UNIVERSIDADE FEDERAL DE PERNAMBUCO

Programa de Pós-Graduação em Inovação Terapêutica

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APLICAÇÃO DA CITOMETRIA DE FLUXO NO DIAGNÓSTICO E CRITÉRIO DE CURA DA LEISHMANIOSE TEGUMENTAR AMERICANA

Recife

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ANDRESA PEREIRA DE OLIVEIRA-MENDES

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Inovação Terapêutica da Universidade Federal de Pernambuco, para a obtenção do Título de Doutor em Inovação Terapêutica.

Orientadora: Profa. Dr[a]. Valéria Rêgo Alves Pereira

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"Dê o primeiro passo na fé.
Você não precisa ver a escada inteira.
Apenas dê o primeiro passo."
Martin Luther King, Jr

RESUMO

OLIVEIRA-MENDES, Andresa Pereira de. Aplicação da citometria de fluxo no diagnóstico e critério de cura da leishmaniose tegumentar americana. 2015. 147f. Tese (Doutorado). Universidade Federal de Pernambuco, Recife, Pernambuco, Brasil.

A leishmaniose tegumentar americana (LTA) é um problema de saúde pública, que afeta a produtividade e a vitalidade das pessoas. Embora estudos avaliem a resposta humoral na LTA, ainda não está completamente esclarecido o papel de anticorpos específicos na imunidade contra *Leishmania*. Além dos desafios sócio-econômicos que agravam ainda mais o problema da LTA, o diagnóstico da doença demonstra dificuldades, sendo freqüentemente necessário à correlação de vários elementos para se chegar ao diagnóstico definitivo. Dessa maneira, o objetivo desse estudo, foi avaliar o uso da citometria de fluxo, como uma metodologia alternativa na avaliação diagnóstica em indivíduos com LTA ativa (AT), como critério de cura pós-terapêutica em indivíduos após o tratamento (PT), naqueles com cura clínica espontânea (CE) e em indivíduos com outras doenças (doença de Chagas – DC, leishmaniose visceral–LV, hanseníase e esporotricose). A reatividade relatada pela citometria de fluxo, utilizando promastigotas vivas e fixadas de *Leishmania (Viannia) braziliensis* foi respectivamente, 86% e 90% de porcentagem de parasitas fluorescentes positivos (PPFP). Por análise comparativa, entre citometria de fluxo e imunofluorescência indireta, utilizando os pacientes AT, 1, 2 e 5 anos PT, verificou-se que a citometria de fluxo mostrou sensibilidade de 86% e especificidade de 77%, enquanto a IFI teve uma sensibilidade de 78% e especificidade de 85%. Contudo esta técnica teve confirmada a sua aplicabilidade no critério de cura da LTA. Analisando os resultados apresentados pelos pacientes CE, obtivemos um desempenho com 100% de especificidade. O diagnóstico diferencial da LTA que utiliza soros de pacientes DC e LV demonstrou reação cruzada, revelando resultados falso-positivos. No entanto, a utilização de soros de pacientes com esporotricose, tuberculose e hanseníase, demonstrou potencial para o uso da citometria de fluxo no diagnóstico diferencial. O estudo mostrou que os ensaios realizados utilizando anticorpos IgG, detectados por *Leishmania (V.) braziliensis* na citometria de fluxo, representam uma ferramenta alternativa para o diagnóstico da LTA e também abrem perspectivas para a utilização no monitoramento e critério de cura da LTA.

Palavras-chave: Leishmaniose Tegumentar Americana. IgG. Citometria de fluxo. Leishmania (Viannia) braziliensis.

ABSTRACT

OLIVEIRA-MENDES, Andresa Pereira de. Aplication of flow cytometry on the diagnosis and cure criterion of Americano f American Tegumentary Leishmaniasis. 2015. 147f. Thesis (Doctorate). Universidade Federal de Pernambuco, Recife, Pernambuco, Brazil.

American tegumentar leishmaniasis (ATL) is a public health problem, affecting the productivity and vitality. Although there are studies to assess the humoral response in the ATL, is not yet fully understood the role of specific antibodies in immunity against Leishmania. In addition to the socioeconomic challenges that further aggravate the problem of ATL, the diagnosis shows difficulties and is often necessary to the correlation of various elements to reach a definitive diagnosis. Thus, the aim of this study was to evaluate the use of flow cytometry as an alternative methodology in the diagnostic evaluation in patients with active ATL before treatment (BT), as post-therapy cure criteria in individuals after treatment (AT), those with spontaneous clinical cure (EC) and in individuals with other diseases (Chagas disease - CD, visceral leishmaniasis-LV, leprosy and sporotrichosis). The reactivity reported by flow cytometry, using live and fixed promastigotes of Leishmania (V.) braziliensis were respectively 86% and 90% percentage of positive fluorescent parasites (PPFP). A comparative analysis of flow cytometry and indirect immunofluorescence using patient AT, 1, PT 2 and 5 years, it has been found that the flow cytometry showed a sensitivity of 86% and specificity of 77% and had a sensitivity IIF of 78% and specificity of 85%. Though this technique has confirmed its applicability in the healing criterion of ATL, analyzing the results presented by the EC patients achieved a performance with 100% specificity. The differential diagnosis of ATL using sera from patients of CD and LV demonstrated cross-reactivity, revealing false-positive results. Moreover, the use of sera from patients with sporotrichosis, tuberculosis and leprosy showed potential for the use of flow cytometry in the differential diagnosis. The study showed that testing performed using IgG antibodies, detected by Leishmania (V.) braziliensis in flow cytometry, are an alternative tool for the diagnosis of ATL and also open up prospects for use in monitoring and in its cure criterion.

Keywords: American Tegumentary Leishmaniasis. IgG. Flow Cytometry. *Leishmania (Viannia) braziliensis*.

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LISTA DE ABREVIAÇÕES E SIGLAS

BCG	Bacillus Calmette-Guérin
CE	Cura Espontânea
CF	Citometria de Fluco
DC	Doença de Chagas
ELISA	Ensaio imunoenzimático
CF- AAPF -IgG	Anticorpos IgG de anti- promastigotas fixadas
CF- AAPV -IgG	Anticorpos IgG de anti- promastigotas vivas
FITC	Isotiocianato de fluoresceína (*Fluorescein isothiocyanate*)
FSC	Detector de Dispersão Frontal (*Forward Scatter*)
HIV	Vírus da Imunodeficiência Humana
IDRM	Intradermorreação de Montenegro
IFI	Imunofluorescência Indireta
IFN-γ	Interferon-gama
Ig	Imunoglobulina
IL	Interleucina
LC	Leishmaniose Cutânea
LD	Leishmaniose Difusa
LM	Leishmaniose Mucocutânea
LTA	Leishmaniose Tegumentar Americana
LVA	Leishmaniose Visceral Americana
L. (V.)	*Leishmania (Viannia)*
NNN	Meio de cultura Novy MCNeal Nicole
PBS	Solução tampão fosfato (*Phosphate buffer solution*)
PCR	Reação em cadeia da polimerase(*Polymerase chain reaction*)
PPFP	Porcentagem de parasitos fluorescentes positivos
ROC	Característica Operativa do Receptor (*Receiver Operating Characteristic*)
SFB	Soro fetal bovino
SSC	Detector de Dispersão Lateral (*Side Scatter*)
TGF-β	Fator de crescimento tumoral (*Tumor growth factor*)
TNF-α	fator de necrose tumoral - alfa

SUMÁRIO

1 INTRODUÇÃO

A leishmaniose tegumentar americana (LTA) é um problema de saúde pública, que afeta a produtividade e a vitalidade dos indivíduos. Essa doença infecciosa apresenta distribuição mundial e acomete, principalmente, aqueles com menor status socioeconômico de países menos desenvolvidos. Nas Américas, a LTA está distribuída do sul dos Estados Unidos ao Norte da Argentina, com exceção do Chile e Uruguai. Devido a sua ampla distribuição, registrando casos em todas as regiões brasileiras, é uma das afecções dermatológicas de maior magnitude no Brasil. Em Pernambuco, é uma doença endêmica e 60% dos casos concentram-se na Zona da Mata. Suas perspectivas de controle são ainda muito dependentes do progresso nas pesquisas científicas, visando obter estratégias mais efetivas para o monitoramento de casos e do vetor (BRANDÃO-FILHO et al., 2003; DESJEUX, 2004; BRITO et al., 2009; 2012; MS, 2010; OMS, 2010).

Os protozoários causadores da LTA são organismos dimórficos, caracterizados por apresentarem durante seu ciclo de vida duas formas evolutivas. A forma promastigota ou flagelada infectante, encontrada na luz do tubo digestivo dos insetos vetores, os flebotomíneos fêmeas, que transmitem a infecção aos hospedeiros vertebrados. Já a forma amastigota, é aflagelada, arredondada e imóvel, se multiplica obrigatoriamente dentro de células do sistema monocítico fagocitário desses hospedeiros vertebrados, estando envolvidas na evolução ou cura da doença (CROFT; COOMBS, 2003; MS 2010; TIUMAN et al., 2011).

As manifestações clínicas da LTA variam desde as lesões cutâneas localizadas, a forma mais comumente encontrada, até graves lesões mucocutâneas desfigurantes, devido a destruição das regiões mucosas. Até então, quatorze espécies de *Leishmania* foram descritas e estão divididas entre os subgêneros *Leishmania* e *Viannia*, causando uma variedade de lesões no homem. A *Leishmania (Viannia) braziliensis (L. (V.) braziliensis)*, principal espécie envolvida na transmissão da LTA até o momento em Pernambuco, é considerada uma das espécies mais importantes para a saúde pública, não só por ser responsável pela maioria dos casos de LTA no país, como também pela sua capacidade de causar as infecções dermatológicas descritas acima (BRANDÃO-FILHO et al., 2003; SILVEIRA; LAINSON; CORBETT, 2004; BRITO et al., 2009; 2012; GOTO; LINDOSO, 2010).

O tratamento da LTA apresenta limitações, as drogas disponíveis são tóxicas e muitas vezes causam reações adversas graves e, além disso, não existe um critério de cura efetivo. O critério de cura é baseado na cura clínica pela reepitelização completa das lesões

ulceradas ou não, regressão total do infiltrado e eritema (GONTIJO; MAYRINK et al., 2006; MS, 2010; OMS, 2010). Desta forma, torna-se relevante a busca de um critério de cura pós-tratamento específico para LTA, (SCHUBACH et al., 1998; MENDONÇA et al., 2004).

Embora estudos mostrem que as células T e a imunidade mediada por células contribuem para a patogênese das diferentes manifestações clínicas da LTA, ainda não está completamente esclarecido o papel de anticorpos específicos na imunidade contra *Leishmania* (TRUJILLO et al., 1999; SOUZA et al., 2005).

Segundo Rodriguez et al. (1996), há correlação das subclasses de IgG com as manifestações clínicas da LTA. Assim, níveis elevados de anticorpos dos isotipos IgG1, IgG2 e IgG3 e baixos níveis ou ausência de anticorpos do isotipo IgG4 podem ser detectados no soro de pacientes com LC (leishmaniose cutânea). No soro de pacientes com LM (leishmaniose mucocutânea) tem-se altos níveis de IgG1, enquanto os níveis de IgG2, IgG3 e IgG4 são comparados aos encontrados no soro de pacientes com LC. Já nos pacientes com LD, os níveis de IgG4 são bastante elevados, enquanto os níveis de IgG1 e IgG2 são semelhantes aos encontrados no soro de pacientes com LC e LM (RODRIGUEZ et al., 1996; REIS et al., 2006). Em 2001, Brito e colaboradores observaram níveis aumentados de IgG em pacientes curados espontaneamente, sugerindo a importância do achado para a dinâmica da resposta de anticorpos e evolução da LTA.

Além dos desafios sócio-econômicos que agravam o problema da LTA, o diagnóstico da doença demonstra dificuldades, pois o mesmo é realizado por associações clínicas, epidemiológicas e laboratoriais (KAR, 1995; BRITO et al., 2000; 2008, TAVARES et al., 2003; MS, 2010; ELMAHALLAWY et al., 2014). Os testes sorológicos convencionais mais utilizados no diagnóstico laboratorial da LTA são a reação de imunofluorescência indireta (IFI) e o ensaio imunoenzimático (*enzyme linked immunosorbent assay* - ELISA). Entretanto, esses métodos apresentam limitações, pois não correlacionam os níveis de anticorpos circulantes com o estágio da doença e podem apresentar reações cruzadas com outros tripanosomatídeos (KAR, 1995; VEXENAT et al., 1996; BRITO et al., 2001; OMS, 2010).

Um dos grandes desafios enfrentados pelos pesquisadores tem sido a escolha da preparação antigênica ideal para análise sorológica, tanto no que se refere ao estudo dos mecanismos moduladores/indutores de doença, bem como para o diagnóstico, prognóstico e critérios de monitoração da infecção (BRITO et al., 2000; 2001; GONÇALVES et al., 2002; ROCHA et al.,2002; CELESTE et al., 2004; ROCHA et al., 2006). Pelas limitações dessas técnicas, abordagens imunológicas alternativas vêm sendo empregadas. Uma delas é a

citometria de fluxo (CF), tecnologia usada na análise quantitativa de anticorpos e vem sendo utilizada no diagnóstico sorológico da LTA (ROCHA et al., 2002; 2006). Em paralelo, alguns estudos avaliam diminuição dos níveis de anticorpos em pacientes após o tratamento com antimoniais e a possibilidade de utilizar testes sorológicos, como a citometria de fluxo, não apenas para o diagnóstico, mas como critério de cura da LTA (AMATO et al., 1998; BRITO et al., 2001).

O presente estudo apresenta o uso da citometria de fluxo nesses dois aspectos, ou seja, para o diagnóstico e para critério de cura. Nesse contexto, apresentamos os resultados da avaliação de antipromastigotas de *L. (V.) braziliensis* vivas e fixadas detectadas pelo anticorpo IgG através da CF, com a finalidade monitorar a cura pós terapêutica nos pacientes comparando a reatividade de IgG antes a após o tratamento. Foi feita uma comparação do método de CF com o ensaio imunológico de imunofluorencência indireta no diagnóstico e critério de cura pós terapêutica da LTA, utilizando *L. (V.) braziliensis* fixadas, detectadas pelo anticorpo IgG. A avaliação das antipromastigotas de *L. (V.) braziliensis* fixadas, utilizando soro de pacientes com LTA, curados espontaneamente e com outras doenças permitiu verificar que a CF apresenta aplicabilidade e atende as necessidades básicas da rotina laboratorial para identificar LTA. Foi possível a identificação da cura clínica em pacientes curados espontaneamente. Embora o uso de formas promastigotas fixadas de *Leishmania (V.) braziliensis* tenha favorecido o aparecimento de reações cruzadas com estes organismos tripanosomatídeos, a utilização de soros de pacientes com esporotricose tuberculose e hanseníase demonstrou o potencial da CF no diagnóstico diferencial.

Portanto, esse estudo mostra a importância dessa técnica no diagnóstico da doença e avaliação da combinação com conjugados FC-AAPV-IgG (anticorpos IgG de anti-promastigotas vivas) e FC-AAPF-IgG (anticorpos IgG de anti-promastigotas fixadas), para detecção de promastigotas vivos e fixados, respectivamente, de *L. (V.) braziliensis*, no monitoramento da evolução clínica dos pacientes com leishmaniose cutânea, da cura pós-terapêutica, e sugeri a aplicação da CF como teste alternativo no diagnóstico da LTA e critério de cura em nossa região.

2 OBJETIVOS

2.1- Geral

Avaliar o uso da citometria de fluxo como uma técnica alternativa na avaliação diagnóstica e como critério de cura de portadores de leishmaniose tegumentar americana em Pernambuco.

2.2- Específicos

2.2.1- Comparar o uso de promastigotas *Leishmania (Viannia) braziliensis* vivas e fixadas na detecção do anticorpo IgG através da citometria de fluxo

2.2.2- Avaliar a reatividade da *Leishmania (Viannia) braziliensis* viva e fixada no monitoramento da cura clínica dos pacientes antes e 1, 2 e 5 anos após o tratamento quimioterápico

2.2.3- Comparar as técnicas de citometria de fluxo, na reação de imunofluorescência indireta e na identificação dos pacientes antes e 1, 2 e 5 anos após o tratamento quimioterápico.

2.2.4- Avaliar a aplicabilidade da citometria de fluxo como critério de cura pós-terapêutica.

2.2.5- Analisar o desempenho da citometria de fluxo na identificação de pacientes com cura espontânea.

2.2.6- Verificar a sensibilidade e especificidade da citometria de fluxo, comparando-se o soro dos pacientes com LTA com o soro de pacientes portadores de outras doenças.

3 REVISÃO DA LITERATURA

3.1- Aspectos Gerais da Leishmaniose Tegumentar Americana

A LTA é uma doença infecciosa negligenciada e que acomete principalmente aqueles com menor *status* socioeconômico e com limitada capacidade de assumir os custos da doença (diagnóstico, hospitalização, tratamento) e suas consequências, que envolvem desde lesões simples até deformidades marcantes (OMS, 2010; MS, 2010; CASTRO, 2013). A maior parte das pessoas afetadas vive em aldeias pobres em áreas rurais, e estão permanentemente expostas aos fatores de risco da doença, normalmente por razões ocupacionais (OMS, 2010; MS 2010; BRITO et al 2012). Dessa maneira, a LTA é uma doença que merece maior atenção devido à sua magnitude, alta morbidade e possibilidade de assumir formas que podem determinar lesões destrutivas, desfigurantes e também incapacitantes, com grande repercussão no campo psicossocial do indivíduo (GONTIJO; CARVALHO, 2003; ANDRADE et al., 2005; NEITZKE-ABREU et al., 2014).

Atualmente, a LTA ocorre de forma endêmica em 82 países e estima-se a incidência de 1,5 milhão de novos casos notificados por ano (figura 1). Cerca de 90% desses casos ocorrem no Marrocos, na Etiópia, Tunísia, Afeganistão, Paquistão, Irã, Iraque, Arábia Saudita, Síria, Brasil, Bolívia, Colômbia, Equador, Peru e Venezuela. Nas Américas, a LTA ocorre desde o Sul dos Estados Unidos até o norte da Argentina, com exceção do Uruguai e do Chile (GONTIJO; CARVALHO, 2003; GOTO; LINDOSO, 2010; OMS, 2010). Em decorrência do seu caráter epidêmico e alto coeficiente de detecção, a LTA é considerada uma das dez endemias mundiais prioritárias. Ocupando o segundo lugar entre as seis doenças infecto-parasitárias mais frequentes do mundo, caracteriza-se ela como problema de saúde pública, tanto pela sua expansão geográfica quanto pela tendência de urbanização (GAZOZAI et al., 2010; DOENÇAS NEGLIGENCIADAS, 2010).

No Brasil, a LTA apresenta-se distribuída por todos os Estados, avança na Região Centro-Oeste e já se encontra na periferia das grandes cidades do Nordeste e do Centro-Oeste. No ano de 2013, foram notificados 19. 652 novos casos da doença, no Sistema de Informação de Agravos de Notificações (SINAN Net), em todo o país (tabela 1). Com expressivo número de casos, a LTA também é caracterizada por apresentar uma importante difusão espacial. Nesse contexto, a LTA apresenta importante heterogeneidade, relacionada às diferentes espécies de *Leishmania* envolvidas em sua etiologia, às formas clínicas associadas e ao

padrão de transmissão envolvido (LAINSON; SHAW, 1998; BRANDÃO-FILHO, 1999; MS, 2010; DATASUS, 2014; MENEZES-SOUZA et al., 2014).

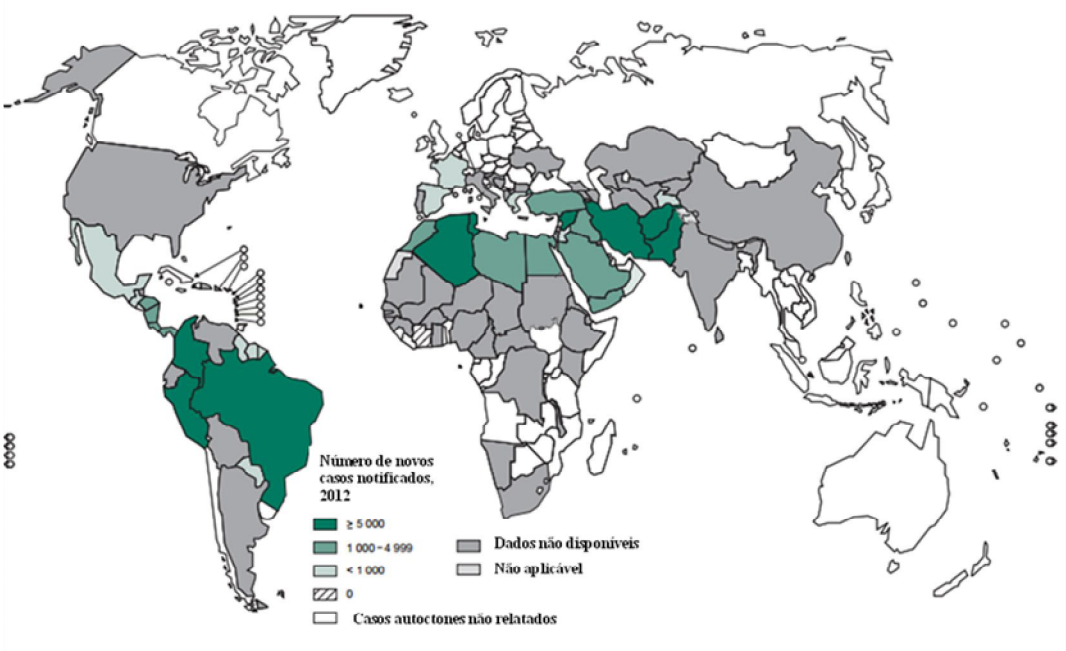


Figura 1: Distribuição mundial da leishmaniose tegumentar.
Fonte: OMS, 2014.

A LTA apresenta sua incidência acentuada em Pernambuco, por todas as regiões do Estado, principalmente na Zona da Mata, onde estão concentrados cerca de 60% dos casos registrados (BRANDÃO-FILHO et al., 1999; BRITO et al., 2008). É importante também registrar o aumento de sua ocorrência no Sertão do Estado (ANDRADE et al., 2009). No biênio 2008-2009 foram confirmados 889 casos novos de LTA em Pernambuco, distribuídos em aproximadamente 34% dos municípios. Para demonstrar o aumento e a força da infecção nesta região, foram realizados inquéritos epidemiológicos, caracterizando a distribuição da infecção e da doença na população. Os estudos mostraram que, em Pernambuco, a LTA ocorre em ambos os sexos e em todas as faixas etárias. O aumento da incidência e a persistência da transmissão neste Estado revela um importante problema de saúde pública devido à expansão espacial (BRANDÃO-FILHO et al., 1999; 2003; BRITO et al., 2008; ANDRADE et al., 2012).

Tabela 1: Casos confirmados notificados no sistema de informação de agravos de notificação – Sinan Net, no período de 2007 a 2013, no Brasil.

Ano de diagnóstico	Casos confirmados
2013	19.652*
2012	25.647
2011	23.054
2010	23.493
2009	23.318
2008	21.581
2007	22.556

Fonte: DATASUS, 2015.
* Dados atualizados até 24/09/2014.

O agente etiológico da LTA é um protozoário intracelular, que apresenta DNA extranuclear, cinetoplasto e uma organela mitocondrial, e que pertence à ordem Kinetoplastida, família *Trypanosomatidae*, gênero *Leishmania*, e divide-se em dois subgêneros, *Leishmania* e *Viannia*. Durante o seu ciclo de vida, esse parasito apresenta-se sob duas formas evolutivas (figura 2), a primeira forma é a promastigota, forma alongada e com o flagelo livre, encontrada no trato digestivo dos hospedeiros invertebrados, os flebotomíneos. A outra forma, a amastigota, forma arredondada ou ovalada sem flagelo, é encontrada no interior de monócitos, histiócitos e macrófagos, que são células do sistema fagocítico mononuclear do hospedeiro mamífero (VEGA-LOPEZ, 2003; BASANO; CAMARGO, 2004; MS, 2010).

