, Kruskal-Wallis and Dunn Tests) was observed. There was a correlation between the presence of *Leishmania* antigen and CD4⁺ T cells ($R = 0.69$, $p < 0.001$, Spearman test) (Fig. 4a), and CD4⁺ and CD8⁺ T cells ($R = 0.60$, $p < 0.001$, Spearman test) (Fig. 4b) in glomeruli, but correlation between *Leishmania* antigen and CD8⁺ T cells was not observed (data not shown). *Leishmania* antigen in the noninfected control dogs and in the case of chronic glomerulonephritis was not present.

Detection of adhesion molecules in renal tissue

Adhesion molecules were searched in 20 infected dogs and in 5 noninfected control animals. ICAM-1 and P-selectin were present in glomeruli in all infected dogs, but LFA-1 was not detected in these animals. In non-infected control dogs the adhesion molecules were also detected but in minimal intensity and P-selectin was observed in only one animal. Both ICAM-1 (Fig. 5a) and P-selectin (Fig. 5b) were localized in the endothelium of the glomerular capillaries, in the mesangium and Bowman's capsule.

Semi-quantitative analysis revealed significantly greater presence of ICAM-1 ($p = 0.003$, Mann-Whitney tests) and P-selectin ($p = 0.01$, Mann-Whitney tests) in infected dogs as a whole when compared with the non-infected control dogs.

In all cases where P-selectin was detected ICAM-1 was also present, presenting positive correlation ($R = 0.56$, $p < 0.005$, Spearman test). Where ICAM-1 and P-selectin were present, T cells, mainly $CD4^+$ T cells were also detected, except in 2 cases of focal segmental glomerulosclerosis and in a case of membranoproliferative glomerulonephritis. There was also correlation between the presence of *Leishmania* antigen and ICAM-1 ($R = 0.54$, $p < 0.05$, Spearman test) (Fig. 6a) and P-selectin ($R = 0.55$, $p < 0.05$, Spearman test) in infected dogs (Fig. 6b).

Discussion

Since the accepted pathogenic mechanism of glomerulonephritis in visceral leishmaniasis is immune complex deposition, immunoglobulin, C_3b and *Leishmania* antigen were searched initially. In the present study immunoglobulins and complement deposits were not present in greater quantity in glomeruli in infected dogs

when compared with the non-infected control dogs. Therefore these findings suggested that, among infected dogs as a whole, immunoglobulin and complement do not have any importance in the pathogenesis of glomerulonephritis, at least in the period in that the animals were examined. In experimental visceral leishmaniasis in hamster, IgG deposits were observed in greater intensity than in control cases in certain phases of infection (Mathias et al., 2001), therefore we can not totally discard their participation in the pathogenesis in other time period in canine VL. However, there are also other studies where immune complex is not considered important in the pathogenesis of glomerulonephritis, reinforcing our findings. Levels of immune complex detected in the bloodstream in dogs or humans with visceral leishmaniasis has not been correlated with the nephropathy of VL (Jubb et al., 1985; Margarito et al., 1998, Dutra et al, 1985; Poli et al, 1991). Absence of IgG, IgA and IgM deposits in the kidney were reported in human VL (Caravaca et al., 1991). Further, immunoglobulins have been detected in sample of control kidney in the study in human VL (Brito et al., 1975).

Role of immunocomplex is doubtful, but if it has any participation, the *Leishmania* antigen does not seem to take part in the complex. In the present study *Leishmania* antigen, immunoglobulins and C₃b were detected in different localization in glomeruli. While *Leishmania* antigen was seen in phagocytic cells, granular IgG and C₃b deposits were localized mainly along the capillary wall.

