RELATÓRIO TÉCNICO CIENTÍFICO
PROJETO DE PESQUISA

"A influência do parasitismo esplênico na resposta imune local e sistêmica e suas implicações imunopatológicas na leishmaniose visceral canina".

LINHA DE PESQUISA
"O Cão como Modelo da Resposta Imune"

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OBS: No período em que o projeto foi enviado o Dr. Rodolfo era estudante de Doutorado, Wendel estava desenvolvendo o Mestrado (atualmente doutorando da UFMG). Além disso, Bruno e Rodrigo Dian eram alunos de Iniciação Científica (atualmente Mestrando nas Ufop) sob minha orientação.
RESUMO

O baço possui grande quantidade de tecido linfóide do organismo, e encontra-se em posição privilegiada estando interposto à circulação sanguínea. A pesquisa da celularidade e dos aspectos imunopatológicos em diferentes compartimentos na leishmaniose visceral canina (LVC) é um tópico pouco investigado. A grande parte dos estudos até o presente momento concentra-se nas descrições das alterações clínicas, histológicas e anatomo patológica. Neste contexto, não há até então pesquisas que busquem o entendimento dos processos imunopatológicos no compartimento esplênico de cães naturalmente infectados com *L. chagasi*, e a provável influência do parasitismo esplênico e cutâneo sobre estas alterações. Considerando a disseminação visceral do parasita por via hematógena e/ou linfática, torna-se bastante relevante o estudo do baço como o local onde os eventos imunológicos de ativação celular ocorrem durante a infecção por *L. chagasi*, visto que este órgão encontra-se intensamente parasitado na LVC. Devido à importância do baço na LV, o presente projeto se propõe à avaliação do impacto do parasitismo esplênico de cães correlacionando às suas possíveis alterações imunológicas e morfológicas através de análises histopatológicas e imuno-histoquímica. Além disso, será utilizada uma moderna abordagem através da imunofenotipagem por citometria de fluxo com o intuito de caracterizar a resposta imune sistêmica e compartimentalizada na LVC. Assim, foi também avaliada a alteração imunofenotípicas de esplenócitos envolvidos nos processos imunopatológicos do baço nas diferentes formas clínicas da LVC e em cães com diferentes graus de parasitismo esplênico. Durante a infecção por *Leishmania*, o parasitismo tecidual difere dependendo do local avaliado e pode implicar em perfis imunopatológicos distintos no decorrer da infecção. Além disto, neste estudo, foi avaliada a relação entre a densidade parasitária esplênica e cutânea e o perfil fenotípico de linfócitos, monócitos e granulócitos em 40 cães naturalmente infectados por *L. (L.) chagasi* categorizados de acordo com três diferentes densidades parasitárias. Vinte cães não infectados, utilizados como grupo controle, foram sorologicamente e parasitologicamente negativos para *L. (L.) chagasi*. 
Os principais achados descrevem a densidade parasitária esplênica como sendo mais estreitamente relacionada às alterações fenotípicas em linfócitos do sangue periférico do que o parasitismo cutâneo durante a LV canina. Os dados obtidos mostraram uma diminuição no percentual de linfócitos T CD5⁺ circulantes em cães com alto parasitismo esplênico em comparação com cães de baixo e médio parasitismo. Foi observada ainda, uma maior razão de linfócitos T/B (CD5⁺/CD21⁺) em cães com menores densidades parasitárias no baço. Estes dados são semelhantes aos observados para linfócitos T Thy-1⁺, sugerindo que as células T CD5⁺ e Thy-1⁺ são as responsáveis pela manutenção e estabelecimento da interação parasito/hospedeiro. A associação entre parasitismo esplênico e células T CD8⁺ re-enfatiza o papel da resposta imune mediada por células T nos mecanismos de resistência durante a LVC. Além disso, durante a avaliação hematológica foi observada uma diminuição nos valores absolutos de monócitos circulantes como uma característica marcante em cães com alto parasitismo, sugerindo que ocorre um recrutamento de monócitos para os tecidos linfóides, durante a LVC ativa, onde eles podem desempenhar um papel importante em reações imunes locais durante a apresentação antigênica. Na avaliação de granulócitos, foi evidenciado um aumento na expressão de MHC-II em eosinófilos, no grupo com alto parasitismo, quando comparado a cães não infectados, sugerindo a participação destas células como apresentadoras de antígeno. Além disto, estes dados sugerem que a eliminação do parasito pode ocorrer via mecanismos fagocíticos no sangue de cães com alto parasitismo esplênico e cutâneo. Desta forma, acredita-se que os resultados obtidos neste projeto acrescentam informações importantes para uma melhor compreensão da patogênese da LVC que poderão auxiliar de forma significativa em futuros estudos estratégicos para o desenvolvimento e testes de medicamentos e vacinas.
1. INTRODUÇÃO E REVISÃO DA BIBLIOGRAFIA

Uma das primeiras investigações histopatológicas da leishmaniose visceral foi realizada por Christophers (1904, segundo Meleney, 1925), em pacientes indianos com esplenomegalia, onde relacionou as alterações teciduais à presença e multiplicação do parasito. Estes estudos apontam para o comprometimento deste órgão parasitado e consequentemente suas alterações fisiopatológicas. Além disto, parece que o processo imunopatológico é mediado por macrófagos e outras células inflamatórias. O baço possui grande quantidade de tecido linfoide, sendo o único órgão linfoide interposto à circulação sanguínea. Esta posição privilegiada o torna de fundamental importância para a resposta imune. Neste sentido, a avaliação esplênica em Hamsters infectados com L. donovani evidenciou expansão secundária da polpa vermelha com macrófagos parasitados por amastigotas e atrofia da polpa branca. (Mangou et al., 1997). Conforme estudo realizado por Veress et al. (1977), o baço de pacientes humanos que vieram a óbito, apresentaram redução significativa da polpa branca pela análise morfométrica, mostrando-se desorganizada e algumas vezes com fibrose e necrose da área central do fóliculo, destruindo sua arquitetura normal. A redução da polpa branca foi relacionada à perda de pequenos linfoцитos. Outro achado interessante foi o acúmulo de material eosinofílico PAS-positivo no espaço extracelular dos foliculos esplênicos (Meleney, 1925, Andrade & Andrade, 1966; Veress et al., 1977). Na LVC, o baço estudado por Triphonas et al. (1977), apresentou trabéculas preeminentes e na polpa branca, observou aumento dos foliculos linfoïdes, sendo que alguns tinham centros germinativos distintos e muitos com hialinose intrafolicular. Segundo Genaro (1993), cães experimentalmente infectados com L. chagasi apresentaram hiperplasia da polpa branca, confluência dos foliculos de Malpighi e acentuado parasitismo. Segundo Triphonas et al. (1977), a polpa vermelha apresentou aumento da celularidade, devido ao aumento do número de macrófagos, formando granulomas, sem a presença de células gigantes, mas circundado por linfoцитos, linfoblastos e plasmócitos. Este autor relata que o baço foi o órgão mais densamente parasitado.
Em estudo realizado por Veress et al. (1977) foi observado na polpa vermelha grande número de plasmócitos e histiócitos parasitados. Segundo Genaro (1993) cães com calazar apresentaram congestão da polpa vermelha. A presença de material hialino, de densidade intermediária entre a substância amiloide e o líquido de edema, foi encontrado no centro dos foliculos linfóides do baço, entre as traves das células hepáticas e as paredes dos sinusóides. A presença deste material foi correlacionada, com a intensidade da plasmocitose e quantidade de globulina plasmática (Andrade & Andrade, 1966). Tais achados reforçam as hipóteses da intensa atividade policlonal de células B, refletindo em hipergamaglobulinemia e nas figuras histopatológicas do baço e linfonodo, com aumento hiperplásico da região B dependente.

A grande parte dos estudos até o presente momento concentra-se nas descrições das alterações histológicas, não havendo até então estudos que busquem o entendimento dos processos imunopatológicos no compartimento esplênico de cães naturalmente infectados com L. chagasi. A pesquisa da cellularidade em diferentes compartimentos na LVC é um tópico pouco investigado. Considerando a disseminação visceral do parasito por via hematogênica e/ou linfática, torna-se bastante relevante o estudo do baço como o local onde os eventos imunológicos de ativação celular podem ocorrer durante a infecção por L. chagasi, visto que este órgão encontra-se intensamente parasitado na LVC. Além disso, o baço é um importante sítio da resposta imune a antígenos presentes no sangue circulante, sendo considerado um órgão de proliferação de linfócitos e de maturação de monócitos. O baço é o maior órgão linfóide interposto entre a circulação sistêmica e portal e o seu comprometimento pode ser a ponte das manifestações hepatoesplênicas, frequentemente observadas na fase sintomática da LVC. Além disso, quando consideramos o fato de que a migração celular para o baço ocorre durante a evolução da doença, o baço passa a ser considerando como um reflexo do sistema imune tornando-se altamente relevante no estudo do processo infeccioso que exerce tal impacto no sistema imunológico do hospedeiro, como é no caso da LVC. Neste contexto, o estudo da imunopatologia na LVC se destaca em importância pelo fato do cão ser um importante paciente na clínica.
veterinária, sendo o calazar canino uma das principais causas de consulta na clínica de pequenos animais. Além disto, o cão representa o modelo que mais se assemelha aos aspectos imunopatológicos observados leishmaniase visceral humana (Moreno & Alvar, 2002).

Devido à importância do baço, o presente projeto se propõe à avaliação do impacto do parasitismo esplênico em cães correlacionando às suas possíveis alterações imunológicas e morfológicas através de análises histopatológicas. A avaliação imunopatológica migração e ativação celular envolvida com a gênese da hepatoesplenomegalia será estudada. Neste sentido, pretendemos avaliar através de anticorpos monoclonais os seguintes marcadores de adesão e ativação celular: CD11a, CD11b, CD11c, CD11d, CD18, CD44, CD62 e CD54. Esta estratégia permitirá o estudo dos eventos iniciais de migração e ativação celular, relacionados com a interação que ocorre entre a superfície de leucócitos e do endotélio vascular. Inicialmente, esta ligação pode ser mediada por selectinas, dentre as quais o CD62, expressa constitutivamente na superfície da maioria dos leucócitos (Tedder et al., 1990). A ampla expressão do CD62 torna esta molécula fundamental durante o tráfego de todas as linhagens de leucócitos.

A próxima etapa da migração para o sítio inflamatório envolve os processo de adesão celular com a participação de integrinas. Neste sentido, se destaca em importância o CD18, CD11a, CD11b, CD11c e CD11d no reconhecimento de moléculas de adesão intercelular (ICAM). Estas proteínas são expressas na maioria dos leucócitos (Marlin & Springer, 1987). De forma similar, o CD54 é expresso tanto em leucócitos como em células endoteliais, apresentando papel crucial na adesão junto aos leucócitos via CD11a e CD11b.

Por fim, uma vez estabelecida a ligação entre as integrinas e seus ligantes endoteliais, as células são capazes de responder ao estímulo quimiotático, iniciando o processo de diapedese, migrando em direção ao sítio inflamatório. A partir de então, os eventos de interação e ativação celular passam a predominar no local da inflamação. O processo de adesão celular mediada pelo CD11a pode facilitar a apresentação antigênica aos linfócitos T além de fornecer co-estimulação a estas células (Gaglia et al., 2000). De forma similar, a ligação entre
as integrinas e o CD54 também pode gerar sinalização co-estimulatória para a produção de citocinas, podendo ser importante na prevenção da apoptose e da manutenção de um estado anêrgico celular (Abraham & Miller, 2001), bem como na geração de linfócitos T citolíticos (Boyd et al., 1988). Esta abordagem pretende esclarecer os mecanismos imunopatológicos envolvidos no estabelecimento e desenvolvimento da esplenomegalia nos diferentes grupos clínicos da LVC.

De posse da metodologia de imunofenotipagem por citometria de fluxo padronizada e considerando a importância deste órgão no desenvolvimento da LVC, este projeto se propõe também avaliar as alterações imunofenotípicas de esplenócitos envolvidos nos processos imunopatológicos, abrindo novas perspectivas na área do conhecimento imunológico da leishmaniose visceral canina e na imunologia veterinária. A utilização da citometria de fluxo permitirá realizar uma análise detalhada de marcadores de superfície presentes em diferentes subpopulações de esplenócitos. Além disso, também serão estudados a expressão de (CD45RA+, CD45RB+ e MHC II+), nestas populações em cães portadores de diferentes formas clínicas e em cães com diferentes graus de parasitismo esplênico. Neste contexto, pretendemos avaliar por citometria de fluxo o fenótipo de esplenócitos de cães naturalmente infectados por L. chagasi, tanto no contexto ex vivo bem como no contexto in vitro através do estímulo específico com antígenos solúveis de L. chagasi. Marcadores de ativação celular e de memória serão avaliados com o objetivo de melhor compreender os eventos envolvidos na resposta imune destes animais.
2. OBJETIVOS

2.1 Objetivo Geral:

Avaliar a influência do parasitismo esplêntico na resposta imune local (no baço) e sistêmica (no sangue periférico) em cães naturalmente infectados por *L. chagasi*, e suas implicações imunopatológicas na leishmaniose visceral canina.

2.2 Objetivos Específicos:

1) Categorizar 45 cães naturalmente infectados por *L. chagasi*, portadores das formas clínicas assintomática (n=15), oligossintomática (n=15) e sintomática (n=15) e 15 cães não infectados com sorologia negativos pelos testes de ELISA e RIFI e exames parasitológicos negativos (pele e medula óssea) e mielocultura;

2) Categorizar 45 cães naturalmente infectados por *L. chagasi*, em animais com baixo (n=15), médio (n=15) e alto (n=15) parasitismo esplêntico conforme os resultados obtidos pela pesquisa da densidade parasitária esplênica pelo índice de "Leishman Donovan Units";

3) Avaliar por citometria de fluxo os fenótipos de células mononucleares: linfócitos T (Thy1+ e CD5+), subpopulação de linfócitos T (CD4+ e CD8+) monócitos (CD14+) e linfócitos B (CD21+) nas populações de esplenócitos totais e de linfócitos totais do sangue periférico de cães naturalmente infectados por *L. chagasi* e de cães não infectados;

4) Avaliar por citometria de fluxo a expressão de marcadores de ativação (MHC II+) e de memória (CD45RA+ e CD45RB+) nas populações de esplenócitos totais e de linfócitos totais do sangue periférico de cães naturalmente infectados por *L. chagasi* e de cães não infectados;

5) Avaliar o impacto do estímulo antigênico na resposta imune celular *in vitro*, pelo teste de proliferação de esplenócitos totais de cães naturalmente infectados por *L. chagasi* e de cães não infectados.
3. METODOLOGIA E ESTRATÉGIA DE AÇÃO DO PROJETO

3.1 – Animais

Para a realização deste projeto foram utilizados 15 cães não infectados e 45 cães naturalmente infectados por L. chagasi, provenientes de centros de controle de zoonoses da região metropolitana de Belo Horizonte, Minas Gerais. Esses animais serão mantidos no canil de experimentação em Leishmanioses (recentemente inaugurado) do Biotério da Universidade Federal de Ouro Preto (UFOP). Os cães passarão por um período de quarentena, com acompanhamento diário por um veterinário, com um rigoroso esquema de medicação vermífuga, banho carrapaticida, e vacinação anti-rábica. Após o período de quarentena os cães foram classificados clínicamente em assintomáticos, oligossintomáticos e sintomáticos, conforme Mancianti et al. (1988) e Reis et al., 1997, e subdivididos em quatro grupos clínicos, entre eles:

**Grupo I** – Cães não infectados (CNI): representado por 15 cães sadios, nascidos, criados e mantidos no canil de experimentação em Leishmanioses da UFOP. Estes animais apresentaram exames sorológicos e parasitológicos negativos.

**Grupo II** – Cães assintomáticos (CA): composto por 15 cães com sorologia positiva pela reação de imunofluorescência indireta que não apresentaram sinais sugestivos da infecção por L. chagasi após exame clínico.

**Grupo III** – Cães oligossintomáticos (CO): representado por 15 cães com sorologia positiva apresentando no máximo até três dos sinais clínicos da doença, entre eles: opacificação da pelagem, alopecia localizada e emagrecimento moderado.
Grupo IV – Cães sintomáticos (CS): compreendido por 15 cães com sorologia positiva e sinais clínicos clássicos da doença, entre eles: emagrecimento acentuado, onicogríse, lesões cutâneas, apatia e ceratoconjuntivite.

Grupo V – Baixo Parasitismo (CB): compreendido por 15 cães com sorologia positiva e LDU = 10 amastigotas/1000 células nucleadas.

Grupo VI – Médio Parasitismo (CB): compreendido por 15 cães com sorologia positiva e LDU = 11 a 250 amastigotas/1000 células nucleadas.

Grupo VI – Alto Parasitismo (CB): compreendido por 15 cães com sorologia positiva e LDU = > 250 amastigotas/1000 células nucleadas.

Após o término das análises, os cães serão submetidos à eutanásia, conforme normas estabelecidas pelo COBEA (Colégio Brasileiro de Experimentação Animal), e procedido imediatamente à necropsia.

Para confirmação do diagnóstico sorológico serão utilizadas reações de RIFI e ELISA conforme descritas abaixo.

3.2 Reação de imunofluorescência indireta (RIFI)

A RIFI foi realizada de acordo com a técnica descrita por CAMARGO (1966) com a finalidade da detecção de anticorpos IgG nos soros dos cães. A técnica foi realizada descongelando os soros à temperatura ambiente sob lâminas contendo antígeno. Em seguida foram colocadas as amostras testemunhos positivos e negativos. As lâminas foram incubadas em câmara úmida, na estufa a 37°C por 30 minutos, seguido por lavagem com PBS e depois foram cobertas com PBS por cinco minutos, seguido de lavagem com água destilada. Posteriormente, foi adicionado em cada poço das lâminas o conjugado total anti-IgG de cão (conjugação na diluição 1:60 + azul de Evans na diluição 1:50, a partir de uma solução estoque de 1% + PBS Tween) sendo a seguir incubadas por 30 minutos.
na estufa à 37°C. As lâminas foram lavadas e cobertas com PBS por cinco minutos, lavadas com água destilada e seca à temperatura ambiente. Por último, foi aplicada sobre as lâminas a glicerina tamponada e as lamínulas. A leitura das lâminas foi realizada em microscópio de imunofluorescência. Animais com títulos de IgG iguais ou superiores à 1:40 foram considerados positivos. Os resultados foram expressos em títulos de anticorpos, obtidos por diluição seriada do soro e do eluato por fator dois até o encontro do resultado negativo ou ponto de titulação (GENARO, 1993). Como conjugado para a RIFI, foi utilizado uma anti-imunoglobulina de cão, fração IgG, marcada com isoticianato de fluoresceina (Biomanguinhos, RJ) obtida de soro imune de coelho.

3.3 O teste Imunoenzimático (ELISA)

Foi padronizado o teste de ELISA empregando antígenos lisados de promastigotas de L. amazonensis e L. chagasi e com o recombinante rK39, para verificar qual a concentração do antígeno, titulação do conjugado e do soro que melhor discriminasse as amostras de cães positivos e negativos para L. chagasi, de acordo com a técnica descrita por RAJASEKARIAH et al. (2001).

O teste de ELISA para detecção de anticorpos IgG anti-L. chagasi foi realizado segundo o teste descrito por VOLLER et al. (1979) com algumas modificações. Como conjugado imunoenzimático foi usado uma anti-imunoglobulina de cão, fração IgG, obtido de soro imune de coelhos e marcada com peroxidase (Sigma-Company USA).

3.4 - Avaliação Parasitológica - Pesquisa do parasito por mielocultura e em esfregaços por aposição de tecidos

A avaliação parasitológica constou de análises em lâminas submetidas à coloração pelo Giemsa (nos esfregaços de tecidos) para quantificação do parasito. Medula óssea: a pesquisa de parasitos na medula óssea foi realizada por exame direto do aspirado medular em lâmina e indiretamente por cultura, após o desafio
mensalmente. Os animais foram submetidos à punção de medula óssea realizada na extremidade inferior do esterno ou na epífise tibial, sob anestesia geral com tiopental sódico (Thionembutal®), a uma concentração de 33%, diluído em solução fisiológica estéril, para uma concentração final de 30mg/ml. Cada animal recebeu 0,5 ml/Kg de peso desta solução, por via endovenosa.

Pele, linfonodo poplitéo, figado e BAÇO: Fragmentos de pele, linfonodo poplitéo, figado e BAÇO foram obtidos e esfregaços por aposição em lâminas de microscopia foram corados pelo Giemsa e exaustivamente examinados ao microscópio óptico, no intuito de identificar formas amastigotas de *Leishmania chagasi*.

### 3.5 - Avaliação Imuno-Histopatológica

Os tecidos foram cortados com espessura entre 4 e 5µm para serem utilizados na reação de imuno-histoquímica e coloração por hematoxilina & eosina (HE). Conforme procedimentos de rotina do Laboratório de Imunopatologia do Núcleo de Pesquisas em Ciências Biológicas (NUPEB)/ICEB II/UFOP. Os fragmentos foram processados rotineiramente (desidratação, diafanização e inclusão em parafina). Foram realizados três cortes histológicos para cada órgão, com espessura entre 4 e 5 µm, sendo destinados a coloração por HE e a reações imuno-histoquímicas anti-*Leishmania* e anti-marcador de superfície celular. A avaliação histopatológica em microscopia óptica foi realizada de forma semiquantitativa, utilizando os seguintes parâmetros: ausente (-), leve (+), moderada (+++) e intensa (+++). Para a avaliação imuno-histoquímica anti-marcador de superfície celular utilizaremos o programa KS300, para a quantificação das células imuno-marcadas em área de 20 campos microscópicos, objetiva de 40x, em tela de 640x480 pixels, conforme Caliari (1997).
3.6 - Avaliação Imuno-Histoquímica anti-Leishmania chagasi

A reação de imuno-histoquímica foi empregada como metodologia auxiliar para detecção de formas amastigotas de *L. chagasi*. Os tecidos após serem parafinados, foram avaliados pela reação de imuno-histoquímica por meio da técnica modificada de estreptoavidina-peroxidase desenvolvida por Sternberger (1986) e conforme descrito abaixo:

- Foi realizada a hidratação dos cortes histológicos em bateria de álcoois decrescentes (absoluto, 90º, 80º, 70º), após os cortes histológicos estarem fixados em lâmina de vidro, procedendo posteriormente com banhos em PBS;
- O bloqueio da peroxidase endógena foi realizado com auxílio de peróxido de hidrogênio (30 volumes) a 4% por 30 minutos em temperatura ambiente, e a seguir adicionado ao banho de PBS;
- Reações inespecíficas foram bloqueadas pela incubação de soro normal de cabra (1:50), em câmara úmida, durante 30 minutos a temperatura ambiente;
- Foi realizada então a incubação de soro hiperimune (anticorpo primário), proveniente de um cão naturalmente infectado por Leishmania, de João Pessoa, Paraíba, com alto título sorológico (1:800), na concentração de 1:100 diluído em BSA 0,1%. Foi utilizada quantidade suficiente do soro para cobrir completamente o fragmento tecidual, procedendo posteriormente à incubação durante 18 a 24 horas em câmara úmida a 4ºC;
- Posteriormente o material foi incubado por 30 minutos em câmara úmida a temperatura ambiente com estreptoavidina-peroxidase complexada ao anticorpo anti-camundongo e anti-coelho biotinilado (Kit Dako Corporation, Carpinteria, CA 93013, USA);
- A peroxidase foi revelada em solução de Diaminobenzidina (DAB) com peróxido de hidrogênio 0,024%, durante 5 minutos em temperatura ambiente;
- Em seguida foram realizadas etapas de lavagem das lâminas em PBS e contra-coloração com hematoxilina de Harris por três segundos;
- Finalmente foi realizada a lavagem em água corrente, desidratação em álcool crescentes, diafanização em xilol e montagem com Etellan® sintético (Merk® 107961.0100 – Alemanha).

- Para o controle negativo, o anticorpo primário foi substituído por soro de cão negativo para Leishmania na diluição 1:100 e/ou PBS, soro normal de camundongo e coelho.