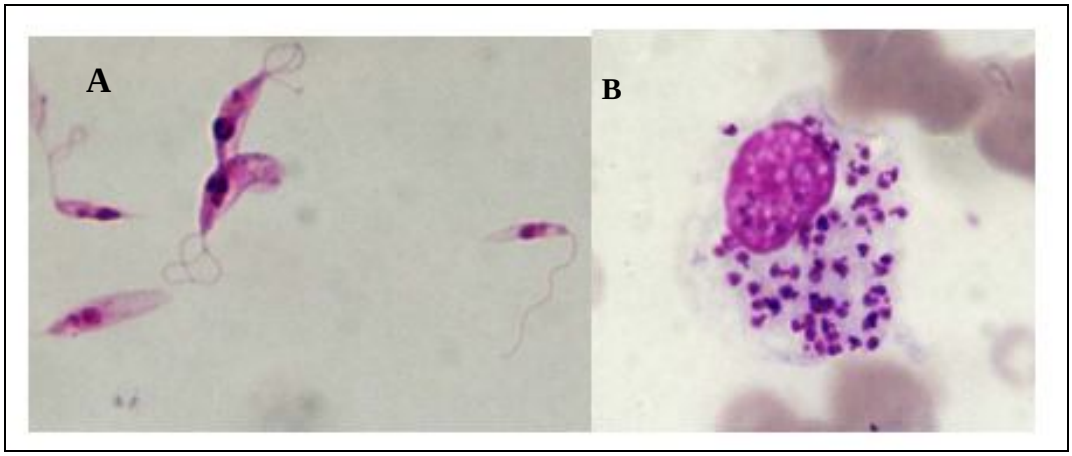


Figura 2: Formas da *Leishmania* : **A-** promastigotas, **B-** amastigotas dentro de monócito.
Fonte: CITADIN, 2008.

O ciclo de vida da *Leishmania* (figura 3) tem início, em figura 3A, quando a fêmea de um inseto vetor, o flebotomíneo, se alimenta de um hospedeiro vertebrado contaminado, pois as formas amastigotas são ingeridas durante o repasto sanguíneo. No intestino do flebotomíneo-fêmea (figura 3B), as formas amastigotas sofrem divisões binárias e se transformam em formas flageladas, as promastigotas. Essas formas migram para a faringe e o esôfago do inseto, onde maturam para formas promastigotas metacíclicas infectantes. Quando o flebotomíneo-fêmea realiza um novo repasto sanguíneo em um hospedeiro vertebrado (figura 3C), libera as formas promastigotas. Em seguida, as formas promastigotas metacíclicas são fagocitadas pelos macrófagos e transformam-se em amastigotas (figura 3D). As formas amastigotas se multiplicam por divisão binária, até o rompimento da membrana celular do macrófago, que as libera para serem fagocitadas por outros macrófagos (figura 3E). O ciclo se reinicia (figura 3A) quando uma fêmea do vetor, ao realizar um repasto sanguíneo, ingere o sangue e, com ele, macrófagos parasitados (DESJEUX, 2004; REITHINGER et al., 2007; NEVES et al., 2009; OMS, 2014; PACE, 2014).

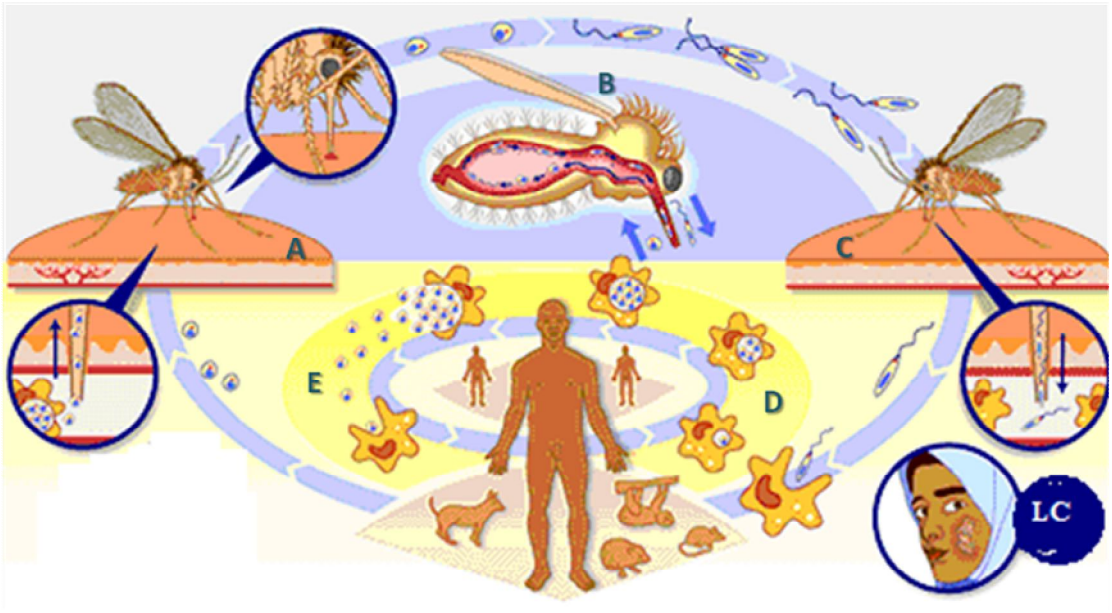


Figura 3: Ciclo de vida da *Leishmania* sp. LC - leishmaniose cutânea.
Fonte: Adaptado de OMS, 2014.

A epidemiologia da LTA é extremamente diversificada, os ciclos de transmissão implicam um grande número de parasitos, reservatórios e vetores envolvidos. Nas Américas, são atualmente reconhecidas 11 espécies dermotrópicas de *Leishmania* causadoras da doença

humana, e oito espécies descritas somente em animais (figura 4). No entanto, no Brasil, já foram identificadas sete espécies envolvidas na etiologia da LTA, sendo seis do subgênero *Viannia* e uma do subgênero *Leishmania*. As três principais espécies são: *L. (V.) braziliensis*, *L. (V.) guyanensis* e *L. (L.) amazonensis* e, mais recentemente, as espécies *L. (V.) lainsoni, L. (V.) naiffi, L. (V.) lindenberg* e *L. (V.) shawi* que foram identificadas em estados das Regiões Norte e Nordeste (DESJEUX, 2004; BRITO et al., 2009; OMS, 2010). A distribuição da infecção e da doença na população foi bem caracterizada na Zona da Mata de Pernambuco, em inquéritos epidemiológicos realizados entre 1991 e 1996. E, desde então, amostras isoladas de pacientes dessa região apresentaram perfis isoenzimáticos de variantes para a principal espécie circulante, que é a *L. (V.) braziliensis* (BRITO et al., 1993; BRANDÃO-FILHO et al., 1999; MARTINS et al., 2010; MENEZES-SOUZA et al., 2014).

3.2 - Manifestações Clínicas da Leishmaniose Tegumentar Americana

A LTA apresenta manifestações clínicas diversas, que variam de acordo com a espécie envolvida na transmissão, com a susceptibilidade da população e com seu nível de exposição. Fatores relacionados aos indivíduos também influenciam na apresentação da doença, e nesses podemos incluir o *status* imunológico do hospedeiro, relacionado às interações do seu sistema imune inato e adaptativo, assim como ao estado nutricional e aos fatores genéticos do mesmo (REITHINGER et al., 2007; CASTELLANO et al., 2009; BRITO et al., 2012; BRELAZ et al., 2012). Embora a apresentação clínica da LTA esteja frequentemente relacionada a uma determinada espécie ou subgênero de *Leishmania*, nenhuma se restringe à mesma. Ademais, uma significativa, porém variável, proporção de infecções é assintomática, ao passo que, sendo sintomáticas, as lesões cutâneas apresentam-se nas formas localizada, disseminada, difusa, e mucocutânea, a mais agressiva (figura 5) (DESJEUX, 2004; AMEEN, 2010; GOTO; LINDOSO, 2010).

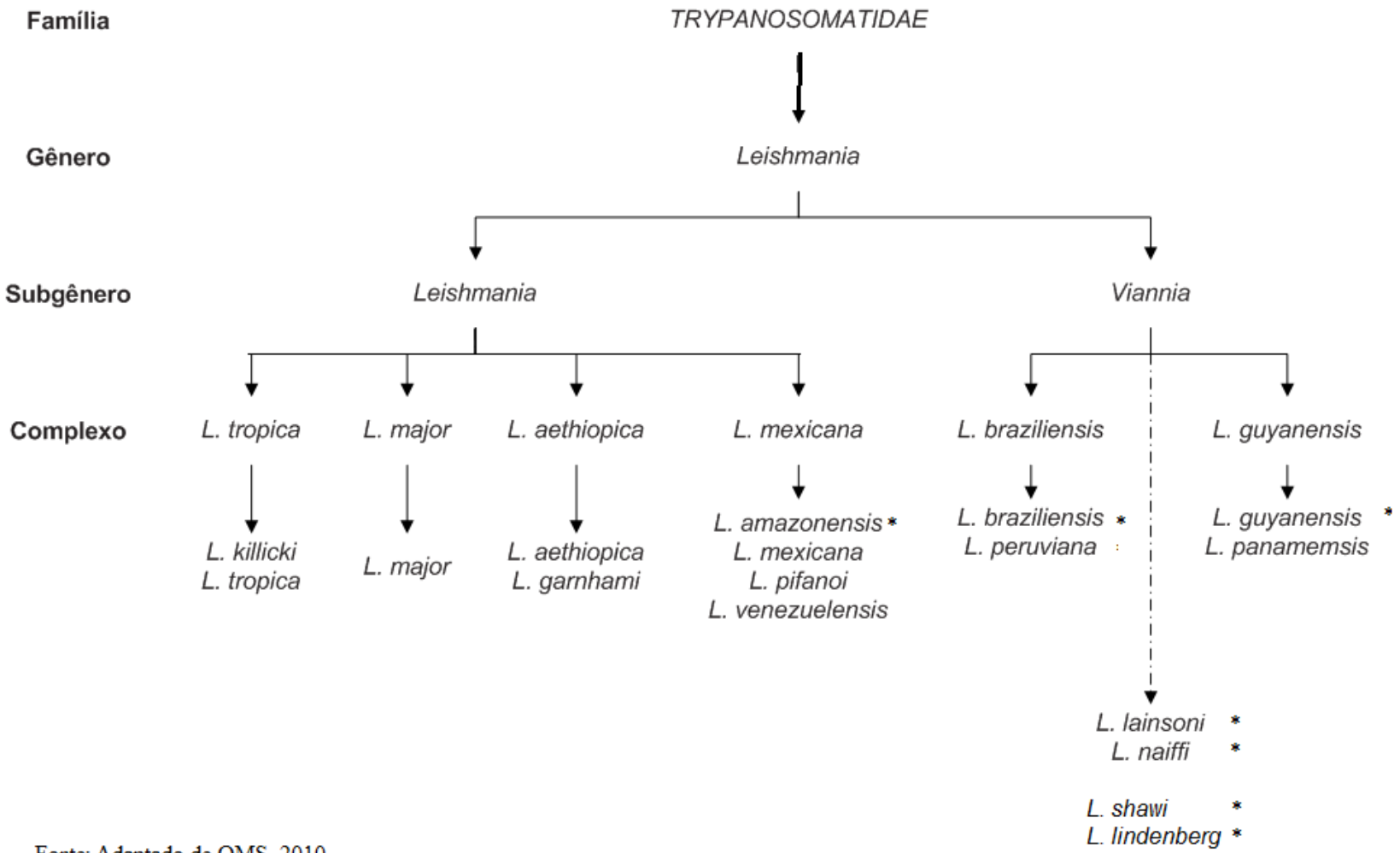
Podendo evoluir para a cura espontânea, a forma cutânea localizada (LC) é a manifestação mais recorrente da doença, em tal caso, não é menos perigosa. As lesões são únicas ou múltiplas e assumem a forma típica com bordas elevadas, sendo a úlcera indolor, arredondada ou oval (REITHINGER et al., 2007; GOTO; LINDOSO, 2010). A densidade de parasitos nas bordas da lesão é grande nas fases iniciais da doença, no entanto, diminuem nas lesões crônicas. Todas as espécies dermotrópicas de *Leishmania* são capazes de desencadear a LC (figura 5A), todavia, no Brasil, o principal agente etiológico é a *L. (V.) braziliensis*, que

tem a disseminação por via hematogênica como a principal complicação. Além disso, ela pode evoluir para a leishmaniose mucocutânea (MS, 2010; GOTO; LINDOSO, 2010; MANSUETO et al., 2014).

Com numerosas lesões nodulares ou ulceradas, a forma disseminada (figura 5B) tem sido descrita em associação com *L. (V.) braziliensis, L. (V.) panamensis, L. (V.) guyanensis* e infecções *L. (L.) amazonensis.* A apresentação dessa forma varia de 10 a 300 lesões, em partes do corpo contínuas ou não. Além disso, podem ocorrer com ou sem envolvimento das mucosas. Essa forma difere da forma anérgica cutânea difusa (figura 5C), onde lesões múltiplas, nodulares, não ulceradas são encontradas por todo o corpo. A leishmaniose cutânea difusa, no Brasil, tem sido associada com a *L. (L.) amazonensis*. O desenvolvimento dessas lesões está relacionado à capacidade particular que a *L. amazonensis* tem de interferir negativamente em vários mecanismos imunológicos, necessários à geração de uma resposta imune efetiva, dessa maneira, levando à proliferação dos parasitos e à ocorrência de metástases do parasito de um sítio para o outro, através dos vasos linfáticos ou da migração de macrófagos parasitados, tornando a leishmaniose difusa uma doença rara (REITHINGER et al., 2007; GOTO; LINDOSO, 2010; OMS, 2010; AMEEN, 2010; MANSUETO et al., 2014).

Comumente conhecida como “úlcera de bauru” ou “espúndia”, a forma grave mucocutânea (figura 5D) da leishmaniose ocorre como consequência do desenvolvimento secundário da forma cutânea localizada, e é caracterizada pela destruição da cavidade oral-nasal, faringe e laringe, em virtude de uma reação imunológica exagerada. O agente etiológico causador da leishmaniose mucocutânea (LM), em nosso país, é a *L (V.) braziliensis*; entretanto, já foram citados casos na literatura atribuídos à *L (L.) amazonensis* e à *L (V.) guyanensis.* A LM, normalmente, surge da evolução crônica da LC, de casos com cura espontânea sem tratamento ou de casos em que o tratamento foi inadequado, desenvolvendo lesões secundárias destrutivas em mucosas e cartilagens. O processo infeccioso começa indolor e com infiltrado discreto no septo nasal, que evolui para a formação de crostas, epistaxe, disfagia, odinofagia, rouquidão e tosse, dificuldades respiratórias, além de dificuldade na fala e na alimentação do doente. A LM pode também atingir as conjuntivas oculares, as mucosas dos órgãos genitais e do ânus. Na fase final, ocorre mutilação grave, com obstrução e destruição do nariz, faringe e laringe. A LM praticamente nunca cicatriza espontaneamente, e as infecções secundárias são frequentes, sendo a pneumonia intercorrente a causa mais comum de morte (NEVES et al., 2009; AMEEN, 2010; GOTO; LINDOSO, 2010; OMS, 2010; NUNES et al., 2011; MANSUETO et al., 2014; MIGNOGNA et al., 2014).

Figura 4: Taxonomia da *Leishmania*.



Fonte: Adaptado de OMS, 2010.
* Espécies envolvidas na etiologia da LTA no Brasil.

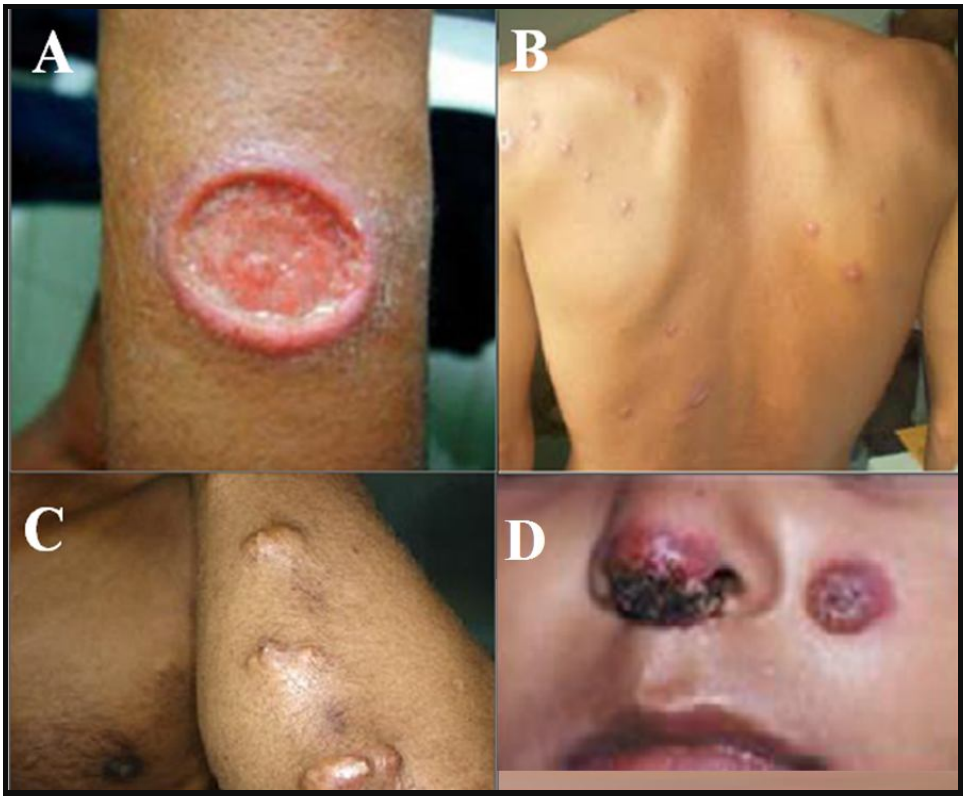


Figura 5: Formas clínicas da leishmaniose tegumentar americana. A - leishmaniose cutânea localizada; B - leishmaniose cutânea disseminada; C - Leishmaniose cutânea difusa; D - leishmaniose mucocutânea.
Fonte: A e B, Ministério da Saúde, 2007; C e D, Almeida, 2013.

3.3 - Tratamento e Controle da Leishmaniose Tegumentar Americana

O tratamento tem como propósito acelerar a cura, reduzir o risco de formação de cicatrizes, prevenir a progressão da doença, e a sua escolha, advém do tamanho e localização da lesão, do número e do potencial de disseminação das lesões. Em todas as formas clínicas da LTA, as drogas de primeira escolha são os antimoniais pentavalentes (Sb^{+5}), cujos esquemas terapêuticos são padronizados pela Organização Mundial de Saúde. As doses são calculadas em mg/Sb^{+5}/kg/dia e há dois tipos de antimoniais pentavalentes utilizados: o antimoniato de N-metilglucamina (Glucantime®) e o estibogluconato de sódio (Pentostam®). O primeiro é distribuído pelo programa de controle de LTA do Ministério da Saúde do Brasil e é indicado como primeira escolha para o tratamento de todas as formas de LTA, exceto para pacientes coinfectados com HIV e para gestantes. Já o segundo, estibogluconato de sódio, não é comercializado no Brasil (GONTIJO; CARVALHO et al., 2003; MS, 2010; AMEEN, 2010; OLIVEIRA et al., 2011).

O esquema terapêutico, com o Sb^{+5}, é composto por doses de 15 a 20 mg/Kg/dia através de injeções intramusculares ou por via endovenosa, em ciclos de vinte a trinta dias (GONTIJO; CARVALHO, 2003; MS, 2010). Sabe-se que, na dose de 20mg Sb^{+5}/kg/dia, o antimonial pode atingir seu limiar de toxicidade, levando a alterações cardíacas, pancreáticas ou renais que obriguem a suspensão do tratamento. O índice de sucesso após o primeiro ciclo de antimoniato de N-metilglucamina é variável, e, no Brasil, a falha no tratamento foi observada em 16% dos pacientes (GOTO; LINDOSO, 2010). O longo tempo de duração do tratamento e a aplicação intravenosa ou intramuscular ocasionam diminuição no número de pacientes que aderem a ele. Além disso, apesar de os antimoniais serem as principais drogas contra a *Leishmania*, e de estarem disponíveis desde o início do século XX, até o momento não se sabe em detalhe o mecanismo de ação dessas drogas (REITHINGER et al., 2007; GOTO; LINDOSO, 2010; MS, 2010; OLIVEIRA et al., 2011).

As drogas de segunda escolha são: a Anfotericina B, um antibiótico poliênico com excelente atividade *in vitro* na destruição de *Leishmania* intra e extracelular; e o isotionato de pentamidina, que são diamidinas aromáticas usadas no tratamento de LTA em áreas endêmicas dos continentes americano, asiático e africano. A anfotericina B é administrada por via endovenosa, gota a gota, na dose diária ou em dias alternados de 1 mg/Kg/dia, sem ultrapassar a dose de 50mg em cada aplicação, e um total de 1- 1,5g para LC, e de 2,5 a 3g para LM, durante todo o tratamento. Ela é dissolvida em soro glicosado 5%, com tempo de infusão de 3 a 4 horas, e apresenta inúmeros efeitos adversos por ser um medicamento altamente tóxico para as células do endotélio vascular. Durante a infusão lenta, pode ocorrer cefaleia, febre, calafrios, astenia, dores musculares, vômitos e hipotensão; e durante a infusão rápida pode ocorrer alterações cardiovasculares e até parada cardíaca. Em razão de complicações renais, observadas em diferentes graus, que ocorrem em praticamente todos os pacientes ao longo do tratamento, a Anfotericina B só é usada, principalmente, em caso de falha de outros tratamentos (GONTIJO; CARVALHO, 2003; REITHINGER et al., 2007; GOYONLO et al., 2014).

O isotionato de pentamidina é usado na dose de 4 mg/Kg/dia, por via intramuscular profunda, sendo aplicado a cada dois dias, não ultrapassando a dose total de 2g. Devido ao medicamento ter ação no metabolismo da glicose, pode haver hipoglicemia seguida de hiperglicemia, quando do seu uso. Semelhante à Anfotericina B, o isotionato de pentamidina é uma droga igualmente tóxica e, muitas vezes, causa reações adversas graves, como diabetes mellitus, hipoglicemia, miocardite e complicações renais; sendo unicamente empregada

quando não se obtém resposta ao tratamento com o antimonial (MS, 2010; MITROPOULOS et al., 2010).

Perante as dificuldades encontradas no tratamento da LTA, limitando o uso dessas drogas em diversos grupos de pacientes, como crianças pequenas, idosos, gestantes, pacientes com problemas renais, hepáticos e com outras doenças crônicas, o estudo para o desenvolvimento de novos compostos candidatos à terapêutica, ou que a complementem, tem se tornado indispensável (AMEEN, 2010; GOTO; LINDOSO, 2010; GOYONLO et al., 2014). Distintas linhas de tratamento com drogas alternativas vêm sendo avaliadas. A Miltefosina, originalmente utilizada para o tratamento do câncer, teve a sua atividade leishmanicida revelada e é agora usada no tratamento da LTA por via oral. Outras drogas, como compostos da classe imidazol (cetoconazol) e triazol (fluconazol, itraconazol) são agentes antifúngicos que demonstram resultados controversos para o tratamento da LTA no Velho Mundo; e no Novo Mundo, o seu uso ainda está sob investigação (AMEEN 2010; MITROPOULOS et al., 2010; OLIVEIRA et al., 2011).

Alguns fármacos, como paramomicina, azitromicina, cetoconazol, imiquimod, são empregados no tratamento tópico para LTA, contudo, expressam eficácias variadas e risco de evolução para forma mucosa. Em virtude da termosensibilidade das formas promastigotas, modalidades físicas, como o tratamento com frio e calor são empregados na LTA. O calor infravermelho, ou com luz ultravioleta, e a crioterapia com CO_2 ou nitrogênio líquido têm sido utilizados com sucesso. Ainda assim, as taxas de eficácia da crioterapia e da termoterapia variam amplamente, tornando difícil a avaliação dos seus méritos relativos. Por conseguinte, esses estudos ainda precisam de maiores investigações para assegurar a efetividade e a aplicabilidade desses novos candidatos à terapêutica da LTA (CROFT et al., 2006; GOTO; LINDOSO, 2010; AMEEN, 2010; MITROPOULOS et al., 2010).

Uma alternativa terapêutica para algumas situações clínicas, que vem sendo desenvolvida, é a imunoterapia. Esta se baseia numa tentativa de aumentar a resposta imune específica do paciente contra o parasito. Essa ideia é baseada na observação de que pacientes, ao se recuperarem da leishmaniose, apresentam uma sólida imunidade contra a reinfecção (EL-ON, 2009; GOTO; LINDOSO, 2010; SINGH; SUNDAR, 2014). Antígenos de *Leishmania*, sozinhos ou combinados com outros, como BCG, vêm sendo utilizados no tratamento da LTA com sucesso parcial. Nessa pesquisa, as preparações contendo promastigotas inteiras foram administradas por via intramuscular e obtiveram a cura em 76-94% dos indivíduos. O antígeno recombinante de *Leishmania* também tem sido testado como candidato à imunoterapia. A combinação de antimoniais e imunomoduladores poderá ser uma

alternativa de tratamento para pacientes refratários ao tratamento com o antimonial. Embora essa modalidade de tratamento seja crescente, ensaios clínicos são ainda necessários para demonstrar o seu benefício na rotina clínica (CONVIT et al., 2003; 2004; EL-ON, 2009; GOTO; LINDOSO, 2010; AMEEN, 2010).