If immunocomplex deposition is not the pathogenic mechanism, other mechanisms would be operating. The finding of focal segmental glomerulosclerosis, a pattern that is not caused by immune complex (Gibson and More, 1998) reinforces the possible existence of other mechanism of glomerular lesion. Currently there is growing evidence that T cells (Fillit and Zabriskie, 1982; Mccluskey and Bhan, 1982;

van Alderwegen et al., 1997) and adhesion molecules as well and their endothelial ligands play a fundamental pathogenic role in some immunologically-mediated glomerulonephritis (Lhotta et al., 1991; Hill et al., 1994ab; Bonventre e Colvin, 1996; Ootaka et al., 1997; Kootstra et al., 1998; Rui-Mei et al., 1998). In the present study, we observed an expressive presence of T cells in glomeruli in 46 (83,6%) infected dogs as a whole, mainly CD4⁺ T cells, but absent in non-infected control dogs. CD8⁺ T cells were less frequent. This finding unquestionably defines the role of CD4⁺ T cells in the pathogenesis of glomerulonephritis in canine VL suggested in our preliminary study (Costa et al., 2000). It is known that the participation of immune elements depends on the pathologic process and of the histopathological patterns of glomerulonephritis (Cunard and Kelly, 2003). In some other diseases the cell population implied has been CD4⁺ T cells (Bolton et al., 1987; Tipping et al., 1998), in others CD8⁺ T cells (Penny et al., 1998), and still concomitantly antibodies and T cells (Bhan et al., 1978; Tipping et al., 1985). Seemingly it depends on the ethiology of the process, and also on the histopathological pattern of glomerulonephritis (Cunard and Kelly, 2003). In experimental crescentic glomerulonephritis in CD4⁻ or CD8⁻ mouse participation of CD4⁺ T cells but not CD8⁺ T cells were shown to be crucial (Tipping et al., 1998).

A significant increase in glomerular area ($P > 0.001$, Mann-Whitney tests) and cell number per glomerulus ($P > 0.001$, Mann-Whitney tests) in proliferative patterns of glomerulonephritis, at least partially may be attributed to the presence of CD4⁺ T cells that are non resident cells of the glomerulus. In another study we have observed that the hypercellularity in glomerulus in canine VL is not due to in situ proliferation, since almost no stain was seen with antibodies for Ki-67, a proliferative marker (personal communication).

Detection of *Leishmania* antigen in glomeruli in 90.9% of the infected dogs as reported in our previous study (Costa et al., 2003) confirms that the glomerular lesions were caused by leishmaniasis. In addition correlation between the presence of *Leishmania* antigen and CD4⁺ T cells was observed in this study suggesting that *Leishmania* antigen is guiding inflammatory infiltrate of CD4⁺ T cells in glomeruli in canine VL.

The presence of P-selectin and ICAM-1 were also analyzed, and in all infected cases we detected both molecules in glomeruli in canine VL. The non-infected control cases have shown a discrete expression of ICAM-1 what has also been observed in human (Müller et al., 1991) and in experimental glomerulonephritis (Hill et al., 1994a). The significantly greater expression of ICAM-1 and P-selectin in infected dogs than in the non-infected control group suggests their role in the pathogenesis of nephropathy in naturally acquired canine visceral leishmaniasis. In human glomerulonephritis (Müller et al., 1991, Lhotta et al., 1991; Cavalcanti, 1995; Ootaka et al., 1997) and in murine malaria (Rui-Mei et al., 1998) ICAM-1 is similarly the expressed.

Strong expression of P-selectin, mainly, in mesangium but also in glomerular capillaries and Bowman capsule was observed in the present study. Similar findings have been reported in other human and experimental glomerulonephritis (Xiao et al., 1997, Tipping et al., 1994; Zachem et al., 1997). The detection of P-selectin in mesangium associated with the strong presence of CD4⁺ T cells but absence of polymorphonuclear leukocytes in glomeruli, suggests a possible presence in glomeruli of newly migrated platelets besides CD4⁺ T cells that are able to express P-selectin . It is reported that interaction occurs between P-selectin and some sub-populations of lymphocytes (Hébert and Brady, 1997). In addition, platelets have been considered as

an important modulators of injury in glomerulonephritis (Hayslett, 1984), and therefore as a marker of activation of platelets in glomerulus. With their aggregation they concur for the infiltration of inflammatory cells and to intraglomerular cell proliferation (Xiao et al., 1997).

In this study was also observed correlation between the presence of *Leishmania* antigen and ICAM-1 and P-selectin indicating important role of the *Leishmania* antigen and these molecules in the pathogenesis of the nephropathy in canine VL.