- Para o controle positivo da reação, utilizaremos uma lâmina com corte histológico de baço de um cão positivo para Leishmania com intenso parasitismo tecidual.

3.7- Avaliação da resposta celular nos contextos ex vivo e in vitro

Os ensaios de imunoﬂuorescência para células do sangue total foi realizado segundo protocolo padronizado por Reis et al, 2005. Todos os anticorpos monoclonaux (AcM) primários utilizados em nossos experimentos foram adquiridos em sua forma pura, sem marcação. Uma vez que a maioria dos anticorpos anti-CD de células caninas foram produzidos em ratos, utilizaremos como anticorpo secundário, um anticorpo IgG- anti-rato marcado com FITC. Como controle foi utilizado apenas os anticorpos secundários para cada ensaio respectivamente. O sangue dos cães foi coletado, em seringas descartáveis estéreis de 20mL, através de venopunção preferencialmente na veia jugular, ou da radial e em seguida serão transferidos 5ml de sangue para tubos estéreis contendo EDTA, destinados a realização de hemograma e para imunofenotipagem, e 10ml em tubos sem anticoagulante, destinados aos testes sorológicos. Do sangue colhido em EDTA, será retirado 1ml e transferido para um tubo de 15ml fundo em V (FALCON®). Sobre este material foi adicionado lentamente 5ml de solução de lise (FACS LISE SOLUTION – BECTON DICKINSON) para remover os eritrócitos da preparação. O sobrenadante foi desprezado e ressuspensido em 13ml de PBS pH 7.2, homogeneizado, com o intuito de lavar as células que foram submetidos à lise. Antes de iniciar o protocolo de imunofenotipagem, era realizado um teste de controle de qualidade da suspensão celular: Este tem como objetivo ajustar o
número de eventos celulares para 1.500 eventos/segundo por tubo além de avaliar antecipadamente a qualidade do perfil celular da amostra após a lise com relação ao tamanho e a granulosidade das células.

3.8- Resposta celular in vitro em células mononucleares do sangue periférico

A imunidade celular foi também avaliada através da reação de proliferação de linfócitos obtidos a partir de células mononucleares do sangue de cães. A partir da suspensão de PBMCs obtida com descrita anteriormente, foi realizado o plaqueamento das células em dois sistemas distintos.

3.9 - Reação de proliferação linfocitária

No primeiro sistema foi realizada para cada cão culturas triplicadas em placas de 96 orifícios na presença de antígeno e de mitógeno e na ausência de qualquer estímulo, depositando-se 15μl da suspensão de células com 1x10⁷ cels/ml, ou seja, 150.000 células/orifício nos orifícios que correspondiam ao controle e ao estímulo do mitógeno, nas culturas com a presença do antígeno serão adicionados 25μl/orifício da suspensão de células com 1x10⁷ cels/ml, ou seja 250.000 células/orifício. Foi adicionado 25μl do antígeno de *L. chagasi* diluído em meio de RPMI-1640 (80μg/ml), em uma concentração final de 10μg/orifício do antígeno. O agente mitogênico (ConA-Concavalina-A-Sigma Chemical Co., EUA) foi adicionado 25μl da solução de uso diluída em RPMI-1640 nos respectivos orifícios da placa, destinados a avaliação e o controle de viabilidade celular e da capacidade linfoproliferativa. A ConA foi utilizada na concentração final de 2μg/orifício. Como controle de proliferação na ausência de estímulo, as células também serão cultivadas na ausência de qualquer estímulo em ambas situações (antígeno e ConA). As células foram estimuladas com ConA por 3 dias e com antígeno por 5 dias, mantidas a 37°C em incubadoras de CO₂ (Forma Scientific) com 5% de CO₂. Nas últimas 6 horas de incubação será adicionado 1 μCi de [3H] timidina (Sigma Chemical Co., EUA). As células serão isoladas em papel de fibra
de vidro (modelo 943-AH-Wathman, EUA) através de um coletor automático de células (Titertek Cell Harvester, Flow Laboratories, EUA), e a incorporação de [3H] timidina será determinada por contagem em líquido de cintilação em um contador de cintilação.

3.10 - Imunofenotipagem de linfócitos após cultivo in vitro

No segundo sistema de cultura foi adicionado 600µl de meio de cultura (CM-Blast), específico para cultivo de células caninas; em todos os orifícios de uma placa de 24 orifícios, em 12 orifícios desta placa será adicionado 100µl do antígeno solúvel de L. chagasi na concentração de 80µg/ml, de modo que a concentração final deste antígeno no sistema será de 10µg/ml e nos orifícios controles 100µl de RPMI 1640. Por fim foi adicionado nos orifícios da placa 100µl da suspensão de células com 1x10⁷ cels/ml, ou seja, 1x10⁶ cels/ml em cada orifício da placa. As culturas foram então incubadas por 5 dias a 37ºC/5% de CO₂.

3.11 - Análise estatística dos dados

Os cálculos estatísticos foram realizados com o apoio instrumental dos softwares MINITAB FOR WINDOWS; EpilINFO.6, empregados na análise de variância, seguido de Teste T de Student. Também foi utilizado o teste de comparação e proporção entre as médias x² (Teste Qui-quadrado). Os dados obtidos foram considerados estatisticamente significativos quando o valor de p for menor que 0,05.
4 – PRINCIPAIS DOS RESULTADOS OBTIDOS:

Durante a execução deste estudo que aborda aspectos parasitológicos e imunopatológicos da pele, baço e sangue periférico foram obtidos diversos resultados. Algumas publicações em periódicos internacionais foram geradas e pelo menos três dissertações de mestrado de forma direta. Além disto, vários estudantes de iniciação científica se beneficiaram dos resultados gerados pelo grupo, colaborando e aprendendo muito durante a vigência do projeto. O projeto trouxe grandes benefícios para o laboratório de Imunopatologia permitindo inaugurar o sistema de cultivo celular no LIMP. Hoje este sistema (capela de fluxo e estufa CO₂) já não atende a atual demanda interna, o que significa que foi um grande investimento. O LIMP cresceu de forma significativa e tornou-se um grande laboratório na Ufop anteriormente coordenado por dois pesquisadores e atualmente atende diretamente a cinco pesquisadores. Desta forma, os benefícios (material permanente) gerados por este projeto hoje atende a mais que o dobro de pesquisadores que inicialmente havia se planejado. Desta forma, eu considero um ótimo investimento gerado pela aprovação deste projeto.

A apresentação dos resultados obtidos neste projeto encontra-se abaixo sumarizadas na seguinte ordem: avaliação de parâmetros hematológicos, parâmetros fenotípicos de células de cães naturalmente infectados, associada à intensidade parasitária tecidual (pele e baço) na LVC. A obtenção destes resultados nos permitiu a relacionar as seguintes evidências:

➢ **Parâmetros Hematológicos:**

- A anemia foi mais pronunciada em cães com alto parasitismo esplênico em função da queda no número de hemácias, da concentração de hemoglobina e do hematócrito;
- Cães com alto e médio parasitismo de baço e pele apresentaram eosinopenia e monocitopenia.
- **Parâmetros Fenotípicos celulares:**
  - Cães com alto parasitismo esplênico apresentaram uma diminuição de linfócitos T circulantes (Thy-1+ e CD5+) e esplenócitos T (Thy-1+), acompanhado de uma diminuição na subpopulação de linfócitos T (CD8+). Além disso cães com baixo e médio parasitismo esplênico tiveram aumento da razão T/B, enquanto a razão CD4+/CD8+ apresentou-se diminuída em todos os grupos de cães infectados;
  - As correlações mais fortes foram observadas entre as células T CD8+ (correlação negativa), razão CD4+/CD8+ (correlação positiva) e o parasitismo esplênico.
  - Cães com médio parasitismo esplênico apresentaram diminuição de esplenócitos T CD45RA+;
  - Cães com baixo parasitismo esplênico apresentaram aumento de monócitos CD45RB+;
  - Cães com alto parasitismo cutâneo apresentaram aumento de esplenócitos T CD45RB+ e eosinófilos CD45RA+;
  - Maior expressão de esplenócitos T CD8+ e de MHC-II por eosinófilos foi observada em cães com alto parasitismo esplênico e cutâneo.
Diagrama das principais alterações fenotípicas observadas em cães naturalmente infectados por *L. chagasi* com diferentes densidades parasitárias de baço e pele.

![Diagrama de Parasitismo de Baço e Parasitismo de Pele](image)

**Legenda:** LT (Linfócitos T), ET (Eosophócitos T), Mon. (Monócitos), Eos. (Eosinófilos). CNI= Cães Não Infectados, BP= Baixo Parasitismo, MP= Médio Parasitismo e AP= Alto Parasitismo. As alterações associadas ao parasitismo esplênico estão representadas no círculo azul, enquanto aquelas associadas ao parasitismo cutâneo encontram-se no círculo amarelo e as alterações relacionadas a ambos os parasitismos encontram-se na intersecção em verde.

- Avaliações da Expressão de Citocinas tipo I e II no Baço de cães naturalmente infectados.

Para compreender melhor a expressão de citocinas no baço foi avaliada de 5 estratégias diferentes, onde todas as avaliações foram feitas relacionando o grupo de cães não infectados com as outras 5 variáveis a serem estudadas. A
primeira variável a ser avaliada foi à presença da doença, onde este grupo foi determinado pela positividade da reação de imuno fluorescência indireta (RIFI). A segunda variável foi à presença do parasito em cães doentes, onde cães doentes apresentavam teste parasitológico positivo e outros negativo. Uma terceira variável foi à presença de sinais clínicos nos cães doentes, onde os cães doentes que não apresentavam sinais clínicos foram denominados assintomáticos (CA) e os que apresentavam, denominados de sintomáticos (CS). A quarta variável a ser avaliada foi a gravidade da doença, onde os cães foram divididos em três grupos de acordo com a gravidade da doença, cães com nenhum sinal clínico aparente, denominados assintomáticos (CA); cães com poucos sinais clínicos, denominados oligosintomáticos (CO); e cães com vários sinais clínicos, denominados de sintomáticos (CS). Uma última e quinta variável foi a densidade de parasitas encontrados no baço dos cães, onde o grupo denominado Baixo Parasitismo (BP) inclui cães apresentando contagem de LDU de 1 a 10; o grupo Médio Parasitismo (MP) inclui cães com LDU de 11 a 250 e o grupo Alto Parasitismo (AP) inclui cães com LDU maior ou igual a 251.

As avaliações de citocinas foram feitas em nível de frequência e de semi-quantificação, onde na frequência o critério de positividade ou não era a presença da expressão da citocina, no qual era percebida pela presença da banda específica amplificada. No caso da semi-quantificação o critério foi a razão da densidade ótica da banda da citocina específica pela densidade ótica da Beta-actina, o qual representa uma idéia de quantidade da expressão de mRNA da citocina.

Com este estudo foi possível observar um aumento na expressão de INF-gama no grupo de cães sintomáticos em relação ao grupo controle, bem como um elevado aumento de IL-10 neste mesmo grupo. O parasitismo também influência muito na expressão diferencial de citocinas em cães doentes em relação aos cães saudáveis com um aumento significativo na expressão de IL-10 nos animais com alto parasitismo em relação aos cães com baixo parasitismo. Estes resultados demonstram um possível envolvimento de uma resposta do tipo II nos cães sintomáticos. Além disso encontramos diversas alterações no estudo de classes e
subclasses de imunoglobulinas por ELISA. Observamos que cães assintomáticos apresentam maiores níveis de IgG1 em relação aos animais sintomáticos, este aumento se reproduz quando avaliamos o grau de intensidade parasitária no baço (manuscrito submetido à publicação). Também observamos aumento significativo de IgE, IgA e IgG2 nos animais sintomáticos em relação aos cães oligo e assintomáticos. Estes resultados reforçam a hipótese de que cães com alto parasitismo e cães sintomáticos apresentam um perfil do tipo II enquanto que cães assintomáticos apresentam um perfil do tipo I. Estes resultados são de fundamental importância para o presente projeto uma vez que a aplicação destas metodologias conjuntamente com o entendimento da resposta imune em cães naturalmente infectados irão auxiliar na compreensão do desenvolvimento da resposta imune em cães vacinados.

Neste projeto também avaliamos a detecção de bandas majoritárias no antígeno total que constitui a vacina e no antígeno de glândulas de flebotomíneos. Estes resultados iniciais são de fundamental importância no rastreamento de futuros antígenos recombinantes.

4.1 Justificativa pelo não cumprimento de parte dos objetivos específicos:

Os estudos com os anticorpos de migração não foram totalmente concluídos. Atualmente estamos padronizando estas reações para citometria de fluxo e imunohistoquímica. Partes dos anticorpos que adquirimos não funcionaram com o Kit de revelação da Imunohistoquímica. Acreditamos que até o final do presente ano já alcançaremos também estes objetivos.
4.2 Produtos/Processos/Serviços:


4.3 Principais Produtos Gerados:

Artigos

Foram gerados diretamente da proposta deste projeto cinco artigos que seguem abaixo listados:

1) REIS, A. B ; Teixeira-Carvalho, A ; Vale, A.M ; Marques, M.J ; Giunchetti, R.C ; Mayrink, W ; Guerra, L.L ; Andrade, R.A ; Corrêa-Oliveira, R ; Martins-Filho, O.A . Isotype patterns of immunoglobulins: Hallmarks for clinical and parasitic status in brazilian dogs naturally infected by Leishmania (Leishmania) chagasi. Veterinary Immunology and Immunopathology, USA, v. 112, n. (3-4), p. 102-116, 2006.

2) REIS, A. B ; Martins-Filho, O.A ; Teixeira-Carvalho, A ; Carvalho, M.G ; Mayrink, W ; França-Silva, J.C ; Giunchetti, R.C ; Genaro, O ; Corrêa-Oliveira, R . Parasite density and impaired biochemical/hematological status are associated with severe clinical aspects of Canine Visceral Leishmaniasis. Research in Veterinary Science, USA, v. 81, n. 1, p. 68-75, 2006.

3) Lage, R.S ; Oliveira, G.C ; Buzek, S.C.U ; Guerra, L.L ; Giunchetti, R.C ; Corrêa-Oliveira, R ; REIS, A. B . Analysis of the cytokine profile in spleen cells from dogs

4) REIS, A. B ; Martins-Filho, O.A ; Teixeira-Carvalho, A ; Carneiro, C.M ; Giunchetti, R.C ; Mayrink, W ; Tafuri, W.L ; Corrêa-Oliveira, R . Systemic and Compartimentalized Immune Responses in Canine Visceral Leishmaniasis. Veterinary Immunology and Immunopathology, 2008.

5) Guerra, L.L ; Teixeira-Carvalho, A ; Giunchetti, R.C ; Martins-Filho, O.A ; REIS, A. B ; Corrêa-Oliveira, R . Evaluation of the influence of tissue parasite density on haematological and phenotypic cellular parameters of dogs naturally infected by Leishmania (Leishmania) chagasi. Parasitology Research, 2008.

5 REFERÊNCIA BIBLIOGRÁFICA RELEVANTE


REIS, A. B. ; MARTINS FILHO, Olindo Assis ; CARVALHO, Maria das Graças ; GIUNCHETTI, Rodolfo Cordeiro; GENARO, Odair ; MAYRINK, Wilson ; OLIVEIRA, Rodrigo Corrêa. Parasite density and impaired biochemical/hematological status


Isotype patterns of immunoglobulins: Hallmarks for clinical status and tissue parasite density in Brazilian dogs naturally infected by *Leishmania (Leishmania) chagasi*

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Abstract

The role of anti-leishmanial immune response underlying the susceptibility/resistance during canine visceral leishmaniasis (CVL) has been recognized throughout ex vivo and in vitro investigations. Recently, we demonstrated that immunoglobulin levels (Igs), as well as the parasite load are relevant hallmarks of distinct clinical status of CVL. To further characterize and upgrade the background on this issue, herein, we have evaluated, in *Leishmania (Leishmania) chagasi* naturally infected dogs, the relationship between tissue parasitism (skin, bone marrow, spleen, liver and lymph node), the CVL clinical status (asymptomatic (AD), with no suggestive signs of the disease, oligosymptomatic (OD), with maximum three clinical signs—opaque bristles; localized alopecia and moderate loss of weight; symptomatic (SD), serologically positive with severe clinical signs of visceral leishmaniasis), and the humoral immunological profile of anti-*Leishmania* immunoglobulins (IgG, IgG1, IgG2, IgM, IgA and IgE). Our major statistically significant findings revealed distinct patterns of tissue parasite density

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Parasite density and impaired biochemical/hematological status are associated with severe clinical aspects of canine visceral leishmaniasis

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Abstract

We have performed a detailed investigation in 40 dogs naturally infected with \textit{Leishmania infantum} (syn. \textit{chagasi}), subdivided into three groups: asymptomatic (AD = 12), oligosymptomatic (OD = 12) and symptomatic (SD = 16), based on their clinical features. Twenty non-infected dogs (CD) were included as control group. Serological analysis, performed by IFAT and ELISA, demonstrated higher antibodies titers in SD in comparison to the AD. A positive correlation was found between parasite density in the spleen and skin smears as well as the bone marrow parasitism with clinical status of the infection. We observed that the progression of the disease from asymptomatic to symptomatic clinical form was accompanied by intense parasitism in the bone marrow. It is likely that this led to the impaired biochemical/hematological status observed. Finally, we believe that the follow-up of these parameters could be a relevant approach to be used as markers during therapeutic and vaccine evaluations.

Keywords: Canine visceral leishmaniasis; \textit{Leishmania infantum} syn. \textit{chagasi}; Parasite density; Biochemical/hematological status

1. Introduction

Visceral leishmaniasis (VL) is one of the most relevant and emergent diseases worldwide, reaching 98\% of mortality in non-treated human cases (Tesh, 1995). Beside its broad epidemiological spectrum, VL is an important zoonosis widely spread in tropical and subtropical areas of the globe. In the last 16 years, 37,294 new human cases of VL were reported in Brazil (Vieira and Coelho, 1998).

From the epidemiological standpoint, the canine visceral leishmaniasis (CVL) is considered to be more important than the human disease, due to its higher prevalence and the fact that both asymptomatic and symptomatic dogs are equally infectious to the vectors (Molina et al., 1994). Many asymptomatic animals, in endemic areas, have been detected with parasites in the skin (Marzochi et al., 1985), and from 1980 to 1997, a total of 414,168 sero-positive dogs were identified in Brazil (Vieira and Coelho, 1998).

The CVL may evolve from asymptomatic cases to a systemic disease, which mostly culminates in death. From the
Analysis of the cytokine profile in spleen cells from dogs naturally infected by *Leishmania chagasi*

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Abstract

Recent studies suggest that asymptomatic dogs infected with canine visceral leishmaniasis (CVL) develop a Th1 immunological profile whilst oligosymptomatic and symptomatic CVL-infected animals present a Th2 profile. In the present study, an RT-PCR method has been standardized and employed to evaluate the frequency and the semi-quantitative level of expression of the cytokines IL-4, IL-10, IL-12, INF-γ and TNF-α in splenocytes of 30 dogs naturally infected with *Leishmania chagasi* and of 7 non-infected dogs (NID). An increase in the level of expression of IL-12 (\(p = 0.059\)) was detected in all CVL-infected dogs compared with NID. In dogs exhibiting high parasitism, the frequency of expression of IL-10 was higher (\(p = 0.011\)) than in animals presenting low parasitism or medium parasitism (MP) and in NID animals, whilst the level of expression of IL-10 was higher (\(p = 0.0094\)) than in animals exhibiting MP and in the NID group. Positive correlations between the levels of expression of IL-10 with respect to the progression of the disease (IL-10: \(r = 0.3510\); \(p = 0.0337\)) and the levels of expression of IL-10 and INF-γ increase in parasitism (IL-10: \(r = 0.3428\); \(p = 0.0438\) and INF-γ: \(r = 0.4690\); \(p = 0.0045\)) were observed. Such data suggest that CVL is marked by a balanced production of Th1 and Th2 cytokines, with a predominant accumulation of IL-10 as a consequence of an increase in parasitic load and progression of the disease, and INF-γ was related with the increase in parasitic load.

Keywords: Cytokines; RT-PCR; Canine diseases; Visceral leishmaniasis; *Leishmania chagasi*

1. Introduction

Leishmaniasis, which comprises a complex of diseases exhibiting significant clinical and epidemiological diversity, continues to represent a severe threat to public health. Whilst the prevalence of leishmaniasis is increasing significantly, the development of appropriate measures for its control is far from complete and
Systemic and compartmentalized immune response in canine visceral leishmaniasis

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ABSTRACT

Human visceral leishmaniasis (VL) and canine visceral leishmaniasis (CVL) are the most important emerging diseases with high prevalence in Latin American countries and are mainly caused by Leishmania (L.) chagasi (Syn. L. infantum). CVL has a great impact on Brazilian public health because domestic dogs are the most important VL peri-domicile reservoirs in both urban and peri urban areas. Our findings highlight the complexity of cellular immunological events related to the natural infection from dogs by L. chagasi, additionally correlating major peripheral blood phenotypic markers with clinical status and tissues parasite density. Our main results demonstrated that lower frequency of circulating B cells and monocytes are important markers of severe CVL, whereas increased levels of CD8+ lymphocytes appear to be the major phenotype feature of asymptomatic disease. Determination of the isotypes patterns during CVL demonstrated that asymptomatic dogs and those with low parasitism are associated with an increase of IgG1, while the symptomatic dogs and those with high parasitism are associated with an increase of IgG, IgG2, IgM, IgA and IgE immunoglobulins. Pioneer findings obtained by our group showed a correlation between clinical status of CVL with degree of tissue parasitic density. This data demonstrated that asymptomatic dogs presented low parasitism while symptomatic dogs are associated with high parasite load in various tissues such as skin, bone marrow and spleen. We have also investigated the association between tissue parasitism and CVL clinical forms. Regardless of clinical status, skin and spleen are the major sites of high parasite density during ongoing CVL. Furthermore, we demonstrated that bone marrow and spleen parasite density are the most reliable parasitological markers to decode the clinical status of CVL. In this article, we have reviewed some aspects of parasitological markers to decode the clinical status of CVL.
Evaluation of the influence of tissue parasite density on hematological and phenotypic cellular parameters of circulating leukocytes and splenocytes during ongoing canine visceral leishmaniasis

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Abstract During Leishmania infection, tissue parasitism at different sites may differ and imply distinct immunopathological patterns during canine visceral leishmaniasis (CVL). For this reason, we have assessed by flow cytometry the impact of spleen and skin parasite density on the phenotypic profile of splenocytes and circulating leukocytes of 40 Brazilian dogs naturally infected by Leishmania chagasi categorized according to spleenic and cutaneous parasitic load. Our major statistically significant findings demonstrated that dogs with spleenic high parasitism presented a significant decrease in absolute counts of CD8 T lymphocytes in comparison with dogs presenting splenic medium parasitism. Moreover, a decrease in the absolute number of circulating monocytes was observed as a hallmark of high parasitism. The increased frequency of CD56+ cells is associated with low spleenic parasitism during CVL. Although we did not find any significant differences between the immunophenotypic analysis performed in circulating lymphocytes according to cutaneous parasitic load, there were negative correlations between CD56+ and CD8+ T cells and cutaneous parasite density, which reemphasizes the role of I cell-mediated immune response in resistance mechanisms during ongoing CVL. These results add new insights about the pathogenesis of CVL and may help in the establishment of additional tools for future studies on drugs and vaccine approaches.