Em virtude da diversidade de agentes etiológicos, de reservatórios, de vetores e da situação epidemiológica da LTA, várias abordagens são utilizadas para o controle dessa endemia. Dentre essas, ressalta-se a vigilância epidemiológica com identificação precoce dos casos e tratamento; medidas de atuação na cadeia de transmissão, impedindo a infecção de vetores, hospedeiros e reservatórios; medidas educativas, incluindo a utilização de mosquiteiros, telas finas nas janelas e portas, repelentes e roupas que protejam áreas expostas. Outras abordagens incluem medidas administrativas, como saneamento (evitando o acúmulo de lixo e de detritos que possam atrair roedores e pequenos mamíferos, que podem servir como reservatório). Melhorias das condições de habitação e capacitação de profissionais de saúde também são essenciais. O desenvolvimento de uma vacina eficiente e operacional seria uma medida definitiva e eficaz em termos de custos e de prevenção (BASANO; CAMARGO, 2004; DESJEUX, 2004; OMS, 2010).

A LTA é uma doença complexa e heterogênea, que está se tornando cada vez mais prevalente, tanto nos países onde ocorre de forma endêmica, quanto nos que ocorre de forma não endêmica. Além disso, um número crescente de casos é visto em viajantes. Por conseguinte, a corrente situação exige novas opções de tratamento e controle, incluindo a imunoterapia e o progresso contínuo no desenvolvimento de vacinas. (NOAZIN et al., 2008; OKWOR; UZONNA, 2009; AMEEN, 2010; MANSUETO et al., 2014). A tentativa de vacinação contra a leishmaniose cutânea remonta a centenas de anos, e é alvo de experiências na América Latina desde o início do século vinte. As duas principais tentativas de vacinas avaliadas no Brasil foram a pentavalente de Mayrink e colaboradores, conhecida como Leishvacin®, e a vacina monovalente simplificada de *L. amazonensis*. Os estudos foram inconclusivos pela ausência de um grupo placebo e a eficácia da vacina não pôde ser avaliada (MAYRINK et al., 2002; NOAZIN et al., 2008; GOTO; LINDOSO, 2010).

A presença de uma resposta imune induzida no indivíduo pela infecção confirma a viabilidade da prevenção da LTA através da vacinação profilática (MUTISO et al., 2013). Embora não haja nenhuma vacina licenciada contra qualquer forma de leishmaniose humana para uso geral, tem havido numerosas tentativas para desenvolver uma vacina eficaz contra a leishmaniose e há várias categorias de vacinas candidatas. Dessa maneira, as que estão em desenvolvimento estão divididas em: vacina *Leishmania* viva, a Leishmanization; vacinas de

primeira geração que consistem de *Leishmania* inteira morta ou frações do parasito; as vacinas de segunda geração, que incluem vacinas de proteínas recombinantes, vacinas de DNA e as suas combinações; e as vacinas de *Leishmania* vivas atenuadas (HANDMAN, 2001; NOAZIN et al., 2008; OKWOR; UZONNA, 2009; COSTA et al., 2011; MUTISO et al., 2013). Os mecanismos imunológicos da LTA humana ainda não são totalmente compreendidos e as respostas observadas na proteção por vacinação em modelos experimentais de infecção podem não refletir as respostas necessárias para a sua eficácia em áreas endêmicas (HANDMAN, 2001; NOAZIN et al., 2008; MUTISO et al., 2013). Existem ainda muitos desafios para que vacinas para prevenção da LTA se tornem uma realidade.

3.4 - Critério de Cura da Leishmaniose Tegumentar Americana

Na forma cutânea da LTA, o critério de cura é baseado no reestabelecimento clínico pela reepitelização completa das lesões ulceradas ou não, regressão total da infiltração e eritema, até três meses após o tratamento (figura 6). Na forma mucosa, é definido pela regressão de todos os sinais, comprovado pelo exame otorrinolaringológico, até seis meses após o tratamento (GONTIJO; CARVALHO, 2003; MAYRINK et al., 2006; MS, 2010; OMS, 2010).

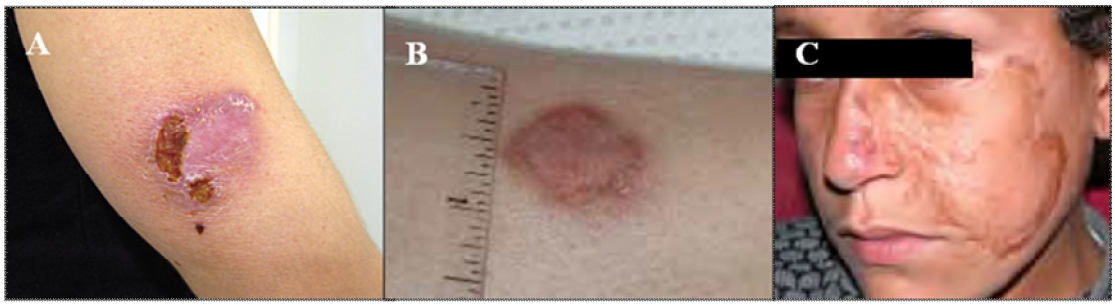


Figura 6: Cicatrizes deixadas pelas lesões da leishmaniose tegumentar americana. A- lesão em processo de cicatrização, B e C- lesões completamente reepitelizadas.
Fonte: figura A, Sorci et al., 2003; figura B, Pimentel et al., 2011; figura C, OMS, 2010.

Distintas abordagens têm sido sugeridas como critério de cura após o tratamento da LTA, dentre elas, temos a utilização de testes parasitológicos, como o cultivo e o isolamento do parasito (AMATO et al., 1998; SCHUBACH et al., 1998; MENDONÇA et La., 2004). À vista disso, supondo que a resposta imune humoral possa desempenhar um papel modulador

da resposta celular durante a infecção, Bitterncourt et al (1968) sugeriram a possibilidade da pesquisa de anticorpos no monitoramento de cura da LTA. Os autores estudaram a reatividade de anticorpos anti-*Leishmania* através da sorologia convencional pela IFI e sugeriram como critério de cura pós-terapêutica na LTA.

Ao avaliar a possibilidade de utilizar a IFI como critério de cura da LTA, estudos apontaram a diminuição dos níveis de anticorpos em pacientes após o tratamento com antimoniais, propondo a existência de correlação entre a cura clínica das lesões e a negativação dos títulos da IFI (AMATO et al., 1998; SCHUBACH et al., 1998). Em estudos análogos, a atenção tem se voltado para a perspectiva de utilização do teste de ELISA, na avaliação do valor preditivo da persistência de anticorpos IgG, durante o desenvolvimento da LTA (MENDONÇA et al., 2004; ROMERO et al., 2005). A demonstração da persistência parasitária após a cura clínica da LTA desperta para o desenvolvimento de pesquisas voltadas para a evolução clínica, epidemiológica e para o controle da LTA. Contudo, a utilização desses testes para avaliação do critério de cura da LTA trata-se ainda de um assunto controverso, uma vez que ocorre a demonstração da persistência de positividade nos ensaios após cura, espontânea ou pós-terapêutica (MARTINS-FILHO et al., 1995; ROCHA et al., 2002; 2006; OLIVEIRA et al., 2013).

Até então, a dinâmica da produção de anticorpos após o tratamento com antimoniais não é conhecida o bastante, e valores preditivos de baixos ou altos níveis de anticorpos dirigidos contra antígenos específicos continuam indefinidos (OLIVEIRA et al., 2013; PEREIRA et al., 2012; ROMERO et al., 2005). A dificuldade pode ser atribuída à falta de uma preparação antigênica padrão, para ser usada na detecção de anticorpos específicos. Com isso em mente, Rocha et al. (2002), utilizando a citometria de fluxo, perceberam suas aplicabilidades para o diagnóstico. A técnica surge como uma nova abordagem, para ser empregada na pesquisa de anticorpos, dado que possui um sistema isento de variabilidades metodológicas inerentes ao analista, e tem eficácia superior aos diferentes protocolos de detecção convencionais. Ademais, é cabível ao diagnóstico e critério de cura pós-terapêutica de diferentes infecções, dentre elas a LTA.

3.5- Resposta Imune na Leishmaniose Tegumentar Americana

A resposta imunológica desempenha um importante papel na cura clínica da doença ou na sua progressão. Embora altos títulos de anticorpos possam ser encontrados em todas as manifestações clínicas da LTA, essa patogênese, parece depender da resposta imune mediada por células no hospedeiro, influenciando o desenvolvimento da doença, seja pelo tipo de linfócitos T efetores envolvidos, ou pelo perfil de citocinas secretadas (MOUGNEAU; BIHL; GLAICHENHAUS, 2011; DA-CRUZ et al., 2005).

Enquanto os primeiros estudos demonstraram um papel crítico das células T CD4+ na imunidade dirigida à *Leishmania*, trabalhos recentes têm proporcionado uma nova visão sobre o papel das células da imunidade inata, tais como neutrófilos, monócitos, NK e células dendríticas (MOUGNEAU; BIHL; GLAICHENHAUS, 2011). Os neutrófilos são uma das primeiras células a chegar ao local da infecção, sendo infectados pelas *leishmanias.* Eles também podem ser considerados como “cavalos de troia", já que facilitam a infecção silenciosa dos macrófagos ao fagocitarem neutrófilos contaminados (NYLEEN; GAUTAM, 2010; NYLÉN; EIDSMO, 2012; SANTOS; BRODSKYN, 2014). Junto com fagócitos, as células NK, células exterminadoras naturais, representam a primeira linha de defesa contra os patógenos, através da produção de citocinas pró-inflamatórias TNF e IFN- γ. As células dendríticas são um importante componente de ligação da resposta imune inata e adaptativa, através do reconhecimento da infecção parasitária e da apresentação celular dos parasitos e/ou de seus antígenos (NYLEEN; GAUTAM, 2010; SOUZA et al., 2013; SANTOS; BRODSKYN, 2014). Além desses mecanismos de resposta imune inata, tem-se também o sistema complemento, que desempenha papel pró-inflamatório, sendo ativado pela via clássica e alternativa na leishmaniose (MOUGNEAU; BIHL; GLAICHENHAUS, 2011).

A maior parte dos conhecimentos de imunologia na LTA, sobretudo em relação à interação parasito-hospedeiro, são provenientes dos estudos de infecções em modelos animais, e o camundongo é o modelo extensivamente recorrido, na tentativa de se identificar e dissecar a contribuição de genes modificadores da doença para a patogênese (MOUGNEAU; BIHL; GLAICHENHAUS, 2011; LORÍA-CERVERA; ANDRADE-NARVÁEZ 2014). Embora, o camundongo apresente a doença semelhantemente aos humanos, existem limitações, como a variação genética generalizada presente na população humana que não se limita à variação genética nos camundongos puros. Além de que, não existe um modelo para LM. Ademais, a via de inoculação é diferente em condições experimentais, que é por via subcutânea ou intravenosa e que não pode ser comparada à picada do flebotomíneo vetor, na

qual ocorre a inoculação de grande número de parasitos e existe a presença da saliva ativando o sistema imune (SAKTHIANANDESWAREN; FOOTE; HANDMAN 2009; MOUGNEAU; BIHL; GLAICHENHAUS, 2011). Em estudos realizados em camundongos, a resposta imune mediada por células T na infecção por *L (V.) braziliensis* é pouco caracterizada. Isso acontece em razão da resistência natural de muitas linhagens de camundongos frente à infecção, sendo necessária uma grande carga parasitária para desencadear a mesma. Consequentemente, devido à dificuldade em estabelecer modelos animais adequados para se estudar a *L. (V.) braziliensis*, surgiram os estudos com pacientes (LIMA et al, 1999; MOURA et al, 2005; REIS et al, 2006).

Na leishmaniose humana e experimental, e em todas as formas clínicas da doença, a imunidade é predominantemente mediada por linfócitos T, de maneira que, a resposta linfocitária na LTA é caracterizada principalmente pelo aumento de células T CD4+. Há mais de 20 anos, se aceita que a diferença entre a resistência e a susceptibilidade à infecção está associada ao nível de expansão de células T CD4+, com direcionamento para um perfil de citocinas Th1 e Th2, respectivamente (MOUGNEAU; BIHL; GLAICHENHAUS, 2011; SOUZA et al., 2013). A interleucina-12 (IL-12), produzida por macrófagos e células dendríticas, o interferon-gama (IFN-γ) produzido pelas células NK, e as células T previamente ativadas promovem o desenvolvimento de células Th1, enquanto que a IL-4 induz o desenvolvimento de células Th2. Linfócitos T CD4+ que apresentam o perfil Th1 produzem IFN-γ e fator de necrose tumoral - alfa (TNF-α), assim como também expressam o fator de transcrição T-bet. Por outro lado, as células que apresentam o perfil Th2 produzem IL-4, IL-5, IL-10, IL-13, e expressam o fator de transcrição GATA-3 (SAKAGUCHI et al., 2008; REIS et al., 2006; SOUZA et al., 2013).

Outros subtipos de células T CD4+, como células Treg e células Th17, também parecem apresentar papel importante na susceptibilidade e resistência à LTA. As células T regulatórias (Treg), que se acumulam no local da lesão, estão relacionadas à regulação das células efetoras locais, e podem ser naturais ou induzidas. Citocinas, como IL- 10 e TGF-β, e substâncias como o ácido retinoico facilitam a diferenciação de células T *naïve* em Treg induzidas que expressam o fator de transcrição Foxp3 (NYLÉN; EIDSMO, 2012; SANTOS; BRODSKYN, 2014). Estudos recentes têm evidenciado também o subtipo celular, conhecido como Th17, relacionado com a produção de IL-17. A princípio, a IL-17 está envolvida com a patogênese de doenças inflamatórias crônicas ou autoimunes (BACELLAR et al., 2009; NYLEEN; GAUTAM, 2010; NYLÉN; EIDSMO, 2012; SANTOS; BRODSKYN, 2014).

Embora estudos avaliem a resposta humoral na LTA, ainda não está completamente esclarecido o papel de anticorpos específicos na imunidade contra *Leishmania* (TRUJILLO et al., 1999; SOUZA et al., 2005). Na última década, estudos apresentados, principalmente em modelos experimentais, apontam que as células B, e seus anticorpos contribuem para a susceptibilidade e doença na LTA. Entretanto, os anticorpos, em geral, não têm sido considerados como fator importante na resistência à doença (SACKS et al., 1984; SACKS; NOBEN-TRAUTH, 2002; KEDZIERSKI; EVANS, 2014). Sacks et al., (1984), constataram que a depleção de células B, observando anticorpos anti-IgM, resultou em melhora na resistência para *Leishmania* em camundongos BALB/c. Além disso, o estudo também implica que, durante a infecção por *Leishmania*, em camundongos com depleção de células B, células T supressoras não são produzidas. A importância das células B para o desenvolvimento e susceptibilidade de respostas de células Th2 foi observada a partir da capacidade dessas células em apresentar antígenos a células T. Além disso, tem sido demonstrado que a IL-10 produzida por células B pode desempenhar um papel na susceptibilidade à infecção cutânea por inibição da produção de IL-12 por células dendríticas *in vitro* (RONET et al., 2008; 2010; KEDZIERSKI; EVANS, 2014).

Alguns tipos celulares, como macrófagos, possuem receptores de superfície para a porção Fc dos anticorpos IgG, os receptores Fc. A associação dos receptores Fc-IgG com macrófagos induz a produção de IL-10 e impulsiona o desenvolvimento alternativo de macrófagos ativados com fraca capacidade leishmanicida (NYLÉN; EIDSMO, 2012). Diante disso, é de conhecimento que a classe IgG não só oferece proteção contra o parasito, como também contribui para a progressão da infecção (SOUZA et al., 2013).

Há um consenso geral de que as células T e a imunidade mediada por células contribuem para a patogênese das diferentes formas de LTA. Isso acontece devido à influência do padrão de citocinas na ativação, proliferação e diferenciação de linfócitos B em células produtoras de imunoglobulinas (TRUJILLO et al., 1999; SOUZA et al., 2005; SOUZA et al., 2013). Estudos realizados em humanos, sugerem que citocinas do perfil Th1, produtoras de IFN-γ, induzem a produção da imunoglobulina G1 (IgG1) e IgG3 em humanos (OZBILGE et al., 2006). Quando associado a IL-6, o IFN- γ induz a produção de IgG2 e antagoniza a produção de IgG1 (KAWANO et al., 1994). É notório, que a produção de IgG3, IgG4 e IgE está associada à IL-4, e sua inibição está associada ao IFN-γ, enquanto o aumento dos níveis de IgG1 e IgG3 está associado ao IL-10 (BUXBAUM, 2008; SKEIKY et al.,1997). No entanto, os níveis de IgA dependem de TGF-β (STAVNEZER; KANG, 2009).

Nas leishmanioses cutânea (LC) e mucocutânea (LM), a imunidade celular e a predominância de isotipos IgG1, IgG2 e IgG3 têm sido associadas à resposta do tipo Th1; já o perfil Th2 tem sido relacionado à leishmaniose cutânea difusa (LD), com presença de IgG4 (SOUZA et al., 2005), direcionando a atenção para a correlação das subclasses de IgG com as manifestações clínicas da LTA. Estudos demonstram que todos os isotipos específicos anti-*Leishmania*, exceto IgD, são detectados no soro de pacientes com LTA. A frequência de detecção das subclasses de IgG na LTA, de acordo com Pissinate et al., (2008), é IgG1 > IgG3 > IgG2 = IgG4. Os pacientes com um maior tempo de evolução da doença apresentam altos níveis de IgE, e nos pacientes com a forma mucocutânea, os níveis de IgA se mostram aumentados (O'NEIL et al., 1993). A intensidade da resposta humoral parece estar relacionada com a carga parasitária e a cronicidade da infecção, e podem ser observados altos títulos de anticorpos em todas as manifestações clínicas da LTA (TRUJILLO et al., 1999; OZBILGE et al., 2006).

A análise da resposta imune humoral na LTA tem abordado o papel das imunoglobulinas em mecanismos imunopatológicos envolvidos na resistência e/ou patogênese da infecção; além disso, também avaliam o uso em investigações sorológicas no diagnóstico e no monitoramento da eficácia pós-tratamento (REIS et al., 2006; PEREIRA et al., 2012; OLIVEIRA et al., 2013). A associação entre o perfil da resposta imune humoral e a patogênese de um dado processo infeccioso tem sido feita em várias doenças, tais como hanseníase, AIDS, filariose e malária (KURNIAWAN et al. 1993, HUSSAIN et al. 1995, OUAAZ et al. 1996, RODRIGUEZ, 1996; PERLMANN et al. 1997). No entanto, recentemente, tem-se observado que os métodos imunossorológicos disponíveis para pesquisa de anticorpos na LTA apresentam resultados controversos, devido a sua baixa sensibilidade e especificidade (RIBEIRO et al., 2007; BOURDOISEAU et al., 2009; MUKBEL et al., 2006). Diante disso, estudos têm mostrado a vantagem do uso de anticorpos específicos no diagnóstico da leishmaniose visceral (SOUZA et al., 2013; PASSOS et al., 2005; OZBILGE et al., 2006; CHATTERJEE et al., 1998).

3.6 - Diagnóstico Clínico da Leishmaniose Tegumentar Americana

A infecção humana por LTA pode ser assintomática ou apresentar um espectro de formas clínicas que podem variar, aparecendo desde lesões cutâneas localizadas, disseminadas, difusas ou nodulares, até as graves lesões mucocutâneas (Figura 4)

(GRIMALDI; TESH, 1993). As lesões cutâneas podem ainda apresentar-se morfologicamente como: impetigoide, liquenoide, tuberculosa ou lupoide, nodular, vegetante e ectimatoide, o que dificulta ainda mais o diagnóstico clínico. Nas lesões mucocutâneas, podem ser observadas úlceras infiltrantes ou úlceras vegetantes (ANDRADE et. al, 2005). As lesões podem ser confundidas tanto com doenças infecciosas, comoparacoccidioidomicose, histoplasmose e sífilis; quanto com doenças neoplásicas, como carcinoma e linfoma (ÁVILA et. al, 2004).

O diagnóstico clínico da LTA pode ser feito com base nas características da lesão associadas à anamnese, onde os dados epidemiológicos são de grande importância. São frequentes as ulcerações com bordas elevadas em moldura, fundo com tecido granuloso e indolores. A apresentação clínica exibe polimorfismo e o espectro de gravidade dos sinais e sintomas é variável, o que torna o diagnóstico clínico difícil (GONTIJO; CARVALHO, 2003; MS, 2007). Devido à dificuldade encontrada para o diagnóstico clínico seguro, os métodos laboratoriais fazem parte do arsenal de investigação.

3.7 – Diagnóstico Laboratorial da Leishmaniose Tegumentar Americana

As diferentes espécies de *Leishmania* envolvidas, as formas clínicas e o tempo de evolução das lesões levam à variabilidade das manifestações da infecção e direcionam o diagnóstico para os métodos laboratoriais. A associação das evidências clínicas, epidemiológicas e laboratoriais é necessária para se chegar ao diagnóstico final da LTA, pois ainda não se dispõe de um método que possa ser usado como padrão-ouro para detecção e diagnóstico de infecção por *Leishmania* (BRITO et al., 2000; REIS, 2007). Os exames laboratoriais podem ser divididos em técnicas de detecção do parasito, técnica molecular e técnicas de imunodiagnósticos (Figura 7).

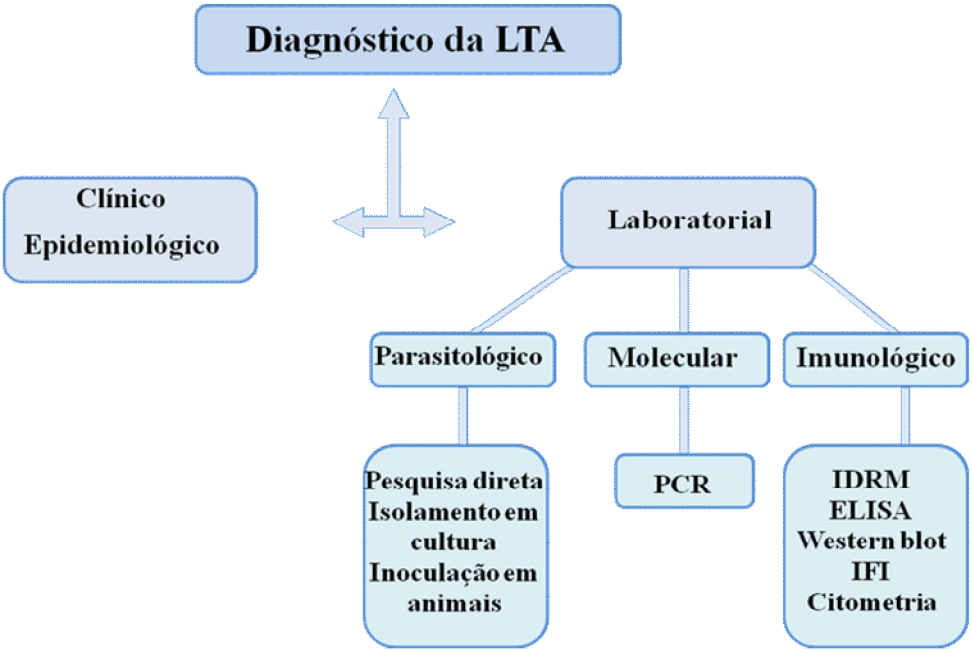


Figura 7: Organograma com diagnóstico da LTA.
Fonte: Da autora.

O diagnóstico de certeza de um processo infeccioso é feito pelo encontro do parasito, através da pesquisa direta, isolamento em cultura, inoculação em animais experimentais (MACHADO, 2004; MS, 2007). A pesquisa direta do parasito pode ser realizada em material obtido da lesão por escarificação, aspiração, ou biópsia da borda da lesão. A visualização em microscópio óptico da forma amastigota de *Leishmania* sp é possível após coloração pelo método de Giemsa ou Leishman (GENARO, 2000). Essa técnica tem sensibilidade de 50 a 70% e depende do número de parasitos presentes na lâmina (GOTO; LINDOSO, 2010). A positividade do teste é inversamente proporcional ao tempo de evolução da lesão cutânea, sendo rara após um ano. Embora seja uma técnica simples, rápida e de baixo custo, não é capaz de detectar parasitos em todos os pacientes (BENSOUSSAN et al., 2006; VEGA-LÓPEZ, 2003).