The data of the present study suggest important participation of CD4⁺ T cells but not imunoglobulins and C₃b in the pathogenesis of the glomerulopathy in the chronic phase in canine visceral leishmaniasis. Presumably, *Leishmania* antigen, P-selectin and ICAM-1 are important in the triggering and maintenance of the pathogenic mechanisms and glomerular infiltration by CD4⁺ T cells.

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Figure Legends

Figure 1. Kidney. Naturally infected dog with *Leishmania (L.) chagasi*. Semi-quantity analyses of the presence of immunoglobulins and complement in the glomerular capillary (median of escors and interval between percentis 25 e 75) in noninfected control and infected dogs. A) IgG. B) IgM. C) IgA. D) C₃b. N = N°. of animals per group.

Figura 2. Kidney. Naturally infected dog with *Leishmania (L.) chagasi*. IgG (A) and C3b (B) deposit outlining the glomerular capillary wall and in the mesangium with granulous aspect of meduim intensity. *Leishmania* antigen deposit (C) on mesangial cells. Immunoperoxidase. Bar = 25 µm.

Figure 3. Kidney. Naturally infected dog with *Leishmania (L.) chagasi*. Presence of CD4⁺ (A) and CD8⁺ (B) T cells in glomerulus of severe intensity in membranoproliferative glomerulonephritis. Immunoperoxidase. Bar = 25 µm.

Figure 4. Kidney. Naturally infected dog with *Leishmania (L.) chagasi*. (A) Correlation between number of cells expressing *Leishmania* antigen and CD4⁺ T cells per glomerulus. (B) Correlation between number of CD4⁺ T cells and CD8⁺ T cells per glomerulus. 50 glomeruli analyzed per animal. $p < 0,001$ (Spearman test).

Figure 5. Kidney. Naturally infected dog with *Leishmania (L.) chagasi*. Presence of ICAM-1 (A) and P-selectin (B) in segmental focal glomerulosclerosis of severe moderately intensity. Immunoperoxidase. Bar = 25 µm.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Apoptosis in nephropathy of naturally acquired canine visceral leishmaniasis

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Short Title: Apoptosis in glomerulonephritis in canine visceral leishmaniasis

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Abstract

Renal involvement in visceral leishmaniasis (VL) in humans and in dog is very frequent. In canine VL we previously observed different patterns of glomerulonephritis (GN) with predominance of proliferative patterns with presence of T cells, predominantly CD4⁺ cells, in glomeruli. Since apoptosis is known to participate in some proliferative processes in kidney, in the present study we analyzed the participation of apoptosis and related cytokines TNF- α and IL-1 α in the renal lesions in dogs with naturally acquired VL from Northeast of Brazil. We studied 36 dogs with naturally acquired VL and 5 non infected controls from the same area. Apoptosis was present in glomerulus in dogs with VL as well as in non infected control animals. The amount of apoptotic cells was less in infected animal group than in control animals. The expression of TNF- α in animals with naturally acquired VL was less than in non infected control animals. When groups with different patterns of GN was analyzed, the groups with proliferative GN presented lower TNF- α expression than non infected control animals. The expression of IL-1 α did not present any significant difference between animals with naturally acquired VL and non infected control animals. The proliferation was also studied by searching the expression of cell proliferation associated antigen Ki-67. It was very discrete and not significant in any pattern. These results showing no proliferation in glomeruli suggest that the inflammatory cells in glomeruli in canine VL are constituted by migrated cells. In addition, decreased apoptosis suggest that it may account for the persistence of increased inflammatory cells in glomeruli that seemed related to low production of TNF- α in canine VL.

Introduction

Canine visceral leishmaniasis (VL) is highly prevalent around the world. In Brazil it is caused by the protozoa *Leishmania (Leishmania) chagasi* and it is present in 18 out of 27 Brazilian States.^(23,25,31) Northeast of Brazil is endemic area for VL and nowadays it is also spreading to other urban areas in the South and Southeast region where the disease was not endemic before.⁽³⁵⁾ In the infectious cycle of VL the dog is the most important domestic reservoir.^(7,26)