Introduction

Visceral leishmaniasis (VL), which is caused by Leishmania donovani (syn. Leishmania chagasi), is endemic in over 80 countries within Europe and Latin America and is transmitted by the bite of the female sand fly (Phlebotomus). The major prophylactic practice to control this human disease, as recommended by the World Health Organization, involves a systematic treatment of human cases besides vector control by insecticide and elimination of the domestic reservoir, mainly seropositive infected dogs (Tesh 1993). In this context, canine visceral leishmaniasis (CVL) has high epidemiological relevance, considering its increased incidence in the last decade, in addition to the intense cutaneous parasitic density reported in both asymptomatic and symptomatic dogs which may contribute to the spread of disease (Molina et al. 1994; Tesh 1995; Giunchetti et al. 2000; Reis et al. 2004a, b).
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IM12 - Histopathological and Immunohistochemical Investigations of the Hepatic Compartment Associated with Parasitism and Biochemical changes in Canine Visceral Leishmaniasis

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Canine visceral leishmaniasis (CVL) is a disease caused by the protozoan Leishmania chagasi and transmitted by the bite of phlebotomine sand flies. The main clinical findings are skin lesions, hepatosplenomegaly, lymphadenopathy, generalized cachexia and anemia. Studies on histopathology in CVL present only report microscopic lesions. In this context, the aim of the present study was to make immunohistochemical study in spleen biopsies from asymptomatic (AD, n=12), oligosymptomatic (OD, n=12) and symptomatic (SD, n=16) CVL-bearing dogs as well as 11 healthy controls (NID). These results of microscopic analysis of the spleen were made association statistical analysis with different histopathological results and parasitism level by immunohistochemical. In addition, we evaluated the correlation between histopathological picture of spleen lesions (parasitism and leukocytes in peripheral blood and spleen) and CD14+ , CD64+ , CD83+ , Thy-1+ , CD11c+ , CD24+ + expression by flow cyrometry. Our data demonstrated that frequency in all infected dogs presented histopathological pattern consistent with white pulp depletion with macrophage infiltrated, red pulp hypertrophy and hyperplasia in comparison to NID. Statistical analysis suggested that cell reactivity in different compartments in spleen may be stimulated by parasite load. The correlation was presented among peripheral blood and histological parameters by AD in CD14+ , CD64+ and CD83+ . Taken together these correlations may be associated between splenocytes and histological parameters by AD in CD14+ and CD24+ and OD in CD24+ . These findings suggest participation reaction in leukocytes emigration from peripheral blood to distinct compartment in spleen reporting of the mononuclear cells regarding immune pathological events associated with CVL outcome.

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IM13 - The spleen in canine visceral leishmaniasis

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In this work, we evaluated the humoral response of patients with Cutaneous Leishmaniasis, against total antigens and lipopolysaccharide (LPS) from Leishmania before and after treatment with immunotherapy (IT) or conventional
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**Título:** IMPACTO DO PARASITISMO DO BAÇO E FIGADO NA IMUNOPATOLOGIA DA LEISHMANIOSE VISCERAL CANINA

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**Área de Conhecimento:** Parasitologia

**Resumo**

A leishmaniose viscral consiste numa das principais doenças parasitárias do mundo e da frequência com que são acometidos, neste estudo propomos avaliar a densidade parasitária pela análise histológica da biópsia pulmonar. A análise histológica hepática apresentou diferença significativa (p<0,05) na inflamação da cápsula, granuloma e hipertrofia de células de Kupffer entre os grupos CNI e os demais grupos clínicos CA, CO e CS. Similarmente, identificou-se maior frequência (p<0,05) na inflamação da cápsula, granulomas e hipertrofia de células de Kupffer nos grupos CA, CO e CS em relação ao CNI. Na análise parasitológica encontramos maior frequência e intensidade (p<0,05) de parasitismo no grupo CS quando comparado ao grupo CA. No baço foi observado que a fibrose e inflamação de células espécies apontam aumento da frequência destas alterações conforme evolução clinica da LVC. Os grupos CA, CO e CS apresentaram maior frequência de depilação do placa branca, hipertrofia e hipercelularidade da polpa vermelha e congestão em relação ao grupo CNI. Encontramos uma maior frequência (p<0,05) do parasitismo espécies quando comparados os grupos CO e CS em relação a CA. Também foi observada a associação (p<0,05) entre a hipertrofia e hipercelularidade da polpa vermelha e congestão com parasitismo. Estes resultados sugerem que um estudo de reatividade inflamatória podendo ser influenciado pelo parasito, caracterizado pela migração diferencial de células mononucleares nos diferentes compartimentos hepatoespênicos. Estas células caracterizaram o infiltrado celular como do tipo plasmocêutico e flogistico na transcrição do processo patológico, estando estas alterações histológicas relacionadas com a imunopatogênese da leishmaniose viscral canina.
Systemic and compartmentalized immune response in canine visceral leishmaniasis

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Abstract

Human visceral leishmaniasis (VL) and canine visceral leishmaniasis (CVL) are the most important emerging diseases with high prevalence in Latin American countries and are mainly caused by Leishmania (L.) chagasi (Syn = L. infantum). CVL has a great impact on Brazilian public health because domestic dogs are the most important VL peri-domestic reservoirs in both urban and peri-urban areas. Our findings highlight the complexity of cellular immunological events related to the natural infection from dogs by L. chagasi, additionally correlating major peripheral blood phenotypic markers with clinical status and tissues parasite density. Our main results demonstrated that lower frequency of circulating B cells and monocytes are important markers of severe CVL, whereas increased levels of CD8+ lymphocytes appear to be the major phenotypic feature of asymptomatic disease. Determination of the isotypes patterns during CVL demonstrated that asymptomatic dogs and those with low parasitemia are associated with an increase of IgG1, while the symptomatic dogs and those with high parasitemia are associated with an increase of IgG2, IgG, IgA and IgE immunoglobulins. Pioneer findings obtained by our group showed a correlation between clinical status of CVL with degree of tissue parasite density. This data demonstrated that asymptomatic dogs presented low parasitemia while symptomatic dogs are associated with high parasite load in various tissues such as skin, bone marrow and spleen. We have also investigated the association between tissue parasitemia and CVL clinical forms. Regardless of clinical status, skin and spleen are the major sites of high parasite density during ongoing CVL. Furthermore, we demonstrated that bone marrow and spleen parasite density are the most reliable parasitological markers to decode the clinical status of CVL. In this article, we have reviewed some aspects...
1. Impact of canine control on the epidemiology from canine and human visceral leishmaniasis

Visceral leishmaniasis (VL) is one of the most important emerging diseases with high prevalence in Latin American countries (Tesh, 1995). VL and canine visceral leishmaniasis (CVL) are mainly caused by *Leishmania* (*L.* chagasi) Syn = *L.* infantum in South America and Europe (Mauricio et al., 2000). Peri-domestic sand flies acquire the etiological agent by feeding on infected wild/domestic reservoirs and transmit it, causing severe disease in humans (Tesh, 1995; WHO, 2000). Nowadays, VL is currently expanding worldwide, mainly in Brazil, where recently, the typically rural outline has shifted to a progressively urbanized profile (Palatnik-de-Sousa et al., 2001). VL and CVL have a great impact on Brazilian public health because *domestic dogs are the most important* VL peri-domicile reservoirs in both urban and peri-urban areas (Reithinger and Davies, 1999; Palatnik-de-Sousa et al., 2001). The major prophylactic practice to control VL, as recommended by WHO, involves a systematic treatment of human cases, besides vector control by insecticide and *Leishmania*-seropositive dogs euthanasia (Tesh, 1995; Palatnik-de-Sousa et al., 2001). Control of CVL remains not completely established mainly because there is no effective vaccine available to be used for immunoprophylaxis (Gradoni, 2001; Giunachetti et al., 2007; 2008b, c, d). In the past 5 years, more than two million dogs were screened and more than 160,000 seropositive dogs were eliminated, but the incidence of human VL has not been reduced to an acceptable level (Braga et al., 1998). Moreover, drug treatment of infected dogs is expensive and unfortunately, different treatment strategies have failed to achieve a consistent parasitological cure for CVL owing to the permanence of latent *Leishmania*-infected cells (Baneth and Shaw, 2002; Noli and Auxilia, 2005). Based on either the similarity of clinical signs observed in human and dogs and the evolution of natural history of the disease, CVL has been suggested as a good model to better understand the pathogenesis of the human disease (Sanchez et al., 2004; Reis et al., 2006a).

2. Clinical and biochemical/hematological biomarkers of progression in CVL

Canine visceral leishmaniasis manifests itself as a broad clinical spectrum ranging from asymptomatic infection to patent severe disease, which mostly culminates in death (Reis et al., 2006b). According to Manclanti et al. (1988), asymptomatic dogs (AD) do not show visible clinical signs because they are apparently healthy animals (Fig. 1A), whereas oligosymptomatic dogs (OD) present some signs such as cutaneous ulcerations, frequently observed on the tip of the ears and on the periorbital areas, moderate weight loss and localized alopecia (Fig. 1B and C). Symptomatic dogs (SD) may show one or more typically clinical signs of CVL, such as cutaneous ulcerations all over the body, blindness, severe weight loss (anorexia), onychogryphosis, furfuraceous dermatitis, general alopecia, keratoconjunctivitis, progressing towards general morbidity state and death (Fig. 1D–J). From the localized cutaneous infection, the parasite can be disseminated via lymphatic or blood vessels, infecting macrophages of the bone marrow, lymph node (LN), liver and spleen, as well as kidney and gastrointestinal tract (Tryphos et al., 1977; Keenan et al., 1984a). Initial clinical signs are hypertrophy of lymph node, dermatitis and periportal and nasal dermatitis that could be disseminated. The opaque bistles, onychogryphosis and edema of the paws could be also found. Other signs such as fever, apathy, diarrhea, intestinal hemorrhage, weight loss, hepatosplenomegaly, hyperkeratosis, cutaneous ulceration, particularly on the nose, ears, tails and keratoconjunctivitis are frequent, although not necessarily present in all animals (Genaro et al., 1988; Dias et al., 1999).

The CVL presumptive diagnosis is generally performed by serological tests, such as indirect immunofluorescence assay test (IFAT) and enzyme-linked immunosorbent assay (ELISA), associated with clinical and epidemiological records. The major problem regarding clinical diagnosis is the fact that CVL signs are very similar to those observed in other infectious diseases (Costa et al., 1991). The chronic aspect of the disease and its long incubation period may generate a delay or failure in clinical diagnosis (Cardoso and Cabral, 1998). Despite their high sensitivity, serological tests present a broad range of cross-reactions with other protozoan (Costa et al., 1991; Grimaldi and Tesh, 1993). However, the parasitological diagnosis presents generally low sensitivity when the tissue parasitism is scarce (Reis et al., 2006a). Considering the clinical forms of the disease, it was possible to define various biomarkers closely related to the parasitological, biochemical and hematological findings observed during clinical progression of the CVL. These findings include normocytic/normochromic anemia and increase of total serum protein levels. It seems that biochemical alterations are linked to a polyclonal humoral immune response, which leads to a raised protein levels in serum (Marzochi et al., 1985; Reis et al., 2006a), characterized by hyperglobulinemia and hypoalbuminemia, besides of decreased albumin/globulin ratio (Cardoso and Cabral, 1998; Almeida et al., 2005; Strauss-Ayali et al., 2007). An impaired hematological status is associated with severe clinical aspects of CVL to be evidenced by marked anemia and leukopenia in decorrence of lymphopenia, eosinopenia and monocytopenia (Reis et al., 2006a, c).
3. Tissues parasite load and clinical progression in CVL

Evaluation of parasite load by "leishman donovan units" (LDU), number of *Leishmania* amastigote by 1,000 nucleated cells (Stauber, 1955 modified by Reis et al., 2006a), and anti-*Leishmania* detection by immunohistochemistry are important parasitological tools to verify parasite density in different lymphoid compartments (Tafuri et al., 2001, 2004; Sanchez et al., 2004; Reis et al., 2006a,b,c; Giunchetti et al., 2006, 2008a,b). In studies performed by our group, tissue parasitism for each compartment was initially classified as low (LP), medium (MP) or high (HP) parasitism based on tissue-specific LDU values statistically categorized into tertiles (Reis et al., 2006a,b). The data demonstrated that AD group presented low parasitism while SD group presented high parasite load in various tissues (skin, bone marrow, spleen, liver and lymph node) (Reis, 2001; Reis et al., 2006a,b,c; Giunchetti et al., 2006, 2008a,b). In this scope, our data showed correlations between AD group with low parasitism and SD group with high parasitism in skin, bone marrow and spleen (Reis et al., 2006b). Previous data obtained by our group has also investigated the association between tissue parasitism in distinct compartments and CVL clinical forms. Despite the clinical status, skin and spleen are the major sites of high parasite density during ongoing CVL (Reis et al., 2006b). Furthermore, we demonstrated that bone marrow and spleen parasite density are the most reliable parasitological markers to decode the clinical status of CVL (Reis et al., 2006b). More recently, Francino et al. (2006) proposed the quantitative real-time PCR (qPCR) to elucidate the status of dogs that are positive for *Leishmania* by conventional PCR, especially in endemic areas. The qPCR turned out to be also very useful to follow-up the parasite load in order to estimate the efficacy of the treatment, vaccines trials or the CVL evolution.

4. Systemic immunological biomarkers of clinical progression in CVL

In the last decade, various research groups have concentrated efforts studying immunopathology of dogs naturally and experimentally infected by *L. chagasi*. *L. infantum* (Cabral et al., 1992; Martinez-Moreno et al., 1993, 1995; Pinelli et al., 1994, 1995, 1999a,b; Brandonisio et al., 1996; Bourdineau et al., 1997; Nieto et al., 1999; Alvar et al., 2004; Tafuri et al., 2004; Solano-Gallego et al., 2004, 2005; Giunchetti et al., 2006; Reis et al., 2006a,b,c; Strauss-Ayali et al., 2007; Lage et al., 2007; Cardoso et al., 2007; Rodriguez-Cortes et al., 2007; Giunchetti et al., 2008a,b). After the pioneering studies lead by Pinelli et al., some authors have searched immunological markers according to *in vivo*, *ex vivo* and *in vitro* context to evaluate clinical progression of CVL (Pinelli et al., 1994, 1995).

The *in vivo* analysis of the intradermal delayed hypersensitivity test, the leishmanin skin test (LST) (Montenegro, 1926), is an useful tool for both clinical diagnosis and epidemiological studies of human VL. The test is negative during the acute stage but it becomes and remains positive following the resolution of clinical symptoms (Reed et al., 1986; Badaró et al., 1986, 1996; Carvalho et al., 1992). The LST was also used for the
diagnosis from a dog population in an endemic region of VL (Cardoso et al., 1998; Cabral et al., 1998). These authors showed that the prevalence of the infection increases considerably using LST to detect Leishmania-specific cellular immunity, in comparison with the prevalence obtained only by culture and serology, confirming that the prevalence and incidence of CVL have still been underestimated (Dye et al., 1993; Tesh, 1995). The use of the LST to evaluate in vivo immune status to distinguish asymptomatic and symptomatic dogs is also controversial, because the SD group might react in a similar manner to the AD group (Reis, 2001). Baleeiro et al. (2006) tested various antigens obtained by L. chagasi, Leishmania braziliensis and Leishmania amazonensis in VL endemic area in Brazil. These authors showed that the use of antigens from different Leishmania species might interfere with the results of the immunological tests performed in dogs naturally infected by L. chagasi. Further investigations will be necessary in an endemic area to define the role of the LST in natural and experimental Leishmania infections and its applications in vaccine trials to CVL.

In symptomatic CVL, cellular immune response is impaired, as indicated by studies showing that peripheral blood mononuclear cells (PBMC) from dogs fail to respond to parasite antigens both in vitro and in vivo. Protective immunity has generally been associated with a distinct cellular immune response, manifested by a strong proliferative response of PBMC to leishmanial antigens (Cabral et al., 1992; Carvalho et al., 1981; Sacks et al., 1987; Pinelli et al., 1999a,b) accompanied by the IFN-γ and TNF-α production, which are required for macrophage activation and killing of intracellular parasites (Kemp et al., 1993; Liew and O'Donnell, 1993; Nacy et al., 1991; Pinelli et al., 1995).

The role of anti-leishmanial cellular and humoral immunity during systemic and compartmentalized immune response underlying the susceptibility/resistance during CVL has been recognized throughout ex vivo and in vitro investigations (Pinelli et al., 1994, 1995; Bourdouzeau et al., 1997; Solano-Gallego et al., 2001a, 2005; Reis et al., 2006a,b,c; Giunchetti et al., 2006, 2008a,b; Lage et al., 2007). In order to assess the cellular immune response, we used the immunophenotypic approaches by flow cytometry to study immunological features of circulating leucocytes as immunological markers for clinical status and bone marrow parasite density in dogs naturally infected by L. chagasi (Fig. 2) (Reis et al., 2006c). T cells analysis in the peripheral blood showed high levels of CD8+ T cells in AD and OD groups as compared to SD group. On the other hand, the SD group presented low levels of CD4+ T cells as compared to AD group. AD and OD groups presented an increase of CD8+ T cells as compared to SD

Fig. 2. Clinical status versus cellular and humoral immune response profile in the peripheral blood, bone marrow and skin from dogs naturally infected by L. chagasi; CVL, canine visceral leishmaniasis; AD, asymptomatic dogs; OD, oligosymptomatic dogs; SD, symptomatic dogs; LDU, leishman donovan units; Ig, immunoglobulins.

and control groups. Interestingly, increased levels of CD8+ lymphocytes appear to be the major phenotypic feature of asymptomatic disease. A decrease in the number of B cells in SD group as compared to AD and control groups were also observed (Reis et al., 2006c). Moreover, a decrease in the number of circulating CD14+ monocytes in SD group as compared to control group was detected (Fig. 2). Furthermore, bone marrow parasite density is related closely to major phenotypic changes reported for peripheral blood leukocytes and pointed out as hallmarks of the clinical status of CVL. We observed a lower number of CD5+ T cells, their subpopulations (CD4+ and CD8+) as well as a lower number of B cells and monocytes mainly in dogs with high parasitism (Reis et al., 2006c). Several reports have focused on the relationship between distinct clinical forms of CVL, disease progression and the IgG isotype levels, in both experimental and natural L (L) infantum/L. (L) chagasi infections (Solano-Gallego et al., 2000, 2001a, b; Leandro et al., 2001; Vercammen et al., 2002, 2007; Quinell et al., 2003; Cordeiro-da-Silva et al., 2003). Although the majority of these investigations have been performed based on well-established ELISA and Western-blot protocols, controversial data on immunoglobulin isotype profiles are frequently documented. Increased levels of IgG and IgG2 have been indiscriminately reported for AD and SD groups as described by Bourdouise et al. (1997) and Vercammen et al. (2002). However, according to Deplazes et al. (1995), Nieto et al. (1999) and Solano-Gallego et al. (2001a, b), SD group showed considerably higher anti-Leishmania IgG1 antibodies in comparison to asymptomatic carriers. Additionally, Courtenay et al. (2002) and Quinell et al. (2003) reported that higher levels of anti-Leishmania IgG, IgG1 and lower levels of IgG2 were also observed in SD group (Day, 2007). However, Leandro et al. (2001) and Cordeiro-da-Silva et al. (2003) have documented increased levels of IgG2 in sera samples from infected animals, particularly in the case of SD group. Despite these controversial findings regarding the immunoglobulin isotype profile associated with CVL, it is clear that during canine Leishmania infection a dichotomous humoral immune response is triggered, similarly with the human infection (Anam et al., 1999). Recently, our group has studied the isotype patterns of immunoglobulins as hallmarks for clinical status and tissue parasite density from Brazilian dogs naturally infected by L. chagasi (Reis et al., 2006b). We have observed that AD and LP groups are associated with an increase of IgG1, while the SD and HP groups are associated with an increase of IgG, IgG2, IgM, IgA and IgE immunoglobulins (Fig. 2).

5. Compartmentalized immune response in different lymphoid organs in CVL.

Analysis of the immune response in different compartments during chronic infection is a useful new scientific strategy for the study of the immune response in parasitic infectious diseases. This approach allows simultaneous investigation of the immunological events observed in the peripheral blood and may indicate whether they are representative of those occurring in the lymphoid tissues (Teixeira-Carvalho et al., 2002).

Although CVL is known to be a severe systemic disease, there are a few studies describing detailed histopathological features of distinct host compartments affected by the parasite. Aiming to better understand events related to compartmentalized immunopathology of CVL, several research groups have performed a broad investigation focusing on histopathological, parasitological and immunological aspects of skin, spleen, liver and lymph nodes (LN) in dogs naturally and experimentally infected by L. chagasi.

The skin is the first point of contact with Leishmania from sand fly vectors. and recent investigations demonstrated that in endemic regions a large population of parasitized asymptomatic dogs is living (Berrahal et al., 1996; Cabral et al., 1998; Solano-Gallego et al., 2000, 2001a, b). Earlier reports of CVL described various macroscopic aspects of skin lesions (desquamation, alopecia, pustular dermatitis, ulcerative dermatosis and nodular disease), which may be associated with immune response (Adler and Theodor, 1932; Cunha, 1938; Torres, 1941; Ferrer et al., 1988). The histopathologic evaluation of the skin biopsies revealed that in all Leishmania-infected dogs the predominant inflammatory cells were macrophages. Lymphocytes and plasma cells were the second most frequent cell types.

Dos-Santos et al. (2004) showed that inflammatory infiltrates are a common histological finding in the skin of dogs regardless of the presence of parasites or evidence of infection with Leishmania. However, skin parasitism in CVL was always associated with inflammation. Macrophages infiltrates were more frequently associated with the presence of Leishmania and a higher parasite burden than pleomorphic focal or diffuse inflammatory infiltrate was found.

We have described that SD group presented intense dermal inflammatory infiltrate characterized as diffuse with a high parasitic burden, decrease in the levels of collagen type I and increase in collagen type III (Giunchetti et al., 2006) (Fig. 3A and B). Furthermore, sections stained with hematoxylin and eosin demonstrated a higher intensity of inflammatory changes in SD group than AD group. Correlation between cellular phenotypes and histological changes seemed to reflect cellular activation and leucocytes migration from peripheral blood to the skin, mediated by antigenic stimulation. The results suggested that chronic dermal inflammation and cutaneous parasitism were directly related to the severity of clinical disease (Giunchetti et al., 2006) as related by Martinez-Moreno et al. (1995), Tarantino et al. (2001) and Solano-Gallego et al. (2004).

Brachelente et al. (2005) demonstrated that the local immune response in CVL includes Th1 as well as Th2 cytokine subsets suggesting that increased expression of the Th2 cytokine-IL-4 is associated with both severe clinical signs and a high parasite burden in the skin lesions. In addition, Guarga et al. (2000) demonstrated that dogs naturally infected by L. infantum presenting lower count of CD4+ cells were more capability to infect sand fly vector.

Considering the spleen as one of the major affected organs during CVL, significant alterations in its morphology and immunological aspects become a relevant...
investigation, because it is a site where cell activation might occur during the infection. Since the contribution of the immune response in the genesis of splenomegaly during CVL is still unclear, we have developed a morphological and phenotypic analysis of spleen biopsies from dogs with CVL. In Fig. 3G and H, the splenic tissue presents capsule inflammation more frequently in infected dogs as compared to control group. These alterations are more intense in OD and SD groups as compared to AD group. We also observed hypertrrophy and hyperplasia in red pulp from spleen in all infected groups, characterized by mononuclear infiltrate cells, mainly plasmocytes. The white pulp presented the substitution of macrophage by lymphocytes in decurrence of hypertrrophy and hyperplasia of this region (Fig. 3G and H).

Recently, studies referring to the evaluation of cytokines profile in spleen cells from dogs naturally/experimentally infected by L. chagasi/L. infantum were conducted (Lage et al., 2007; Strauss-Ayali et al., 2007). Lage et al. evaluated the cytokines expression in splenocytes from dogs naturally infected by L. chagasi and did not detected differences on the TNF-α, IL-12, IL-4, IFN-γ and IL-10 levels when the dogs were classified by different clinical forms. However, when the dogs were categorized by different parasite load, the authors observed that hundred percent of dogs with high parasitemia expressed IL-10 as compared to those with low and medium parasitemia. These data suggest that CVL is marked by a balanced production of Th1/Th2 cytokines, with a predominant accumulation of IL-10, probably as a consequence of an increase in parasite load and progression of the disease. These data still showed a possible relationship between intensity of splenic parasitism and evolution of the clinical manifestations of CVL (Lage et al., 2007).