O isolamento do parasito pode ser feito através da cultura em meios apropriados, como o meio bifásico Novy e McNeal modificado por Nicolle-NNN (Novy & MacNeal 1903, Nicolle 1908), Liver Infusion Tryptose- LIT (Camargo 1964) e/ou Schneiders, suplementado com soro fetal bovino inativado, a partir de material obtido por punção aspirativa ou biópsia das lesões dos pacientes. A sensibilidade do isolamento em cultura é geralmente baixa, em torno de 20 a 40%. A baixa sensibilidade da cultura está relacionada, em muitos casos, à escassez do parasito nas lesões, principalmente *L.* (*V.*) *braziliensis* (BRANDÃO-FILHO et al., 1999; BENSOUSSAN et al., 2006; RODRÍGUEZ-GONZÁLEZ et al., 2006; REITHINGER

et al., 2007). A inoculação em animais susceptíveis para cultivo *in vivo* tem o hamster (*Mesocricetus auratus*) como animal mais usado. Porém, o longo período de acompanhamento até a formação da úlcera no animal e os elevados custos para manutenção dos animais limitam o método às instituições de pesquisa científica; tornando-o de pouco valor prático para a rotina laboratorial (RODRIGUES, 2000; BENSOUSSAN et al., 2006; MS, 2007).

A Reação em Cadeia da Polimerase PCR convencional é uma técnica que permite a amplificação de segmentos específicos de DNA a partir de oligonucleotídeos iniciadores *(primers)* pareados especificamente nas margens da região alvo, permitindo o seu uso como instrumento específico para o diagnóstico de diversas doenças infecciosas (GOMES et al., 1999; RODRIGUES, 2000; 2002; SINGH, 1997). Embora várias abordagens moleculares tenham sido desenvolvidas para o diagnóstico da leishmaniose e a PCR seja uma técnica altamente sensível e específica, trata-se de um teste sofisticado para uso na rotina laboratorial, com exigências técnicas e custo elevado, limitando-se a laboratórios de referência e/ou a clínicas médicas (REITHINGER et al., 2007; SOUSA et al., 2013).

O diagnóstico imunológico baseia-se na avaliação da resposta imune celular utilizando a técnica de Intradermorreação de Montenegro (IDRM) e/ou na avaliação da resposta imune humoral utilizando as técnicas de imunofluorescência indireta (IFI), ensaio imunoenzomático (ELISA), *western blot* e citometria de fluxo. A detecção da imunidade celular através da IDRM tem sido empregada como o importante recurso no diagnóstico imunológico da LTA, dado a sua grande sensibilidade e especificidade. O teste consiste na injeção de uma suspensão de antígeno preparado com promastigotas para a indução de resposta de hipersensibilidade tardia, onde a reação é lida após 48 a 72 horas (MACHADO, 2004; GOTO; LINDOSO, 2010). Embora apresente resultado positivo na maioria dos casos de LTA (90%), o resultado é negativo em lesões recentes, na forma cutânea difusa e em pacientes imunodeprimidos. Em áreas endêmicas, é comum o teste ser positivo devido à ocorrência de infecções subclínicas (VEGA-LÓPEZ, 2003; REIS, 2007). Além disso, o teste não diferencia infecção de doença e nem doença ativa de pregressa (WEIGLE et al., 2002; REITHINGER et al., 2007; GOTO; LINDOSO; 2010).

A avaliação da resposta imune humoral na LTA tem sido geralmente abordada através de testes sorológicos, e os mais utilizados atualmente são a IFI, o ELISA e o *Western blot*. Esses testes apresentam controvérsias no diagnóstico LTA, principalmente considerando a baixa sensibilidade, especificidade e pouca reprodutibilidade. Além disso, podem apresentar reações cruzadas com outros tripanosomatídeos. Sabe-se ainda que baixos níveis de

anticorpos são observados por essas técnicas, e que ocorre ausência de correlação entre níveis de anticorpos circulantes com a presença de infecção ativa (KAR, 1995; PASSOS et al., 2000; BRITO et al., 2000; 2001; SCHALLIG; OSKAM, 2002; SAVANI et al., 2003; ALVES; BEVILACQUA, 2004).

A IFI é uma técnica amplamente utilizada no diagnóstico da LTA, na qual são observadas, frequentemente, reações cruzadas com outros tripanosomatídeos, ocorrendo falso-positivos com soros de pacientes portadores de doença de Chagas e leishmaniose visceral, por exemplo (VEXENAT et al. 1996, BRITO 2008), bem como com soros de pacientes portadores de hanseníase e de tuberculose (KAR, 1995). Além disso, ocorre grande variabilidade de resultados em técnicas sorológicas que dependem da natureza do antígeno, que pode estar associada aos diferentes antígenos utilizados e, principalmente, pela forma como foram produzidos e purificados (TAVARES et al. 2003). O ELISA consiste em uma metodologia de realização simples e rápida, bastante utilizada nas investigações científicas. Embora apresente maior sensibilidade em relação à IFI, tem sido menos utilizado na rotina laboratorial por apresentar custo mais elevado e exigências laboratoriais superiores à IFI (MACHADO, 2004; ROMERO et al., 2005). O *Western blot* tem sido mais utilizado no diagnóstico da leishmaniose visceral (KAR, 1995). Em estudo comparativo, Brito (1999) demonstrou que a técnica de *Western blot* apresentou maior sensibilidade e especificidade em relação às técnicas de IFI e ELISA.

3.7.1 – Citometria de Fluxo no diagnóstico da Leishmaniose Tegumentar Americana

Um aspecto limitante em relação ao diagnóstico da LTA no homem é a ausência de um teste capaz de identificar com eficiência os casos com formas leves da doença (tabela 2). Testes diagnósticos eficientes, capazes de proporcionar um diagnóstico precoce e preciso, são essenciais para determinar a escolha do tratamento na vigilância epidemiológica de doenças infecciosas; e as abordagens sorológicas para detectar anticorpos específicos contra um agente infeccioso constituem-se em uma alternativa valiosa para um diagnóstico precoce. Diante disso, vários esforços têm sido feitos objetivando o desenvolvimento de uma abordagem sorológica confiável para o diagnóstico da LTA usando preparações de antígenos para detectar anticorpos anti-*Leishmania* (SOUSA et al., 2013; KOTRESHA; NOORDIN, 2010; PASSOS et al., 2005; GONÇALVES et al., 2002; RYAN et al., 2002; SILVEIRA et al., 2001). Um método confiável para o diagnóstico baseado na citometria de fluxo para detecção de anticorpos antiformas de *L. (V.) braziliensis* foi descrito por Rocha et al. (2002; 2006).

Tabela 2: Vantagens e desvantagens dos métodos utilizados para o diagnóstico laboratorial da LTA.

TESTES LABORATORIAIS	VANTAGENS	DESVANTAGENS
PESQUISA DIRETA	Técnica simples, rápida e de baixo custo; Sensibilidade de 50 a 70%; Visualização do parasito.	Não detecta parasitos em todos os pacientes; Sensibilidade depende do número de parasitos na lâmina; Sensibilidade inversamente proporcional ao tempo de evolução da lesão cutânea.
ISOLAMENTO DO PARASITO EM CULTURA	Visualização do parasito	Sensibilidade geralmente baixa, em torno de 20 a 40%. Escassez dos parasitos nas lesões.
INOCULAÇÃO EM ANIMAIS	Visualização do parasito	Longo período de acompanhamento até a formação da úlcera no animal; Elevados custos para manutenção dos animais; Pouco valor prático para a rotina laboratorial.
PCR	Permite a amplificação de segmentos específicos de DNA; Técnica sensível e específica;	Teste sofisticado para uso na rotina laboratorial; Exige técnicas e custo elevado; Limitado a laboratórios de referência e/ou a clínicas médicas.
IDRM	Grande sensibilidade e especificidade; Resultado positivo na maioria dos casos de LTA (90%).	Resultado negativo em lesões recentes, na forma cutânea difusa e em pacientes imunodeprimidos; Em áreas endêmicas, é comum o teste ser positivo devido à ocorrência de infecções subclínicas; Não diferencia infecção de doença; Não diferencia doença ativa de pregressa.
ELISA, IFI e WESTERN BLOT	Avaliação da resposta humoral; A IFI é amplamente utilizada no diagnóstico da LTA; O ELISA é de um teste com realização simples e rápida.	Baixa sensibilidade, especificidade e pouca reprodutibilidade dos testes; Reações cruzadas com outros tripanosomatídeos; Ausência de correlação entre níveis de anticorpos circulantes com a presença de infecção ativa; Reações cruzadas com outras doenças, como hanseníase e tuberculose, pela IFI; O Western blot é mais utilizado no diagnóstico da leishmaniose visceral; O ELISA menos utilizado na rotina laboratorial por apresentar custo mais elevado e exigências laboratoriais superiores à IFI.
CITOMETRIA DE FLUXO	Análise rápida, objetiva e quantitativa de células em suspensão; As células são marcadas com anticorpos monoclonais específicos ligados a fluorocromos; Identificação e a quantificação de células pelo tamanho, granulosidade e intensidade de fluorescência; Várias vantagens para os imunoensaios, tais como capacidade de produção elevada possibilidade de quantificação do analito, volume de amostra reduzida, elevada reprodutibilidade e sensibilidade, uma ampla faixa dinâmica e, sobretudo, potencial para multiplexagem; Método de maior sensibilidade e especificidade; Aplicação diagnóstica e como critério de cura na LTA.	

A citometria de fluxo tornou-se uma ferramenta importante no diagnóstico e prognóstico de diferentes doenças, pois é um recurso que permite uma análise rápida, objetiva e quantitativa de células em suspensão (FALDYNA et al., 2001;STETLER-STEVENSON, 2003; NAKAGE et al., 2005). As células da amostra em suspensão são marcadas com anticorpos monoclonais específicos ligados a fluorocromos, que permitem a identificação e a quantificação de células pelo tamanho, granulosidade e intensidade de fluorescência. Essa técnica apresenta várias vantagens para os imunoensaios, tais como capacidade de produção elevada, possibilidade de quantificação do analito, volume de amostra reduzida, elevada reprodutibilidade e sensibilidade, uma ampla faixa dinâmica e, sobretudo, potencial para multiplexagem (SOUSA et al., 2013; JANI et al., 2002).

Considerando as aplicabilidades da citometria de fluxo descritas por Martins- Filho et al., (1995), o presente trabalho busca uma metodologia alternativa através da pesquisa de anticorpos IgG de *L. (V.) braziliensis*. Dessa forma, acreditamos que a aplicação da citometria de fluxo, na detecção de LTA em pacientes antes e após-tratamento, e também curados espontaneamente, possa contribuir para a obtenção de um método de maior sensibilidade e especificidade, com aplicação diagnóstica e como critério de cura na LTA.

4 MATERIAL E MÉTODOS

4.1- Tipo de Estudo

O estudo foi do tipo experimental ou ensaio clínico não randomizado. Este ensaio baseou-se na comparação entre um grupo de participantes sujeitos à intervenção, e outro grupo formado por sujeitos não expostos à intervenção, denominado "controle" (figura 8). Ambos foram escolhidos a partir de critérios de disponibilidade ou de conveniência (ROUQUAYROL; ALMEIDA FILHO, 2003).

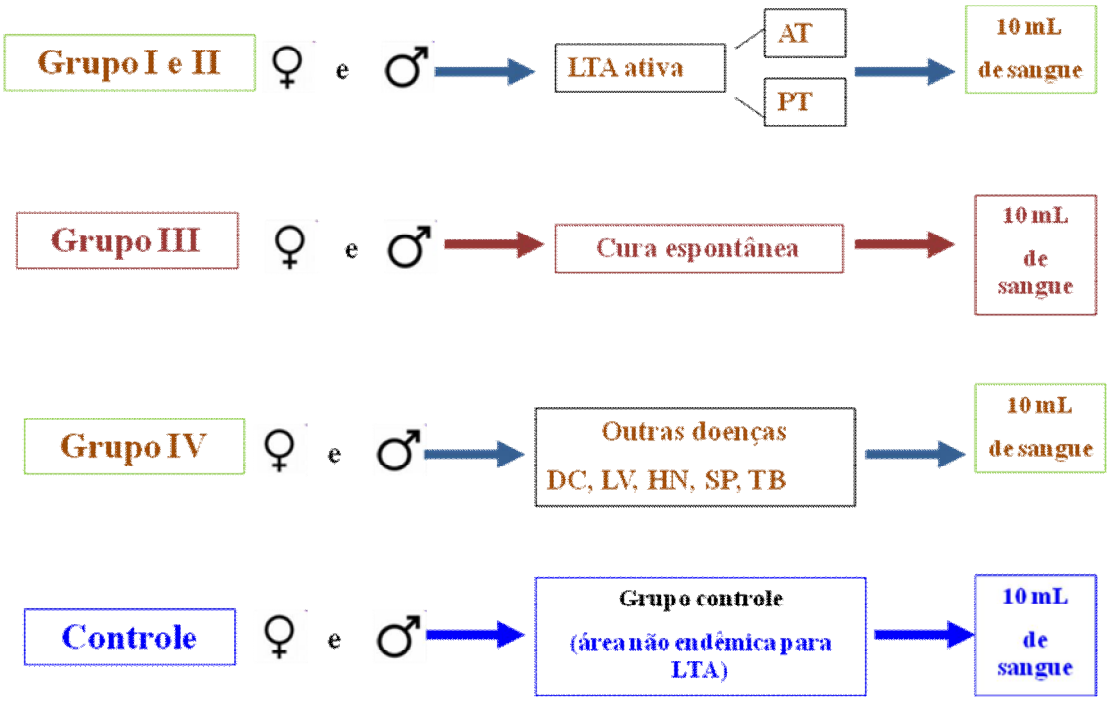


Figura 8: Fluxograma de coleta de amostras. AT= antes do tratamento; PT= após o tratamento; DC= doença de chagas; LV= leishmaniiose visceral; HN= hanseníase; SP= esporotricose; TB= tuberculose.

4.2- Critérios de Inclusão

Foram considerados critérios de inclusão, a presença de lesão cutânea ativa, e ausência de tratamento quimioterápico prévio. A confirmação do diagnóstico foi realizada por critérios clínicos, epidemiológicos e pelo menos dois testes laboratoriais. O histórico de doença

pregressa e apresentação de cicatriz característica da lesão (aspecto de pergaminho), e ausência de quimioterapia foram os critérios para inclusão no grupo de cura espontânea.

4.3- Critérios de Exclusão

Como critérios de exclusão foram considerados a ausência de cicatriz, tratamento quimioterápico prévio para LTA, e presença de lesão de outras doenças dermatológicas.

4.4- População de estudo

Os pacientes do presente estudo foram procedentes de Moreno, e municípios vizinhos, como Camaragibe (Região Metropolitana de Recife- RMR), Vitória de Santo Antão e Vicência, áreas endêmicas de LTA em Pernambuco. Foram selecionados os pacientes suspeitos que apresentavam lesão e história clínica compatível com a doença, os quais foram encaminhados ao ambulatório de Dermatologia do Hospital Universitário de Pernambuco. Os pacientes foram esclarecidos do objetivo do estudo e foram realizados os procedimentos de biópsia, punção aspirativa, escarificação na borda da lesão. Foram feitas as coletas de 10 ml de sangue antes e após tratamento quimioterápico com Glucantime® (antimoniato de N-metilglucamina), e em pacientes que tiveram a cura espontânea e que não receberam tratamento. O critério de cura clínica para os pacientes tratados ou curados espontaneamente foi estabelecido considerando-se a completa cicatrização da lesão. O tratamento quimioterápico foi realizado nos postos de saúde dos municípios deste estudo, utilizando-se o Glucantime® (antimoniato de N-metilglucamina), administrado por via intramuscular. O tratamento foi feito em ciclos de 20 dias em doses diárias de 15mg/Kg e acompanhado durante três meses até a completa cicatrização. Foram formados quatro grupos de pacientes: i) portadores de lesões ativas com faixa etária superior a 13 anos; ii) pacientes com cura clínica após (um, dois e cinco anos) de tratamento; iii) pacientes com cura clínica espontânea, ou seja, sem tratamento prévio com quimioterápico e iv) pacientes com outras doenças não leishmanióticas (Doença de Chagas, leishmaniose visceral, esporotricose, tuberculose e hanseníase). O material coletado foi processado no Laboratório de Imunogenética do Departamento de Imunologia do Centro de Pesquisas Aggeu Magalhães-FIOCRUZ, na cidade de Recife-PE. Os protocolos experimentais foram submetidos e aprovados pelo Comitê de

Ética do CPqAM/FIOCRUZ, parecer nº 011/2013, registro no CAEE 11083812.7.0000.5190 (anexo 1).

4.5- Grupo Controle

O grupo controle foi constituído por indivíduos considerados saudáveis, residentes em área não endêmica, não gestante, sem tratamento crônico, não fumante, não receptores de transfusão sangüínea e sem história prévia da doença.

4.6- Exames Laboratoriais de Avaliação dos Pacientes

Além da avaliação clínica e epidemiológica, os pacientes foram submetidos a alguns procedimentos laboratoriais para confirmação da doença. Os exames foram realizados em colaboração com o Serviço de Referência em Leishmanioses CPqAM/FIOCRUZ/PE e incluíram: pesquisa direta; punção aspirativa; biópsia; teste de intradermorreação de Montenegro (IDRM); reação em cadeia de polimerase (PCR) que utilizou *primers* específicos para o subgênero *Viannia*: B1 (5'- GGGGTTGGTGTAATATAGTGG-3') e B2 (5'-CTAATTGTGCACGGGGAGG-3') (DE BRUJIN; BARKER, 1992; SAMBROOK; FRITSCH; MANIATIS, 1989) e a e imunofluorescência indireta (IFI).

4.7- Obtenção de Soro

A partir do sangue coletado, o soro foi obtido após centrifugação a 400 X g por 5 minutos na temperatura ambiente. As amostras de soros foram inativadas a 56°C por 30 minutos e centrifugadas a 4°C, 1000 X g por 5 minutos. Após a centrifugação, o sobrenadante foi aliquotado e estocado a -20°C até sua utilização.

4.8- Reação de Imunofluorescência Indireta

As lâminas contendo a suspensão antigênica de promastigotas de *Leishmania* fixadas (*L. major* -like promastigotas / MHOM/BR/71/49) foram revestidas com 20 µl de amostras de soro diluídas na proporção de 1:20 a 1:320 em 0,01 M PBS pH 7,2. Dois soros padrão foram

adicionados (positivo e negativo) e incubados numa câmara úmida durante 30 minutos a 37 °C. Após a incubação, o excesso de soro foi removido das lâminas, lavando-as por imersão em PBS pH 7,2, três vezes com intervalos de 10 minutos. As lâminas foram secas com uma toalha de papel e incubadas numa diluição de 1:100 do anti-IgG humano (específicos da cadeia pesada) conjugado com isotiocianato de fluoresceína - FITC (Bio -Mérieux), preparado com o azul de Evans (40 mg), previamente diluído 1:10 em solução tampão de PBS. Após a reação, as lâminas foram lavadas três vezes em PBS durante 10 minutos e secadas com toalhas de papel. A montagem foi feita com glicerina tamponada pH 8,5 e, em seguida, a lâmina foi observada no microscópio de fluorescência, com objetiva de 250 X. Uma parte das reações de IFI foi realizada em colaboração com o laboratório de Soroepidemiologia e Imunobiologia do Instituto de Medicina Tropical da Universidade de São Paulo.

4.9- Obtenção das Formas Promastigotas Vivas e Fixadas de *L. (V.) braziliensis*

Inicialmente, formas promastigotas da cepa de referência (MHOM/BR/75/M2903) mantidas *in vitro* foram expandidas em meio Schneider´s até atingir a fase exponencial. Em seguida, os parasitos foram transferidos para tubos de polipropileno de 50mL, e foram submetidos a baixa centrifugação (7 X g) , 25°C por 10 minutos. Em seguida, os parasitos foram recuperados do sobrenadante, após 10 minutos em repouso na temperatura ambiente, e o sobrenadante foi transferido para outro tubo de 50mL e o sedimento desprezado. Os parasitos foram lavados em Salina tamponada contendo 10% de SFB, por três vezes, a 4°C, 871 X g durante 10 minutos, e o sedimento formado foi homogeneizado cuidadosamente. A alíquota de parasitos da suspensão foi contada e a concentração ajustada para os ensaios de imunofluorescência indireta e citometria de fluxo.

As formas promastigotas de *L. (V.) braziliensis* fixadas foram obtidas a partir das formas promastigotas vivas. Para o processo de fixação, o sedimento formado foi ressuspendido em solução fixadora MFF (10g de paraformaldeído, 10,2g de cacodilato de sódio, 6,65g de cloreto de sódio/L) e estocado 24h a 4°C. Após incubação, os parasitos foram lavados em Salina tamponada contendo 10% de SFB, por duas vezes. Em seguida, a concentração foi ajustada para o ensaio de citometria de fluxo.

4.10- Citometria de Fluxo

A análise da reatividade de anticorpos antiformas promastigotas de *Leishmania (V.) braziliensis* foi feita inicialmente pela seleção desses parasitos no gráfico FSC (canal de dispersão frontal) versus SSC (canal de dispersão lateral) em escala logarítmica, avaliando-se os parâmetros de tamanho e granulosidade ou complexidade interna, respectivamente (Figura 8). As formas promastigotas apresentaram uma distribuição característica e homogênea em gráficos de tamanho x granulosidade, permitindo o posicionamento do marcador sobre a região correspondente à população de interesse (R1). Utilizando histogramas de intensidade de fluorescência em função do número de parasitos, foi analisada a intensidade de fluorescência relativa apresentada pela população selecionada. Para cada amostra individual foram adquiridas informações relativas aos parâmetros de tamanho, granulosidade e intensidade relativa de fluorescência de 20.000 parasitos. Neste estudo, foi empregado o anticorpo marcado com FITC, correspondente à fluorescência do tipo 1 (FL1-fluorescência verde). Os resultados das análises de fluorescência apresentados pelos parasitos após incubação com soros obtidos de pacientes portadores leishmaniose tegumentar e soros controle foram expressos sob a forma de percentual de parasitos fluorescentes positivos (PPFP), observados para cada teste individual em relação ao controle do conjugado. O PPFP foi determinado para cada amostra através do estabelecimento de um limiar de negatividade em função da curva de fluorescência, obtida para o controle da ligação inespecífica do conjugado. Para cada experimento foi estabelecido um limiar de reatividade de, no máximo, 2% de PPFP para o controle interno da reação (controle do conjugado). Em seguida, empregando-se o mesmo marcador, foram obtidos os valores de PPFP para amostras individuais.

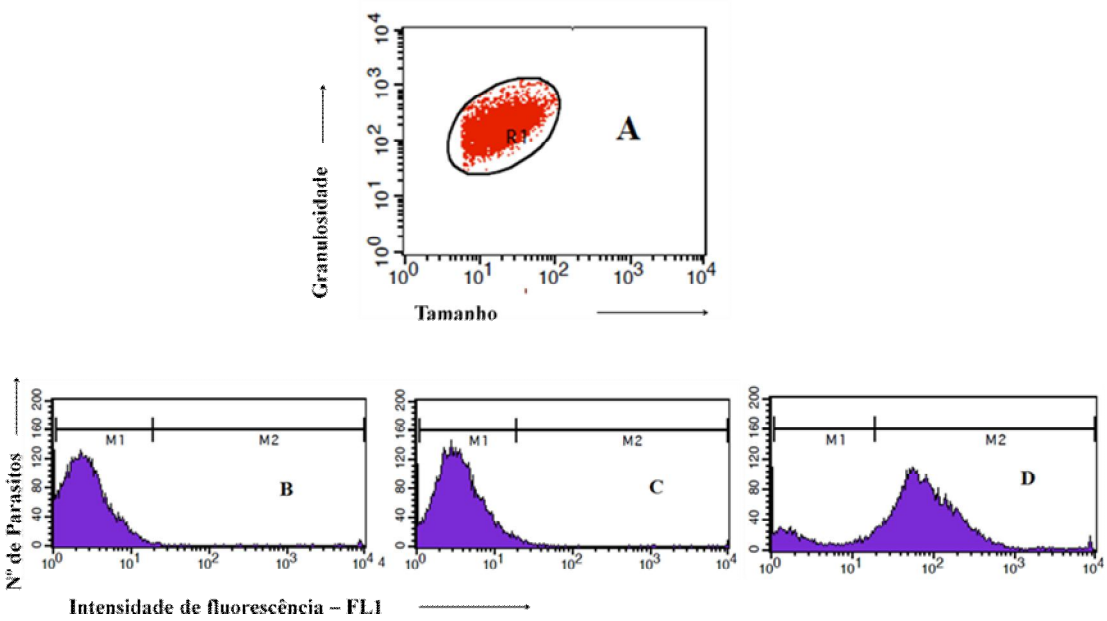


Figura 9: Seleção da população de formas promastigotas de *Leishmania (Viannia) braziliensis*, utilizando-se os parâmetros de tamanho e granulosidade (A). Histogramas individuais representando o percentual de parasitos fluoresecentes positivos (PPFP) obtidos com controle interno da reação (B), após a incubação com um soro de um indivíduo não infectado (C) e um soro de um paciente portador de LTA (D).