Leishmania is an obligatory intracellular parasite of mononuclear phagocytes. During infection several organs of the host are affected including the kidney. Nephropathy of VL has been reported both in humans and in dogs, and the renal involvement in dog is very frequent.^(4,27,36) However, the histopathological patterns of the lesions present in VL only recently we have clearly established in dogs with naturally acquired VL from the Northeast of Brazil. In this study, the histopathological analysis of kidney samples revealed glomerulonephritis in all 55 animals with VL that was distributed in six morphological patterns: minor glomerular abnormalities (14.5%), focal segmental glomerulosclerosis (18.2%), diffuse mesangial proliferative glomerulonephritis (30.7%), diffuse membranoproliferative glomerulonephritis (32.7%), crescentic glomerulonephritis (1.8%) and chronic glomerulonephritis (1.8%).⁽⁹⁾ Presence of proliferative process is identified in some of these patterns of glomerulonephritis in other animal models and human disease, such as diffuse mesangial proliferative glomerulonephritis and crescentic glomerulonephritis using immunohistochemical markers of mitosis.^(32,44)

Apoptosis has been reported in the course of renal injury, including glomerulonephritis, both in animal models and clinical kidney diseases.⁽³³⁾ Apoptosis, a type of cell death, is considered important in the pathophysiological processes in

many tissues to clear the undesired cells without eliciting inflammation.^(8,24) In glomerulonephritis apoptosis plays an essential role in the recovery of the original glomerular structure determining the regression of cell numbers during the repair process.^(3,40) Several cytokines and inflammatory mediators induce apoptosis. The absence of survival factors IGF-I, IGF-II and bFGF, collagen IV and laminin or the presence of lethal cytokines TNF- α Fas ligand (FasL), IL-1 α , and oxygen radicals can predispose renal cells to apoptosis.^(21,27,33,39,42)

Since in canine visceral leishmaniasis we observed patterns where proliferation and/or inflammatory cell migration process is present, and apoptosis is known to have a role in repair processes in glomerulonephritis, in the present study we analyzed the participation of proliferative process, apoptosis and related cytokines TNF- α and IL-1 α

ANIMALS AND METHODS

Animals and diagnosis of visceral leishmaniasis

The dogs that presented positive serology for leishmaniasis during the survey performed by Center of Control of Zoonosis of Terezina city were initially selected and 36 male and female adult dogs with different age and breed were chosen randomly from this population during the period from May 1996 through May 1998. The diagnosis of VL was confirmed by detection of anti-*Leishmania* antibodies in the sera by indirect immunofluorescence assay and ELISA, and by detection of *Leishmania* in smears of skin, spleen and popliteal lymph nodes and/or culture of material from external bone marrow, spleen and popliteal lymph nodes. The characterization of the parasite was performed at Instituto Evandro Chagas, in Belém, Pará State in samples of two dogs using species-specific monoclonal antibodies. Five

dogs of the same endemic area without VL were used as control. All *Leishmania*-infected dogs were routinely sacrificed by Center of Control of Zoonosis for the control of transmission of visceral leishmaniasis. The non-infected animals used as control in this study were street dogs collected to be sacrificed for control of rabies.

The animals were handled and sacrificed under general anesthesia using 25 mg/kg i.v. thiopental sodium (Sigma-Aldrich, St. Louis, MO) that was injected with 0.01M, pH 7.4 phosphate-buffered 10% formalin (buffered formalin) through the carotid artery during 12 to 15 minutes.⁽⁹⁾ All *Leishmania*-infected dogs were routinely sacrificed by the Center for Zoonosis Control to avoid VL transmission. The noninfected control animals were stray dogs, captured to be sacrificed for rabies control. All procedures involving animals were performed according to the Brazilian guide for care and use of laboratory animals (Projeto de lei 3.964/97-www.planalto.gov.br), and all experimental protocols used were previously approved by the Ethics Committee of the Federal University of Piauí. The kidneys were removed and renal tissues were fixed in buffered formalin, and embedded in paraffin, and 3 µm thick sections of kidney were prepared and submitted to various immunohistochemical staining and apoptosis analysis.