Strauss-Ayali et al. developed a study investigating the immune response in splenocytes of L. infantum-infected dogs following experimental and natural infection. Increased levels on IFN-γ, T-bet, IP-10 and RANTES were observed in all evaluated groups. IL-4 levels increased as early as one month after experimental infection, while IL-5 was high at later stages. IL-10 and TGF-β did not change during the infection. The study indicated that both type-1 and type-2 immune responses occur in the spleen during CVL and suggested that the early elevation of IL-4 might
have a role in the persistence of parasites in the presence of high IFN-γ expression.

The immunopathological evaluation of the hepatic compartment associated with parasitism and biochemical findings have been also performed in order to better understand the genesis of hepatomegaly in CVL (Giunchetti et al., 2008b). Intense reaction of the Kupffer cells, capsule and portal inflammation and the presence of intralobular granulomas were observed in the different clinical groups (Fig. 3C and D). SD group presented a higher frequency of parasitism as compared to AD group. Inflammatory alterations were more intense in the SD group and were associated with parasitism. Moreover, the results indicated an association between histological liver changes (inflammation of the hepatic capsule, portal inflammation, and hypertrophy/hyperplasia of the Kupffer cells) and the enhancement of biochemical alterations (plasmatic globulin) according to progression of clinical forms of CVL.

Despite LN is one of the most relevant lymphoid tissues involved in the parasite-host interface during L. chagasi infection, the cellular and molecular basis of the compartmentalized immune response as well as histopathology into LN is not completely established. There are only few studies focusing on the LN during CVL (Keenan et al., 1984a,b; Martinez-Moreno et al., 1993; Tafur et al., 2001; Lima et al., 2004; Giunchetti et al., 2008a). A detailed histopathologic analysis was performed by our group aim to increment information about parasite load and major immunophenotypic features of the LN in L. chagasi-infected dogs (Giunchetti et al., 2008a). Our major histopathological findings highlighted that hypertrophy/hyperplasia of LN cortical and medullary zones were the principal characteristics observed in AD group, whereas atrophy of LN cortical zone was predominant feature in SD group (Fig. 3E and F).

In CVL, lymphadenopathy is usually defined as an increase in LN size (enlargement of LN), usually described as a regional or generalized alteration (Rogers et al., 1993). It has been demonstrated that all LN from L. chagasi-infected dogs display a chronic lymphadenitis, regardless of the anatomical region analyzed, with hypertrophy/hyperplasia of cortical and medullary zones (Lima et al., 2004). However, the clinical status or the tissue parasitic load might not be directly related to the intensity of the lesions, once previous data demonstrated that AD group presented higher LN parasitism than OD or SD groups (Giunchetti et al., 2008a).

6. Conclusion and new challenges

Our findings highlight the complexity of cellular immunological events related to natural infection from dogs by L. chagasi, correlating major peripheral blood phenotypic markers with clinical status and tissues parasite density. Our data suggest that the sustained T cell compartment (both CD4+ and CD8+ T cells) observed in AD and LP groups may be resultant from the high activity of the host immune system to perform antigen presentation and to remove parasites from infected sites. Lower frequency of circulating B cells and monocytes are important markers of severe CVL, whereas increased levels of CD8+ lymphocytes appear to be the major phenotypic feature of asymptomatic disease. Despite the clinical status, skin and spleen is the major sources of high parasite density during ongoing CVL. Furthermore, we demonstrated that bone marrow and spleen parasite density are the most reliable parasitological markers to decode the clinical status of CVL.

Additional studies on the specificities of the activated cells and their both cytokine and chemokine profiles may provide important information that will lead to a better understanding of the immunological/inflammatory events that take place in the distinct lymphoid compartments during canine visceral leishmaniasis.

Conflict of interest

None.

Acknowledgements

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References


Evaluation of the influence of tissue parasite density on hematological and phenotypic cellular parameters of circulating leukocytes and splenocytes during ongoing canine visceral leishmaniasis

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Abstract During Leishmania infection, tissue parasitism at different sites may differ and imply distinct immunopathological patterns during canine visceral leishmaniasis (CVL). For this reason, we have assessed by flow cytometry the impact of spleen and skin parasite density on the phenotypic profile of splenocytes and circulating leukocytes of 40 Brazilian dogs naturally infected by Leishmania chagasi according to splenic and cutaneous parasite load. Our major statistically significant findings demonstrated that dogs with splenic high parasitism presented a significant decrease in absolute counts of CD8+ T lymphocytes in comparison with dogs presenting splenic medium parasitism. Moreover, a decrease in the absolute number of circulating monocytes was observed as a hallmark of high parasitism. The increased frequency of CD8+ T cells is associated with low splenic parasitism during CVL. Although we did not find any significant differences between the immunophenotypic analysis performed in circulating lymphocytes according to cutaneous parasite load, there were negative correlations between CD8+ and CD8+ T cells and cutaneous parasite density reemphasizes the role of T cell-mediated immune response in resistance mechanisms during ongoing CVL. These results add new insights about the pathogenesis of CVL and may help in the establishment of additional tools for future studies on drugs and vaccine approaches.

Introduction

Visceral leishmaniasis (VL), which is caused by Leishmania (Leishmania) infantum [syn. Leishmania (Leishmania) chagasi], is endemic in over 88 countries within Europe and Latin America and is transmitted by the bite of the female sand fly (Desjeux 2004). The major prophylactic practice to control this human disease, as recommended by the World Health Organization, involves a systematic treatment of human cases besides vector control by insecticide and elimination of the domestic reservoir, mainly seropositive infected dogs (Tesh 1995). In this context, canine visceral leishmaniasis (CVL) has high epidemiological relevance, considering its increased incidence in the last decade, in addition to the intense cutaneous parasite density reported in both asymptomatic and symptomatic dogs which may contribute to the spread of disease (Molina et al. 1994; Tesh 1995; Giunchetti et al. 2006; Reis et al. 2006a, b).
Considering the pathological similarities between dogs and humans in many diseases caused by infectious agents, several studies have utilized these animals as an alternative model to evaluate parasite–host interaction, immune responses, and development of new methods focusing on the diagnosis, prognosis, and the evaluation of therapeutic and vaccine protocols for use in routine clinical veterinary (Cobbold and Mecalfe 1994; Williams 1997). Moreover, dogs constitute an excellent model to study leishmaniasis (Moreno and Alvar 2002; Alvar et al. 2004), and for this reason, the natural history of CVL has been recently described, particularly in relation to the parasite load in different tissues and to the immunopathological changes concerning the progression of clinical forms (Chamizo et al. 2005; Giunchetti et al. 2006a, b; Reis et al. 2006b, c; Lage et al. 2007; Giunchetti et al. 2008a, b).

In this context, the clinical status of CVL is highly variable. Some infected dogs may develop symptomatic infection resulting in death, while others remain asymptomatic or develop one or more mild symptoms and are classified as oligosymptomatic (Mancianti et al. 1988; Barbieri 2006; Reis et al. 2006a, b, c). Dogs infected with the *Leishmania* parasite present high titers of anti- *Leishmania* antibodies and a depression of the T cell-mediated response (Pinelli et al. 1994; Martinez-Moreno et al. 1995; De Luna et al. 1999; Campino and Abranches 2002).

Aiming to further characterize the immunopathological pattern in several animal species, flow cytometry has been shown to be a promising tool to evaluate a variety of cell subsets with a great interest and emphasis on studies in canine clinical veterinary (Reis et al. 2005). Moore et al. (1992) studied specific monoclonal antibodies for canine CD4 and CD8 that are expressed in T lymphocytes subpopulations and verified a high CD4 expression in canine neutrophils. Pinelli et al. (1995), in an immunophenotypic study of canine cells, observed a decrease of CD4 and CD8 lymphocytes in peripheral blood cells of dogs with VL. Moreover, Reis et al. (2006c) observed a lower frequency of circulating B cells (CD21) and monocytes (CD14) as important markers of severe CVL, whereas increased levels of B (CD21) and T (CD4 and CD8) lymphocytes appeared to be the major phenotypic feature of asymptomatic disease.

Despite these findings regarding major lymphocytes subsets in dogs with different clinical forms of VL, there is a great gap in the understanding of the role of the tissue parasite density during CVL immune response. Recently, in a pioneer study, our group demonstrated that the spleen and skin are the most relevant sites of high parasitism during ongoing CVL (Reis et al. 2006b). The skin is the first point of contact with organisms of the genus *Leishmania* from sand flies vectors (Giunchetti et al. 2006) and its parasite density has been associated with a granulomatous inflammatory pattern in dogs with VL (Dos Santos et al. 2004). This tissue was considered by Abranches et al. (1991) to be an important reservoir compartment for parasites in healthy and sick *Leishmania*-infected dogs and the important role of dogs in VL transmission is supported by the high parasite loads found in the skin of infected animals (Deane and Deane 1962). The spleen is the main site of lymphoid cells interposed into the blood stream. Moreover, there is a large amount of circulating *Leishmania* antigens in the spleen, besides high local parasite density frequently in contact with splenocytes that lead to strong cellular and humoral immune responses (Reis et al. 2006b).

In this current study, we have assessed the impact of spleen/skin parasite density on the phenotypic profile of mononuclear cells in Brazilian dogs naturally infected by *L. chagasi* categorized according to three different parasite densities. Our major findings describe spleen parasite density as being related more closely to major phenotypic changes in peripheral blood leukocytes than skin during CVL. Moreover, the association between spleen parasitism and CD8+ T cells reemphasizes the role of T cell-mediated immune response in resistance mechanisms during ongoing CVL.

### Materials and methods

#### Animals

Sixty mixed-breed adult dogs of both genders, 2–6 years old, were provided by the Control Zoonosis Center in Belo Horizonte City Council, Minas Gerais state, Brazil after clinical preselction and maintained in the kennels of the Institute of Biological Sciences of Federal University of Minas Gerais, Minas Gerais state, Brazil. Prior to the inclusion in this study, all animals were treated for intestinal helminthic (Endal plus®) infections and immunized against parvovirus, leptospirosis, distemper, parainfluenza, and hepatitis (HTLP SCV-L vaccine Pfizer®). The animals were kept in quarantine with drinking water and a balanced feed given ad libitum. The dogs used in this study were stray or domiciled mongrel dogs naturally infected with *L. chagasi*, selected based on their serological results on indirect immunofluorescence assay test (IFAT), used as a gold standard virological test for diagnosis of CVL. Animals presenting IFAT titers ≥1:40 were considered positive and included into the *L. chagasi*-infected groups. Animals with IFAT negative (<1:40) were considered noninfected and included as a control group. Infection with *L. chagasi* was confirmed serologically in all IFAT-positive dogs, including enzyme-linked immunosorbent assay (ELISA) extract and ELISA r-K39, as described previously (Reis et al. 2005) and/or at least one parasitological examination performed at
two different tissue sites (spleen and skin), as described below.

Ethics

All procedures in this study were according to the guidelines set by the Brazilian Animal Experimental College (COBEA). This study was approved by the Ethical Committee for the use of Experimental Animals of the Federal University of Minas Gerais, Minas Gerais state, Brazil (CETEA).

Parasitological evaluation

Assessment of parasitological parameters was performed by diagnoses in tissue smears (spleen and skin) carried out after euthanasia performed by intravenous overdose of barbiturate and necropsy of the animals. The smears were stained by Giemsa and examined under optical microscopy for the identification of amastigote forms of Leishmania. Parasite density evaluation was performed in the spleen and skin and the results were expressed as Leishman Donovan units (LDU index), according to Reis et al. (2006b), modified from Stauber (1955) which corresponds to the number of Leishmania amastigotes by 1,000 nucleated cells. Tissue parasitisms for both sites were classified initially as low (LP), medium (MP), and high (HP) parasitism based on spleen- and skin-specific LDU values categorized statistically into tertiles according to Reis et al. (2006b), as follows: LP (zero to 11 in the spleen; zero to nine in the skin); MP (12–170 in the spleen; ten to 130 in the skin), and HP (184–2,564 in the spleen; 133–7,246 in the skin). The number of animals included on each subgroup was approximately 13–14 animals.

Noninfected dogs (CG, n=20) displaying negative parasitological and serological examination for Leishmania were considered in this study as the control group.

Blood sample collection

Five milliliters of peripheral blood from the brachiocephalic vein were collected into tubes containing ethylenediamine tetraacetate acid (EDTA; final concentration: 1 mg/mL). A hemogram was performed in each sample (Coulter MD18; Luton, UK). All samples were maintained at room temperature up to 12 h prior to processing.

Immunophenotyping by flow cytometry

Immunophenotyping analyses of peripheral blood through flow cytometry were undertaken as described by Reis et al. (2005). Briefly, 1 mL of EDTA whole blood was submitted to prefixed and erythrocyte lysis by the slow addition of 13 mL of lysis solution (FACS lysing solution; Becton Dickinson, San Diego, CA, USA) followed by incubation for 10 min at room temperature (RT). After centrifugation (450×g for 10 min at RT), the pellet was resuspended in 500 μL phosphate-buffered saline (PBS) supplemented with 10% of fetal bovine serum (FBS-10%). In 96-well U-bottom plates (Limbro Biomedicals, Aurora, OH, USA), 30 μL of prefixed leukocyte suspension were incubated at RT for 30 min in the dark in the presence of 30 μL of anticanine cell surface marker monoclonal antibodies (mAbs) diluted previously in PBS-10% in indirect immunofluorescence procedures. A range of cell surface markers that define major canine leukocyte subpopulations were used, including diluted purified antilog CD5 1:800 (rat IgG2a, clone YKIX322 3), antilog CD4 1:12,500 (rat IgG2a, clone YKIX302 9), antilog CD8 1:800 (rat IgG1, clone YCATE55 9), anti-MHCII 1:200 (rat IgG2b, clone YKIX334 2), anti-CD45RA 1:200 (rat IgG2b, clone YKIX753 22 2), and anti-CD45RB 1:800 (rat IgG2b, clone YKIX716 13), all purchased from Serotec (Oxford, UK). Cells were additionally incubated in the same conditions, in the presence of 60 μL of previous diluted fluorescein isothiocyanate (FITC)-conjugated sheep antirat IgG antibody.

Five microliters of undiluted FITC-labeled mouse antihuman CD21 (mouse IgG1, clone IOB1a; Immunotech, Marseille, France) and 50 μL of previously diluted PE/Cy-5-conjugated mouse antihuman CD14 1:200 (mouse IgG2a, clone TUK4; Serotec Oxford, UK) were used in direct immunofluorescence procedures.

For the achievement of splenocytes, fragments of spleen were collected and transferred to a glass macerator. In this procedure, heparinized RPMI 1640 and Ficoll-Hypaque (Histopaque® 1.077-Sigma, USA) was used according to standardized protocol. In the immunofluorescence assay to evaluate the phenotypic expression of splenocytes obtained in the ex vivo context, 2–3 mL of the cellular suspension of each spleen were submitted to lysis, using the same solution described above.

Before flow cytometric data collection and analysis, labeled cells were fixed for 30 min with 200 μL of FACS FIX solution (10.0 g/L paraformaldehyde, 10.2 g/L sodium cacodylate, and 6.65 g/L sodium chloride, pH 7.2).

Flow cytometric acquisition and data analysis

Flow cytometric measurements were performed on a FACScan instrument (Becton Dickinson, Mountain View, CA, USA). The CellQuest software package was used in both data acquisition and analysis. A total of 10,000 events was acquired for each preparation. Canine whole blood leukocytes and splenocytes were identified on the basis of their specific forward (FSC) and side (SSC) light-scatter properties. Following FSC and SSC gain adjustments, the
lymphocytes were selected based on their characteristic FSC versus SSC gain distribution. Fluorescence was evaluated based on the spectra of FITC and Cy5-PE on FL1 or FL3 single-histogram representation, respectively. The monocytes were analyzed by fluorescence intensity detection on single histograms directly on ungated leukocytes. For data analysis, a marker was set on the internal control in order to confine over 98% of the unlabeled cells.

The results were expressed in absolute counts (cell number per cubic micrometer) that allow the normalization of data from groups whose overall leukocytes counts may differ. The absolute counts for lymphocyte subsets were calculated as the product of the percentage of positive cells (CD5<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, CD21<sup>+</sup>) within gated lymphocytes by the absolute lymphocyte counts derived from the hemogram. The absolute counts for monocytes were obtained as the product of CD14<sup>+</sup> cells obtained within ungated leukocytes by the total white blood cell counts derived from the hemogram. Semiquantitative analyses were performed to evaluate differential expression of cell surface markers presenting unimodal distribution (MHC-II, CD45RA, and CD45RB). In these cases, the results were expressed as the mean fluorescence channel (MFC).

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 4.03 software package (San Diego, CA, USA). In the parametric data, one-way analysis of variance was used for the comparative study between groups, followed by Tukey’s test. In the nonparametric data, Kruskal-Wallis test was used for between group comparative study, followed by Dunn’s test for multiple comparisons. Spearman’s rank correlation was also computed to investigate associations between expression of phenotypic features and spleen and skin parasite densities. In all cases, the differences were considered significant when the probabilities of equality, P values, were <0.05.

Results and discussion

Marked anemia, eosinopenia, and monocytopenia are hallmarks of hematological dysfunction associated with high spleen parasite density during canine visceral leishmaniasis.

Recently, some studies focusing on the immunopathological pattern in CVL had been performed considering different tissues and distinct clinical forms (Chamizo et al. 2005; Giunchetti et al. 2006; Reis et al. 2006a, b, c; Lago et al. 2007; Giunchetti et al. 2008a, b). Although immunophenotypic changes in peripheral blood cells of dogs classified into different clinical forms have been previously reported, there are very few studies regarding these changes in dogs according to their parasite load in different organs (Sanchez et al. 2004; Reis et al. 2006c; Giunchetti et al. 2008b). It has been proposed that the spleen and the skin are the major sites of high parasite density during ongoing CVL (Reis et al. 2006b), and for this reason, the immunophenotypic alterations in dogs according to their splenic or cutaneous parasite load were analyzed in the present study.

The assessment of hematological parameters demonstrated severe anemia in dogs with splenic HP (Table 1). We observed significant decreases in erythrocytes of MP and HP dogs compared to the control group (p<0.001) as well as in hemoglobin and hematocrit values in HP dogs compared to the LP group (p<0.05) (Table 1). Dogs with high cutaneous parasite densities showed only decreased

<table>
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<tr>
<th>Hematological parameters</th>
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<td>CG</td>
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<td></td>
<td>Spleen</td>
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<td></td>
<td>LP</td>
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<tr>
<td>Erythrocytes (million/mm&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>6.8±0.8</td>
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<tr>
<td>Hemoglobin (g/dL)</td>
<td>15.7±1.9</td>
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<tr>
<td>Hematocrit (%)</td>
<td>46.4±5.4</td>
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<tr>
<td>Leukocytes (×10&lt;sup&gt;3&lt;/sup&gt;/mm&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>12.8±2.7</td>
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<tr>
<td>Neutrophils</td>
<td>7.3±3.1</td>
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<tr>
<td>Eosinophils</td>
<td>1.8±0.8</td>
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<tr>
<td>Lymphocytes</td>
<td>2.7±1.5</td>
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<tr>
<td>Monocytes</td>
<td>1.1±0.5</td>
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The results are shown as the average values/standard deviation. The letters (a and b) represent statistically significant differences for the control and low parasitism dogs, respectively, when p<0.05. LP low spleen and skin parasite density, MP medium spleen and skin parasite density, HP high spleen and skin parasite density, CG control group.
hemoglobin values in comparison to the LP dogs \( (p<0.05) \). These data are in agreement with Reis et al. (2006c) which also observed these hematomatological alterations associated with severe disease. In addition, white blood cell counts of dogs with both parasitized compartments demonstrated significant decrease of eosinophils \( (p<0.001) \) in MP and HP dogs as well as decrease in the absolute number of monocytes \( (p<0.05) \) in HP dogs compared to the control group (Table 1). This finding is in accordance with a decrease \( (p<0.05) \) in the absolute number of CD14\(^+\) cells observed in the HP group in comparison with the MP group (Fig. 1). According to Reis et al. (2006a, c), the decrease in absolute values of circulating monocytes in symptomatic and high parasitism dogs may suggest, during active CVL, the recruitment of these cells to lymphoid tissue where they might play an important role in immunological connections throughout antigen presentation. Giunchetti et al. (2006) showed a reduction in CD14\(^+\) monocytes counts in the skin of symptomatic dogs and a positive correlation between chronic dermal inflammation score and CD14\(^+\) monocytes, demonstrating a potential activation of this cell population and migration to the dermis, but with scant contribution to the resistance for *L. chagasi* infection.

Increased frequency of CD8\(^+\) T cells is associated with low splenic parasitism during CVL. In order to evaluate whether the parasite load during CVL reflects in the differential immunophenotypic profile of peripheral blood, we conducted an analysis of circulating leukocytes of *L. chagasi*-infected and control dogs. Our data demonstrated that dogs with splenic HP presented a significant decrease \( (p<0.05) \) in absolute counts of CD5\(^+\) T lymphocytes in comparison with the MP group (Fig. 1). Moreover, analysis of the CD5\(^-\)/CD21\(^+\) cell ratio revealed an increase \( (p<0.05) \) in the LP and MP groups compared to the control group.

Analysis of T cell subpopulations showed a decrease \( (p<0.05) \) in CD4\(^+\) T lymphocytes in the HP group when compared to the MP group and an increase \( (p<0.05) \) in CD8\(^+\) T lymphocytes in the LP and MP groups compared to the control group (Fig. 1). In order to explore more effectively the balance between the T cell subpopulations, we have also reported a decreased \( (p<0.05) \) CD4\(^+\)/CD8\(^+\) cell ratio in infected dogs presenting LP and MP parasitism compared to the control group (Fig. 1), reinforcing the role of CD8\(^+\) T cells in tissue parasite control.

Typically, high CD8\(^+\) T cells counts have been observed in the peripheral blood of asymptomatic dogs and are associated with low bone marrow parasitism, suggesting that these subpopulations create a microenvironment efficient to remove the parasites in hosts bearing asymptomatic or mild disease and with lower parasite densities (Reis et al. 2006c). In addition, CD8\(^+\) T cells are thought to play an important role in the development of an effective immune response to *Leishmania* sp., possibly through cytotoxic mechanisms, which act as potent protective events during CVL (Pinelli 1997). In fact, different studies have correlated the level of CD8\(^+\) T cells with protection during *L. infantum* *L. chagasi* infection (Pinelli 1997; Reis et al. 2006c) or during the immunogenic response after vaccine administration against CVL (Giunchetti et al. 2007; Giunchetti et al. 2008c).

Expression of MHC-II, CD45RA, and CD45RB by peripheral blood lymphocytes was evaluated through semiquantitative analyses in order to verify whether parasite load may be associated with an altered pattern of these constitutive cell surface markers. Our data did not show any statistically significant differences for MHC-II or CD45RA and CD45RB considered as isolated parameters (Fig. 1) or even in the CD45RB/CD45RA expression index (data not shown). Although important differences in the expression of these markers were already found by our group when we evaluate them according to distinct clinical forms of CVL (Reis et al. 2006c), herein, we did not find any statistically significant differences for these cell markers, suggesting that these parameters were not influenced by splenic or cutaneous parasite density during CVL.

High splenic parasite density is correlated with a lower frequency of circulating T cell subsets

To confirm and extend our findings, we performed correlation analyses between the expressions of major lymphocyte subsets counts in the peripheral blood with splenic parasite density (Fig. 2). Our data showed an association between low cell counts and high spleen parasite densities during CVL. Negative correlations between T cell subsets (CD5\(^+\), \( r=-0.4344, p=0.0057 \); CD4\(^+\), \( r=-0.3223, p=0.0454 \); and CD8\(^+\) cells, \( r=-0.5012, p=0.0012 \)) as well as the CD5\(^-\)/CD21\(^+\) cell ratio \( (r=-0.3457, p=0.0311) \) and spleen parasite density were observed. We found either a positive correlation between CD4\(^+\)/CD8\(^+\) cell ratio \( (r=0.5455, p=0.0003) \) and spleen parasite density (Fig. 2).