4.11- Análise Estatística

A análise estatística foi realizada para a avaliação do desempenho dos testes diagnósticos em estudo, para a definição do ponto de corte através da construção da *receiver operating characteristic curve* – curva ROC, avaliação dos índices de desempenho e intervalo de confiança. Os testes foram realizados utilizando o programa estatístico *MedCalc Statistical*. Todas as conclusões foram tomadas no nível de significância de 5%.

5 RESULTADOS E DISCUSSÃO

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Parasitology

Evaluation of anti-lived and anti-fixed *Leishmania* (*Viannia*) *braziliensis* promastigote IgG antibodies detected by flow cytometry for diagnosis and post-therapeutic cure assessment in localized cutaneous leishmaniasis ☆

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Resumo

Este estudo tem como objetivo investigar a metodologia de citometria de fluxo baseado no desempenho para detecção de anticorpos IgG de anti- promastigotas vivas (CF- AAPV -IgG) e fixadas (CF- AAPF -IgG) *Leishmania (Viannia) braziliensis* como um meio de monitorar cura pós- terapêutica de pacientes com leishmaniose cutânea localizada (LCL). As amostras de soros de 30 pacientes LCL infectados com *L. (V.) braziliensis* foram analisadas, comparando a reatividade de IgG antes e após o tratamento específico com antimonial pentavalente. A reatividade foi relatada com a porcentagem de parasitas fluorescentes positivos (PPFP), utilizando um PPFP de 60 %, como o ponto de corte. Na diluição 1:1.024 de soro, a porcentagem positiva de LCL amostra de soro para o CF- AAPV -IgG e CF-AAPF-IgG foi de 86% e 90%, respectivamente, antes do tratamento. Análise do ΔPPFP que representa a diferença entre PPFP antes e depois do tratamento apareceu como uma nova abordagem para controle pós-terapêutico da reatividade de IgG em LCL. Nossos dados apoiam a perspectiva do uso de CF- AAPV e CF- AAPF como uma ferramenta útil para o diagnóstico sorológico e para o acompanhamento pós-terapêutico de pacientes com LCL.

Abstract

This study aims to investigate a flow cytometry performance-based methodology to detect anti-live (FC-ALPA-IgG) and anti-fixed (FC-AFPA-IgG) *Leishmania* (*Viannia*) *braziliensis* promastigote IgG as a means to monitor post-therapeutic cure of patients with Localized Cutaneous Leishmaniasis (LCL). Serum samples from 30 LCL patients infected with *L. (V.) braziliensis* were assayed, comparing the IgG reactivity before and after specific treatment with pentavalent antimonial. Reactivities were reported as the percentage of positive fluorescent parasites (PPFP), using a PPFP of 60% as a cut-off. In the serum dilution 1:1,024, the positive percentage of LCL serum sample for FC-ALPA-IgG and FC-AFPA-IgG was 86% and 90%, respectively, before treatment. Analysis of ΔPPFP that represents the difference between PPFP after and before treatment appeared as a new approach to monitor post-therapeutic IgG reactivity in LCL. Our data support the perspective of using FC-ALPA and FC-AFPA as a useful serological tool for diagnosis and for post-therapeutic follow-up of LCL patients.

Keywords: Flow Cytometry, FC-ALPA, FC-AFPA, IgG, *L. (V.) braziliensis*, LCL.

1 Introduction

Leishmaniasis encompasses multiple clinical syndromes, most notably, visceral, cutaneous, and mucosal forms. Localized Cutaneous Leishmaniasis (LCL) is a disease associated with infections caused by several species of the genus *Leishmania* (Grimaldi; Tesh, 1993). Clinical manifestations depend on the parasite factors, the epidemiological characteristic of the vector and the host genetic and immunological constitution (Rogers et al., 2002). Cutaneous Leishmaniasis is a serious public health problem and is endemic to Brazil, particularly in the State of Pernambuco, where *Leishmania (Viannia) braziliensis* is known as the major circulating species (Brito et al., 2009).

At present, there is not a gold-standard test for Cutaneous Leishmaniasis and, frequently, a combination of different diagnostic techniques is needed to obtain more precise results. Thus, diagnosis is performed by the association of clinical, epidemiological and laboratorial aspects. These techniques include amastigote identification by tissue immunocytochemical techniques, “imprints” (printing by biopsy apposition), in aspirated

lesion and in histopathological evaluation, besides promastigote identification *in vitro*. The indirect immunofluorescence (IIF), ELISA (Enzyme-linked immunosorbent assay) and Western-blot, based on the presence of specific antibodies against parasite antigens, are the serological methods used. Montenegro skin test is a late hypersensitivity test based in the immunity mediated by cells. At the same time, there has been notable improvement in techniques such as polymerase chain reaction (PCR), Real-time PCR and flow cytometry with the objective of increasing sensitivity and specificity (Vega-Lopez, 2003).

The traditional laboratory methods have several limitations and present difficulties. Furthermore, immunoassays can show low antibody titers, due to a cross reactivity with *Trypanosoma cruzi* that depends on the antigen, as well as the lack of well-standardized procedures used to detect the specific antibodies. Moreover, antibody production after treatment is not yet clear and the predictive value of lower or higher levels against specific antigenic fractions during follow-up is not well defined (Brito et al., 2001).

Chemotherapy treatment is based, primarily, on the administration of pentavalent antimonials. These antimonials are highly toxic and administered via intramuscular injections for a prolonged period. Besides toxicity, one of the limitations in the treatment is the absence of an objective cure criterion. The cure criterion adopted by many authors is the complete healing of the lesion. Nevertheless, this is an unsatisfactory criterion, as the reactivation of the lesions may occur even after the treatment (Gontijo & Carvalho, 2003; Mendonça et al., 2004; Schubach et al., 1998). Therefore, these facts point to the need of new tools that can help determine prognostics and efficient diagnosis of Cutaneous Leishmaniasis.

More recently, a flow-cytometry analysis has been described for diagnosis purposes. The anti-live promastigote antibodies (FC-ALPA-IgG) detected through this method showed high sensitivity and specificity applicable in the diagnosis of LCL (Rocha et al., 2002; 2006). This study opens new perspectives for the use of FC-ALPA-IgG in the response evaluation of novel therapeutic protocols.

Based on that, in the present study we report the use of flow cytometry-based methods for the assessment of humoral response against live and fixed *Leishmania (Viannia) braziliensis* promastigote forms. Herein, we propose to investigate the performance of FC-ALPA-IgG and FC-AFPA-IgG (anti-fixed promastigote antibodies) to monitor post-therapeutic cure of LCL patients, comparing the IgG reactivity before and after specific treatment.

2 Population, Materials and Methods

2.1 Study Population

A total of 30 patients with Localized Cutaneous Leishmaniasis -LCL (ages ranging from 14 to 83 years old, mean=26±16.5 years, including 20 males and 10 females) from endemic areas of the State of Pernambuco (Amaraji and Moreno municipalities) were evaluated on clinical, epidemiological features and laboratorial criteria. All patients showed positive diagnoses for LCL, including direct parasite detection, Montenegro skin test, indirect immunofluorescence and PCR. Serum samples were collected from all LCL patients before the treatment onset.

All patients received the standard treatment for LCL, carried out with N-methyl glucamine antimoniate (Glucantime®) and the therapeutic scheme was made of doses of 20 mg/Kg/day through subcutaneous injections during thirty days. The occurrence of adverse reactions was monitored throughout the treatment period. After treatment, sera sample were collected from all patients, in a cross-sectional investigation with intervals ranging from 1 to 24 months, further grouped in 1-3 months (n = 4), 4-7 months (n= 14) and 12-24 months (n=12). All patients underwent blood collection twice: prior to and after the end of chemotherapy treatment with Glucantime®.

Eight serum samples from healthy individuals from non-endemic areas and without previous LCL infection were used as a control for non-infected individuals (NI). All of them signed the “Term of Free and Informed Consent” and the CPqAM / Fiocruz Research Ethics Committee (Protocol no 27/04) approved the experimental protocols.

2.2 Parasite Preparation

Leishmania (Viannia) braziliensis promastigote forms (MHOM/BR/75/2903) were cultivated in liver infusion tryptose medium (LIT) at 24 ± 1°C temperature. After serial passage *in vitro*, the parasites, in stationary-phase, were collected and homogenized in a low-rotation vortex to dissolve clumps. All procedures were performed aseptically in a biohazard hood. The homogenate was centrifuged at 100 X g, for 10 min to remove cell debris. Prior to the recovery of the parasite suspension, the supernatant was left to rest for 10 min at room temperature. Then, the supernatant was transferred to another tube and centrifuged at 1000 X g for 10 min, at 4°C. The pellet of parasites was suspended in buffer pH 7.2, containing 10%

heat-inactivated fetal bovine serum (PBS-10% FBS) (Gibco, Grand Island, NY) and centrifuged at 1000 X g for 10 min. Two distinct parasite preparations were used to evaluate the anti-*L. (V.) braziliensis* IgG reactivity referred to as "live" and "fixed" promastigote suspensions. For the live promastigote suspension, parasites were immediately re-suspended in PBS 10% FBS and used for the immunofluorescence by flow cytometry. For the fixed promastigote suspension, the parasites were re-suspended in equal volume of PBS and fixing solution (per liter, 10g of paraformaldehyde, 10.2 g of sodium cacodylate and 6.65 g of sodium chloride, pH 7.2; Sigma Chemical Corp., St Louis, Mo) and stored overnight at 4ºC. The fixed promastigotes were washed in PBS and stored at 4°C until use. Both parasite suspensions were counted in a Neubauer hemacytometer chamber and the concentration adjusted to 5 X 10^6/ml. The live or fixed promastigotes were used separately in the immunofluorescence assays by flow cytometry.

2.3 Immunofluorescence by Flow Cytometry

The immunofluorescence assays to detect anti-live (FC-ALPA-IgG) and anti-fixed (FC-AFPA-IgG) *Leishmania (V.) braziliensis* IgG antibodies were performed as described by Rocha *et al*. (2002). Suspension of live or fixed parasites (2.5 x 10^5/well) were incubated in 96-wells round bottom polystyrene plates, at 37°C for 30 min in the presence of different dilutions (1:128 to 1:16,384 for FC-ALPA-IgG and 1:256 to 1:32,768 for FC-AFPA-IgG) of serum samples. After incubation with the test serum samples, the parasites were washed twice with 150□l of PBS-10%FBS (at 1,000 X g for 10 min at 4°C). Afterwards, the parasites were re-incubated at 37°C for 30 min in the dark, in the presence of fluorescein isothiocyanate (FITC)-conjugated anti-human IgG antibody (Sigma Chemical Corp., St. Louis, MO) diluted at 1:1,200 in PBS-10% FBS for live parasites and at 1:16,000 for fixed parasites. After a second wash procedure, the FITC-labeled parasites were fixed for 30 min with a FACS fixing solution (per liter, 10 g of paraformaldehyde, 10.2 g of sodium cacodylate and 6.65 g of sodium chloride, pH 7.2; Sigma Chemical Co.) before being analyzed in the cytometer. Flow cytometry measurements were performed in a maximum period of 24 hours after parasite fixation. Each assay included an internal control of nonspecific binding in which parasites, not exposed to human serum, were incubated with FITC-conjugated anti-human IgG. In all experiments, positive and negative LCL control samples were included. The positive control chosen was a patient's serum with parasitological, immunological and molecular diagnostics all positive for LCL. The optimum working concentration of sera and anti-human antibody

conjugates were determined with the checkerboard titration method as previously proposed by Rocha *et al*. (2002). All FC-ALPA and FC-AFPA tests were repeated and confirmed, assuring the repeatability of the test.

2.4 Flow Cytometry Data Acquisition and Analysis

Flow cytometry acquisition was performed using a Becton Dickinson FACSCalibur™ equipment. The Cell-Quest™ software package was used in both data storage and analysis. Stained parasites were run in the cytometer, and 10,000 events per sample were acquired. Promastigotes were identified based on their specific forward (FSC) and side (SSC) light scattering properties. Following FSC and SSC gain adjustments, parasites were found by assuming a characteristic FSC X SSC dot plot distribution. The relative FITC fluorescence intensity of each event was analyzed with a single histogram representation. A marker was set up on the histogram representation of the FITC-conjugated internal control and used in all data analysis reported here to determine, for each sample, the percentage of positive fluorescent parasites (PPFP) as previously described by Rocha *et al*. (2002). The receiver operating characteristic curve - ROC curve (Greiner *et al.* 2000) was used to select the cut-off value to discriminate between positive and negative results of FC-ALPA-IgG and FC-AFPA-IgG. The analysis of paired ΔPPFP, defined as the difference between PPFP after treatment and PPFP before treatment was used as a new approach to monitor post-therapeutic IgG reactivity in LCL as proposed by Lemos *et al*. (2007) to monitor patients with Visceral Leishmaniasis.

3 Results

3.1 Initial Serum Screening

In the first step of this study, we analyzed the anti-*Leishmania braziliensis* IgG reactivity profile for live (FC-ALPA) and fixed (FC-AFPA) antigen preparations, aiming to characterize the titration curve and the serum dilution to differentiate Localized Cutaneous Leishmaniasis patients and non-infected individuals. Data analysis showed that both antigen preparation FC-ALPA-IgG and FC-AFPA-IgG have the same titration curve and patterns of reactivity (PPFP values). This analysis identified the specific serum dilution (1:1.024 for FC-ALPA-IgG and FC-AFPA-IgG) that best segregates the PPFP values of LCL patients and

non-infected individuals. As observed in Figure 1, the results were classified as positive with PPFP≥ 60% and negative, PPFP≤ 60%. This percentage was done according to Martins-Filho et al.(1995), from the analysis of antibody titration curves of individual sera, expressed by the mean of PPFP values. The initial screening showed that sera were considered negative (PPFP≤ 60%) when reacting in dilution 1:128 to 1:16,384 for FC-ALPA-IgG and 1:512 to 1:32,768 for FC-AFPA (Figure 1). In view of these results, FC-ALPA-IgG and FC-AFPA-IgG were able to discriminate the IgG reactivities of patients compared to the control group.

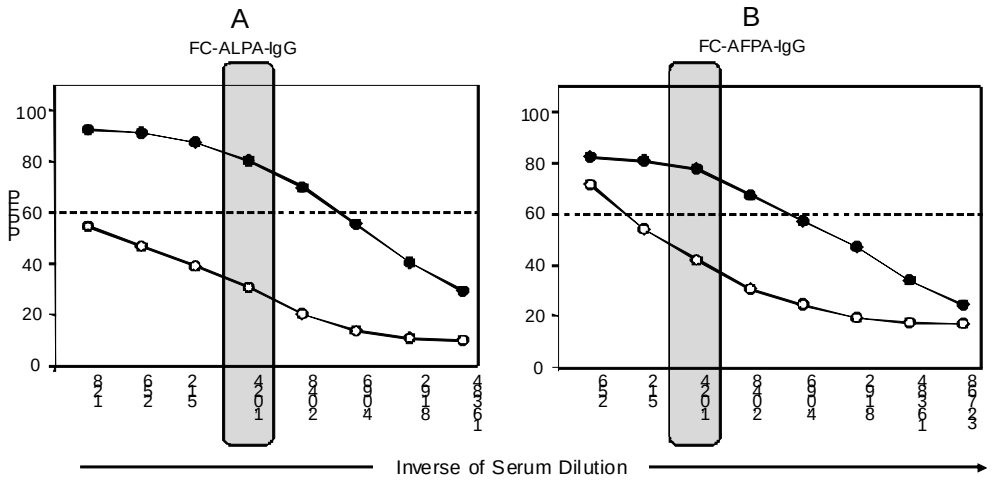

Figure 1: Anti-live FC-ALPA-IgG (A) and anti-fixed FC-AFPA-IgG (B) *L.* (*V.*) *braziliensis* IgG reactivity in serum samples from patients with Localized Cutaneous Leishmaniasis (LCL= ●) and Non-Infected individuals (NI= ○). The results are expressed as mean percentage of positive fluorescent parasites (PPFP) at sera dilutions 1:128 to 1:16,384 for FC-ALPA-IgG and sera dilutions 1:256 to 1:32,768 for FC-AFPA-IgG. The rectangles represent the selected serum dilution of the higher segregation range between patients and negative control (1:1,024 for FC-ALPA-IgG and FC-AFPA-IgG).

3.2 Performance of Anti-live and fixed *L. (V.) braziliensis* IgG Reactivity to identify active LCL

In this step, we evaluated the test performance in the identification of patients with active LCL. Thus, sera were separated and reactivity was determined in the dilution 1:1,024 and the cut-off of PPFP-60% (Figure 1). Using this approach, the sensitivity of FC-ALPA-IgG and FC-AFPA-IgG was 86% and 90%, respectively. The specificity was 78%, showing cross-reactivity of 22% for both parasite preparations (live and fixed) (Figure 2). The results

obtained in this evaluation showed that, despite the cross-reactivity observed for NI samples, the FC-ALPA-IgG and FC-AFPA-IgG has important value in identifying LCL cases.

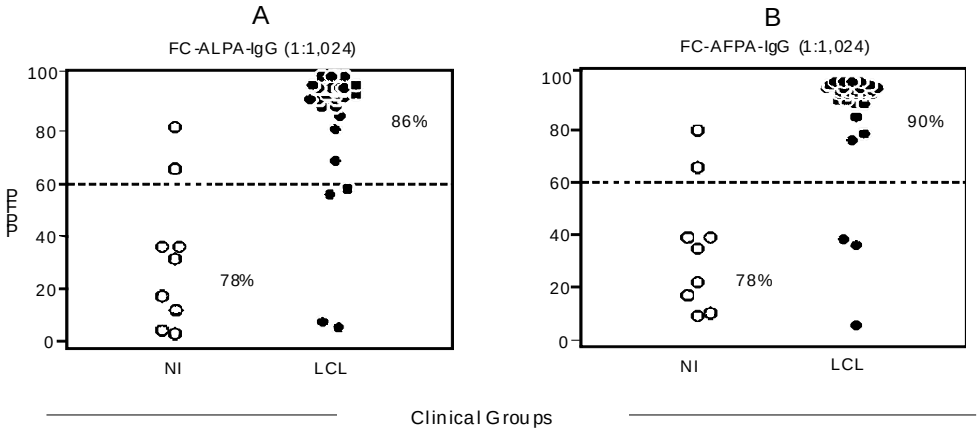

Figure 2: Anti-live FC-ALPA-IgG (A) and anti-fixed FC-AFPA-IgG (B) *L.* (*V.*) *braziliensis* IgG reactivity in serum samples from patients with Localized Cutaneous Leishmaniasis (LCL= ●) and Non-Infected individuals (NI= ○). The results are expressed as individual percentage of positive fluorescent parasites (PPFP) at serum dilutions 1:1,024. The dotted line represents the cut-off between negative and positive results.

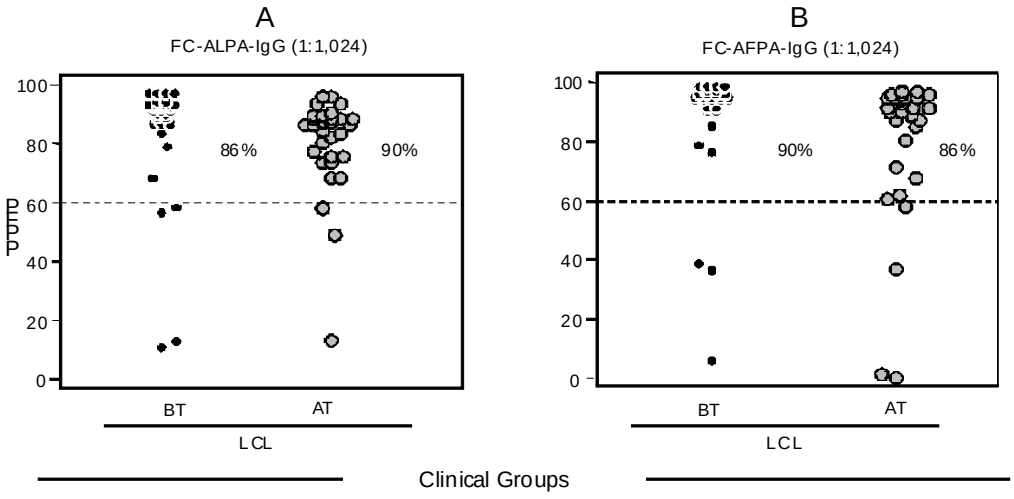

Figure 3: Comparative reactivity of anti-live FC-ALPA-IgG (A) and anti-fixed FC-AFPA-IgG (B) *L.* (*V.*) *braziliensis* IgG reactivity in serum samples from patients with Localized Cutaneous Leishmaniasis before treatment (BT=●) and after treatment (AT= ○). The results are expressed as individual percentage of positive fluorescent parasites (PPFP) at serum dilutions 1:1,024. The dotted line represents the cut-off between negative and positive results.

Additionally, we analyzed the applicability of FC-ALPA-IgG and FC-AFPA-IgG to monitor the post-therapeutic cure of LCL. The analysis of PPFP values performed after treatment did not demonstrate applicability of this parameter for post-therapeutic cure assessment at serum dilution 1:1,024. No significant changes in the PPFP values at serum dilution 1:1,024 were observed after treatment showed reactivity less than 60% to FC-ALPA-IgG and FC-AFPA-IgG (Figure 3).

Further comparative analysis used the PPFP values of paired samples evaluated before and after treatment along the titration curves (1:2,048 to 1:16,384 for FC-ALPA-IgG and 1:2,048 to 1:32,678 for FC-AFPA-IgG). Our data demonstrated that only FC-ALPA-IgG led to differential reactivity when comparing the mean PPFP values observed before and after treatment (Figure 4 -asterisks).

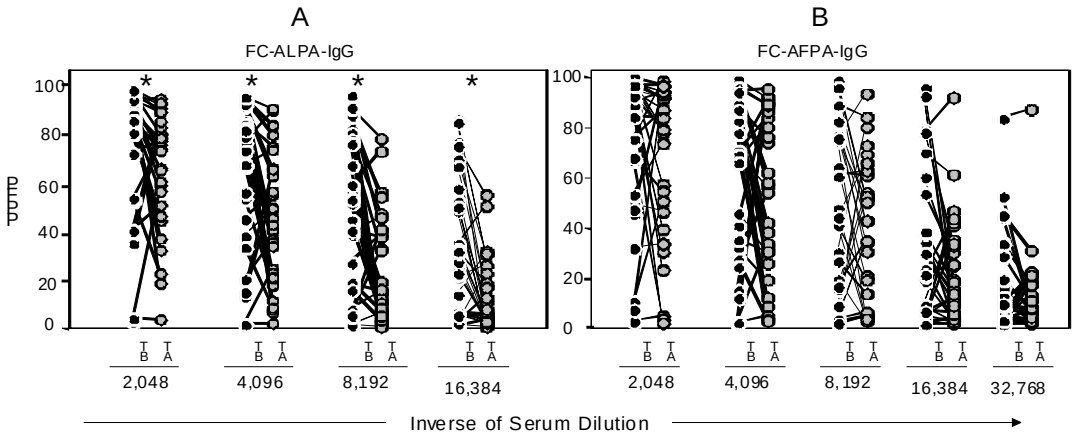

Figure 4: Differential Anti-live FC-ALPA-IgG (A) and anti-fixed FC-AFPA-IgG (B) *L.* (*V.*) *braziliensis* IgG reactivity in serum samples from patients with Localized Cutaneous Leishmaniasis before treatment (BT=●) and after treatment (AT=○). The results are expressed as individual percentage of positive fluorescent parasites (PPFP) for each pair of samples at serum dilutions 1:2,048 to 1:16,384 for FC-ALPA-IgG and 1:2,048 to 1:32,768 for FC-AFPA-IgG. Significant differences between the mean PPFP values before (BT) and after treatment (AT), at $p<0.05$ are highlighted by *.

3.3 Introducing ΔPPFP as a New Strategy Applied to Post-therapeutic Cure Assessment in LCL

As the analysis of PPFP values performed after treatment did not demonstrate any applicability for post-therapeutic cure assessment at a serum dilution 1:1,024, we searched for a new tool to comparatively analyze the IgG reactivity before and after treatment.

The proposed strategy was to analyze the differential PPFP reactivity detected by paired samples (delta reactivity [Δ]). In our case, we have used anti-*Leishmania* IgG reactivity ΔPPFP that represents the difference between PPFP after treatment and PPFP before treatment (ΔPPFP= $PPFP_{AT} – PPFP_{BT}$).

Initially, we evaluated the ΔPPFP values throughout FC-ALPA-IgG and FC-AFPA-IgG titration curves, aiming to identify the serum dilution range 1:2,048 to 1:16,384 for FC-ALPA-IgG and 1:2,048 to 1:32,678 for FC-AFPA-IgG that represent the highest differential reactivities (Figure 5). As shown in Figure 5, the FC-ALPA-IgG at serum dilution 1:8,192 showed that 81% of the treated patients displayed negative ΔPPFP values, demonstrating a decrease in IgG reactivity after treatment. Moreover, the FC-AFPA-IgG at serum dilution 1:4,096 was able to identify 61% of patients with negative ΔPPFP values. Consequently, these dilutions were the best choices to analyze the ΔPPFP values for FC-ALPA-IgG and FC-AFPA-IgG, respectively.