Immunohistochemistry for TNF- α , IL-1 α and Ki-67 (MIB-1) in renal tissue

Formalin-fixed and paraffin-embedded sections of kidneys were deparaffinized in xylene, hydrated in decreasing concentrations of alcohol, then incubated with 0.03% hydrogen peroxide in methanol solution for 30 minutes in the dark to block the endogenous peroxidase. Antigen retrieval was done using Tris-HCl (1.2 mg/ml) pH 1.0 in microwave oven (Sanyo, Brazil), under maximum potency in consecutive cycles of 10 and 5 minutes. After washing in 0.01M phosphate-buffered saline pH 7.2

(PBS) the sections were treated with 'Blocking Kit' (Vector Laboratories, Inc., Burlingame, USA), and with "protein block" (Dako Corporation) following the protocols provided by the manufacturer. Then the tissue sections were incubated with different antibodies: polyclonal goat anti-human TNF- α (10 μ g/ml) (cod-sc-1347, Santa Cruz Biotechnology Corporation, California, USA), monoclonal mouse anti-human IL-1 α (10 μ g/ml) (cod-sc-9983, Santa Cruz Biotechnology Corporation, California, USA) and monoclonal mouse anti-human Ki-67 antigen (1:70) (clone: MIB-1 - code M 7240, Dako Corporation, code K 1500, Carpinteria, USA) antibodies. The sections were incubated overnight with different antibodies at 4°C in humid atmosphere. When mouse antibody was used, the reaction proceeded using catalyzed signal amplification (CSA) system -peroxidase (Dako Corporation, code K 1500, Carpinteria, USA) following protocols provided by manufacturer, and when goat antibody was used the reaction proceeded using secondary biotinylated rabbit antibody anti-goat IgG antibody and streptavidine-peroxidase system (Dako Corporation, code K 1500, Carpinteria, USA). After each incubation step the sections were washed three times in PBS. The reaction was developed using 0.06% hydrogen peroxide and 0.3 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical, USA) in PBS. The counterstaining was done with Harrys' hematoxin (Sigma Chemical, USA).

Detection of apoptosis by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL method).⁽¹⁸⁾ Specific kit for apoptosis detection (BOEHRINGER MANNHEIM, Germany) was used on tissue sections following the protocols provided by the manufacturer. Tissue samples processed as for immunohistochemistry staining, after incubation with 0.03% hydrogen peroxide in methanol solution, were washed in PBS, incubated sequentially with 0.1% Triton X-

100 (Merck; Darmstadt, Germany) in 0.1% sodium citrate for 2 minutes on ice, with 20 µg/ml Proteinase-K in PBS for 15 min at 37°C, with 3% bovine serum albumin and 20% fetal bovine serum (Cutilab, Brazil) in PBS for 30 minutes, and then with the TUNEL mix [terminal deoxynucleotidyl transferase (TdT) and fluorescein isothiocyanate (FITC)-conjugated dUTP] in humidified chamber for 60 min at 37°C. The reaction proceeded with incubation with horse-radish peroxidase-conjugated anti-FITC antibody, Fab fragment, for 30 min at 37°C, and the reaction developed using 0.06% hydrogen peroxide and 0.3 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical, USA) in PBS, and counterstained with Harris' hematoxylin. After each incubation step the sections were washed three times in PBS. Negative control was performed omitting TdT in the reaction. As a positive control the section was incubated with 1mg/ml Deoxyribonuclease I (GIBCO BRL, USA) in 50 mM Tris-HCl pH 7.5, 1 mM MgCl₂, 1mg/ml BSA for 10 minutes, at room temperature.

Semi-quantitative analysis of histochemical staining

Immunohistochemical cytokine and apoptosis staining was analyzed semi-quantitatively and classified according to the following scale: 0 = normal, 1 = minimal or doubtful; 1⁺ = medium; 2⁺ = moderate; 3⁺ = moderately severe; 4⁺ = severe.⁽³⁴⁾ For statistical analysis following scores were attributed to each value of the scale of 0 to 4⁺: 0 to 0; 0.5 to 1; 1.0 to 1⁺; 2.0 to 2⁺; 3.0 to 3⁺, and 4.0 to 4⁺.

Statistical analysis

The semi-quantitative parameters were analyzed by Kruskal-Wallis and Dunn tests for the comparison of multiple groups, and Mann-Whitney test to analyze two groups using Sigma Stat software (Jandel Corporation, USA).