Augmented skin parasite density is related to the low levels of circulating CD8\(^+\) T cells besides high CD4\(^+\)/CD8\(^+\) cell ratio

The immunophenotyping of peripheral blood lymphocytes from dogs categorized according to their skin parasite load did not show any statistically significant differences between the groups (Fig. 3). However, the correlation analyses between expression of phenotypic features and
Fig. 1 Immunophenotypic profile of peripheral blood leukocytes in *L. chagasi*-infected dogs categorised according to their spleen parasite density as low (LP, light gray bars), medium (MP, dark gray bars), and high (HP, black bars) parasitemia dogs. Uninfected dogs were used as a control group (CG, white bars). The results are shown as scattering of individual values and mean absolute cell counts, cell ratio, or MFC. Significant differences at $p<0.05$ are indicated by the letters a and c in comparison to CG and MP respectively.
Fig. 2 Correlation between splenic parasite density (LDU-log) and absolute numbers of peripheral blood leukocyte subpopulations from L. chagasi-infected dogs. The results are expressed as scattering of individual values. Spearman's correlation indexes (r and p values) are shown on the graphs. Connecting lines illustrate positive and negative correlation indexes.
Fig. 3 Immunophenotypic profile of peripheral blood leukocytes in *L. chagasi*-infected dogs categorized according to their skin parasite density as low (LP, light gray bars), medium (MP, dark gray bars), and high (HP, black bars) parasitemia dogs. Uninfected dogs were used as a control group (CG, white bars). The results are shown as scattering of individual values and mean absolute cell counts, cell ratio, or MFC.
Fig. 4 Correlation between skin parasite density (LDU-log) and absolute numbers of peripheral blood leukocyte subpopulations from *L. clugesi*-infected dogs. The results are expressed as scattering of individual values. Spearman’s correlation indexes (r and p values) are shown on the graphs. Connecting lines illustrate positive and negative correlation indexes.
Fig. 5 Immunophenotypic profile of splenocytes in *L. chagasi*-infected dogs categorized according to their spleen parasite density as low (LP, light gray bars), medium (MP, dark gray bars), and high (HP, black bars) parasitism dogs. Uninfected dogs were used as a control group (CG, white bars). The results are shown as scattering of individual values and mean absolute cell counts, cell ratio, or MFC. Significant differences at \( p < 0.05 \) are indicated by the letter a in comparison to CG.
skin parasite densities showed negative correlations for CD5$^+$ T cells ($r=-0.3218$, $p=0.0429$) and CD8$^+$ T cells ($r=-0.3411$, $p=0.0312$), indicating that, similar to the results obtained with splenic parasitism, dogs with highest cutaneous parasite densities have the lowest levels of both these phenotypes. A positive correlation between the CD4$^+$CD8$^+$ cell ratio ($r=0.3709$, $p=0.0185$) and skin parasite density was also found (Fig. 4), according to the previous finding that reported a positive correlation between chronic dermal inflammation score in CVL and circulating CD4$^+$ T cells (Gianchetti et al. 2006).

In order to evaluate the association between skin and spleen parasite load, we have performed a correlation between splenic and cutaneous parasite density, demonstrating the presence of a significant positive correlation ($r=0.818$, $p=0.000$) between these two tissues parasite densities during CVL (data not shown).

Increased proportions of CD8$^+$ and decreased proportions of CD45RA$^+$ splenocytes are associated with medium splenic parasitism during CVL.

During *Leishmania* infection, there is a large amount of circulating parasite-derived antigens that reach the spleen. In addition, the high local parasite density frequently in contact with splenocytes would lead to a cellular activation and a strong immune response to the parasites (Reis et al. 2006b). In order to analyze the compartmentalized immune response in the spleen, we have performed an immunophenotypic characterization of major splenic lymphocyte populations from *L. chagasi*-infected or control group according to their spleen parasite density (Fig. 5). Our data revealed a significant increase ($p<0.05$) in CD8$^+$ splenocytes in the MP group compared to the control group (Fig. 5). We postulate that CD8$^+$ splenocytes may present a distinct activation status during CVL, possibly associated with immunomodulatory or suppressor cell activity. According to this hypothesis, Perhype-Magalhães et al. (2006) showed, in human active visceral leishmaniasis, that circulating CD8$^+$ T cells showed a mixed cytokine pattern characterized by elevated levels of both intracellular IFN-γ and IL-10. Similar results displaying a mixed pattern of cytokine mRNA in splenocytes during CVL has been published by our group (Lage et al. 2007). In this study, the levels of IFN-γ and IL-10 expressions were positively correlated with the parasite load. In addition, Strauss-Ayali et al. (2007) also observed that both type 1 and type 2 immune responses occur in the spleen during canine *L. infantum* infection.

Furthermore, we observed a significant decrease ($p<0.05$) in the CD45RA expression in the MP group compared to the control group (Fig. 5). According to Tipold et al. (1998), loss of CD45RA was shown to occur in other species upon T cell activation. Therefore, the decrease in the CD45RA expression might suggest that, in the spleen of dogs with medium parasite density, there is still an activation profile of T cells. However, whether the changes in CD45 isoforms expression occur simultaneously or sequentially in different tissues, and how they may affect the development of CVL, needs to be determined.

In conclusion, our findings highlight the importance of quantitative investigations regarding the parasite load at different tissues associated with immunopathological changes during ongoing CVL. The assessment of dogs categorized according to parasite density may contribute as an additional tool for immunological investigations, considering therapeutic and vaccine approaches.

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and low frequency of tumour necrosis factor-alpha monocyties are hallmarks of active human visceral leishmaniasis due to *Leishmania chagasi* infection. Clin Exp Immunol 146:124-132


Analysis of the cytokine profile in spleen cells from dogs naturally infected by *Leishmania chagasi*

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Abstract

Recent studies suggest that asymptomatic dogs infected with canine visceral leishmaniasis (CVL) develop a Th1 immunological profile whilst oligosymptomatic and symptomatic CVL-infected animals present a Th2 profile. In the present study, an RT-PCR method has been standardised and employed to evaluate the frequency and the semi-quantitative level of expression of the cytokines IL-4, IL-10, IL-12, INF-\(\gamma\) and TNF-\(\alpha\) in splenocytes of 30 dogs naturally infected with *Leishmania chagasi* and of 7 non-infected dogs (NID). An increase in the level of expression of IL-12 (\(p = 0.059\)) was detected in all CVL-infected dogs compared with NID. In dogs exhibiting high parasitism, the frequency of expression of IL-10 was higher (\(p = 0.011\)) than in animals presenting low parasitism or medium parasitism (MP) and in NID animals, whilst the level of expression of IL-10 was higher (\(p = 0.0094\)) than in animals exhibiting MP and in the NID group. Positive correlations between the levels of expression of IL-10 with respect to the progression of the disease (IL-10; \(r = 0.3510\); \(p = 0.0337\)) and the levels of expression of IL-10 and INF-\(\gamma\) increase in parasitism (IL-10; \(r = 0.3428\); \(p = 0.0438\) and INF-\(\gamma\); \(r = 0.4690\); \(p = 0.0045\)) were observed. Such data suggest that CVL is marked by a balanced production of Th1 and Th2 cytokines, with a predominant accumulation of IL-10 as a consequence of an increase in parasitic load and progression of the disease, and INF-\(\gamma\) was related with the increase in parasitic load.

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Keywords: Cytokines; RT-PCR; Canine diseases; Visceral leishmaniasis; *Leishmania chagasi*

1. Introduction

Leishmaniasis, which comprises a complex of diseases exhibiting significant clinical and epidemiological diversity, continues to represent a severe threat to public health. Whilst the prevalence of leishmaniasis is increasing significantly, the development of appropriate measures for its control is far from complete and...
remains dependent on further research in order to obtain better tools and a more cost-effective strategy for case management and vector control.

Visceral leishmaniasis (VL), which is caused by *Leishmania (Leishmania) infantum* [syn. *Leishmania (Leishmania) chagasi*], is endemic in over 88 countries within Europe and Latin America, and is transmitted by the bite of the female sand fly (phlebotomine) (Desjeux, 2001, 2004). Canine visceral leishmaniasis (CVL) is one of the most important emerging diseases with a high prevalence in Latin American countries. The main prophylactic procedures recommended by the World Health Organisation for the control of CVL include systematic treatment of human cases, vector control through the use of insecticide, and elimination of the domestic reservoir (Tesh, 1995).

Seropositive infected dogs are the zoonotic reservoir for parasite transmission in CVL. Following transmission, the parasites initially multiply in macrophages in the skin at the site of infection. From such a localised cutaneous infection, the parasite can be disseminated via lymphatic or blood vessels, infecting macrophages of the bone marrow, lymph node, liver and spleen, as well as the kidneys and gastrointestinal tract of the dog (Reis et al., 2006a).

The major signs of CVL include hepatosplenomegaly, lymphadenopathy, cutaneous lesions, keratoconjunctivitis, opaque bristles, alopecia, apathy, onychogryphosis, anorexia and severe weight loss (Reis et al., 2006a). According to Mancianti et al. (1988), CVL can be categorised into three distinct clinical forms on the basis of the major features observed in infected dogs. Asymptomatic (AD) animals show no indicative signs of the disease, those presenting a maximum of three clinical indications, including opaque bristles and/or localised alopecia and/or moderate weight loss, are classified as oligosymptomatic (OD), whilst animals exhibiting the most severe clinical signs of CVL are considered symptomatic (SD). Several epidemiological studies have indicated that about 50% of seropositive dogs are asymptomatic (Acco-Sanchez et al., 1998; Fisa et al., 1999), although these animals are potentially infectious to sand flies (Molina et al., 1994; Giunchetti et al., 2006).

Dogs infected with the CVL parasite present high titres of anti-*Leishmania* antibodies, with a predominant IgG1 response (El Amin et al., 1986) and a depression of the T cell-mediated response (Pinelli et al., 1994; Martinez-Moreno et al., 1995; De Luna et al., 1999; Campino and Abranches, 2002). However, the nature of the cellular immune response in dogs is not fully understood. Studies using peripheral blood mononuclear cells (PBMCs) derived from experimentally infected dogs suggest an association between Th1 immune response and resistance to CVL (Pinelli et al., 1994; Santos-Gomes et al., 2002). On the other hand, a clear association between Th1 immune response, and humoral and cellular responses during the progression of the illness could not be demonstrated in a similar study using bone marrow aspirate from dogs infected with *L. chagasi* (Quinnell et al., 2001). Such studies, however, reveal only a partial description of the cell-mediated immunity and do not necessarily reflect that observed in the affected organs.

Canine experimental models are often used in order to investigate the immune mechanisms involved in diseases such as leishmaniasis and Chagas. Moreover, by virtue of the similarities between CVL and the human disease, the animal model has been of great value in the clinical testing of new drugs and vaccines (Cobbold and Metcalf, 1994). However, the availability of tools and reagents for use in the study of the immunopathological mechanisms in this experimental model is still somewhat limited.

In the present paper, we describe the application of a cytokine-specific RT-PCR method in the determination of the cytokine profile in parasite target organ, such as the spleen, of dogs infected with *L. chagasi*. A balanced production of cytokine of Th1 and Th2 was revealed in splenocytes of infected dogs, with a predominant accumulation of mRNA for IL-10 and INF-γ that was related to the parasitic load and to clinical progression. The results obtained will permit a better understanding of the immune response in organs affected by CVL infection.

2. Materials and methods

Details of the proposed study were presented to and approved by the Ethical Committee for the Use of Experimental Animals (CETEA) of the Universidade Federal de Minas Gerais (UFMG, Belo Horizonte, Minas Gerais, Brazil).

2.1. Animals

The 37 dogs included in the study were selected from a population of stray or domiciled mongrel animals that had been captured by the Zoonosis Control Center in Belo Horizonte (Minas Gerais, Brazil) and confined under quarantine at the kennels of the Institute of Biological Sciences, UFMG. Prior to the commencement of the study, selected animals were treated for intestinal helminth infections (Endal Plus®, Schering-Plough Coopers, São Paulo, SP, Brazil) and immunised
against parvovirus, leptospirosis, distemper, parainfluenza and hepatitis (Vanguard® HTHP 5CV-I vaccine, Pfizer, New York, NY, USA). During confinement, all animals received drinking water and a balanced feed ad lib. After the quarantine period, blood samples were collected using 10 mL disposable sterile syringes, and 5 mL samples were transferred, in the absence of anticoagulant, to specimen tubes. Serum samples were stored in appropriate aliquots at −20 °C until required for use in the serological tests.

2.2. Serological diagnosis of CVL

Diagnosis of CVL was carried out by assay for specific anti-Leishmania IgG reactivity using the indirect immunofluorescence antibody test (IFAT), which is considered to be the “gold-standard” diagnostic tool. Parasites, L. amazonensis (MfHOM/BR/1960/BH6), were maintained in logarithmic growth in liver infusion tryptose (LIT) medium (Mancianti et al., 1988). Serum samples were applied to slides coated with fixed promastigote forms of the parasite, and fluorescent-conjugated anti-dog IgG antibodies (Biomanguinhos-Fiocruz, Rio de Janeiro, Brazil) were used to reveal IgG reactivity. Observations were made using a microscope employing blue and ultraviolet incident light. Samples presenting fluorescence at dilutions ≥1:40 were considered positive. Positive and negative sera were assayed concurrently for control purposes.

2.3. Determination of parasite load index

Seropositive dogs were submitted to parasitological examination for Leishmania and subsequently classified according to parasitic load. Euthanasia was performed by intravenous overdose of barbiturate (Thiopental®); following necropsy, fragments of spleen were collected and imprints prepared on two microscope slides. Slides were air-dried, fixed in methanol, stained with Giemsa, and examined under an optical microscope in order to detect the amastigote forms of Leishmania. Parasite densities were determined according to Stauber (1955), and expressed as Leishman Donovan Units (LDU index), which correspond to the number of Leishmania amastigotes per 1000 nucleated cells. Parasite loads were classified as low (LP; low parasitism) when LDU values were between 0 and 10, medium (MP) for LDU values 11–250, and high for LDU values ≥251. For control purposes, seven dogs who were shown to be non-infected with CVL according to the IFAT assay were also submitted to parasitological examination.

2.4. RT-PCR analyses

Total RNA was extracted from splenocytes obtained from the study dogs using Tri reagent (Sigma), and subsequently treated with deoxyribonuclease I (Invitrogen Brasil) and reverse-transcribed using the SuperScript™ II pre-amplification system with oligo(dT)12-18 as primer (Invitrogen Brasil), all according to the manufacturer’s instructions. Diluted (10×) canine cDNA (2 μL), obtained from 2.0 μg of total mRNA, was amplified by PCR in a reaction mixture containing 2 μL of 10× Taq buffer (Invitrogen Brasil, São Paulo, Brazil), 2.5 mM MgCl2, 0.5 mM dNTP (Invitrogen Brasil), 0.5 pmol of specific primers, 0.16 μL of Taq DNA polymerase (Invitrogen Brasil), and DEPC-treated water to a final volume of 20 μL. Primers were adapted from published sources (Gröne et al., 1998) using canine sequences from GenBank with the accession numbers: IL-4, AF239917; IL-10, U33843; IL-12, U49100; TNF-α, S74068; INF-γ, AF126247; and β-actin, Z70034. The forward and reverse primers, respectively, were as follows: IL-4, CACTCACCAGATCCTTGGTACCAGG and TTGCAATGTGTCCGTGCCTGTTGTT, IL-10, CCCCCGCTGAGAACCAGGACACG and TGGCCTCTTACACTGTCCTACCG; IL-12, CTACAGTACGTCATCTCC and CACTGCTTTCCCTGACACTCC; TNF-α, CCAAGTGTACACCAGTACGAGTAC; INF-γ, CCAGATTACGACCCAGCAGTACG and TTCCTTATGAGTACGACCCAG; and β-actin, GACCCGTAATACCCATATTGCA and CTGTGCAAGTGTTGGTTCGAGATG. PCR amplification was performed in an MJ Research model PTC-100 thermocycler over 40 cycles (45 cycles for IL-4) each consisting of 1 min at 94 °C, 2 min at 56 °C for IL-4, INF-γ and TNF-α (59.5 °C for β-actin, IL-10 and IL-12), and 1 min at 72 °C, followed by a final extension at 72 °C for 5 min. Amplified fragments were analysed by electrophoresis on 6% polyacrylamide gel containing 0.5 μg/mL ethidium bromide (Sigma) (Fig. 1B). A set of primers corresponding to constitutive canine β-actin was used as positive controls and to adjust the efficiency of the cDNA synthesis.

In order to semi-quantify the intensity of the ethidium bromide signals, the gel images were captured using an Eagle Eye II (Stratagene, Cedar Creek, TX, USA) gel imaging system and analysed using a Stratagene image analysis software package with integrated density programme (Zero-Dscan version 1.3). The results obtained were expressed as cytotoxic area (pixels)/β-actin area (pixels) × 1000, and designated semi-quantitative levels of expression of cytokine.
2.5. Statistical analysis

Statistical analyses were performed using Minitab 9.2 (Minitab Inc., State College, PA, USA) and Prism 3.0 software packages (Prism software, Irvine, CA, USA). The χ² test was used to determine the frequency of cytokine expressing cells. The Wilcoxon Signed Rank and the Mann-Whitney tests were used, respectively, to analyse cytokines IL-12 and IFN-γ, and IL-10 and TGF-β with respect to disease diagnosis, whilst the Kruskal–Wallis test was used to analyse cytokines in relation to parasite load and clinical status. Correlations between clinical status, parasite load and levels of IL-10 and IFN-γ were evaluated using the Spearman rank correlation test (r coefficient). In all cases, differences were considered significant for p-values ≤ 0.05.

3. Results

Thirty of the study dogs exhibited anti- Leishmania IgG antibody titres higher than 1:40 and were considered
Table 1: Semi-quantitative determination by RT-PCR of the levels of expression of canine cytokines in splenocytes of non-infected dogs (NID) and animals naturally infected by *Leishmania chagasi* (CVL-infected dogs tested positive in IFAT, ELISA and ELISA rK39 assays).

| Groups | Level of expression of cytokines
<table>
<thead>
<tr>
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<th></th>
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<tbody>
<tr>
<td></td>
<td>IL-10</td>
</tr>
<tr>
<td>NID</td>
<td>0 (0–21)</td>
</tr>
<tr>
<td>CVL</td>
<td>27 (0–455)</td>
</tr>
</tbody>
</table>

* Median ratio of the expression levels of mRNA as determined from [cytokine area (pixels)/β-actin area (pixels)] × 1000; values labelled * are significantly different at *p* ≤ 0.05.

b 25th–75th percentiles.

seropositive for CVL. Infection with *L. chagasi* was confirmed in all IFAT-positive dogs by at least one additional serological test, including ELISA-extract and ELISA rK39 assays previously described (Reis et al., 2006a), and/or by parasitological examination. CVL-infected dogs were clinically classified, according to the presence/absence of clinical signs (Mancianti et al., 1988), into groups AD (*n* = 8), OD (*n* = 10), and SD (*n* = 12). Seven non-infected dogs (NID), each of whom exhibited negative IFAT results at 1:40 dilution and presented negative results in the parasitological tests for *Leishmania*, were included as a control group.

RT-PCR analysis of splenocytes of the study animals was employed in order to determine the frequency and the level of expression of the cytokines IL-4, IL-10, IL-12, TNF-α and IFN-γ, as well as β-actin, in non-infected dogs and those naturally infected with *L. chagasi*. The frequency of expression of a cytokine was determined as the percentage of dogs in each group, classified according to the disease diagnosis, clinical status and parasitic load, expressing the cytokine. With respect to the NID group, the splenocytes showed expression of IL-12, IL-10, and TNF-α (Fig. 1A). None of the animals of this group exhibited expression of IL-4.

![Graphs](image_url)

Fig. 2. Frequency of expression (% of animals testing positive in each category) of canine cytokines in splenocytes from 7 non-infected dogs (NID) and 30 dogs naturally infected by *Leishmania chagasi* and classified according to their clinical status as asymptomatic (AD, *n* = 8), oligosymptomatic (OD, *n* = 10) and symptomatic (SD, *n* = 12). Key to cytokines: TNF-α (□); IL-12 (■); IFN-γ (●); IL-4 (▲); IL-10 (■).
or IFN-γ. On the other hand, in CVL-infected dogs (n = 30), 6 (20.0%) animals showed expression of II-4, and 8 (26.6%) of IFN-γ, whilst 5 animals (16.6%) exhibited expression of II-12, 22 (73.3%) of II-10, and 30 (100%) of TNF-α. However, statistical analysis did not reveal any significant differences (p ≤ 0.05) in the frequencies of cytokine expression between the infected and the non-infected groups (Fig. 1A).

The levels of cytokine expression, as determined semi-quantitatively by RT-PCR, in splenocytes of infected dogs were higher for IFN-γ (p = 0.014) and II-12 (p = 0.059) than in splenocytes of the NID group. The corresponding differences between the two groups with respect to the expression of II-10 (p = 0.117) and TNF-α (p > 0.05) were not, however, statistically significant (Table 1).

Fig. 2 shows the frequency of cytokine expression in splenocytes of CVL-infected dogs distributed according to the clinical status of the animals. In the AD group, expression of II-4, II-10, II-12, IFN-γ and TNF-α was observed, respectively, in one (12.5%), three (37.5%), two (25.0%), two (25.0%) and eight (100%) of the animals. Within the OD group, positive expression of II-4, II-12 and IFN-γ occurred in less than 30% of the animals, whilst more than 59% of the dogs showed expression of II-10 and TNF-α. In the SD group, expression of II-4, II-10, II-12, IFN-γ and TNF-α was observed, respectively, in 2 (16.7%), 8 (66.7%), 2 (16.7%), 4 (33.3%) and 12 (100%) of the animals.

The levels of expression of selected cytokines, as determined semi-quantitatively by RT-PCR analysis of splenocytes of infected dogs, are presented in Table 2 according to the clinical status and parasitic load of the study animals. No statistically significant differences were observed between clinical groups with respect to II-10 (p = 0.321), II-12 (p = 0.428), IFN-γ (p = 0.547) and TNF-α (p > 0.05). However, there was a statistically significant increase in the accumulation of mRNA for II-10 in dogs showing high LDU values (HP group) compared with the NID and MP groups (p = 0.009). Moreover, when the frequency of expression of the cytokines was distributed according to parasitic load (Fig. 3), it was observed that expression of II-10 mRNA occurred in 100% of animals in the HP group, a value that was significantly different from those determined in the NID, LP and MP groups (p = 0.011).

The results of an analysis on the correlations between semi-quantitative levels of expression of cytokines in splenocytes from CVL-infected dogs, clinical status and parasitic load are shown in Table 3. A positive correlation between the clinical status and levels of expression of II-10 (r = 0.3501; p = 0.0337) was revealed. Furthermore, there was a positive correlation between the levels of expression of II-10 (r = 0.3428; p = 0.0438) and IFN-γ (r = 0.4690; p = 0.0045) and parasitic load.

4. Discussion

The clinical manifestations that develop following infection with Leishmania are a consequence of complex interactions between the parasite and the immune response of the host. In this context, CVL presents a spectrum of clinical forms that have been shown to be correlated with immune mechanisms dependent on T cell and macrophage activities, and also on cytokine balance (Santos-Gomes et al., 2002). Whilst the expression of cytokines has been evaluated in humans, hamsters and several strains of mice (Awasthi et al., 2004), the cytokine response in CVL has received very little attention. Apart from one investigation that involved bone marrow (Quinnell et al., 2001), previous studies have been concerned primarily with cytokine expression in PBMCs (Pinelli et al., 1994; 1999; Chamizo et al., 2001, 2005; Santos-Gomes et al., 2002). Thus, whilst the spleen is responsible for the major immune response in leishmaniasis, and is also an important infection target in VL, our present knowledge of the cellular response in this immune compartment is very limited.
Initial reports concerning the profile of cytokine expression in CVL induced by *L. infantum* and/or *L. chagasi* have suggested a balance between Th1 and Th2 cytokines, with only a slight tendency towards one or the other (Pinelli et al., 1994, 1999; Chamizo et al., 2001, 2005; Santos-Gomes et al., 2002). Santos-Gomes et al. (2002), for example, investigated the expression of cytokines in active CVL and observed low expression during the asymptomatic phase, with the occurrence of a Th1 response, and low expression of both Th1 and Th2 during the symptomatic phase. The observed balance between cytokines reflects the complexity of the human and canine infection compared with systems studied in murines.