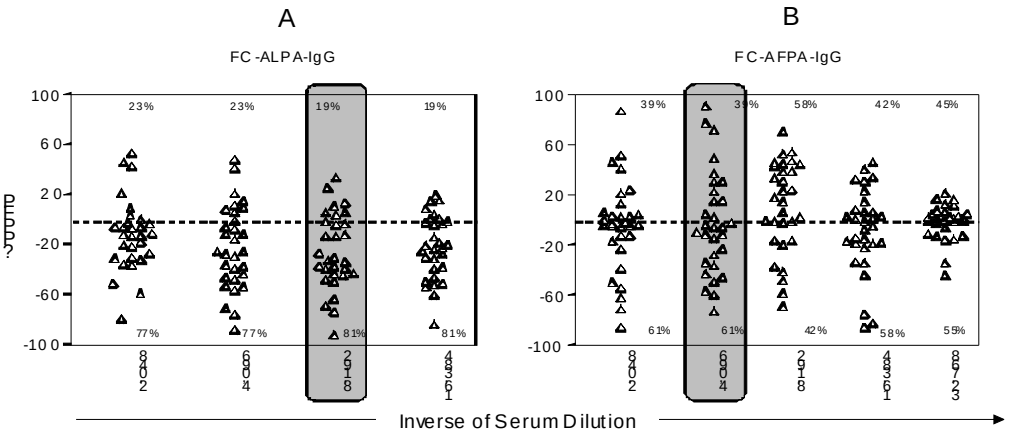

Figure 5: Differential anti-*L.* (*V.*) *braziliensis* IgG reactivity of paired samples detected by FC-ALPA-IgG (A) and FC-AFPA-IgG (B). The results are expressed as differential percentage of fluorescent positive parasites (ΔPPFP) for each pair of samples from LCL patients (△) evaluated before and after treatment. The ΔPPFP was defined as the difference in the PPFP values observed after treatment, taking the PPFP before treatment as the reference (ΔPPFP= $PPFP_{AT} – PPFP_{BT}$). The gray rectangle identifies the serum dilution that yielded the higher frequency of negative ΔPPFP values. The dashed line represents the cut-off edge of ΔPPFP=0 referring to unaltered serological profile.

After setting the specific serum dilutions to monitor the ΔPPFP values, we investigated whether the time after treatment would interfere in the performance of this new strategy of post-therapeutic follow-up. For this purpose, the treated patients were categorized into three subgroups based on the time after treatment when they were evaluated, including 1-

3 mAT; 4-7 mAT; and 12-24 mAT. Data analysis was performed after the establishment of a gray zone corresponding to the first quartile of the ΔPPFP range (cut-off edge of 25%, considering PPFP values from 0 to 100%) according to Lemos et al, 2007. We believe that the use of this gray zone would give further strength to data interpretation, since it would avoid interference regarding the possible intrinsic flow cytometry measurement variability. Our data demonstrated that 75% of the patients evaluated at 1-3mAT showed negative ΔPPFP values detected by FC-ALPA-IgG whereas 25% of them showed negative ΔPPFP values detected by FC-AFPA-IgG. Patients evaluated at 4-7mAT displayed 43% and 29% of negative ΔPPFP values detected by FC-ALPA-IgG and FC-AFPA-IgG, respectively. When ΔPPFP reactivity was evaluated 12-24mAT, 58% and 33% of the patients presented negative results in the FC-ALPA-IgG and FC-AFPA-IgG, respectively (Figure 6).

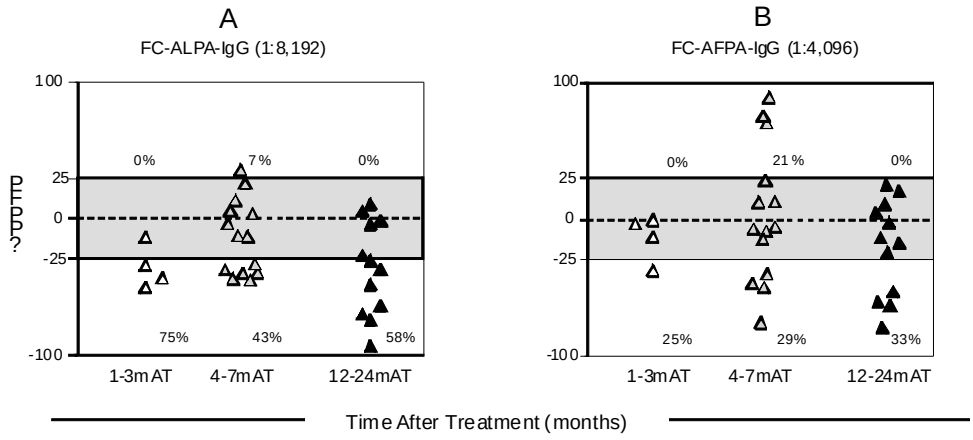

Figure 6: Differential anti-*L.* (*V.*) *braziliensis* IgG reactivities of paired samples detected by FC-ALPA-IgG (A) and FC-AFPA-IgG (B) defined as differential percentage of fluorescent positive parasites (ΔPPFP) for pairs of samples from LCL patients evaluated before and after treatment. Patients were categorized into three groups based on the time (months) after treatment (mAT) and referred as 1-3 mAT (△), 4-7 mAT (▲) and 12-24 mAT (▲). The results are expressed as ΔPPFP for each pair of samples. Data analysis was performed after the establishment of a gray zone (the gray rectangle) corresponding to the first quartile of the ΔPPFP range (cut-off of 25%).

4 Discussion

One of the major limitations for the use of serological approaches in the diagnosis and cure assessment of Cutaneous Leishmaniasis is the scarcity of sensitive methods to discriminate the IgG reactivity during active infection and the residual serological reactivity

after effective treatment. In general, the diagnosis and the cure criterion are based on clinical and epidemiological findings and complete therapeutic effectiveness is considered when complete lesion healing is observed. Nevertheless, this is an unsatisfactory criterion, as lesion reactivation may occur even after treatment and complete healing of the initial lesion. Although cutaneous lesions caused by *Leishmania* (*Viannia*) *braziliensis* are usually susceptible to antimonial treatment and healing occurs at the end of therapy, the occurrence of more than 90% of relapses up to one year in Cutaneous Leishmaniasis strengthens the suggestion of clinical control for at least one year post treatment (Coutinho et al., 1996).

Given this context, it has been suggested that the decrease in anti-*Leishmania* antibodies titer in serum of patients after treatment could be an alternative method to monitoring specific post-therapeutic cure (Chiari et al., 1973, Mendonça et al., 1988). Moreover, the presence of anti-*Leishmania* antibodies after treatment could indicate the persistence of the parasite and be a predictive factor in Cutaneous Leishmaniasis recurrence (Saravia et al., 1989). Associated with these aspects, the existence of various parasite species and vectors, as well as parasite-host relation represent important elements related to Cutaneous Leishmaniasis complexity. This fact points to the need for new tools that help to determine prognostics and an efficient diagnosis.

Serological tests to diagnose Cutaneous Leishmaniasis may also present limitations, such as low sensitivity, low specificity, low reproducibility, low antibody titers, the absence of correlation between the circulating antibody levels with the disease stage and they can show crossing reactions with other species of the Trypanosomatidae family (Brito et al., 2000;2001; Kar, 1995).

In this study, we have evaluated the applicability of an indirect immunofluorescence assay based on flow cytometry methodology to detect anti-live and anti-fixed *Leishmania (Viannia) braziliensis* promastigote IgG for the serological diagnosis of LCL. In addition, we have verified the applicability of serological reactivity to assess post-therapeutic cure in cured patients after different periods following treatment with pentavalent antimonial chemotherapy. The rationale underlying our experimental design was the fact that the live promastigote preparation would represent a useful tool to work with a selective set of antigens (outer membrane epitopes) and contribute to a more refined analysis. However, the labile nature of this antigenic preparation is the major concern about its use in clinical laboratory practice. On the other hand, although the use of fixed promastigotes represents a feasible way to produce and store a bulk amount of pre-fixed antigen that contributes to large scale production, it would contribute to the development of a larger amount of intracytoplasmatic

epitopes and complicate the observation of minor changes in the serological reactivity following therapeutic intervention (Pissinate et al., 2008).

Another important aspect of the serological methods applied to Cutaneous Leishmaniasis diagnosis is the choice of antigen sources, which still represents a relevant obstacle. When total promastigotes are used as antigen, it is common to find false positive reactions due to cross-reactions with other diseases. It is important to investigate alternative preparations to detect *Leishmania* antibodies (Celeste et al., 2004, Gonçalves et al., 2002). In the present investigation we have chosen the *L.* (*V.*) *braziliensis* promastigotes as the antigenic source to access IgG reactivity in LCL patients. Although this species is very difficult to grow *in vitro*, requiring the use of axenic cultures, complex medium composition and fine pH and temperature controls (Lemesre et al., 1988), *L. (V.) braziliensis* is the most important LCL causative agent in Brazil and especially in Pernambuco.

A diagnostic method based on flow-cytometry to detect anti-live *L.* (*V.*) *braziliensis* antibodies has been described by Rocha et al. (2002, 2006). They demonstrated 96% sensitivity for FC-ALPA-IgG *L. braziliensis* in active patients. Using fixed *L. amazonensis* promastigotes Pissinate et al. (2008), showed a good performance of FC-AFPA-IgG in the serological diagnosis of LCL. However they found cross-reactivity with other co-endemic diseases, like tripanosomatid infections. Our data demonstrated that both methods display low specificity and still require methodological adjustments in order to improve their performance as confirmatory diagnostic tools. In fact, Rocha et al. (2002) and Pissinate et al. (2008) have already demonstrated that the occurrence of false positive results in the FC-ALPA-IgG and FC-AFPA-IgG in endemic areas are mostly related to the cross-reactivity of serum samples from patients with Chagas disease and Visceral Leishmaniasis, co-endemic diseases generally observed in areas of prevalent Cutaneous Leishmaniasis.

In our study, we have found that FC-ALPA-IgG using *L. (V.) braziliensis* displayed 86% sensitivity whereas FC-AFPA-IgG showed 90% sensitivity for the diagnosis of LCL. This difference obtained could be explained by the heterogeneity of *L.* (*V.*) spp. in Brazil. Brito et al (2009) demonstrated the presence of 10 circulating zymodemes in the well-defined "Zona da Mata" of Pernambuco. The heterogeneity observed among *L.* (*V.*) *braziliensis* parasites from this region is noteworthy, particularly in contrast to the homogeneity of parasites isolated from other regions of Brazil (Brandão-Filho et al., 2003, Brito et al., 1993, Cupolillo et al., 2003).

The results obtained showed that both techniques (FC-ALPA-IgG and FC-AFPA-IgG) are useful for the sero-diagnosis of LCL as compared to the conventional

immunofluorescence assay. Although FC-AFPA-IgG display a slight higher sensitivity in the diagnosis of LCL, the FC-ALPA-IgG seems to be more reliable for cure monitoring, being able to identify more differences between IgG reactivity before and after treatment when assessed by ΔPPFP. We found 81% of the treated patients with negative ΔPPFP results for FC-ALPA-IgG compared to 61% negative ΔPPFP for FC-AFPA-IgG. These results show that FC-ALPA-IgG represents a better performance than FC-AFPA-IgG for post-therapeutic monitoring of LCL patients. We have a general belief that the FC-ALPA-IgG represents better performance than the FC-AFPA-IgG, as previous studies of our group have demonstrated a real advantage of using live, instead of fixed, parasites in serological approaches applied to the diagnosis and cure assessment of human protozooses (Martins-Filho et al., 1995, 2002, Vitelli-Avelar et al., 2007, Pissinate et al., 2008). In fact, the use of live promastigotes seems to represent a better tool to achieve better performance of serological approaches since in this antigenic preparation only the outer membrane epitopes are available for IgG binding in contrast with the fixed antigenic preparation on which the cytoplasmic antigens are also available for IgG recognition. The use of a selected set of outer membrane antigens is a good strategy to work with a more restricted IgG repertoire that would potentially find slight differences resulting from the loss of B-cell clones early after effective etiological treatment.

We also investigated whether performance would be influenced if the test was performed at different times after treatment. For this purpose serum samples collected after treatment were segregated into three groups referred to as 1-3mAT, 4-7mAT and 12-24mAT. The FC-ALPA-IgG and FC-AFPA-IgG were assayed and the ΔPPFP values generated to monitor sero-reactivity at different times following treatment. Our findings demonstrated an overall low performance of ΔPPFP to demonstrate differential reactivity according to the time after treatment. However, the FC-ALPA-IgG still demonstrated better performance as compared to the FC-AFPA-IgG, leading to higher frequency of cases with negative ΔPPFP. It is important to mention that the low performance of ΔPPFP to detect differential reactivity in this cross-sectional investigation should not be considered the end point of using this parameter for cure assessment in LCL since this approach would be better evaluated in a longitudinal investigation in order to generate more accurate data for cure assessment in LCL.

Although most flow cytometry-based methods still represent higher cost compared to conventional methods, such as immunosorbent and immunofluorescence assay, the possibility of working with a microplate serological approach has reduced the final cost of a given test.

Moreover, at the present time, several clinical laboratories in developing countries are considering the acquisition of flow cytometers. Therefore, in the near future, the implementation of new flow cytometry-based tests will became routine as will the interchange between research centers and clinical laboratories. In our experience, flow cytometry-based serological approaches present good reproducibility and outstanding concordance among independent analysts (Garcia et al., 2009).

In conclusion, our data suggested that the new flow cytometry-based methodology has promising potential to identify active LCL clinical cases in patients. Further longitudinal studies are currently under investigation in order to better characterize the approach for monitoring post-therapeutic cure, as well as to obtain the clinical values of this new approach and to validate its use in medical studies.

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Research paper

Comparison of flow cytometry and indirect immunofluorescence assay in the diagnosis and cure criterion after therapy of American tegumentary leishmaniasis by anti-live *Leishmania* (*Viannia*) *braziliensis* immunoglobulin G

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Resumo

O objetivo deste estudo foi comparar as técnicas de imunofluorescência indireta (IFI) e citometria de fluxo para avaliação clínica e laboratorial dos pacientes antes e depois da cura clínica e avaliar a aplicabilidade da citometria de fluxo no acompanhamento pós- terapêutico de pacientes com leishmaniose tegumentar americana (LTA). Soros de 14 pacientes antes do tratamento (AT), 13 pacientes um ano após o tratamento (PT), 10 pacientes 2 e 5 anos foram avaliadas. Os resultados de citometria de fluxo foram expressos em níveis de reatividade de IgG, com base na percentagem de parasitos fluorescentes positivos (PPFP). A diluição 1:256 permitiu a diferenciação entre os indivíduos AT e PT. A análise comparativa dos IFI e citometria de fluxo pela curva ROC (Receiver Operating Characteristic Curve) apresentaram, respectivamente, a AUC (área sob a curva) = 0,8 (IC 95% = 0,64-0,89) e AUC = 0,90 (IC 95% = 0,75 -0,95), demonstrando que a citometria de fluxo teve acurácia equivalente. Nossos dados demonstraram que 20% de PPFP foi o melhor ponto de corte identificado pela curva ROC para o ensaio de citometria de fluxo. Este teste mostrou sensibilidade de 86% e especificidade de 77%, enquanto a IFI teve uma sensibilidade de 78% e especificidade de 85%. A triagem após o tratamento, através da análise comparativa dos índices de desempenho das técnicas, 1, 2 e 5 anos PT, mostraram um desempenho igual da citometria de fluxo em

comparação com a IFI. No entanto, a citometria de fluxo mostrou ser uma melhor alternativa diagnóstica quando aplicada ao estudo do critério de cura na LTA. As informações obtidas neste trabalho abrem perspectivas para o monitoramento de cura pós terapêutica na LTA.

Abstract

The aim of this study was to compare the techniques of indirect immunofluorescence assay (IFA) and flow cytometry to clinical and laboratorial evaluation of patients before and after clinical cure and to evaluate the applicability of flow cytometry in post-therapeutic monitoring of patients with american tegumentary leishmaniasis (ATL). Sera from 14 patients before treatment (BT), 13 patients 1 year after treatment (AT), 10 patients 2 and 5 years AT were evaluated. The results from flow cytometry were expressed as levels of IgG reactivity, based on the percentage of positive fluorescent parasites (PPFP). The 1:256 sample dilution allowed us to differentiate individuals BT and AT. Comparative analysis of IFA and flow cytometry by ROC (Receiver Operating Characteristic Curve) showed, respectively, AUC (area under curve) = 0.8 (95% CI = 0.64-0.89) and AUC = 0,90 (95% CI = 0,75-0.95), demonstrating that the flow cytometry had equivalent accuracy. Our data demonstrated that 20% was the best cut-off point identified by the ROC curve for the flow cytometry assay. This test showed a sensitivity of 86% and specificity of 77% while the IFA had a sensitivity of 78% and specificity of 85%. The after-treatment screening, through comparative analysis of the techniques performance indexes, 1, 2 and 5 years AT, showed an equal performance of the flow cytometry comparing with the IFA. However, flow cytometry shows to be a better diagnostic alternative when applied to the study of ATL in the cure criterion. The information obtained in this work opens perspectives to monitor cure after treatment of ATL.

Keywords: Flow Cytometry, Indirect Immunofluorescence Assay, IgG, *L. (V.) braziliensis*, American Tegumentary Leishmaniasis.

1 INTRODUCTION

The american tegumentary leishmaniasis (ATL) is a disease that can be caused by several species of unicellular protozoa of the genus *Leishmania* (Basano; Camargo, 2004). ATL has an increased incidence in Pernambuco, a State in the Brazilian northeast, where we can

highlight the Zona da Mata region, with more than 60% of the total reported cases (Brandão-Filho et al. 1999; Brito et al., 2008). The increasing incidence and transmission persistence in Brazil revealed that ATL is a preoccupying public health problem, where *Leishmania (Viannia) braziliensis* is the main responsible specie circulating in Pernambuco (Brito et al., 2008, Andrade et al., 2009).

So far the cure criterion is based on the clinical cure, seen by the complete reepithelialization of the skin wound, ulcerated or not, and by the total regression of the infiltration and erythema, observed usually three months after treatment in the cutaneous form (Gontijo; Carvalho, 2003; Mayrink et al. 2006). Several approaches have been suggested as cure criteria after treatment of ATL. However, assuming that the humoral immune response may have a modulator role on the cellular response during the infection, (Bitterncourt et al 1968), suggested the possibility of researching antibodies as a way to monitor the ATL cure. The dynamics of the antibody production after treatment is still not well known, and the levels of antibodies against specific antigens remain undefined (Romero et al., 2005). The problem can be attributed to the lack of a standard antigenic preparation to detect specific antibodies.

Although high titers of antibodies can be found in all clinical manifestations of ATL, it is generally accepted that the protective immune response in *Leishmania* infection is primarily cellular, demoting the antibodies participation to background (Liew, O'Donnell, 1993). Studies related to the analysis of the humoral immune response in ATL have approached the role of immunoglobulins in immunopathologic mechanisms involved on the resistance and / or pathogenesis of the infection. In addition, they have also evaluated the use of serological investigations in diagnosis and monitoring the post-treatment efficacy (O'Neil et al. 1993; Molesleh et al. 1995; Chiari et al., 1973). Studies have shown that all anti-*Leishmania* specific antibodies isotypes, except IgD, are detected in the serum of patients with ATL. The frequency detection of IgG subclasses in ATL, according to Pissinate et al. (2008), is IgG1> IgG3> IgG2 = IgG4. Patients with a longer time of disease evolution show high levels of IgE, while IgA levels are increased in patients with the mucocutaneous form (O'Neil et al., 1993). The intensity of the humoral response seems to be related to the parasite load and chronicity of the infection. High antibody titers can be observed in all clinical manifestations of ATL (Trujillo et al., 1999). Available studies with immunoserologic methods for antibodies research in ATL are controversial, due to their low sensitivity and specificity (Bourdoiseau et al. 2009; Mukbel et al., 2006).

Since there is no diagnostic test considered as the gold standard for ATL, the diagnosis is done by the association of clinical, epidemiological and laboratorial aspects (Brito et al.,

2008, Kar, 1995). The clinical diagnosis is not always easy or immediate, because the ATL injuries can be mistakenly identified as lesions of other diseases, such as chromomycosis, sporotrichosis, some types of cancer, paracoccidioidomycosis, syphilis, cutaneous tuberculosis and lepromatous leprosy (Gontijo; Carvalho, 2003; Marzochi 1992). Moreover, there are limitations in conventional diagnostic methods. The indirect immunofluorescence assay (IFA) and enzyme immunoassay - ELISA, and conventional serological tests that are more widely used, do not correlate the levels of circulating antibodies with the stage of the disease, and may have crossed reactions with other trypanosomatids (Kar, 1995; Vexenat et al., 1996). Due to the limitations of these techniques, alternative immunological approaches have been employed.

One of them is the flow cytometry, a technology that simultaneously measures and analyzes various physical characteristics of individual particles, as the fluid flow passes through a light beam, thereby allowing the detection of anti-*Leishmania* antibodies (Rocha et al., 2002, 2006). Considering the applicability of the flow cytometry, as a system free from methodological variability inherent in the analyst and with superior applicability to different protocols of conventional detection and revelation; Rocha et al. (2002) brought an alternative by testing IgG antibodies of anti-live *Leishmania (V.) braziliensis,* applicable to the diagnosis and post-therapeutic criteria of cure for ATL.

Regarding the applicability of flow cytometry, as described by Martins-Filho et al. (1995), as a methodology with superior sensitivity to the different protocols proposed for conventional detection, this study sought a better understanding of the humoral immune response linked to the clinical progression of ATL patients. It aimed the flow cytometry technique as an effective diagnostic method and to evaluate its applicability in monitoring post-therapeutic cure of ATL. Thus, the indirect immunofluorescence assay (IFA) and flow cytometry were compared in clinical and laboratorial evaluation of patients before and after clinical cure, and the applicability of flow cytometry was evaluated for the post-therapeutic monitoring of these patients.

2 MATERIALS AND METHODS

2.1 PATIENTS

A total of 14 patients with active localized cutaneous leishmaniasis, from endemic areas of the State of Pernambuco - Brazil were evaluated on clinical, epidemiological features and laboratorial criteria. All patients showed positive diagnosis for ATL, such as: direct research, Montenegro skin test, indirect immunofluorescense and PCR. The treatment was given with N-methyl glucamine antimoniate (Glucantime®) and the therapeutic scheme was made of doses of 20 mg/Kg/day through subcutaneous injections during twenty or thirty days. The cure criterion was clinical, with the total healing of lesions and re-epithelialization of the skin. Sera sample were collected before chemotherapy (n=14) and in different years after treatment: 1 year (n = 13), 2 years (n= 10) and 5 years (n=10). Eight control sera were taken from healthy blood of volunteer donors. All of them signed the "Term of Free and Informed Consent" and the CPqAM / Fiocruz Research Ethics Committee (Protocol n^{o} 27/04) has approved the experimental protocols.

2.2 PARASITE PREPARATION

Leishmania (Viannia) braziliensis promastigotes (MHOM/BR/75/M2903) maintained *in vitro* in the Immunogenetics laboratory / CPqAM / FIOCRUZ / PE, were expanded in Schneider's medium until the exponential phase. Then the parasites were subjected to low-rotation (7 X g), 25 ° C for 10 minutes. Then the parasites were recovered from the supernatant after 10 minutes rest at room temperature. The supernatant was transferred to another tube and the pellet discarded. The parasites were washed in buffer pH 7.2, containing 10% heat-inactivated fetal bovine serum (PBS-10% FBS) three times at 4 ° C, 871 X g for 10 minutes. The sediment formed was thoroughly homogenized. The parasites were then resuspended for fixing, in an equal volume of 1% paraformaldehyde, and incubated overnight. They were washed with buffered saline containing 10% FBS. The aliquot of parasite suspension was counted and the concentration adjusted for the flow cytometry test.

2.3 INDIRECT IMMUNOFLUORESCENCE REACTION

The IFA test was performed in collaboration with the Seroepidemiology and Immunobiology laboratory of the Institute of Tropical Medicine, University of São Paulo. The slides, containing the antigenic suspension of fixed promastigotes of *Leishmania* (*L. major*-like promastigotes / MHOM/BR/71/49) were coated with 20 µl of serum samples diluted in the ratio of 1:20 to 1:320 in 0.01 M PBS, pH 7.2. Two standard sera were added (positive and negative), and incubated in a moist chamber for 30 minutes at 37 °C. After incubation, the excess sera were removed from the slides, washing them by immersion in PBS, pH 7.2 three times at intervals of 10 minutes. The slides were dried with paper towel in a dilution ratio of 1:100 of anti-human IgG (heavy chain specific) conjugated to fluorescein isothiocyanate - FITC (Biolab Mérieux) prepared in Evans blue (40 mg) in buffer solution PBS (diluted at 1:10 ratio in the same buffer) was placed on slides, incubating with same conditions. After the reaction, the slides were washed three times in PBS for 10 min and dried with paper towels. The assembly was made with buffered glycerine, pH 8.5 and then the slide was observed in the fluorescence microscope, with the objective of 250 X.