Results

Thirty six dogs with naturally acquired visceral leishmaniasis from endemic area of Teresina, State of Piauí, showing different patterns of glomerulonephritis, were selected for this study: focal segmental glomerulosclerosis (N=8), mesangial proliferative glomerulonephritis (N=10), membranoproliferative glomerulonephritis (N=10) and minor glomerular abnormalities (N=8). Five non-infected control dogs that have not shown glomerular alterations from the same area were included. Initially we have searched whether hypercellularity was due to either proliferation or infiltration in glomeruli in different patterns. Ki-67 antigen is considered as cell proliferative marker, including in glomerular cells ^(19,32). The expression of Ki-67 was very discrete and not significant in any pattern (Fig. 2).

Apoptosis was observed in glomerular cells (Fig.3). Apoptosis was present in glomerulus in dogs with visceral leishmaniasis as well as in control animals without *Leishmania* infection. However, less apoptotic cells were found in infected animal group than in control group (Fig.4). From thirty six samples of dogs with naturally acquired visceral leishmaniasis, thirty were positive and six negative for apoptosis. In different patterns of glomerulonephritis, apoptosis was observed in seven from eight cases with minor glomerular abnormalities, seven from eight with focal segmental glomerulosclerosis, seven from ten with mesangial proliferative glomerulonephritis and nine from ten cases with membranoproliferative glomerulonephritis. Apoptosis was present in all five non-infected control animals (Fig.3).

Apoptosis was analyzed semiquantitatively and less apoptotic cells were present in dogs with naturally acquired visceral leishmaniasis when compared with non-infected control animals ($p < 0.05$, Mann-Whitney test) (Fig. 9). The group with different patterns of glomerulonephritis were analyzed in relation to non-infected

control group and we could not detect any significant difference ($p > 0.05$, Kruskal-Wallis test), although a tendency was seen with groups with mesangial proliferative glomerulonephritis and membranoproliferative glomerulonephritis (Fig. 10).

TNF- α was detected in endothelium of glomerular capillary cells, mesangium and in mononuclear cells in glomeruli (Fig 5). TNF- α was expressed in twenty five from thirty six animals studied: in seven from eight cases with minor glomerular abnormalities, in six from eight with focal segmental glomerulosclerosis, in five from ten with mesangial proliferative glomerulonephritis and in seven from ten with membranoproliferative glomerulonephritis.

Analysis of semiquantitative data has shown that the expression of TNF- α in animals with naturally acquired visceral leishmaniasis was less than in non infected control animals ($p < 0.05$, Mann-Whitney test)(Fig. 11). When the groups with different patterns of glomerulonephritis was analyzed in relation to non infected control animals, the groups with mesangial proliferative glomerulonephritis and membranoproliferative glomerulonephritis presented lower TNF- α expression than non infected control animals ($p < 0.05$, Kruskal-Wallis and Dunn tests) (Fig. 12).

IL-1 α was detected in endothelium of glomerular capillary cells, mesangium and in mononuclear cells in glomerulus (Fig 8). It was positive in thirty two from thirty six cases studied: in seven from eight cases with minor glomerular abnormalities, in five from eight with focal segmental glomerulosclerosis, in all ten with mesangial proliferative glomerulonephritis and in all ten with membranoproliferative glomerulonephritis. All five non infected control dogs presented a moderate expression. Analysis of semiquantitative data of animals with naturally acquired visceral leishmaniasis and non infected control animals did not

present any significant difference in the expression of IL-1 α ($p>0.05$, Mann-Whitney test).

Leishmania-antigen was not detected in five non infected control dogs. Thirty three infected cases presented the antigen in interstitial infiltrate and in glomeruli (data not shown). In the group of naturally acquired visceral leishmaniasis the detection of *Leishmania*-antigen was significantly higher (Fig. 13) than in the non-infected control group. In the groups with membranoproliferative glomerulonephritis and mesangial proliferative glomerulonephritis the detection of *Leishmania*-antigen was significantly higher than in non-infected control animals (Fig. 14).