A specific cellular immunity that is protective against *L. infantum*/*L. chagasi* has been observed in dogs by several researchers (Cabral et al., 1992; Pinelli et al., 1994; Moreno et al., 1999; Reis et al., 2006c). It has been suggested that such resistance could be associated with low titres of specific anti-*Leishmania* IgG antibodies and with the production of cytokines IL-2 and TNF-α (Pinelli et al., 1994; Reis et al., 2006b), which appear to be related to the Th1 response.
However, the strong immunosuppression induced by the parasite in active CVL does not permit the determination of specific cytokine profiles in cells stimulated by soluble antigens of *Leishmania* sp. (Santos-Gomes et al., 2002). The reduced or absent proliferation response observed *in vitro* may be related either to the inhibition of IL-2 or to the expression of IL-2 and TNF-α receptors that are extremely important for the parasite-specific cytotoxic activity of T cells.

Recently, Chanizo et al. (2005) reported that the PBMCs of asymptomatic CVL-infected dogs, with or without *in vitro* stimulation, presented detectable levels of TNF-α, IL-2, INF-γ, IL-18, IL-4, IL-6 and IL-10 mRNAs, but with a significant reduction in the frequency of detection of mRNA for IL-4. PBMCs of healthy dogs exhibited detectable levels of all cytokines mRNAs except for IL-6.

In the present work, mRNAs for all of the cytokines studied, namely, IL-4, IL-10, IL-12, IFN-γ and TNF-α, were detected in at least one CVL-infected dog (i.e. IFAT + and ELISA + or ELISA rK39 +) in each of the three clinical groups, although expression of IFN-γ and IL-4 was not observed in non-infected dogs. These findings are similar to those reported by other researchers (Quinnell et al., 2001; Santos-Gomes et al., 2002) in which, for example, mRNA for IFN-γ and IL-12 could not be detected in healthy dogs, whilst animals naturally infected with *L. infantum* exhibited detectable levels of expression of IL-18, IL-10, IFN-γ and IL-4. In the present study the frequency of expression of IL-10 in dogs presenting HP was significantly different from that of NID, and LP and MP animals. An increase in IL-10 mRNA was also detected in human tissue (Ghalib et al., 1993; Karp et al., 1993; Kenney et al., 1998), hamsters (Melby et al., 1998) and murines (Engwerda et al., 1996) that had been infected by *L. infantum*. The predominant accumulation of IL-10 may play a role in the modulation of the Th1 response, inhibiting the microbicidal activity of infected macrophages. The consequential splenomegaly could result from immunosuppression in the lymphoid compartments. It is important to note that IFN-γ may also be expressed, although in low quantities, by parasitised dogs, but this finding still needs confirmation.

The results of the serological analysis of CVL-infected dogs indicate an equilibrium between Th1 and Th2 responses since detectable levels of expression of IFN-γ, TNF-α and IL-12 together with IL-4 and IL-10 were found. However, when clinical indications are considered alongside the biochemical data, the Th1 response appears to be predominant since the expression of IL-4 decreased within the SD group, whilst the expression of IL-12 increased within the AD group. Hence the possible role of IL-4 in the clinical evaluation of CVL, and of IL-12 in the maintenance of the asymptomatic form of the disease, cannot be ruled out.

The frequency of expression of IL-12 and IFN-γ within the CVL groups was significantly different from that of the NID group (*p* < 0.05 and *p* = 0.014, respectively), although there were no significant differences between the CVL groups with respect to the expression of these cytokines. These results agree with those reported by Quinnell et al. (2001), who suggested that IFN-γ expression was not an appropriate indicator of resistance since asymptomatic and polysymptomatic dogs accumulated similar levels of this cytokine in tissues. Thus, as is the case in humans, mice and hamsters, the expression of IFN-γ may not be a suitable primary indicator of the development or cure of the disease in dogs. On the other hand, the inhibition of macrophage activation by IL-10 may have an important role in monitoring the progression of CVL.

A diminution in the expression of IFN-γ in Con-A stimulated PBMCs was observed by Santos-Gomes et al. (2002) immediately after infection of the dog, indicating that during this phase the parasite interferes with the ability of lymphocytes to express this cytokine. During the periods of incubation and emergence of infection, the capability of the non-stimulated cells of the animal to express IFN-γ is restored. The difficulty in detecting the expression of cytokines *in vitro* may be related to the capacity of the antigen to inhibit the proliferation of PBMCs in dogs undergoing active CVL, thus leading to a reduction in the expression of IFN-γ. The present study demonstrated that the significant increase in mRNA for IL-10 (*r* = 0.3501; *p* = 0.0337) was correlated with the progression of the disease. However, this effect is not related to IFN-γ deficiency, but is possibly due to the inhibition of the action of this cytokine on the activation of the macrophages, which is mediated by IL-10. Similar results were obtained by Richard et al. (1998).

In leishmaniasis, IL-10 has been associated with the suppression of Th1 cytokines, leading to the development of a Th2 immune response (Mosmann and Moore, 1991) and, consequently, to a reduction in macrophage activation (Bogdan et al., 1991). The demonstration that IL-10 inhibits the production of IFN-γ from IL-12 in PCMCs, stimulated with soluble antigen from *L. chagasi*, derived from patients suffering from VL suggests that this is the main cytokine involved in the development of the disease (Baezellar et al., 1996).

In the present study, the role of IL-10 in *Leishmania* infection was confirmed through the demonstration that
the frequency of expression of IL-10 increased significantly (r = 0.4430; p = 0.0068), along with that for IFN-γ (r = 0.3499; p = 0.0365), with the intensity of parasitism. Moreover, a significant increase in the level of IL-10 expression was found in HP dogs compared with those in the NID and MP groups. In contrast to the findings of Quinell et al. (2001) and Santos-Gomes et al. (2002), our results present evidence for the first time that the increase in mRNA for IL-10 is directly proportional to the increase in clinical symptoms. According to Reis et al. (2006b) a positive correlation was observed between the spleen parasitic load and evolution of the clinical status in CVL (r = 0.5048; p = 0.0010). These results reinforce our data as we observed a positive correlation between IL-10, clinical status severity and parasite load. The co-existence of Th1 cytokines and IL-10 has been reported in cases of VL (Kenney et al., 1998), during the period that precedes the emergence of CVL (Santos-Gomes et al., 2002), and in non-stimulated PBMCs from asymptomatic CVL-infected dogs (Chantzo et al., 2005). In all of these cases, IL-10 may act as a regulator of potential damage occasioned by the increased expression of TNF-α, which occurs as a consequence of a predominant Th1 response (Titus et al., 1989). Furthermore, IL-10 may act as a regulatory factor responsible for maintaining the balance between Th1 and Th2 responses (Kemp et al., 1999). Such a regulatory function has been considered in some detail in a recent review by Awasthi et al. (2004). Initially IL-10 was characterised as a cytokine related to the Th2 response, but recent studies concerning the suppression of T cell populations suggest that the situation is more complex. Thus, IL-10 is not only produced by Th2 T CD4+ cells, but also by macrophages, B cells and mastocytes, and hence the cytokine cannot be considered to be Th2 exclusively (Barral-Neto et al., 1998). Moreover, it is possible that IL-10, rather than IFN-γ, may be the most important macrophage regulator. An inhibitory activity over macrophages may also be attributed to TGF-β, since this cytokine is associated with an increase in IL-10 and with the inhibition of macrophages in murine leishmaniasis, characterising what is known as a Th2 response (Barral et al., 1993; Wilson et al., 1998). Recently, various other roles have been demonstrated for IL-10, such as suppression and regulation of the immune system in autoimmune diseases (Goudy et al., 2003), reaction of a host to organ transplant (Buchler, 2002), and susceptibility to parasitic infections (Kane and Mosser, 2001; Reed et al., 1994). The presence of IL-10 may also be important in explaining the high persistence of parasites in both C57BL/6 resistant mice and BALB/c susceptible mice (Belkaid et al., 2001; Vinha da Costa et al., 2002).

As previously stated, CVL is characterised by the progressive deterioration of body functions, which culminates with the death of the animal. The drugs typically employed in the treatment of leishmaniasis including human VL, i.e. pentavalent antimony and amphotericin B, are not 100% efficient, and return of the disease is common (Baneth and Shaw, 2002; Moreno and Alvar, 2002). The results produced in the present study provide a valuable contribution to the understanding of CVL, and to the immune response against the disease. The most significant aspects of the work are the standardisation of a RT-PCR technique for the determination of cytokine expression in the spleen compartment, and the clarification of the influence of parasitism on the immune response of dogs naturally infected with _L. chagasi._

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Isotype patterns of immunoglobulins: Hallmarks for clinical status and tissue parasite density in brazilian dogs naturally infected by *Leishmania (Leishmania) chagasi*

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Abstract

The role of anti-leishmanial immune response underlying the susceptibility/resistance during canine visceral leishmaniasis (CVL) has been recognized throughout in vivo and in vitro investigations. Recently, we demonstrated that immunoglobulin levels (Igs), as well as the parasite load are relevant hallmarks of distinct clinical status of CVL. To further characterize and upgrade the background on this issue, herein, we have evaluated, in *Leishmania (Leishmania) chagasi* naturally infected dogs, the relationship between tissue parasitism (skin, bone marrow, spleen, liver and lymph node), the CVL clinical status (asymptomatic (AD), with no suggestive signs of the disease; oligosymptomatic (OD), with maximum three clinical signs—opaque birest; localized alopecia and moderate loss of weight; symptomatic (SD), serologically positive with severe clinical signs of visceral leishmaniasis), and the humoral immunological profile of anti-*Leishmania* immunoglobulins (IgG, IgG1, IgG2, IgM, IgA and IgE). Our major statistically significant findings revealed distinct patterns of tissue parasite density

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within *L. chagasi*-infected dogs despite their clinical status, pointing out the spleen and skin as the most relevant sites of high parasitism during ongoing CVL. Parasite density of bone marrow and spleen were the most reliable parasitological markers to decode the clinical status of CVL. Moreover, the parasite density of bone marrow better correlates with the most anti-*Leishmania* IgG reactivity. Additionally, a diagnostic hallmark for canine visceral leishmaniasis was found, highlighting strong correlation between IgG1 and asymptomatic disease, but with IgA, IgE and IgG2 displaying better association with symptomatic disease. The new aspects of this study highlighted pioneer findings that correlated the degree of tissue parasite density (low (LP), medium (MP) and high (HP) parasitism) with distinct patterns of anti-*Leishmania* IgG reactivity. In this scope, our data re-enforced the anti-*Leishmania* IgG but with IgA reactivity as the better marker for overall tissue parasitism. The association between clinical status, Ig profile and the tissue parasitism support a novel investigation on the impact of humoral immune response and susceptibility/resistance mechanism during ongoing CVL.

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**Keywords:** Canine visceral leishmaniasis; Clinical forms; Tissue parasitism; Ig profile; *Leishmania chagasi*

### 1. Introduction

Visceral leishmaniasis caused by *Leishmania* (*Leishmania*) *infantum* syn. *Leishmania* (*Leishmania*) *chagasi* affects wild and domestic animals as well as humans in several parts of the Old and New World. Peridomestic sand flies acquire the etiological agent by feeding on infected wild/domestic reservoirs leading to transmission to humans causing severe disease fatal if not treated immediately after the onset of early symptoms (WHO, 2000). The major prophylactic practice to control this human disease, as recommended by the World Health Organization, involves a systematic treatment of human cases besides vector control by insecticide and elimination of the domestic reservoir, mainly seropositive infected dogs (Tesh, 1995).

**Canine visceral leishmaniasis (CVL)** is one of the most important emerging diseases with high prevalence in Latin American countries (Tesh, 1995). The major signs of CVL include hepatosplenomegaly, lymphadenopathy, cutaneous lesions, keratoconjunctivitis, opaque bristles, alopecia, apathy, onychogryphosis, anorexia and severe weight loss (Bettini and Gradoni, 1986). Hypergammaglobulinemia is also one of the classic signs of CVL as the disease progresses and it is accompanied by a suppression of cellular immune response, both mitogen-triggered and antigen-specific as well as a strong up-regulation humoral response (Pinelli et al., 1994; Cabral et al., 1998). According to Mancianti et al. (1988), CVL can be categorized into three distinct clinical forms, based on major features observed for infected dogs, which can be classified as asymptomatic (AD), with no suggestive signs of the disease, oligosymptomatic (OD), with maximum three clinical signs including opaque bristles and/or localized alopecia, and/or moderate loss of weight and symptomatic (SD), with characteristic clinical signs of visceral leishmaniasis, such as opaque bristles, severe loss of weight, onychogryphosis, cutaneous lesions, apathy and keratoconjunctivitis, showing the most severe signs of CVL.

Several reports have focused attention on the relationship between distinct clinical forms of CVL, disease progression and the IgG isotype levels, in both, experimental and natural *L. (L.) infantum* and *L. (L.) chagasi* infections (Solano-Gallego et al., 2001; Leandro et al., 2001; Quinnell et al., 2003; Cordeiro-da-Silva et al., 2003; Vercammen et al., 2002). Although the majority of these investigations have been performed based on well-established ELISA and Western-blotting protocols, controversial data on immunoglobulin isotype profiles are frequently documented.

Increased levels of IgG and IgG2 have been indiscriminately reported for AD and SD as described by Boudreiseau et al. (1997) and Vercammen et al. (2002). However, according to Deplazes et al. (1995), Nieto et al. (1999) and Solano-Gallego et al. (2001), SD showed considerably higher anti-*Leishmania* IgG1 antibodies in comparison to asymptomatic carriers. Additionally, Courtenay et al. (2002) and Quinnell et al. (2003) reported that higher levels of anti-*Leishmania* IgG1/IgG2 and lower levels of IgG2 were also observed in SD. However, Leandro et al. (2001) and Cordeiro-da-Silva et al. (2003) have documented
increased levels of IgG2 in sera samples from infected animals, particularly in the case of SD.

Despite these controversial findings regarding the immunoglobulin isotype profile associated with CVL, it is clear that during canine Leishmania infection a dichotomous humoral immune response is triggered, similarly to that found in human infection (Anam et al., 1999). However, it is important to mention that the small number of infected animals evaluated on these studies may count for these contradictory results, especially considering the broad spectrum of humoral immune response observed during CVL (Alvar et al., 2004).

In order to further investigate this controversial issue on the context of CVL, herein we have performed a detailed analysis of anti-Leishmania immunoglobulin isotypes on a larger number of infected dogs, focusing attention on the possible association between clinical status and immunoglobulin isotype profile. As a pioneer investigation, we have also assessed the levels of Leishmania-specific antibody isotypes in dogs with visceral leishmaniasis classified by their degree of parasite load. The major goal of this new strategy is to evaluate the possible association between immunoglobulin isotype profiles and tissue parasitism that may offer additional scientific data to support further investigations on the immunopathological progress of CVL.

2. Materials and methods

2.1. Dogs and study design

In the current study, 60 mixed breed adult dogs of both genders aging from 2- to 6-year-old were captured by Control Zoonosis Center in Belo Horizonte City Hall (Minas Gerais state), where the clinical pre-selection was carried out and maintained in quarantine, confined in kennels at the Instituto of Biological Sciences, Universidade Federal de Minas Gerais (UFMG), Brazil. Prior to the inclusion in this study, all animals were treated for intestinal helminthic (Endal plus®) infections and immunized against parvovirus, leptospirosis, distemper, parainfluenza and hepatitis (HTLP 5CV-L vaccine Pfizer®). All animals received drinking water and a balanced feed (Kinus® - BRASWEY-AS) ad libitum.

The dogs inserted in this study were stray or domiciled mongrel dogs, selected based on their serological results on IFAT, used as a "gold standard" immunological test for diagnosis of CVL. Forty dogs presenting IFAT titers ≥1:40 were considered positive and included into one of the groups constituted of infected animals. Twenty non-infected dogs with IFAT negative at 1:40 sera dilution and negative parasitological exams for Leishmania were considered non-infected and included as a control group (CD).

After quarantine, blood samples were collected in 10cc disposable sterile syringes, preferentially from jugular and/or cephalic veins. Five milliliter samples were transferred to a tube with no anticoagulant. The serum samples were stored in aliquots at 20 °C, until use for the serological examinations.

Leishmania infected dogs did not receive any treatment for CVL and were euthanized prior to collection of tissue samples which include skin, spleen, liver and lymph nodes. As chemotherapeutical practices for CVL is not officially allowed in Brazil, all infected dogs must be submitted to euthanasia.

This study was approved by the Ethical Committee for the use of Experimental Animals of the Universidade Federal de Minas Gerais, Brazil (CETEA).

2.2. Clinical evaluations

The mongrel dogs serologically positive by IFAT, were clinically classified according to presence/absence of clinical signs into three distinct categories, including: "asymptomatic" (AD, n = 12), with no suggestive signs of the disease; "oligosymptomatic" (OD, n = 12), with maximum three clinical signs, including opaque bristles and/or localized alopecia, and/or moderate loss of weight; "symptomatic" (SD, n = 16), with characteristic clinical signs of visceral leishmaniasis, such as opaque bristles, severe loss of weight, onychogriphosis, cutaneous lesions, apathy and keratoconjunctivitis.

2.3. Serological diagnosis of CVL—indirect immunofluorescence antibody test

Diagnosis of CVL was made by IFAT through the specific anti-Leishmania IgG reactivity using promastigote forms of Leishmania amazonensis (MHOM/
BR/1960/BH16). The parasites were maintained in logarithmic growth, in liver infusion tryptose (LIT) medium as described by Camargo and Rebonato (1969). FITC-conjugated-anti-dog IgG antibody was used to access the IgG reactivity (Biomanguinhos, FIOCruz, RJ, Brazil). Animals with antibody titration higher than 1:40 were considered to have a positive diagnosis of CVL.

Infection with *L. chagasi* was confirmed in all IFAT positive dogs by at least one additional serological approach, including ELISA-extract and ELISA r-K39, as previously described (Reis et al., 2006) and/or parasitological examination as described below.

2.4. Parasitological analysis—parasite load index

All seropositive dogs were euthanized under sedation (Thiopental®) and submitted to parasitological examinations for *Leishmania* and classified according to the parasite load.

Bone marrow aspiration was undertaken from the inferior region of the sternum or from the iliac crest, under sedation with intravenous doses of sodium thiopental—Thiombental® (8.4 mg/kg of body weight).

Parasitological diagnosis in tissue smears (ear skin, spleen, liver and lymph node) was performed after necropsy of the animals. Tissue samples were randomly collected regardless the presence or absence of lesions. Imprints were performed on two microscopic slides and after air-drying, samples were fixed in methanol, stained with Giemsa, and examined under optical microscopy, for the identification of *Leishmania* amastigote forms. The parasite density evaluation was performed in ear skin, bone marrow, spleen, liver and popliteal lymph node imprints, and the results expressed as “Leishman Donovan Units” (LDU index), according to Stauber (1955), which correspond to the number of *Leishmania* amastigotes per 1000 nucleated cells.

2.5. Immunological study—immunoglobulin isotype profile by enzyme linked immunosorbent assay (ELISA)

The “in-house” ELISA tests were performed to determine the anti-*Leishmania* immunoglobulin pattern and were carried out using soluble *L. chagasi* (MHOM/BR/1972/BH16) promastigotes antigen (SLA) from axenic culture in LIT medium. The parasites were cultured for 7 days (Mancianti et al., 1995), washed three times by centrifugation at 2000 rpm in phosphate buffer solution (PBS) pH 7.2, for 10 min, followed by three ultrasound cycles per min at 40 W on ice bath (Sonifier Cell Disruptor®—Branson Sonic Power Co., USA). The sonicated material was centrifuged at 18,500 rpm for 1 h and 30 min at 4 °C. The supernatant was transferred to dialysis tubes and dialyzed against PBS for 36 h, and submitted to four PBS changes every 6 h. Finally, the remaining material was filtered through disposable sterile filters of 22 μm under aseptic conditions; one aliquot was taken for protein concentration by the method of Lowry et al. (1951), and adjusted to the concentration of 1000 μg/mL and stored in small aliquots at 70 °C prior to use.

Ninety-six-well microplates (MaxiSorp™, Nalge Nunc Int., USA) were coated with SLA at a concentration of 10 μg/well, overnight at 4 °C. After coating and washing procedures, sera samples were added at the dilution 1:80 followed by washes and addition of peroxidase conjugated goat anti-dog IgG1 (anti-heavy chain specific), IgM (anti-μ chain specific), IgA (anti-α chain specific) and IgE (anti-ε chain specific) or sheep anti-dog IgG and IgG2 (both anti-heavy chain specific). All reagents were purchased from Bethyl Laboratories Inc., Montgomery, TX, USA. Wells were then washed, substrate and chromogen (O-Phenylenediamine, Sigma—Aldrich Co., USA) were added and absorbance was read on an automatic ELISA microplate reader (Multiskan™ MCC 340, Labsystems, Helsinki, Finland) at 492 nm. The conjugate concentrations were determined by a block titration method with positive and negative standard sera. The conjugate anti-IgG1, IgM, IgA, IgE was used at dilution 1:1000 and the anti-IgG and IgG2 were used at dilutions 1:8000 and 1:16,000, respectively.

2.6. Statistical analysis

Statistical analysis was performed using the Minitab 9.2 Statistical Software (Minitab Inc., Pennsylvania, USA). One-way analysis of variance (ANOVA) was used for the comparative studies of Immunoglobulins absorbance values between groups.
of clinical form and parasitic status. Student's t-test was used to identify significant differences between the group absorbance averages. In the non-parametric data, Kruskal–Wallis test was used for the comparative study between groups, followed by Dunns test. Comparative analysis between clinical form and parasitic status was carried out by the chi-square test. Spearman's rank correlations (rS) was computed to investigate associations among immunoglobulin levels (Igs), parasite density and clinical status parameters. In all cases, the differences were considered significant when the probabilities of equality, p-values, were ≤0.05.

3. Results

3.1. Distinct patterns of tissue parasite density are observed in dogs infected with *L. chagasi*

We have evaluated the tissue parasitism in different organs from *L. chagasi* naturally infected dogs (skin, bone marrow, spleen, liver and lymph node). Results were expressed as parasite density (LDU) (Fig. 1). Tissue parasitism for each compartment was initially classified as low (LP), medium (MP) or high (HP) parasitism based on tissue-specific LDU values statistically categorized into tertiles, as follow: skin (LP: 0–9; MP: 10–130; HP: 133–7246), bone marrow (LP: 0–0; MP: 2–33; HP: 46–1104), spleen (LP: 0–11; MP: 12–170; HP: 184–2564), liver (LP: 0–1; MP: 6–41; HP: 44–2196), lymph node (LP: 0–1; MP: 3–24; HP: 37–616). This approach strengthens further statistical analyses on similar number of dogs into each subgroup. The number of animals included on each subgroup was approximately 12–14 animals despite the tissue evaluated. Data analysis pointed out the spleen and the skin as the major targets of higher LDU values (Fig. 1), wherein the parasite density was much higher than other evaluated tissues (skin compared to bone marrow, p = 0.0027; liver, p = 0.0035; lymph node, p = 0.0006 and spleen compared to bone marrow, p = 0.0006; liver, p = 0.0011; lymph node, p = 0.0025).

3.2. Despite the clinical status, spleen and skin are the major sites of high parasite density during ongoing canine visceral leishmaniasis

Analysis of LDU values has been further addressed by calculating the 75% percentile for each tissue evaluated (Fig. 2). Our results demonstrated distinct profile of parasite density depending on both the tissue and the clinical form evaluated. In this context, the skin (as compared to bone marrow, p = 0.0027; liver, p = 0.0035; lymph node, p = 0.0006) and the spleen (as compared to bone marrow, p = 0.0006; liver, p = 0.0011; lymph node, p = 0.0025) have been pointed out as the most relevant sites of high parasite density in comparison to all other sites, when taking together all infected dogs.