2.4 FLOW CYTOMETRY AND DATA ANALYSIS

The flow cytometry assay for the detection of *Leishmania (V.) braziliensis* anti-promastigotes was performed according to Rocha et al. (2002). The parasite suspension (2.5×10^5/well) was incubated in 96-well U-bottom plates at 37°C for 30 minutes with different dilutions of inactivated serum samples (1:64 to 1:8192). The parasites were washed twice with 150 µl of PBS-10% FBS (1000 X g for 10 min at 4 °C). Then they were incubated at 37° C for 30 minutes protected from the light with anti-human IgG conjugated to fluorescein isothiocyanate - FITC (Sigma Chemical Corp., St. Louis, MO) diluted 1:400 in PBS -10% FBS. After the second washing, the FITC-labeled parasites were fixed for 30 minutes before being analyzed in the flow cytometer (FACScalibur, Becton Dickinson) and identified using the software “Cell Quest Pro” linked to the cytometer. The results of the samples in the flow cytometer were acquired within a maximum period of 24 hours after fixation of the parasites. Stained parasites were run in the cytometer, and 10.000 events per sample were acquired. The promastigotes were identified based on their specific forward (FSC) and side (SSC) light scattering properties. Following FSC and SSC gain adjustments, the parasites were found by assuming a characteristic FSC X SSC dot plot distribution. The relative FITC fluorescence

intensity of each event was analyzed with a single histogram representation. A marker was set up on the histogram representation of the FITC-conjugated internal control and used in all data analysis reported here to determine, for each sample, the percentage of positive fluorescent parasites (PPFP) as previously described by Rocha *et al.* (2002).

2.5 CUT OFF and *Receiver Operating Characteristic Curve* (ROC Curve)

For the IFA test, results were considered positive, when values were equal to or greater than the titer of 1:20. On the flow cytometry, we used the cut-off suggested by Martins-Filho (1995), in order to analyze the test reactivity, where PPFP> 20% were considered positive for the test. The ROC curve was used to select the cut-off value to discriminate positive and negative results of IFA and flow cytometry.

2.6 STATISTICAL ANALYSIS

The tests performance was investigated determining the results, which were classified into four categories, according to the lesion group (before treatment - BT) or absence of lesion (after-treatment group - AT). These categories are shown in Table 1 and are defined as follows: True-Positive (TP) = lesion and positive test; False Positive (FP) = no lesion and a positive test; False Negative (FN) = lesion and test negative; and true-negative (TN) = no lesion and negative test. Based on these elements, the sensitivity and specificity indexes were calculated. The sensitivity was calculated by the ratio a/(a+c) translating thereby the proportion of patients with ATL (lesion), where IFA tests or flow cytometry were positive. The specificity is related to the proportion of individuals without the clinical manifestations of ATL (no lesion), those who are negative can be determined by the ratio d/(b+d). The receiver operating characteristic curve (ROC curve) was built applying the sensitivity values in the ordinate axis, and the complement of specificity in the abscissa axis. The curve was used to select the cut-off value to discriminate negative from low positive and high positive PPFP results. The test's global accuracy was also evaluated, taking the area under the ROC curve (AUC) according to Swets (1988). All analysis were performed by MedCalc software.

Table 1
Categories for the diagnosis test results classification of the patients.

		ATL	
		BT	AT
Result of test	Positive	**TP(a)**	**FP(b)**
	Negative	**FN (c)**	**TN(d)**
Total		(a+c)	(b+d)

3 RESULTS

3.1 STUDY POPULATION SCREENING

Most patients had ulcerated lesions with raised borders and granulomatous background distributed in uncovered areas of the body. Only one patient presented the disseminated cutaneous form, with more than 20 papular lesions, distributed throughout the body surface. And another patient had the mucocutaneous form. The laboratory diagnosis of ATL was confirmed in collaboration with the CPqAM Leishmaniasis Reference Service and with Seroepidemiology and Immunobiology Laboratory (IMT / USP). Patients were included only after a positive diagnosis in at least one of the tests (Table 2).

Table 2
Characteristics of patients and laboratory results.

Patient	Sex	Age	Number of lesions	Evolution (months)	MST (mm)	Direct search	PCR	Isolation
1	M	17	03	1	5	ND	+	N
2	F	46	03	1	10	+	+	Y
3	M	34	01	2	15	+	+	N
4	F	NI	01	1	15	+	+	N
5	F	15	>20	NI	ND	−	+	N
6	M	46	01	1	10	−	+	Y
7	M	75	01	2	5	+	+	N
8	M	20	02	3	10	−	+	N
9	M	28	01	2	10	+	−	Y
10	M	51	01	1	5	−	+	Y
11	M	30	01	1	15	−	+	N
12	F	24	01	2	−	+	+	Y
13	M	33	01	36	15	ND	+	Y
14	F	13	03	12	6	−	ND	N

F = female; M = male; MST = Montenegro test; PCR = polymerase chain reaction; + = positive; − = negative; ND = not done; NI = not informed; Y = yes; N = no.

3.2 APPLICABILITY OF INDIRECT IMMUNOFLUORESCENCE AND FLOW CYTOMETRY TESTS IN THE IDENTIFICATION OF PRESENCE OR ABSENCE OF ATL IN PATIENTS

Patients who showed titers of 1:20 and above, were considered positive by IFA. We evaluated the applicability of the IFA method to identify clinical activity in ATL by total IgG reactivity of sera from individuals (Figure 1) in terms of sensitivity and specificity. These patients were classified according to the presence of ATL (BT- before treatment) and the absence of ATL (1, 2 and 5 years after treatment – AT). The analyzed patients showed a sensitivity of 92.86%. The specificity in patients 1 year AT was 38.46%; 30% in patients 2 years AT and 50% in patients 5 years AT for the IFA test. On the flow cytometry, we used a cut-off of 20% PPFP, as proposed by Martins-Filho (1995). The reactivity was expressed as the mean of the percentage of positive fluorescent parasites (PPFP), (Figure 2), obtained after incubating the sera at dilutions of 1:64 to 1:8192, with fixed promastigotes under previously standardized conditions. The analysis of the titration curves of IgG antibodies anti-live promastigotes of *L. (V.) braziliensis* present in patients' sera, suggested that 1:256 to 1:2048 dilutions are the regions (R) of reactivity that correspond to the best titers of the differential reaction between BT individuals and 1, 2 and 5 years AT. These dilutions of individual serum samples BT and AT were analyzed to evaluate its applicability to identify the patients' clinical activity for ATL (Figure 3). In serum dilutions of 1:256, the patients showed a sensitivity of 86% and specificity of 77% in patients 1 year AT; specificity of 80% in patients 2 years AT and specificity of 70% in patients 5 years AT on the flow cytometry test.

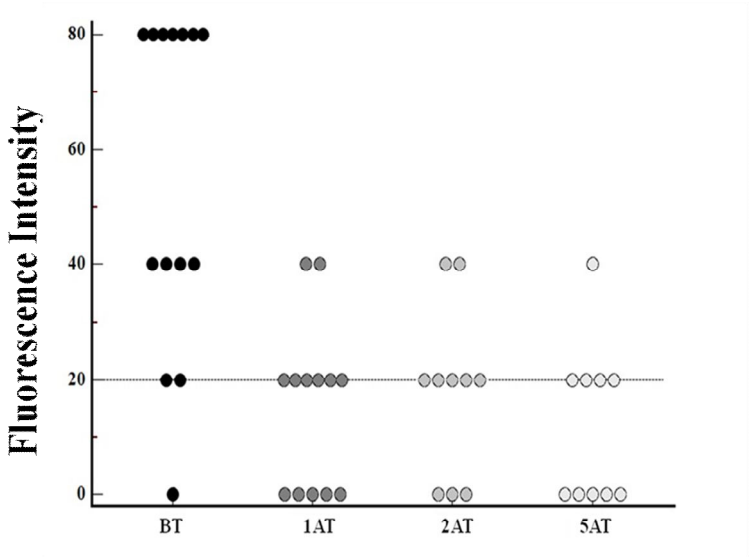


Figura 1: IgG reactivity in sera of ATL patients before and 1, 2 and 5 years after treatment, subjected to indirect immunofluorescence method. The dotted line corresponds to the cutoff of 1:20.

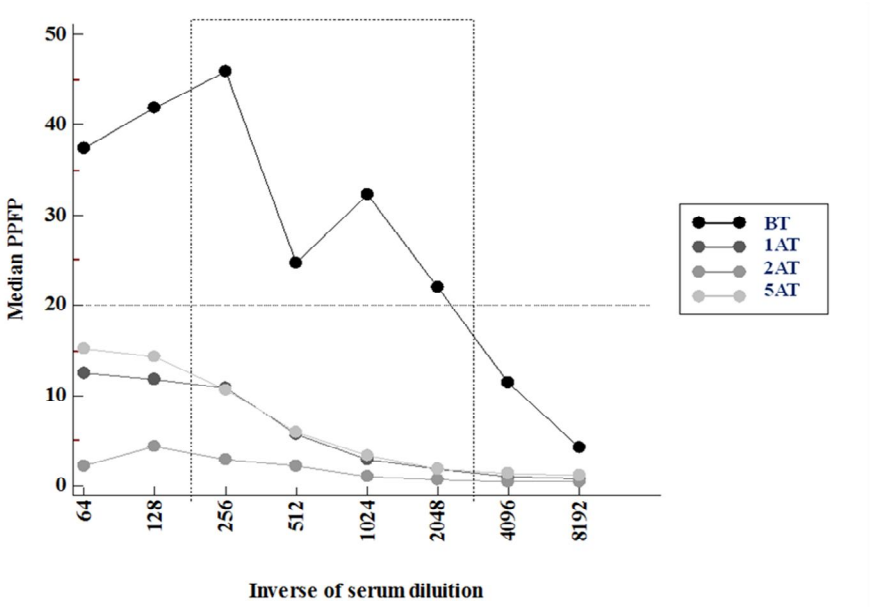


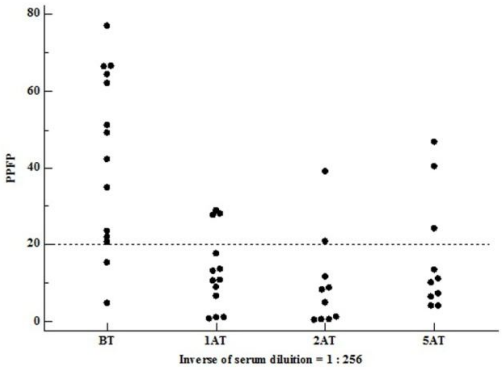
Figura 2: Titration curves of antibodies IgG anti-fixed promastigotes of *L. (V.) braziliensis* present in sera of ATL patients classified as the presence of injury (BT) and the absence of injury (1, 2 and 5 years AT). The dotted line corresponds to a cutoff of 20% PPFP.

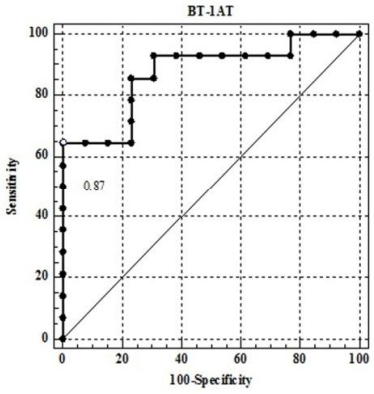
3.3 GLOBAL ACCURACY OF INDIRECT IMMUNOFLUORESCENCE AND FLOW CYTOMETRY TESTS

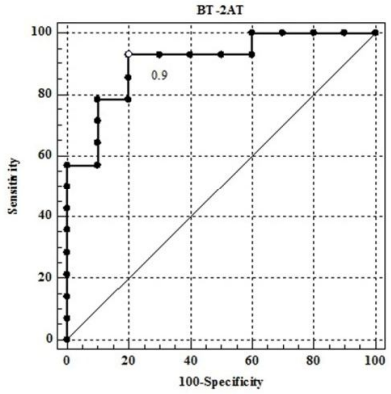
IFA titers and flow cytometry values in serum dilutions of 1:256, were used to build the ROC curve for the tests (Figure 4). With the ROC analysis, we found that the Area Under Curve IFA (AUC = 0.8; 95% CI =0.78 to 0.99) differed from the observed in the flow cytometry (AUC = 0.9; 95% CI= 0.75 to 0.99). Thus, we observed that the accuracy for the IFA test was equivalent to the flow cytometry's. Therefore, the ROC curve showed that a cut-off of 20% of PPFP with sensitivity of 86% and specificity of 77% was to demonstrate the performance of the test using flow cytometry. This result is in agreement with the cut-off of 20% previously described by Martins-Filho et al. (1995). About the cut-off point used for the IFA, the ROC curve showed that the 1:20 titer resulted in a sensitivity of 78% and a specificity of 85%.

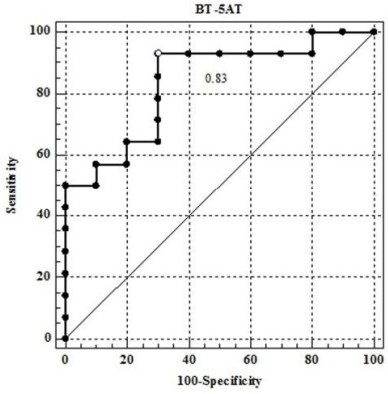
4 DISCUSSION

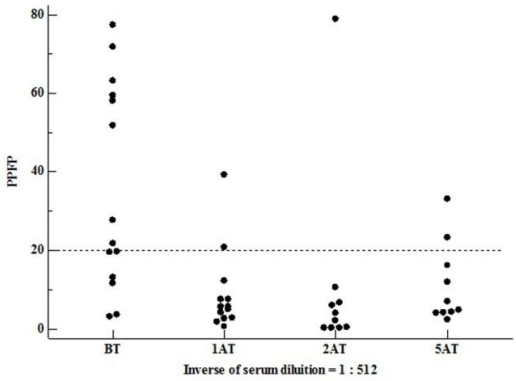
Leishmania (V.) braziliensis is the main species responsible for the disease in Brazil, classified as a species of great importance to the public health. It is responsible for a wide spectrum of lesions, which makes the clinical diagnosis neither simple nor immediate. Moreover, their lesions are often confused with other skin infections, reinforcing the need for a differential diagnosis (Marzochi 1992; Pirmez et al. 1999; Avila et al., 2004). Thus, the socio-economic challenges associated with methodological difficulties in establishing a safe diagnosis of ATL, justifies the development of new diagnostic approaches for the disease. The performance evaluation of individual samples by flow cytometry, at the cut-off point of 20%, demonstrated the segregation of BT patients and individuals in different regions of AT, which had not been possible through the analysis of the titers of IFA. We decided to do a comparative analysis of test performance with the proposed technique IFA, the most conventional serological technique used for detection of antibodies indicated in the diagnosis of ATL by *Leishmania (V.) braziliensis*. Although, in some situations, the IFA serological results present difficulties on finding parasites (Sampaio et al. 2009; Molesleh et al., 1995).

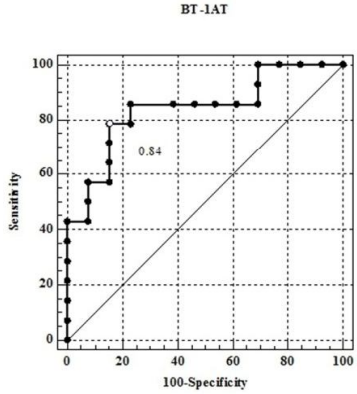

PPFP
80
60
40
20
0
BT
1AT
2AT
5AT
Inverse of serum dilution = 1 : 256

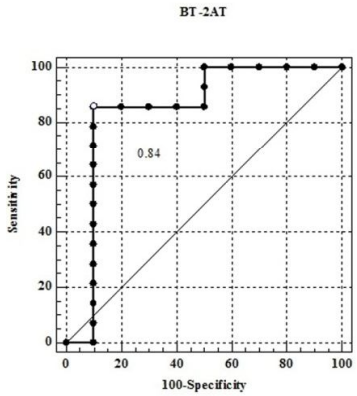

BT-1AT
Sensitivity
100
80
60
40
20
0
0.87
0
20
40
60
80
100
100-Specificity

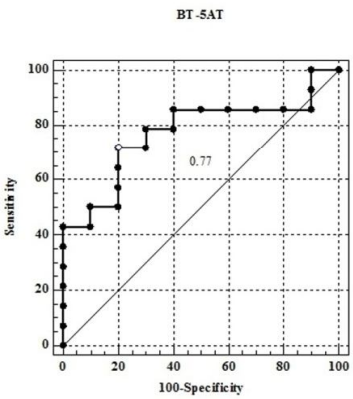

BT-2AT
Sensitivity
100
80
60
40
20
0
0.9
0
20
40
60
80
100
100-Specificity

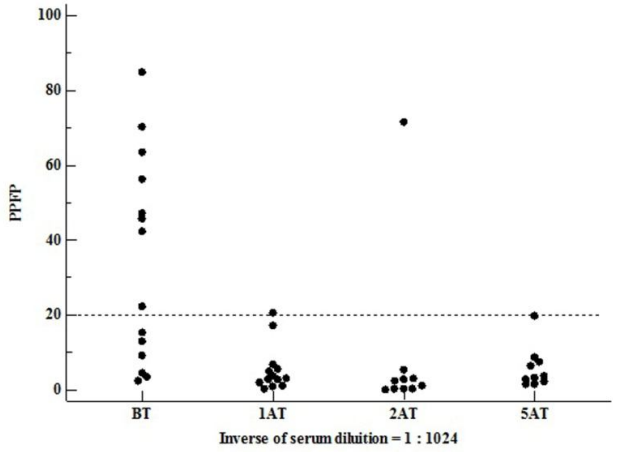

BT-5AT
Sensitivity
100
80
60
40
20
0
0.83
0
20
40
60
80
100
100-Specificity

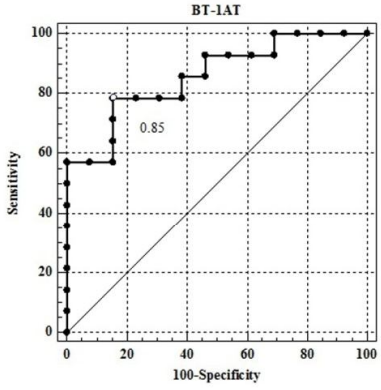

PPFP
80
60
40
20
0
BT
1AT
2AT
5AT
Inverse of serum dilution = 1 : 512

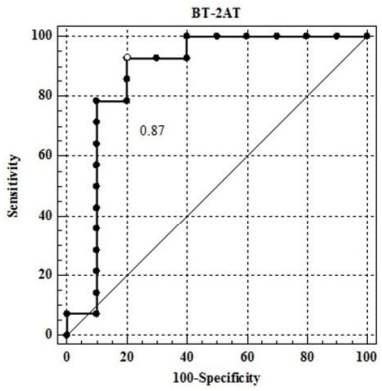

BT-1AT
Sensitivity
100
80
60
40
20
0
0.84
0
20
40
60
80
100
100-Specificity

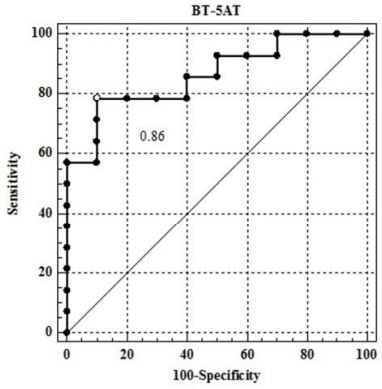

BT-2AT
Sensitivity
100
80
60
40
20
0
0.84
0
20
40
60
80
100
100-Specificity

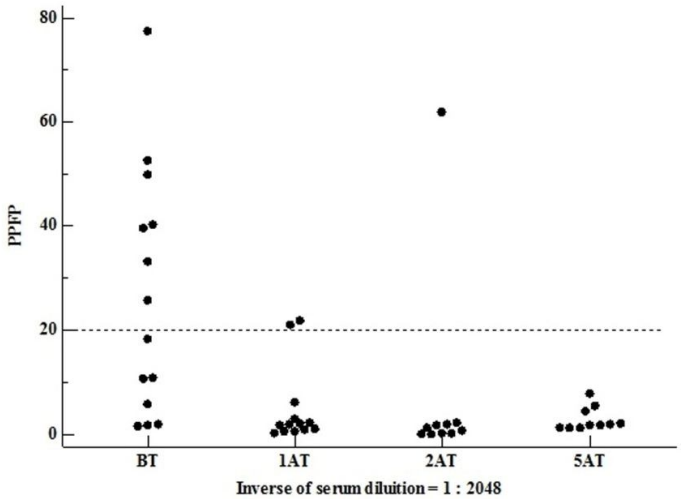

BT-5AT
Sensitivity
100
80
60
40
20
0
0.77
0
20
40
60
80
100
100-Specificity

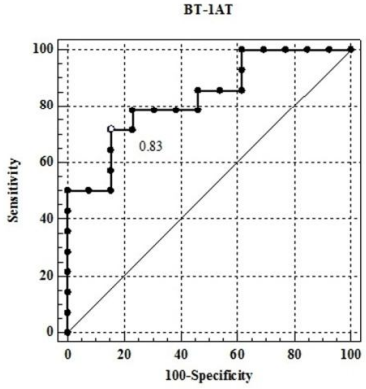

PPFP
BT
1AT
2AT
5AT
Inverse of serum dilution = 1 : 1024

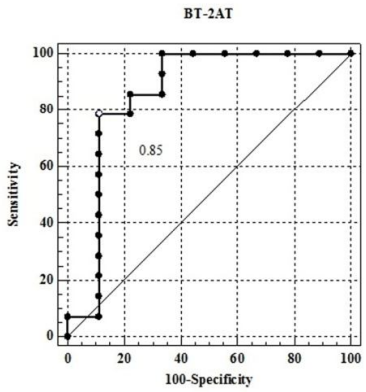

BT-1AT
Sensitivity
100-Specificity
0.85


BT-2AT
Sensitivity
100-Specificity
0.87


BT-5AT
Sensitivity
100-Specificity
0.86


PPFP
BT
1AT
2AT
5AT
Inverse of serum dilution = 1 : 2048


BT-1AT
Sensitivity
100-Specificity
0.83


BT-2AT
Sensitivity
100-Specificity
0.85

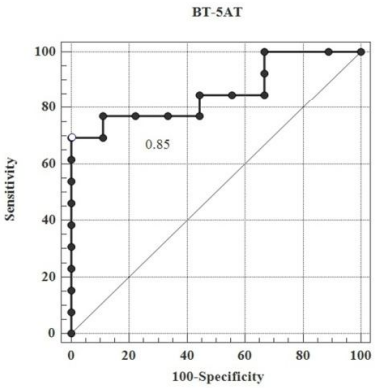

BT-5AT
Sensitivity
100-Specificity
0.85

Figura 3: Evaluation of the flow cytometry assay applicability on identifying patients with active ATL from the sera of patients from the region title with 256, 512, 1024 and 2048 reactivity between BT and 1, 2 and 5 years AT. ROC curve with AUC in different times. The dotted line corresponds to a cutoff of 20% PPFP.

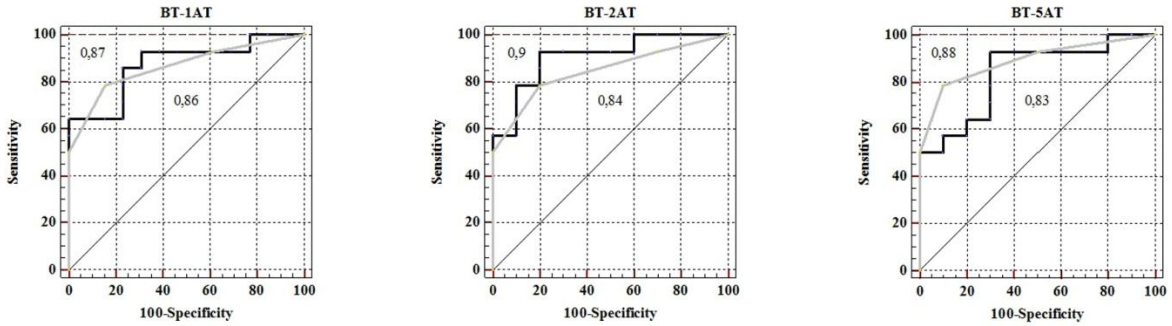


Figure 4: Comparison between ROC curves of IFA (–) and flow cytometry in serum dilutions of 1:256, (--), BT with 1, 2 and 5 years AT, built from the performance, sensitivity and 100 - specificity of the evaluated tests. AUC = area under the curve of flow cytometry and IFA.