Discussion

In our previous study we have shown different patterns of glomerulonephritis in canine visceral leishmaniasis. One of the features of human glomerulonephritis is the proliferation of mesangial cells and consequent glomerular hypercellularity.⁽⁶⁾ Despite the majority of the studies suggest that the hypercellularity in glomerulonephritis is due to the increased cell proliferation^(6,11,12,36), when we investigated the expression of Ki-67 antigen in the renal lesions in 15 dogs with naturally acquired VL, we observed a discrete expression of this antigen, suggesting no important proliferative process ongoing in these cases. This result suggested that the maintenance of glomerular hypercellularity in canine VL must be due to either the prevention of apoptosis in mesangial cells or migration of inflammatory cells or both. In a previous report, we observed mononuclear cells, mainly T CD4⁺ cells, that were not present in controls, in glomeruli in canine VL, indicating migration of these cells into the glomeruli in VL cases.⁽¹⁰⁾

It is known that apoptosis is the major mechanism for resolution of glomerular hypercellularity in experimental mesangial proliferative nephritis, mechanism by which surplus mesangial cells are cleared.^(3,33,40) In this study we detected apoptosis, by TUNEL method, in kidney from dogs with VL. TUNEL method is also supposed to stain proliferative cells in culture, however other studies show that it is rare to stain proliferative cells in tissue, thus being more specific for apoptosis.^(22,30,38) Furthermore, we did not detect any significant proliferation in glomeruli searching the ki67 antigen.

We observed less apoptotic cells in dogs with naturally acquired visceral leishmaniasis than in non infected control animals. In proliferative glomerulonephritis, whether efficient, apoptosis may mediate the resolution of injury; on the contrary, when ineffective, it may contribute to the persistence and progression of renal disease.⁽⁴¹⁾ Our data in glomerulonephritis in canine visceral leishmaniasis suggest that apoptosis occurs in lower degree than in non-infected control animals, determining the persistence of glomerular cell proliferation. However, we have not observed significant difference between non infected control dogs and naturally acquired visceral leishmaniasis animals when different patterns of glomerulonephritis were considered, although a tendency was seen with groups with mesangial proliferative glomerulonephritis and membranoproliferative glomerulonephritis. There are few studies in literature on apoptosis in trypanosomatid infections, and particularly in leishmaniasis. Most of the studies focus on apoptosis of T lymphocyte populations and relates it to the progression of the infection.^(1,13,15) In the lesion, there are studies on experimental canine chagasic myocarditis where abundant apoptosis of myocytes, endothelial cells, and immune effector cells including lymphocytes was observed.⁽⁴⁵⁾ Study on human chronic Chagas' heart disease, apoptosis of

inflammatory cells is observed and it is suggested to be related to the clearing of lymphomononuclear cells in the lesion.⁽³⁷⁾

Many factors such as TNF- α , IL-1 α , IFN α , Fas ligant, oxygen radical species and nitric oxide provide regulation of inflammatory process and also induces apoptosis in cells as observed in renal parenchymal cell and in bovine glomerular endothelial cells.^(15,17,28) We investigated the expression of TNF- α in canine visceral leishmaniasis and we detected TNF- α expression in various cells of glomerulus: mesangial, endothelial, Bowman's capsule cells and inflammatory infiltrate. Our data contrast with the finding of Yamamoto and Loskutoff that detected TNF- α mRNA only in inflammatory cells.⁽⁴³⁾ In dogs with visceral leishmaniasis the TNF- α expression was less than in non infected control animals and when the groups with different patterns of glomerulonephritis were evaluated the expression of TNF- α in membranoproliferative glomerulonephritis and mesangial proliferative glomerulonephritis was less than in non-infected control animals. Decreased production of TNF has been observed in adriamycin- or puromycin aminonucleoside-induced experimental nephrosis in rat in mesangial and epithelial cells in culture.⁽²⁰⁾

IL-1 α expression was also studied in glomerular cells from infected and non-infected animals and no significant difference was observed among the groups, suggesting that IL-1 α has no important role in glomerulonephritis in canine visceral leishmaniasis.