Analysis of LDU values regarding the clinical status confirmed the skin and the spleen as the major parasitic site as compared to bone marrow in AD (p = 0.0313 and p = 0.0313, respectively), bone marrow and lymph node in OD (p = 0.0195, p = 0.0078 and p = 0.0371, p = 0.0039, respectively) and liver and lymph node in SD (p = 0.0102, p = 0.0065 and p = 0.0088, p = 0.015, respectively).

The other evaluated organs, liver and lymph node, showed similar values of parasite density. It is important to notice that bone marrow from AD showed lower parasite density in comparison to OD and SD (Fig. 2).
3.3. Bone marrow and spleen parasite density are the most reliable parasitological markers to decode the clinical status of canine visceral leishmaniasis

The frequency of low, medium and high parasite density in different tissues of *L. chagasi* naturally infected dogs categorized according to their clinical status are shown in Table 1. Data analysis demonstrated that clinical forms of CVL have a strong association with parasite density status, with connection between LP and AD, whereas HP showed to be more associated with SD. Interestingly, bone marrow parasitism pointed out that 75% of dogs with LP display connection with AD group, whereas 25% and 12.5% of animals displaying LP were confined into OD and SD groups, respectively. In contrast, 68.8% of dogs presenting high parasitism belong to the SD
Table 1
Frequency of low, medium and high parasite density (LDU) in different tissues of *L. chagasi* naturally infected dogs categorized according to their clinical status.

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Parasite density (LDU)</th>
<th>Clinical status</th>
<th>AD</th>
<th>% total</th>
<th>OD</th>
<th>% total</th>
<th>SD</th>
<th>% total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin <em>(r = 0.4416, p = 0.0043)</em></td>
<td>LP</td>
<td>6 (50.0%)</td>
<td>15.0</td>
<td>5 (41.7%)</td>
<td>12.5</td>
<td>2 (12.5%)</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MP</td>
<td>3 (25.0%)</td>
<td>7.5</td>
<td>4 (33.3%)</td>
<td>10.0</td>
<td>6 (37.5%)</td>
<td>15.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HP</td>
<td>3 (25.0%)</td>
<td>7.5</td>
<td>3 (25.0%)</td>
<td>7.5</td>
<td>8 (60.0%)</td>
<td>20.0</td>
<td></td>
</tr>
<tr>
<td>Bone marrow <em>(r = 0.6508, p &lt; 0.0001)</em></td>
<td>LP</td>
<td>9 (75.0%)</td>
<td>22.6</td>
<td>3 (25.0%)</td>
<td>7.5</td>
<td>2 (12.5%)</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MP</td>
<td>2 (16.7%)</td>
<td>5.0</td>
<td>7 (58.3%)</td>
<td>17.5</td>
<td>3 (18.8%)</td>
<td>7.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HP</td>
<td>1 (8.3%)</td>
<td>2.5</td>
<td>2 (16.7%)</td>
<td>5.0</td>
<td>11 (68.8%)</td>
<td>27.5</td>
<td></td>
</tr>
<tr>
<td>Spleen <em>(r = 0.5048, p = 0.0010)</em></td>
<td>LP</td>
<td>7 (58.3%)</td>
<td>17.9</td>
<td>4 (36.3%)</td>
<td>10.4</td>
<td>2 (12.5%)</td>
<td>5.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MP</td>
<td>3 (25.0%)</td>
<td>7.7</td>
<td>5 (45.5%)</td>
<td>12.8</td>
<td>5 (31.3%)</td>
<td>12.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HP</td>
<td>2 (16.7%)</td>
<td>5.1</td>
<td>2 (18.2%)</td>
<td>5.1</td>
<td>9 (56.2%)</td>
<td>23.1</td>
<td></td>
</tr>
<tr>
<td>Liver <em>(r = 0.4066, p = 0.0092)</em></td>
<td>LP</td>
<td>7 (58.3%)</td>
<td>18.5</td>
<td>6 (50.0%)</td>
<td>15.0</td>
<td>1 (6.3%)</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MP</td>
<td>2 (16.7%)</td>
<td>5.0</td>
<td>2 (16.7%)</td>
<td>5.0</td>
<td>8 (50.0%)</td>
<td>20.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HP</td>
<td>3 (25.0%)</td>
<td>7.5</td>
<td>4 (33.3%)</td>
<td>10.0</td>
<td>7 (43.7%)</td>
<td>17.5</td>
<td></td>
</tr>
<tr>
<td>Lymph node <em>(r = 0.7995, p = 0.0017)</em></td>
<td>LP</td>
<td>7 (58.3%)</td>
<td>17.5</td>
<td>5 (41.7%)</td>
<td>12.5</td>
<td>2 (12.5%)</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MP</td>
<td>2 (16.7%)</td>
<td>5.0</td>
<td>6 (50.0%)</td>
<td>15.0</td>
<td>4 (25.0%)</td>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HP</td>
<td>3 (25.0%)</td>
<td>7.5</td>
<td>1 (8.3%)</td>
<td>2.5</td>
<td>10 (62.5%)</td>
<td>25.0</td>
<td></td>
</tr>
</tbody>
</table>

LDU, Leishman Donovan Units. The results are expressed as frequency of animals displaying a given parasite density score. Parasite density degree (low (LP), medium (MP) and high (HP) parasitemia); clinical status (asymptomatic (AD), oligosymptomatic (OD) and symptomatic (SD) dogs).

* Differences statistically significant in comparison to AD at *p < 0.05*.

** Differences statistically significant in comparison to OD at *p < 0.05*.

Spearman correlation indexes *(r)* at *p < 0.05* are shown in table.

group, whereas only 8.3% and 16.7% of dogs with HP fit into AD and OD groups, respectively. Analysis of spleen parasitemia demonstrated that 58.3% of dogs showing LP belong to AD group in contrast with 12.5% of dogs from SD group. Similar results were documented in the liver compartment, with 58.3% of dogs with LP fitting into the AD group in contrast with 6.3% of dogs from SD group. Analysis of parasite density of lymph node demonstrated that 58.3% of dogs with LP belong to AD group and 62.5% of dogs with high parasitemia were from SD group, whereas only 8.3% of dogs with HP fit into OD group (Table 1).

In addition, in order to validate the association between tissue parasite load in the organs more affected during CVL, we further performed a correlation analysis between parasite density in specific organs and clinical status of CVL (Table 1). Our results demonstrated that, although parasite density in all organs was positively correlated with the clinical forms of CVL, parasite density of bone marrow and spleen display the stronger correlation with clinical status *(r = 0.6508, p < 0.0001 and r = 0.5048, p = 0.0010, respectively).*

3.4. Analysis of immunoglobulin isotypes point out a prognostic hallmark for canine visceral leishmaniasis highlighting strong correlation between IgG1 and AD but IgA, IgE and IgG2 displaying better association with SD

The reactivity of seric anti-*Leishmania* immunoglobulin isotypes (IgG, IgG1, IgG2, IgM, IgA and IgE) from dogs naturally infected, categorized according to the clinical status of CVL are shown in Fig. 3. Our results demonstrated that all non-infected dogs displayed negative IgG, IgG1 and IgG2 reactivity considering the cut-off edges for the anti-*Leishmania* immunoglobulin reactivity detected by ELISA (0.140 for total IgG; 0.170 for IgG1 and 0.180 for IgG2). Moreover, all infected dogs displayed significant higher anti-*Leishmania* total IgG, IgG2, IgM and IgA in comparison to control group.
Fig. 3. Anti-Leishmania immunoglobulin isotypes reactivity in *L. chagasi* naturally infected dogs categorized according to their clinical status as asymptomatic (AD: □), oligosymptomatic (OD: △) and symptomatic (SD: ■) dogs. Uninfected dogs were used as a control group (CD: □). Results were expressed as scattering of individual values and mean optical density for each immunoglobulin isotype. Significant differences at \( p < 0.05 \) were identified by the letter "a" in comparison to CD. Confirmatory analysis was also performed and the spearman correlation indexes (\( r \)) at \( p < 0.05 \) are shown in figure.

\( p < 0.05 \). Further, only AD and OD groups presented higher levels of anti-Leishmania IgG1 and IgE, respectively, in comparison to control group (\( p < 0.05 \)).

The correlation between immunoglobulins levels and the clinical status of CVL was also evaluated and demonstrated a positive association between most anti-Leishmania immunoglobulin isotypes investigated and the clinical status of CVL, except for IgG1 and IgM: (IgG, \( r = 0.6299; p < 0.0001 \)), (IgA, \( r = 0.4839; p < 0.0043 \)), (IgE, \( r = 0.4508; p < 0.0109 \)) and (IgG2, \( r = 0.3858; p < 0.0184 \)). Interestingly, a
negative correlation was observed between IgG1 and disease morbidity ($r = -0.5085; p < 0.0025$) (Fig. 3).

3.5. Anti-Leishmania IgG and IgA reactivity better reflect the overall tissue parasitism

The analysis of the anti-Leishmania total IgG reactivity in *L. chagasi* naturally infected dogs categorized according to parasite density from different tissues are shown in Fig. 4. Tissue parasitism was classified as LP, MP and HP as previously described. Data analysis revealed that animals displaying MP or HP presented increased levels of IgG in most tissues. Interestingly, only animals displaying HP at bone marrow also presented increased levels of IgG. In addition, the analysis of correlation demonstrated a significant positive association between anti-Leishmania IgG reactivity and parasite density in all evaluated organs: (skin, $r = 0.5146$; $p = 0.0016$), (bone marrow, $r = 0.4816$; $p = 0.0034$), (spleen, $r = 0.5200$; $p = 0.0016$), (liver, $r = 0.4420$; $p = 0.0078$) and (lymph node, $r = 0.4536$; $p = 0.0062$) (Fig. 4).

The evaluation of the other anti-Leishmania immunoglobulin isotypes (IgG1, IgG2, IgM, IgA and IgE) in *L. chagasi* naturally infected dogs categorized according to parasite density in different tissues is shown in Table 2. Our data demonstrated that IgG1 and IgE are not associated with tissue parasitism. Higher IgG2 levels were observed in dogs with MP and/or HP in the liver and lymph node sites, in comparison with LP. On the other hand, although higher IgM levels were also observed in dogs with MP and/or HP in the lymph node, an increase in the IgM levels was detected only in dogs with HP in the liver. Analysis of IgA reactivity also exhibited higher association with tissue parasitism, since higher IgA levels were observed in dogs displaying MP and/or HP in most tissues, except skin (Table 2).

3.6. Bone marrow parasite density better correlates with immunoglobulin reactivity during canine visceral leishmaniasis

In order to identify which organ confirms better association between parasite load and anti-Leishmania immunoglobulin isotype pattern, we have performed additional correlation analysis between tissue parasite density of different organs and anti-Leishmania

![Fig. 4. Anti-Leishmania IgG reactivity in *L. chagasi* naturally infected dogs categorized according to parasite density (LDU) in different tissues as low (LP), medium (MP) and high (HP) parasitism. The results are expressed in box-plot format highlighting the gap of 50% of data set measurement. *Significant differences in comparison to LP at $p < 0.05$. Confirmatory correlation analysis was also performed and the spearman correlation indexes ($r$) at $p < 0.05$ are shown in figure.](image)
immunoglobulin isotype reactivity (Fig. 5). Interestingly, our results pointed out the bone marrow as the top organ showing positive correlation with most immunoglobulin isotypes (IgG, $r = 0.4816; p = 0.0034$; IgG1, $r = -0.4077; p = 0.0185$; IgG2, $r = 0.3602; p = 0.0285$; IgM, $r = 0.3468; p = 0.0306$) and (IgA, $r = 0.4031; p = 0.0290$) (Fig. 5). Positive correlation was also observed between skin, spleen and liver parasite density and levels of anti-Leishmania IgG2, IgM and IgA. Similar data was detected between lymph node and IgG2 levels (table inserted in Fig. 5).

4. Discussion

Besides its relevance as the major reservoir for human visceral leishmaniasis, dogs have been considered to be a relevant model for $L. (L.)$ infantum and $L. (L.)$ chagasi infection, since many aspects resemble those observed for the human disease (Keenan et al., 1984; Hommel et al., 1995; Alvar et al., 2004). The analysis of clinical and pathological features in CVL disease disclosed three major groups of infected animals, as they respond to chronic infection, generally named asymptomatic, oligosymptomatic and symptomatic, both in natural and experimental infection (Pinelli et al., 1994; Keenan et al., 1984; Abranches et al., 1991; Cordeiro-da-Silva et al., 2003). Despite the importance of canines as reservoirs for the human disease, little information is available on the immunological basis of CVL (Alvar et al., 2004). The goal of this present investigation was to evaluate the humoral immune response in naturally $L. (L.)$ chagasi-infected dogs focusing on a possible prognostic marker such as a correlation among immunoglobulin isotypes profile, clinical status as well as tissue parasite density.

Firstly, we have assessed the tissue parasite density of relevant organs in order to identify the major sites of parasite spreading. According to Tafuri et al. (2004), the clinical manifestations of CVL do not necessarily correlate with the parasite load. However, other investigations have reported a good correlation between the parasite load of the spleen and lymph node and the clinical manifestations of CVL (Borruto-Melo et al., 2004; Sanchez et al., 2004). Our findings pointed out the spleen and the skin as the
major targets of higher LDU values (Fig. 1), where high parasite density showed to be distinct from other evaluated tissues. In addition, we have evaluated in L. (L.) chagasi naturally infected dogs, the relationship between tissue parasitism (skin, bone marrow, spleen, liver and lymph node) and CVL clinical status (asymptomatic (AD), oligosymptomatic (OD) and symptomatic (SD)). We have documented that despite the clinical status, the spleen and the skin remain as the top sites of high LDU values (Fig. 2). It was important to notice that bone marrow from AD showed lower parasite density in comparison to OD and SD. Together, these findings re-emphasize our previous observations demonstrating the lower performance of bone marrow impression smears in diagnosing CVL in AD (Reis et al., 2006). Moreover, the higher parasite density of the spleen and the skin also reflects the higher sensitivity of spleen and skin impression smears to CVL diagnosis (Reis et al., 2006). It was interesting to notice that despite parasite density of bone marrow represents the lower LDU values (Fig. 1), additional analysis pointed out this organ as the major site that correlates with the clinical status of CVL (Table 1). Indeed, the present work demonstrated that better correlations between parasite density and clinical forms are documented this way: bone marrow > spleen > lymph node > skin > liver (Table 1). Our data showed also that whereas AD display skin, liver and lymph node parasitism similarly to that observed for symptomatic dogs, SD exhibited intense parasite load in most evaluated tissue a higher density was found in the bone marrow and in the spleen when compared to AD (Fig. 2). Together, these data may explain discordant reports regarding the association between clinical status and tissue parasite load during CVL (Tafuri et al., 2004; Sanchez et al., 2004; Barrouin-Melo et al., 2004). It is possible that a correlation analysis between clinical features and tissue parasitism performed in distinct sites is the major responsible for this controversy. Therefore, it is important to highlight the major contribution of our present investigation to clarify the influence of compartmentalized parasite density when evaluating its association with the clinical status of CVL.
In addition, when searching for laboratory prognosis markers, we have aimed to correlate clinical status and tissue parasite density with humoral immune response of *L. (L.) chagasi*-infected dogs. While some reports have suggested that the sole presence of anti-*Leishmania* antibodies is not a conclusive marker for the disease progression during canine *L. (L.) infantum* infection (Gicheru et al., 1995; Nieto et al., 1999), herein, we have observed that the reactivity of serum anti-*Leishmania* immunoglobulin isotypes from dogs naturally infected with *L. (L.) chagasi*, showed a marked association between clinical status and tissue parasite density and therefore represents a good indicator of disease morbidity. Our data demonstrated that anti-*Leishmania* IgG and IgA reactivities were the best markers for overall tissue parasitism (Fig. 4 and Table 2, respectively). We indeed observed a positive correlation between clinical forms of CVL with the levels of IgG, IgG2, IgA and IgE (Fig. 3) and tissue parasite density with the levels of IgG, IgG2, IgM and IgA (Fig. 5). Although no correlation was observed among the levels of IgM as well as IgA and lymph node parasitism (table inserted in Fig. 5), we have detected an increase in the levels of these immunoglobulins in animals displaying both medium and high lymph node parasite density (Table 2). On the other hand, we verified a negative correlation between the IgG1 reactivity and both clinical status and bone marrow parasitism with (Figs. 3 and 5). Moreover, the bone marrow parasite density strikingly correlated with most immunoglobulin reactivity, except for IgE (Fig. 5).

Abranches et al. (1991) verified that specific antibodies production starts at 1.5–3 months after the *L. (L.) infantum* experimental infection. Although Abranches et al. (1991) did not find any correlation between IgG titers and disease progression, the major findings from Lanotte et al. (1979) and Oliveira et al. (1993) suggested that higher levels of IgG observed in asymptomatic dogs from endemic areas (Oliveira et al., 1993) seem to be consistent with the fact that 90% of these dogs would develop the advanced and symptomatic form of the disease (Lanotte et al., 1979). According to this proposal, it has been reported that dogs infected with *L. (L.) infantum* showed increased levels of IgG anti-*Leishmania* prior to the appearance of the first clinical signals (Hommel et al., 1995). Our data support these observations, since the antibody levels (mainly IgG, IgM, IgA and IgG2) are elevated in all infected dogs, not depending on their clinical status (Fig. 3). Similar findings were also reported by Bourdoiseau et al. (1997), Leandro et al. (2001), Vercammen et al. (2002) and Cordeiro-da-Silva et al. (2003). These authors observed an increase of total IgG level in asymptomatic and symptomatic dogs. Furthermore, the IgG2 levels appeared to be the predominant subclass present in the sera of all infected animals (Bourdoiseau et al., 1997; Vercammen et al., 2002), particularly in the case of symptomatic dogs (Leandro et al., 2001; Cordeiro-da-Silva et al., 2003).

Additional correlation analysis pointed out that antibody response was closely related to CVL morbidity, since as the clinical signals become evident, higher levels of IgG, IgA, IgG2 and IgE are observed (Fig. 3). In the present work, the levels of IgG1 suggest an association with immunoprotector mechanisms of the canine infection, since they were frequently elevated in AD (Fig. 3) as well as in animals bearing low bone marrow parasite density (Fig. 5). In contrast, circulating IgG2 seems to be more associated with CVL morbidity, being positively correlated with more severe clinical status and higher parasite density (Figs. 3 and 5). Previous investigations have postulated an association between high levels of IgG2 and asymptomatic form of CVL, whereas IgG1 levels better correlate with symptomatic disease (Deplazes et al., 1995; Nieto et al., 1999; Solano-Gallego et al., 2001; Courtenay et al., 2002; Quinell et al., 2003). On the other hand, additional investigations failed to demonstrate this trend suggesting that in fact lower levels of IgG1 better associated with symptomatic dogs (Bourdoiseau et al., 1997; Leandro et al., 2001; Vercammen et al., 2002; Cordeiro-da-Silva et al., 2003). Our results resembled in part those obtained by Bourdoiseau et al. (1997) and Vercammen et al. (2002) that described an association between high levels of IgG1 and the establishment/maintenance on asymptomatic chronic disease. We believed that the small number of infected animals enrolled on these studies might account for these controversial results (Solano-Gallego et al., 2001).

Although IgM is classically associated with the acute forms of parasitic diseases, it has been demonstrated that in canine *L. (L.) chagasi* infection, the levels of IgM, detected early after the first month
during acute infection persist throughout chronic infections (Genaro et al., 1992). Our data re-emphasize these findings, since high IgM levels were found in all L. (L.) chagasi-infected dogs, despite their clinical status (Fig. 3). However, despite no correlation between IgM levels and CVL clinical status was detected (Fig. 3), our findings revealed that IgM levels were correlated with skin, bone marrow, spleen, and liver parasite density (table inserted in Fig. 5). The spleen is an important immunologically active compartment, where cells from the immune system interact with parasite-derived antigens during L. (L.) chagasi infection. Considering the spleen as the main site of lymphoid cells interposed into the blood stream, the large amount of circulating Leishmania antigens, besides high local parasite density frequently in contact with splenocytes, would lead to a strong humoral response. Moreover, as the spleen contain the larger amount of B-1 lymphocytes (Tschenderof, 1985), the major source of poly-reactive IgM anti-carbohydrate (Garzelli et al., 1994; Karpahtkin et al., 1995), it is plausible to infer that the increment of IgM reflects the fundamental response to parasite antigens.

Our results also emphasize that disease progression was additionally characterized by increment of other specific immunoglobulins isotypes (mainly IgA and IgE) that may contribute to aggravation of clinical status during CVL, as previously suggested by Iniesta et al. (2005). Our data demonstrated that levels of anti-Leishmania IgA were positively correlated with clinical status (Fig. 3) as well as skin, bone marrow, spleen and liver parasite density (Fig. 5). Interestingly, as IgA deposits are identified in the kidneys during CVL, it has been proposed that IgA may contribute for the genesis of CVL-associated glomerulonephritis (Nieto et al., 1992).

Likewise IgA, but independently of tissue parasite density, we have found that IgE levels were correlated with CVL clinical status (Fig. 3). Similar results were reported by Iniesta et al. (2005), pointing out higher IgE levels in symptomatic animals.

The association between clinical status, Ig profile and the tissue parasitism support a novel investigation on the impact of humoral immune response and susceptibility/resistance mechanism during ongoing CVL. In conclusion, the new aspects of this study highlighted pioneer findings that revealed a correlation between distinct patterns of tissue parasite density and the profile of anti-Leishmania immunoglobulin isotypes during CVL, pointing out the spleen and skin as the most relevant sites of high parasite density and the parasitism in the bone marrow and spleen as the most reliable parameters to decode CVL clinical status. Moreover, the parasite density of bone marrow better correlates with most anti-Leishmania IgG reactivity. An outstanding prognostic marker for canine visceral leishmaniasis was identified, highlighting IgG1 for asymptomatic infection and in relevance IgA, IgE and IgG2 for symptomatic disease. Moreover, our data re-enforce the anti-Leishmania IgG but with IgA reactivity as the better marker for overall tissue parasitism.

Taken together, our findings supported that the association between clinical status, Ig profile and tissue parasitism should be taken into account in further investigations focusing the impact of humoral immune response on susceptibility/resistance mechanism during ongoing CVL.

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References


Parasite density and impaired biochemical/hematological status are associated with severe clinical aspects of canine visceral leishmaniasis

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Abstract

We have performed a detailed investigation in 40 dogs naturally infected with Leishmania infantum (syn. chagasi), subdivided into three groups: asymptomatic (AI = 12), oligosymptomatic (OD = 12) and symptomatic (SD = 16), based on their clinical features. Twenty non-infected dogs (CD) were included as control group. Serological analysis, performed by IFAT and ELISA, demonstrated higher antibody titers in SD in comparison to the OD. A positive correlation was found between parasite density in the spleen and skin smears as well as the bone marrow parasitism with clinical status of the infection. We observed that the progression of the disease from asymptomatic to symptomatic clinical form was accompanied by intense parasitism in the bone marrow. It is likely that this led to the impaired biochemical/hematological status observed. Finally, we believe that the follow-up of these parameters could be a relevant approach to be used as markers during therapeutic and vaccine evaluations.

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Keywords: Canine visceral leishmaniasis; Leishmania infantum (syn. chagasi); Parasite density; Biochemical/hematological status

1. Introduction

Visceral leishmaniasis (VL) is one of the most relevant and emergent diseases worldwide, reaching 98% of mortality in non-treated human cases (Tesh, 1995). Beside its broad epidemiological spectrum, VL is an important zoonosis widely spread in tropical and subtropical areas of the globe. In the last 16 years, 37,294 new human cases of VL were reported in Brazil (Vieira and Coelho, 1998). From the epidemiological standing point, the canine visceral leishmaniasis (CVL) is considered to be more important than the human disease, due to its higher prevalence and the fact that both asymptomatic and symptomatic dogs are equally infectious to the vectors (Molina et al., 1994). Many asymptomatic animals, in endemic areas, have been detected with parasites in the skin (Marzochi et al., 1985), and from 1980 to 1997, a total of 414,168 seropositive dogs were identified in Brazil (Vieira and Coelho, 1998).

The CVL may evolve from asymptomatic cases to a systemic disease, which mostly culminates in death. From the
localized cutaneous infection, the parasite can be disseminated via lymphatic or blood vessels, infecting macrophages of the bone marrow, lymph node, liver and spleen, as well as kidneys and gastrointestinal tract. Initial clinical signs are hypertrophy of lymph node, dermatitis and periocular and nasal dermatitis that can disseminate. The bristles become opaque and fall, together with onychogryphosis and edema of the paws. Other signs such as fever, apathy, diarrhea, intestinal hemorrhage, weight loss, hepatosplenomegaly, hyperkeratosis, cutaneous ulceration, particularly on the nose, ears, tail, and keratoconjunctivitis are frequent, although not necessarily present in all animals (Genaro et al., 1988; Dias et al., 1999). The CVL presumptive diagnosis is generally performed by serological tests, such as indirect immunofluorescence assay test (IFAT) and enzyme linked immunosorbent assay (ELISA), conjunction with clinical and epidemiological records. The major problem regarding clinical diagnosis is the fact that CVL signs are very similar to those observed in other infectious diseases. The chronic aspect of the disease and its long incubation period may generate a delay or failure in clinical diagnosis (Cardoso and Cabral, 1998). Despite its high sensitivity, serological tests present a broad range of cross-reactions with other protozoan (Costa et al., 1991; Grundal and Tesh, 1993). However, the parasitological diagnosis is generally low in sensitivity when parasite density is low.

There are several biochemical and hematological alterations in naturally or experimentally infected dogs. The findings include normocytic/normochromic anemia, and increase of total serum protein levels. It seems that biochemical alterations are linked to a polyclonal humoral immune response, which leads to raised protein levels in serum (Marzochi et al., 1985). This can be observed by an increase of the total serum proteins with hyperglobulinemia and hypoalbuminemia, with decreased albumin/globulin ratio (Cardoso and Cabral, 1998; Strauss-Ayali and Baneth, 2001). Considering the re-emergent aspect of visceral leishmaniasis, in several parts of the world, the epidemiological importance of dogs, we assessed both the clinical and laboratorial aspects from canine visceral leishmaniasis contributing to monitor the clinical status of dogs during infection.

2. Materials and methods

2.1. Animals

Sixty mixed breed adult dogs of both genders aging from 2 to 6 years old were selected. They were maintained in the kennel of the Institute of Biological Sciences of Federal University of Minas Gerais or provided by the Center of Zoonosis (Zoonosis Center - Belo Horizonte City Council) of Minas Gerais state, Brazil. The clinical pre-selection was carried out in the latter location. The animals were kept in quarantine with drinking water and a balanced feed (Kinus® - BRASWEY - AS) given ad libitum.

The dogs inserted in this study were stray or domiciled mongrel dogs, selected based on their serological results on IFAT, used as a "gold standard" immunological test for diagnosis of CVL. Animals presenting IFAT titers \( \geq 1:40 \) were considered positive and included into the infected groups. Animals with IFAT negative at 1:40 were considered non-infected and included as a control group. Leishmania infected dogs did not receive any treatment for CVL and were euthanatized to proceed the organs collections, including skin, spleen, liver and lymph nodes. As chemotherapeutical practices for CVL is not officially allowed in Brazil, all infected dogs must be submitted to euthanasia.

Infection with *Leishmania infantum* was confirmed in all IFAT positive dogs by at least one additional serological and/or parasitological approach used.

2.2. Blood sample collection

After quarantine, blood samples were collected in 20 c.c., disposable sterile syringes, preferentially from jugular and/or cephalic veins. Five milliliter-samples were transferred to tubes with EDTA (in the proportion of 1 mg/ml) for the hemogram and 5 ml were transferred at a tube with no anticoagulant. The serum samples were stored in aliquots at \(-20^\circ\)C, until use in the serological and biochemical tests.

2.3. Clinical and parasitological evaluations

The dogs were clinically classified, according to presence/absence of infection signs: asymptomatic (AD, \( n = 12 \)), with no suggestive signs of the disease, oligosymptomatic (OD, \( n = 12 \)), with maximum three clinical signs including opaque bristles and/or localized alopecia and/ or moderate loss of weight; symptomatic (SD, \( n = 16 \)), with characteristic clinical signs of visceral leishmaniasis, such as opaque bristles, severe loss of weight, onychogryphosis, cutaneous lesions, apathy and keratoconjunctivitis; non-infected dogs (CD), animals for negative serological and parasitological exams for *Leishmania*, considered here as control animals.

The assessment of parasitological parameters was performed by the isolation of the parasite in bone marrow cultures, obtained by aspiration of the bone marrow, in NNN/LIT medium. The cultures were maintained in incubators at 23°C and examined three times every 10 days. Bone marrow aspiration was undertaken from the inferior region of the sternum or from the iliac crest, under sedation (25 mg/kg live weight of sodium tiopental - Thionembutal® - via intravenous). Parasitological diagnoses in tissue smears (ear skin, spleen, liver and popliteal lymph node) were performed after necropsy of the animals. The fragments of the tissues were used in slide smears by apposition of two microscopy slides. The smears were stained by Giemsa and examined under optical microscopy, for the identification of amastigote forms of *Leishmania*. The par-
asile density evaluation was performed, in bone marrow, skin and spleen smears and the results expressed as "Leishman Donovan Units" (LDU index), according to Staubner (1955), which correspond to the number of Leishmania amastigote by 1000 nucleated cells. This study was approved by the Ethical Committee for the use of Experimental Animals of the Universidade Federal de Minas Gerais, Brazil (CETEA).

2.4. Serological assessment

For the IgG assay, IFAT, ELISA-extract and ELISA-rk39 were performed. For IFAT, Leishmania amazonensis promastigote (MHOM/BR/1966/BH16) were used. These parasites were maintained in logarithmic growth in LIT medium, as described by Camargo and Reboutato, 1969. For IFAT, specific anti-canine IgG antibodies FITC-conjugated diluted 1:200, as recommended by the manufacturer (Biomanguinhos, FIOCRUZ, RJ, Brazil). Animals with antibody titration higher than 1:40 were considered to be positive. The results are expressed as antibody titers.

For ELISA-extract tests, soluble antigens from L. infantum (syn. chagasi) promastigote (MHOM/BR/1972/BH46) were used. The parasites were cultured for 7 days in LIT medium (Manchetei et al., 1995), washed three times by centrifugation at 2000 rpm in phosphate buffer solution (PBS) pH 7.2, for 10 min, followed by three ultra-sound cycles of 1 min at 40 W on ice bath (Sonifier Cell Disruptor® – Branson Sonic Power Co., EUA). The sonicated material was centrifuged at 18,000 rpm for 1 h and 30 min at 4 °C. The supernatant was transferred to dialysis tubes and dialyzed through PBS for 36 h, and submitted to four PBS changes every 6 h. Finally, the remaining material was filtered in disposable sterile filters of 22 μm under asep-tic conditions; one aliquot was taken for protein dosage by the method of Lowry et al. (1951), and adjusted to the concentration of 1000 μg/ml and stored in small aliquots at −70 °C prior to use.

The recombinant antigen rk39, specific for Leishmania of the donovanii complex (Badaró et al., 1997) was used.

For ELISA tests, including both ELISA-extract and ELISA-rk39, an anti-canine IgG peroxidase-conjugated was used at 1:8000 dilution (Bethel Laboratories, INC – Montgomery Texas, EUA). Sera samples were tested at the dilution of 1:80 and the optical density obtained with an automated ELISA reader (Bio-Rad – 2530, EUA) at 492 nm.

Results from ELISA-extract test were considered positive when optical density was ≥1.40. Data from ELISA r-k39 were classified considering the optical density ≥90 as the edge between the negative and positive results.

2.5. Biochemical and homological evaluation

Serum proteins were determined by the Biuret reagent and the readings performed in a spectrophotometer in 510 nm (CELM E-22SD). The electrophoretic profile of the serum proteins was carried out using cellulose acetate strips according to the manufacturer instructions (CEN Gel®). The electrophoretic readings were performed in a spectrophotometer and analyzed with the software CS-93101 PC.

Hemogram was performed by conventional technique of counting erythrocytes and leukocytes (Dace and Lewis, 1984) in an automatic cell counter – CELM CC 510.

2.6. Statistical analysis

Statistical analysis was performed using the Minitab 9.2 software package. In the parametric data, one-way analysis of variance (ANOVA) was used for the comparative study between groups. Student's t-test was used for determining the differences between the groups' averages. In the non-parametric data, Kruskal-Wallis test was used for the comparative study between groups, followed by Dunn's test. Spearman rank correlation was calculated to compare results of the different methods. The differences were considered significant when the probabilities of equality, p values, were ≤0.05.

3. Results

3.1. Serological parameters

Total IgG reactivity results in infected animal sera as evaluated by IFAT, ELISA-extract and ELISA rk-39 are shown in Fig. 1. Statistical analysis confirmed that uninfected controls selected by presenting negative IFAT, also differ from the infected animals when tested by ELISA-extract and ELISA-rk39 serology. The mean ELISA reactivity of all CVL carrier groups was higher than that observed for the control group (p < 0.001).

No significant difference was observed ELISA-extract and ELISA rk-39 in total IgG reactivity among the CVL groups. On the other hand, IFAT reactivity revealed that SD displayed a higher serological titer in comparison to AD.

Despite the methodology applied to evaluate the immunological status of infected dogs, a positive correlation was observed between serological reactivity and the clinical status (IFAT r = 0.4207, p = 0.0069; ELISA-extract r = 0.8041, p < 0.0001; ELISA rk39 r = 0.7573, p < 0.001).

3.2. Parasitological parameters

Parasite detection on different tissues from the dog host is shown in Table 2. Although SD displayed higher frequency of positive results in comparison to AD, regardless the tissue evaluated, statistical analysis demonstrated that only AD and OD displayed differences on the frequency of positive parasitological analysis depending on the tissue specimen. In this context, our data demonstrated lower performance of bone marrow impression smears in AD
group, whereas higher sensitivity of skin impression smears was identified for OD in comparison to the other tissues.

Further analysis of parasite load, reported as "Leishman Donovan Units" demonstrated that skin, bone marrow and spleen LDV indexes in AD was lower when compared to SD group. No significant differences in LDV values, including those from the skin, bone marrow and spleen, were observed among the OD and all others infected groups (Fig. 2). When we compared LDV values in the different tissues analyzed we observed that all infected animals display higher skin LDV indexes in comparison to bone marrow. Moreover, our data demonstrated a positive correlation among bone marrow LDV and spleen LDV ($r = 0.6188; p = 0.0319$) in AD dogs, while a positive correlation was observed between skin LDV and spleen LDV ($r = 0.9746; p = 0.0001$) in the OD dogs. Only for SD dogs we observed a positive correlation between skin and bone marrow LDV values ($r = 0.7994; p = 0.0002$).

Correlation analysis of parasite density and serological findings demonstrated that AD showed a positive correlation between skin, bone marrow and spleen LDV and IFAT (skin $r = 0.8304; p = 0.0008$, bone marrow $r = 0.8271; p = 0.0009$, spleen $r = 0.5782; p = 0.0489$).

3.3. Biochemical and hematological parameters

The evaluation of biochemical parameters, related to protein alterations, showed no significant difference in total protein levels among all groups. However, a significant decrease ($p < 0.001$) in albumin concentrations, in the oligosymptomatic and symptomatic dogs, was observed, when compared to control and asymptomatic animals (Fig. 3). Dogs of the oligosymptomatic group presented a significant increase in globulin concentration ($p < 0.001$), when compared to control group, while the symptomatic group presented a significant increase ($p < 0.001$) in globu-

Fig. 1. Reactivity of Total IgG in serum from CVI infected dogs. IFAT results are shown as scattering of individual values and mean serum dilution. ELISA-extract results (cut-off = 140) and ELISA-IFAT (cut-off = 90) are shown as scattering of individual values and mean optical density. The letters "a" and "b" represent statistically significant differences as compared to control (CD) and asymptomatic dogs (AD), respectively. NR = non-reactive.

Fig. 2. Index of parasite density determined by LDV in skin, bone marrow and spleen of naturally infected dogs by L. infantum. The results are shown as median and maximum/minimum values. Asymptomatic dogs = (unstained bars), oligosymptomatic dogs = (gray bars) and symptomatic dogs = (black bars). Results are expressed as number of amastigotes/1000 nucleated cells. The lines represent differences statistically significant for the asymptomatic dogs (AD) in comparison to the symptomatic dogs.
lin concentration, when compared to control and asymptomatic animals. Those dogs from the oligosymptomatic group showed a significant decrease \((p < 0.001)\) in A/G ratio when compared to the control group, while the symptomatic animals presented a significant decrease \((p < 0.001)\) in A/G ratio in comparison to the control and asymptomatic groups (Fig. 3). However, only SD dogs presenting a positive correlation between both total protein and globulin values and IFAT titers \((r = 0.6177; p = 0.0108; r = 0.5947; p = 0.0151\), respectively).

The assessment of hematological parameters demonstrated severe anemia in the symptomatic animals, with a significant decrease \((p < 0.001)\) in the number of erythrocytes, hemoglobin and hematocrit, in relation to the control, asymptomatic and oligosymptomatic groups. As for the white blood cells, statistical analysis showed significant difference among the symptomatic dogs and the other groups in the leukocytes series; however, no significant difference in absolute values of granulocytes neutrophils was observed among the groups of dogs under study. Statistical analysis revealed that the symptomatic dogs showed a significant decrease \((p < 0.025)\) in leukocyte absolute values (leukopenia) when compared to the control group. Data analysis also demonstrated a significant decrease \((p < 0.003)\) in absolute values of the eosinophilic granulocytes subpopulation from symptomatic dogs when compared to the control animals. The symptomatic group showed a significant decrease \((p < 0.017)\) in absolute values of lymphocytes when compared to the control and asymptomatic groups. Data analysis showed also a significant decrease \((p < 0.0017)\) in absolute values of monocytes for the symptomatic dogs when compared to control animals (Table 1).

4. Discussion

All clinical signs observed for the OD and SD groups have been extensively reported in the literature for both natural (Mancianti et al., 1988; Genaro et al., 1997) and experimentally infected dogs (Pinelli et al., 1994, 1995; Abranches et al., 1991). The high frequency of clinical signs presented by infected dogs allow us to observe that the disease gradually evolves from an apparently normal clinical picture, presented by AD, to an active intermediate state, which includes some clinical manifestation (OD) that may evolve to a classical severe and terminal form of the disease (SD), characterized by a larger number of clinical signs. These findings suggested that the sub-clinical or asymptomatic disease may indicate that a good balance in parasite-host interaction is ongoing (Oliveira et al., 1993).

Hypergammaglobulinemia is a remarkable characteristic of CVL (Almeida et al., 2005). Several studies have reported a high antibody production during active CVL,
with elevated levels of IgM and IgA circulating immunocomplexes (Margarito et al., 1998). Many reports have focused special attention to the search of alternative methodologies, as well as the use of specific antigen preparations, aiming to identify a better diagnostic method and prognostic tool for CVL clinical investigation. Some recombinant antigens have been pointed out as relevant candidates for CVL immunodiagnosis (Badard et al., 1997; Soto et al., 1999; Rosário et al., 2005). Herein, the humoral immune response was evaluated by distinct immunological approaches, including IFAT, ELISA-extract, and ELISA rK-39. Our data demonstrated that IFAT was able to identify as positive, all infected animals, despite their clinical status and have been used in this study as the gold standard to diagnosis CVL and select positive animals. ELISA-extract failed to detect IgG reactivity on two IFAT-seropositive animals, one AD and another OD. ELISA rK-39 was not able to detect IgG reactivity on five animals (three from AD and two from OD group). Genaro et al. (1997), using the recombinant protein rK-39 on a immunochromatographic assay (TRALD) to diagnosis CVL in an endemic area, demonstrated 92.1% of sensitivity and 99.5% of specificity, with significantly higher ability to detect Leishmania-specific antibodies when compared to IFAT. However, it was demonstrated that rK-39 was not able to predict the active infection when testing dog samples displaying antibody titers between 1:40 and 1:320, estimated by IFAT (Genaro et al., 1997). Our data are in agreement with these reports since all five seronegative animals detected by ELISA rK-39 displayed serological titers under 1:320 by IFAT. Together, our data demonstrated that ELISA rK-39 was able to confirm seropositivity in 35 out of 40 IFAT-positive animals (87.5% of sensitivity), re-enforcing previous reports of its applicability for CVL diagnosis (Rosário et al., 2005). The profiles of IgG anti-rK-39 presented by naturally infected dogs showed a positive correlation according to the clinical form of the disease. Similar results were also observed with IFAT and ELISA-extract. However, data analysis demonstrated that only IFAT titers were able to discriminate the mean IgG reactivity among infected dogs, with an enhancement of reactivity detected as more severe clinical status of infection was observed, with significant differences detected between AD and SD groups. According to Abranches et al. (1991), as the clinical signs appear, the antibody levels tend to be higher. However, Abranches et al. (1991) have not found a correlation between severity of the disease and total IgG titers. On the other hand, Pinelli et al. (1994) demonstrated that dogs experimentally infected with L. infantum, which also presented clinical signs, showed the highest antibody titers, similar to the results obtained by Genaro et al. (1988) in dogs naturally infected with L. infantum. Our data are in accordance with this later observation, since we found that SD group displayed the highest antibody titers in IFAT. Despite no significant differences on the mean antibody levels detected by ELISA-extract and ELISA rK-39 observed for SD, it was interesting to notice that a positive correlation between severity of disease and the antibody titers was detected by all methods.

Following infection, at the site of parasite inoculation, in the dog skin, the parasite disseminates carried out by histiocytes and Langerhans cells to the lymph node, bone marrow, spleen, liver, kidney, lungs and gastrointestinal tract, been rarely detected on the blood stream (Cardoso and Cabral, 1998). Infected animals presented hyperthermy of the Phagocytic Mononuclear System, leading to splenomegaly, hepatomegaly and generalized adenopathy (Lanotte et al., 1979). During Leishmania infection, tissue parasitism at different sites may differ and therefore is relevant for diagnosis purposes the identification of the major compartments presenting higher parasitemia levels, in order to improve CVL diagnostic sensitivity. In this context, although the majority of authors consider the parasite search in the host tissues as the most reliable diagnostic method, its sensitivity and relative value have been questioned, considering the lack of uniformity of parasite distribution during natural infection. The parallel parasite search, at different affected tissues of the mammalian host organism, could contribute as a definitive criterion, which enables the investigator to choose the most appropriate biological specimen to be analyzed during diagnosis and prognosis practices. According to Cardoso and Cabral (1998), bone marrow biopsies are more sensitive than lymph nodes and therefore are more indicated for diagnosis purposes. Despite Ashford et al. (1995) reporting similar percentage of parasite detection in cultured versus impression bone marrow smear, our findings from AD and SD point out that bone marrow culture showed higher sensitivity as compared to bone marrow impression smears. Abranches et al. (1991) state that bone marrow examination led to positive results only during advanced stages of CVL. Our data confirmed such proposal as 88% of SD in contrast to 25% of AD showed positive results on bone marrow impression smear (Table 2). In general, our data demonstrated that parasitological survey performed on tissue impression smears (skin, spleen, liver and lymph node)

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**Table 2**

<table>
<thead>
<tr>
<th>Tissue/methodology</th>
<th>Number of positive animals (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clinical groups</td>
</tr>
<tr>
<td></td>
<td>AD</td>
</tr>
<tr>
<td>Bone marrow/culture</td>
<td>6 (50.0)</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>3 (25.0) (^{1})</td>
</tr>
<tr>
<td>Skin</td>
<td>5 (42.9)</td>
</tr>
<tr>
<td>Spleen</td>
<td>6 (50.0)</td>
</tr>
<tr>
<td>Liver</td>
<td>5 (42.9)</td>
</tr>
</tbody>
</table>

The letters "\(^{1}\)" and "\(^{2}\)" represent statistically significant differences as compared to AD and OD, respectively.

\(^{1}\) Represents statistical differences within a given clinical group.

\(^{2}\) Results are expressed as number of positive dogs per group; in parenthesis, percentage of positive dogs per group.
as well as cultured bone marrow proved to be more efficient to detect Leishmania organisms in AD than direct bone marrow impression smear, on which was possible to detect amastigotes only in 25% of the specimens analyzed (Table 2). These findings re-enforce that tissue parasitism intensity is parallel with the evolution of the clinical manifestations and point out the importance of the skin parasitism as a useful diagnostic tool when managing asymptomatic animals.

A great advance of parasitological methods is the possibility that such tests provide to evaluate the infection load through the parasite density (LDU). Thus, one may estimate the real parasite density and its contribution to the development of the pathology associated with the infection. Despite the relevance of quantitative parasitological diagnosis and prognostic approaches there are no previous reports regarding the LDU index in natural or experimentally infected dogs. Our data demonstrated that parasite density in skin, bone marrow and splenic compartments were higher according to the severity of clinical manifestations (Fig. 2). Then, AD presented lower tissue parasitism as compared to SD on which LDU would count up to 7246. 1106 and 2564 amastigotes/1000-nucleated cells in skin, bone marrow and splenic sites, respectively. Interestingly, it was observed that all infected dogs, even those AD, presented higher skin parasitism as compared to bone marrow. These findings on the skin LDU indexes re-enforce the importance of cutaneous parasitism density to CVL diagnosis as well as the transmissibility of the infection from AD to susceptible species philobateomy.

It is important to mention that these findings, regarding skin parasite load, described as LDU, is a pioneer report on canine visceral leishmaniasis. Moreover, the results of this current work highlight the importance quantitative investigations regarding the number of amastigote forms at different tissues. In addition, the assessment of parasite density would also contribute as therapeutic tool when measuring leishmanicidal activities of new promising drugs for CVL, regarding the several affected tissues.

Several biochemical and hematological alterations are observed in dogs natural or experimentally infected by L. infantum. A remarkable CVL characteristic is the dysproteinaemia, with serum protein electrophoresis revealing decreased levels of albumin (A) and increased levels of globulin fraction (G), leading to inversion of A/G ratio (Keenan et al., 1984). Herein, we have confirmed these findings and also demonstrated that SD group presented lower A/G ratio in comparison to AD and CD, whereas OD differ only from CD (Fig. 3C). This inversion may occur due to decrease of albumin levels and increase of globulin, remarkably observed in SD. Further analysis demonstrated a positive correlation among both total protein and IFAT titers in the SD. These data revealed a relationship between the presence of exacerbated clinical forms of CVL, serum protein levels and humoral immune response. The factors that contribute to the enhancement of gammaglobulin levels in CVL are not yet completely understood, but this increase is probably due to the polyclonal activation B-lymphocytes triggered by L. infantum antigens as well as the synthesis of non-specific antibodies and the presence of circulating immunocomplexes (Marzochi et al., 1985; Margarito et al., 1998).

Regarding the hematological picture, it is common to observe normocytic/normochromic anemia, whereas, in the white blood cells, there is a leukopenia with moderately or accentuated lymphopenia (Abranches et al., 1991). The results presented in this study showed that during the symptomatic CVL decreased leukocyte counts, due to a drop in monocytes, eosinophils and mainly in lymphocyte population is one of the most relevant hematological finding. Leukopenia, associated to the symptomatic CVL, may be due to multifactorial mechanism on which cellular dysfunction with diminished hematopoiesis, affected by an intense bone marrow parasitism as well as leucocyte recruitment and trapping into several organs are the major events (Alvar et al., 2004). It could also be related to secretion of suppressor cytokines triggered by Leishmania infection (Pinelli et al., 1994, 1995).

In conclusion, our study shows that the clinical evolution of CVL in naturally infected dogs promotes clear alterations in serological, parasitological and biochemical-hematological parameters. Since these alterations are directly correlated with CVL clinical status, they would be taken into account when dealing with diagnosis and prognosis features. The investigation of these laboratorial parameters, associated with the clinical aspect of CVL, is extremely important to be considered in routine clinical follow-up.

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