Leishmania antigen was detected in glomeruli and in renal interstitium in the animals, and in the groups with membranoproliferative glomerulonephritis and mesangial

proliferative glomerulonephritis it's presence was greater than in non-infected control animals.^(9,10)

The mesangial cells can control the renal injury and participate in the resolution of inflammation, and it may occur involving molecules that belongs to TNF- α superfamily that when activated induces apoptosis, although TNF- α may also exert other functions in different receptors for TNF, TNFR1 (TNF p55) and TNFR2 (TNF p75).^(2,3,14) Although the receptors for TNF e IL-1 are different, the post-receptor events are similar for both cytokines.⁽¹⁵⁾ However, our results suggest that TNF, but not IL-1, participates in glomerulonephritis in canine visceral leishmaniasis. It was demonstrated that both TNF-[alpha] and LPS individually are able to induce glomerular endothelial cell death and pointed to a potent induction of apoptotic cell death in glomerular endothelial cells in response to small quantities of LPS. Based on our data, we may speculate on the role of *Leishmania* antigen in the inhibition of TNF production that in some way may downregulate the apoptotic process. However, further studies are necessary to analyze the mechanism that triggers the process, and the involvement of cytokines in apoptosis signaling.

Our data showing no proliferation in glomeruli suggest that inflammatory cells in glomeruli in canine VL are constituted by migrated cells. In addition, decreased apoptosis suggest that it may account for the persistence of increased inflammatory cells in glomeruli that seemed related to low production of TNF- α .

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Figure legends

Figs.1-8. Kidney; 18x21. Ki-67 antigen, apoptosis, expression of TNF- α and IL-1 α in glomeruli. Fig. 1. Ki-67 expression (Arrowhead) in lymph node cells in non-infected control dog. Bar = 25 μ m. Fig. 2. Ki-67 expression (Arrowhead) in glomerular cells in mesangial proliferative glomerulonephritis in canine visceral leishmaniasis. Bar = 25 μ m

Fig. 3. Detection of apoptosis (Arrowhead) in glomerular cells in non-infected control dog. Bar = 25 μ m. Fig. 4. Detection of apoptosis (Arrowhead) in glomerular cells in mesangial proliferative glomerulonephritis in canine visceral leishmaniasis. Bar = 25 μ m. Fig. 5. TNF- α expression (Arrowhead) in endothelium of glomerular capillary cells, and in Bowman's capsule in non-infected control dog. Bar = 25 μ m.

Fig. 6. TNF- α expression (Arrowhead) in glomerular lesions in mesangial proliferative glomerulonephritis in canine visceral leishmaniasis. Bar = 25 μ m. Fig. 7. IL-1 α expression (Arrowhead) in mononuclear cells in glomerulus, in endothelium of glomerular capillary cells, mesangium and Bowman's capsule cells in non-infected control dog. Bar = 25 μ m. Fig. 8. IL-1 α expression (Arrowhead) in focal segmental glomerulosclerosis in canine visceral leishmaniasis. Bar = 25 μ m

Fig. 9-10. Semiquantitative data of apoptotic cells in glomeruli. Fig. 9.

Semiquantitative data (score) of apoptotic cells in dogs with naturally acquired visceral leishmaniasis and non-infected control animals. * $p < 0.05$ (Mann-Whitney test). Fig. 10. Semiquantitative data (score) of apoptosis in groups with different patterns of glomerulonephritis and non-infected control group. $p > 0.05$ (Kruskal-Wallis test).

Fig. 11-12. Semiquantitative data of TNF- α expression in glomeruli. Fig. 11.

Semiquantitative data (score) of TNF- α expression in animals with naturally acquired visceral leishmaniasis and non-infected control animals. * $p < 0.05$ (Mann-Whitney test). Fig. 12. Semiquantitative data (score) of TNF- α expression in groups with different patterns of glomerulonephritis in dogs with naturally acquired visceral leishmaniasis and non-infected control animals. * $p < 0.05$ (Kruskal-Wallis and Dunn tests).

Fig. 13-14. Semiquantitative data of *Leishmania* antigen in glomeruli. Fig.13.

Semiquantitative data (score) of *Leishmania* antigen in animals with naturally acquired visceral leishmaniasis and non-infected control animals. * $p < 0.05$ (Mann-Whitney test). Fig. 14. Semiquantitative data (score) of *Leishmania* antigen in groups with different patterns of glomerulonephritis in dogs with naturally acquired visceral leishmaniasis and non-infected control animals. * $p < 0.05$ (Kruskal-Wallis and Dunn tests).