In a comparative analysis of the performance indexes, the sensitivity values found for the IFA and flow cytometry demonstrated that flow cytometry has applicability to screening ATL in epidemiological studies or as part of initial research in a patient, since it was frequently positive (12/14) in the presence of the disease. The sensitivity found for IFA agrees with other studies (Reis et al., 2008, Romero et al. 2005; Szargiki et al., 2009) and, despite the equivalent sensitivity to flow cytometry, studies report that the sensitivity of IFA for the cutaneous forms *of L. (V.) braziliensis* may present variation from 50 to 80% (Marzochi, 1992; Tavares et al., 2003). Studies show that the sensitivity of a diagnostic method depends on the multifactorial aspects inherent of the antigen nature used as well as the detection system intrinsic to a particular technology (Kar 1995, Romero et al., 2005). Thus, the best performance and reliability of the flow cytometry methodology has been guaranteed by using intact parasites, studying selective membrane antigens, available on the parasites of *L. (V.) braziliensis*.

Regarding the specificity, the data analysis showed that the flow cytometry is better when compared to the IFA, with an average performance and below desired levels. Gontijo et al. (2002) reported a reactivity of 21.5% for IFA in individuals without clinical signs of ATL,

residents of endemic areas for the disease. Therefore, we attributed the false-positive results in flow cytometry to the possibility of re-infection, since those individuals are from endemic areas and immunologically sensitized (Rocha et al., 2006). Although, we could not exclude the possibility of occurring cross-reactivity with other infectious agents. In fact, Pereira et al 2012, demonstrated that methods which have displayed specificity still have required methodological adjustments in order to improve their performance as a tool for a confirmatory diagnostic.

In the ROC curve, the comparative analysis of the overall accuracy of the IFA and flow cytometry demonstrated an approximated accuracy of the flow cytometry when compared to IFA in groups 1, 2 and 5 years after treatment. The ROC curve allowed the identification and selection of a best cut-off point for the flow cytometry with these patients. Thus, through the ROC curve, the value of PPFP = 20%, giving a better counterbalance of sensitivity and specificity. This did not happen with Rocha et al 2002, while studying anti-live L. braziliensis with cut-off of 50% and with others studies, that studying IgG subclasses the cut-off was 60% (Rocha et al, 2006; Pereira et al, 2012). The difference between our study compared to these, is that we have used fixed parasites. The use of fixed promastigotes represents a feasible way to store a bulk amount of antigenic support that contributes to a large scale production. Moreover, there risk of infection during manipulation of living parasites, further support the use of fixed preparations as antigen source for serological tests (Pissinate et al, 2008).

The cure criterion found so far is only clinically, and it is based on the re-epithelialization of the lesions. The conventional serological tests are still considered limited, because they do not distinguish active infection from past infection. The dynamics of the reactivity of antibodies directed against the membrane antigens after treatment of ATL remains unclear (Brito et al. 2001). Considering the applicability of the flow cytometry as a post-treatment cure criterion, from the antibody titration curve, we conducted the follow-up of BT patients, one, two and five years AT. The flow cytometry showed a better performance compared to IFA. We observed a greater decrease in the percentage of positives and above the median values of PPFP, suggesting that there is applicability in the post-therapeutic cure control in ATL. Thus, the test identified cure in most patients, noting that to validate these results a larger number of patients should be evaluated.

Although most flow cytometry-based methods still represent higher cost compared to conventional methods, such as immunosorbent and immunofluorescence assay (Pereira et al 2012), many clinical laboratories, at the present time, have invested in the acquisition of a flow cytometer due to its frequent use for many different sub-areas of biology sciences and

health (Vieira et al. 2003; Nakage et al. 2005; Machado Jr et al., 2006; Thomas, Buxbaum et al., 2008; Santiago et al., 2008). The possibility of working with a microplate serological approach has reduced the final cost of a given test (Pereira et al 2012).Thus, in this context, the expansion of the use of flow cytometry will, in the near future, aid the implementation of new methodologies in conventional laboratories, providing greater exchange between the research centers of technological development and the clinical laboratory routine. This new serologic methodology, which uses flow cytometry to detect anti-*L.(V.) braziliensis*, in our experience, presented good reproducibility and outstanding concordance among independent analysts (Garcia et al., 2009). In addition, it has proved to be an alternative diagnostic applied to the study of ATL and that the information obtained in this study opens the possibility to monitor the after treatment cure of ATL. Furthermore, the preparation of other studies in this line, with a larger sample, would contribute to the understanding of the humoral immune response in patients with ATL.

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5.3- Artigo 3: Manuscrito submetido ao periódico: *Diagnostic Microbiology and Infectious Disease*

Spontaneous cure assessment in American Tegumentary Leishmaniasis by flow cytometry-based serology

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Resumo

Vários esforços foram feitos para desenvolver uma abordagem sorológica confiável utilizando a citometria de fluxo no diagnóstico e monitoramento cura da leishmaniose tegumentar americana (LTA). O presente estudo teve como objetivo demonstrar a aplicação da citometria de fluxo baseada na sorologia para identificar cura espontânea em LTA. Para este propósito, selecionamos pacientes não tratados com LTA e indivíduos com completa cura e reepitelização da pele sem intervenção terapêutica ativa. A análise da média da porcentagem de parasitos fluorescentes positivos (PPFP) ao longo das curvas de titulação de anti-promastigotas fixados de *L. (V.) braziliensis* utilizando anticorpos IgG confirmou a aplicabilidade deste método para monitorar cura espontânea da LTA com excelente índices de desempenho, co-positividade (100%) e co-negatividade (100%). A análise do índice de razão de verosimilhança corrobora o excelente desempenho deste método de avaliação da cura espontânea em LTA. Em uma abordagem complementar este estudo teve como objetivo avaliar o desempenho do teste para o diagnóstico LTA em uma população, incluindo outras doenças infecciosas/parasitárias. Os dados demonstraram um desempenho moderado da prova para efeitos de diagnóstico diferencial com 43% de reatividade cruzada especialmente com leishmaniose visceral, doença de Chagas e hanseníase. Juntos, estes resultados demonstraram a aplicabilidade da citometria de fluxo baseada na sorologia para acompanhar o

monitoramento de cura em pacientes com LTA, mas aconselhamos que ajustes na metodologia ainda sejam necessários, a fim de melhorar o seu desempenho para o diagnóstico diferencial de LTA em áreas co-endémicas para outras doenças infecciosas/parasitárias.

Palavras-chave: LTA, Citometria de fluxo, cura espontânea e outras doenças.

ABSTRACT

Several efforts have been made to develop reliable flow cytometry serological approaches to American Tegumentary Leishmaniasis (ATL) diagnosis and cure monitoring. The present study aimed to demonstrate the application of flow cytometry-based serology to identify spontaneous cure in ATL. For this purpose, we have enrolled untreated patients with active ATL and subjects with complete healing and reepithelialization of the skin without therapeutic intervention. The analysis of the mean percentage of positive fluorescent parasites (PPFP) along the titration curves of anti-fixed promastigote *L. (V.) braziliensis* IgG confirmed the applicability of this method for monitoring spontaneous cure of ATL with outstanding co-positivity (100%) and co-negativity (100%) performance indexes. The analysis of likelihood ratio index corroborate the excellent performance this method for spontaneous cure assessment in ATL. In a complementary approach this study intended to evaluate the performance of the test for ATL diagnosis in a population including other infectious/parasitic diseases. Data demonstrated a moderate performance of the test for differential diagnosis purposes with 43% of cross reactivity especially with visceral leishmaniasis, Chagas disease and hanseniasis. Together, these findings demonstrated the applicability of the flow cytometry-based serology for follow-up cure monitoring of ATL patients but advise that adjustments in the methodology are still required in order to improve its performance for the differential diagnosis of ATL in co-endemic areas for other infectious/parasitic disease.

Keywords: ATL, flow cytometry, spontaneous cure and other diseases.

1. INTRODUCTION

American Tegumentary Leishmaniasis (ATL) is an infectious, chronic, non-contagious disease that affects millions of people worldwide and is still a serious public health problem (Couto, 2014). The most common species in Brazil are *L. (Viannia) braziliensis, Leishmania (Viannia) guyanensis, and L. (Leishmania) amazonensis.* ATL is currently distributed in all of the states, particularly in the state of Pernambuco.

ATL may present diverse clinical forms, from self-limiting cutaneous lesions to mucocutaneous disfiguring lesion forms, depending on the immune status of the patient and species of *Leishmania* (Murback 2011), with the *Leishmania (Viannia) braziliensis* the main species involved in the transmission of ATL so far in Pernambuco (Brito et al, 2009; de Oliveira, 2012).

Since there is no diagnostic test considered as the gold standard for ATL, diagnosis of leishmaniasis is based on criteria that consider epidemiological data, clinical features and laboratory test results (Brito 2008; Kar 1995). The association of some of these elements is frequently necessary to achieve the final diagnosis (Guedes 2008). Diagnosis is reached through direct research, culture in specific medium or histopathological test. Immunological tests, such as Montenegro's intradermal reaction and indirect immunofluorescence, are indirect methods used (Murback, 2011). Timely and adequate treatment is of great importance to prevent the evolution of the disease to destructive and severe forms. Drugs currently employed present high toxicity and frequent adverse effects, and none of them is sufficiently effective (Ourives-Neves, 2011; Lima 2007), besides there is no effective cure criterion which is based on clinical spontaneous cure assessment (Gontijo and Camargo, 2003).

Several efforts have been made to develop reliable flow cytometry serological approaches to ATL diagnosis and cure monitoring by using distinct antigen preparations to detect anti-*Leishmania* antibodies (Gonçalves et al., 2002; Celeste et al., 2004; Ryan et al., 2002; Rocha et al., 2006, Pereira et al. 2012; Oliveira et al, 2013). Together, these approaches have demonstrated that flow-cytometry-based methods are reliable tools applicable to the diagnosis and post-therapeutic cure assessment in ATL. Bittencourt et al., 1968 already suggested the possibility of antibodies search as a way to monitor the ATL cure. In 2001, Brito and colleagues, observed increased levels of IgG in spontaneously cured patients, suggesting a particular interest in evaluating whether the dynamics of the antibody response could be useful to monitor clinical cure of ATL. Pereira and colleagues have demonstrated that although flow cytometry-based serology was not able to detect changes in the anti-

Leishmania IgG reactivity. An early (1month-24 month) and after the end of etiological treatment assessments, employing a novel methodological approach by delta-reactivity pattern would represent a plausible method for post-therapeutic monitoring of ATL. Lately, Oliveira and colleagues, 2013 have demonstrated that flow cytometry can, in fact, be applicable for cure monitoring of ATL when performed later on after the end of etiological treatment. These authors demonstrated that flow cytometric serology was able to distinguish the IgG reactivity observed in patient with active ATL from those observed 1-5 years after the end of ATL treatment.

In the present work, we intended to validate the flow cytometry serology using anti-fixed *Leishmania (Viannia) braziliensis* promastigote IgG antibodies to diagnoses ATL infection and further demonstrate its applicability to identify spontaneous cure by demonstrating differential reactivity as compared to patients with active infection.

2. POPULATION, MATERIALS AND METHODS

2.1 Study Population

A total of 52 individuals were evaluated in this study. The patients with positive diagnosis for American Tegumentary Leishmaniasis (ATL=15) were selected before treatment and had their positivity confirmed in at least two diagnostic tests, including: Montenegro intradermoreaction, indirect immunofluorescence and PCR. Subjects spontaneously cured ATL (CUR=08) showed complete healing and reepithelialization of the skin without therapeutic intervention. Sera samples from 8 healthy individuals with no documented infection or exposure to *Leishmania* parasites were included as non-infected controls (NI). In addition, 21 sera samples were used for the differential diagnosis assessment including Visceral Leishmaniasis (VL=04), Chagas disease (CH=10), Hanseniasis (HN=05), Sporotrichosis (SPOR=01) and Tuberculosis (TB=01). All collected samples were from endemic areas in Pernambuco State, with the exception of CH group samples that are from Minas Gerais State. All samples were evaluated for clinical, epidemiological and laboratorial criteria and all individuals signed the “Term of Free and Informed Consent”. CPqAM/Fiocruz (Protocol no. 011/2013) and CPqRR/Fiocruz (CAAE: 0012.0.245.175-07) Research Ethics Committees has approved the experimental protocols.

2.2 Parasite Preparation

L. (V.) braziliensis promastigote forms (MHOM/BR/75/M2903) were cultivated in Schneider's medium until exponential phase. The parasites were centrifuged at low-speed (100×g), 25°C for 10min and recovered from the supernatant after 10min rest at room temperature. The supernatant was transferred to another tube and washed in buffer pH 7.2, containing 10% heat-inactivated fetal bovine serum (phosphate buffered saline [PBS]-10% FBS) three times at 4°C, 871×g for 10min. The pellet of parasites were thoroughly homogenized, re-suspended for fixing in an equal volume of 1% paraformaldehyde, and incubated overnight. The parasites were washed with PBS-10% FBS. The parasite suspension was counted, the concentration was adjusted for $5.0x10^6$/ml and used for flow cytometric immunofluorescence assay.

2.3 Flow Cytometry and Analysis

Flow cytometry acquisition and analysis for IgG detection of anti-fixed *L. (V.) braziliensis* promastigote was performed according to Oliveira et al (2013). Briefly, the parasite suspension ($2.5×10^5$/well) was incubated in 96-well U-bottom plates at 37°C for 30min in the presence of serial dilutions of inactivated serum samples (from 1:64 to 1:8,192). After incubation with sera, the parasites were washed twice with 150µl of PBS-10% FBS (1,000 ×g for 10min at 4°C) and re-incubated at 37°C for 30 min in the dark with anti-human IgG antibody conjugated with fluorescein isothiocyanate – FITC (Sigma Chemical Corp., St. Louis,MO) previously diluted 1:4,000 in PBS – 10% FBS. The FITC-labeled parasites were washed twice and fixed with FACS fix solution for 30min and stored at 4^oC up to 24h before flow cytometry analyses.

Flow cytometric acquisition (10,000 events per sample) was performed on a FACScalibur, Becton Dickinson and the "CellQuest Pro" software was used for both data storage and analysis. The parasites were identified based on their specific forward (FSC) and side (SSC) light-scattering properties. Parasites were selected by gating on the FSC×SSC dot-plot distribution. The relative FITC fluorescence intensity for each parasite was analyzed by a single histogram representation. A marker was set on the internal control to confine the nonspecific binding of FITC-conjugated antibody up to 2% and used to determine for each sample the percentage of positive fluorescent parasites (PPFP) as previously described by Oliveira et al (2012).

2.4 Statistical Analysis

Performance indexes were calculated to determine the applicability of flow cytometry serology to the diagnosis of ATL as well as spontaneous cure assessment, including "co-positivity" or sensitivity = ([true positive / real positive] x 100) and "co-negativity" or specificity = ([true negative / real negative] x 100). In addition, data were submitted to the ROC curve analysis to identify the area under the curve (AUC) as the global test accuracy. The data set was also analyzed by the two-graph receiver operating characteristic (TG-ROC) and Likelihood ratios - LR (LR+ = Se / (1–Sp) and LR- = (1–Se) / Sp).

Statistical analyses were performed using the MedCalc® statistical software and the Computer Method for Diagnostic Tests – CMDT, version 1.0β (Berlin: Freie Universität Berlin; 1997. Copyright © 1997-1999 Jens Briesofsky).

3. RESULTS

3.1 Applicability of flow cytometry-based serology for spontaneous cure assessment in ATL

Serum samples from 15 patients positive for ATL, 8 spontaneously cured (CUR) and 8 healthy individuals (NI) were tested by flow cytometry. The analysis of the mean PPFP values along the titration curves of anti-fixed promastigote *Leishmania* IgG (from 1:64 to 1:8,192) were used to select the 1/1,024 serum dilution as previously reported by Pereira et al., 2012 and Oliveira et al. 2013. At this serum dilution data also confirmed the higher segregation range among ATL, CUR and NI group (Figure 1A).

The Figure 1B showed the co-positivity and co-negativity performance indexes confirming the selection of 1/1,024 sera dilution as the most promising condition to discriminate the PPFP values of ATL from those provided by CUR and NI sera samples using the PPFP=20% as the cutoff edge. These results suggest the applicability of the method for monitoring spontaneous cure of the disease.

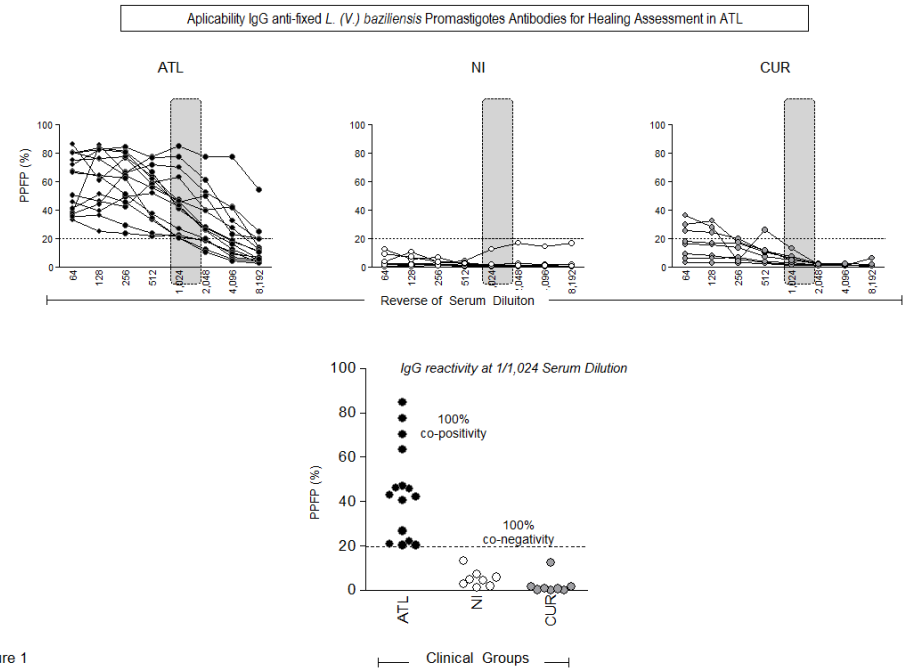

Figure 1

Figure 1: Titration curve average of IgG antibodies anti-fixed *Leishmania braziliensis* promastigotes from American tegumentary leishmaniasis patients (ATL ● n=15), non-infected individuals (NI ○n=8) and spontaneously cured patients (CUR ● n=8) serum samples **(A)**. The IgG antibodies anti-fixed *Leishmania braziliensis* of individual serum samples from ATL, NI and CUR groups at 1/1,024 dilution **(B)**. The results were expressed as percentage of positive fluorescent parasites (PPFP). The dotted line represents the cut-off between negative and positive results (cut-off = 20%).

3.2 Performance indexes of IgG anti-fixed *L. (V.) braziliensis* promastigotes antibodies for spontaneous cure monitoring in ATL

ROC curves were prepared to confirm the selection of 1/1,024 sera dilution as the most capable condition to discriminate the PPFP values among the groups (Figure 2A) as well as to assess the performance of IgG anti-fixed *L. (V.) braziliensis* promastigotes antibodies for spontaneous cure assessment in ATL. The analysis of the area under the ROC curve demonstrated an outstanding performance of the test (AUC=1.0; IC95%:1.0-1.0). The performance analysis of IgG anti-fixed *L. (V.) braziliensis* promastigotes antibodies for spontaneous cure assessment in ATL demonstrated the outstanding performance indexes with 100% of sensitivity (IC95% =79-100) and 100% of specificity (95%IC=78-100) using the PPFP=20% as the cutoff edge (Figure 2B,C). Likelihood ratio (LR) corroborate the performance of IgG anti-fixed *L. (V.) braziliensis* promastigotes antibodies for spontaneous cure assessment in ATL, demonstrating that positive results (PPFP>20%) has infinite times more chance to come from an ATL patient then NI+CUR individuals. On the other hand,

negative results (PPFP<20%) is unlikely to belong to an ATL individual, strongly suggesting the spontaneous cure (Figure 2D).

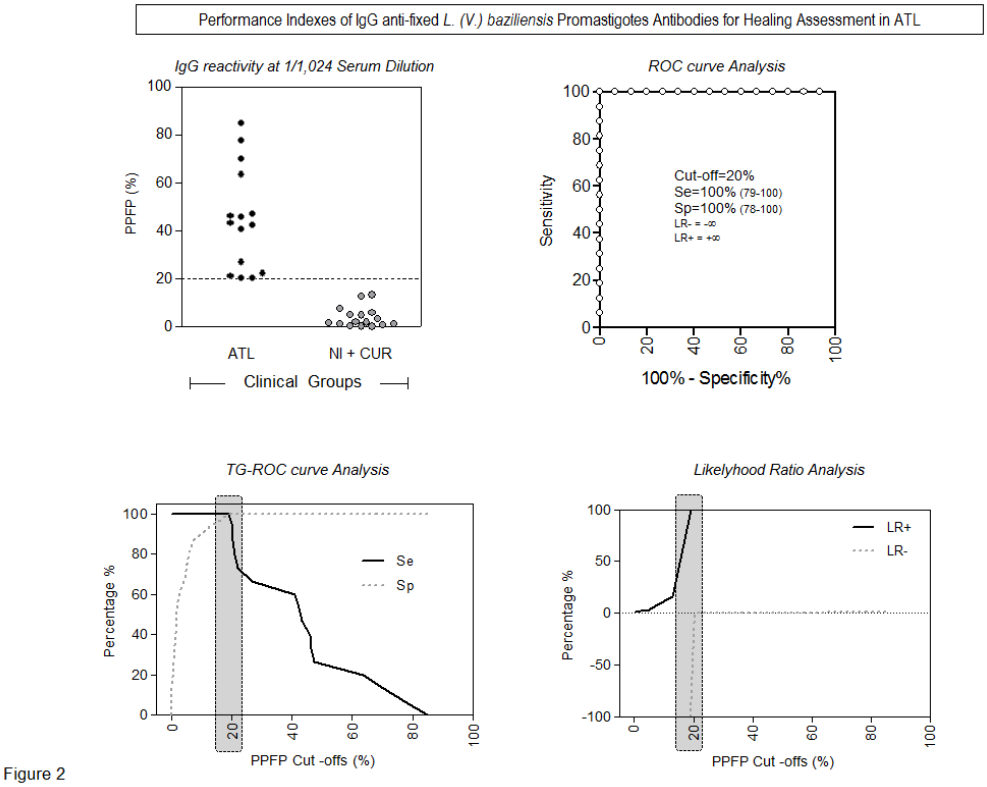

Figure 2

Figure 2: The IgG antibodies anti-fixed *Leishmania braziliensis* of individual serum samples from ATL, NI + CUR groups at 1/1,024 dilution **(A)**. ROC curve analyses confirmed the previously elected cut-off, and demonstrated outstanding performance indexes (Sensitivity-Se; Specificity-Sp; Area under the curve-AUC **(B,C)**; and Positive/Negative Likelihood Ratio-LR+/LR- for ATL **(D)**. The results were expressed as percentage of positive fluorescent parasites (PPFP). The dotted line and gray bars represents the cut-off between negative and positive results (cut-off = 20%).

3.3 Performance indexes of IgG anti-fixed *L. (V.) braziliensis* promastigotes antibodies for ATL diagnosis in a population including other infectious/parasitic diseases

Serum samples from 15 patients positive for ATL, 8 spontaneously cured (CUR) and 8 healthy individuals (NI) together with 21 sera samples for the differential diagnosis assessment including Visceral Leishmaniasis (VL=4), Chagas disease (CH=10), Hanseniasis (HN=05), Sporotrichosis (SPOR=01) and Tuberculosis (TB=01) were tested by flow cytometry. The analysis of the mean PPFP values along the titration curves of anti-fixed

promastigote *Leishmania* IgG (from 1:64 to 1:8,192) permitted to select the 1/1,024 serum dilution for the segregation range among ATL and NON-ATL group (Figure 3A).

The Figure 3B analysis confirmed the 1/1,024 sera titer as a potential dilution to discriminate the PPFP values of ATL and NON-ATL sera samples using the PPFP = 20% as the cutoff edge, although it presented 43% of cross reactivity. The analysis of the area under the ROC curve demonstrated a moderate performance of the test (AUC=0.77; IC95%:0.6-0.9) (Figure 3C). The performance analysis of IgG anti-fixed *L. (V.) braziliensis* promastigotes antibodies for ATL diagnosis in a population including other infectious/parasitic diseases showed 100% of sensitivity (IC95%=78-100) and 57% of specificity (95%IC= 40-73) (Figure 3C). These results suggest the applicability of the method for ATL diagnosis in a population including other infectious/parasitic diseases showing a high sensitivity. However, the specificity was limited showing 43% of crossreactivity.

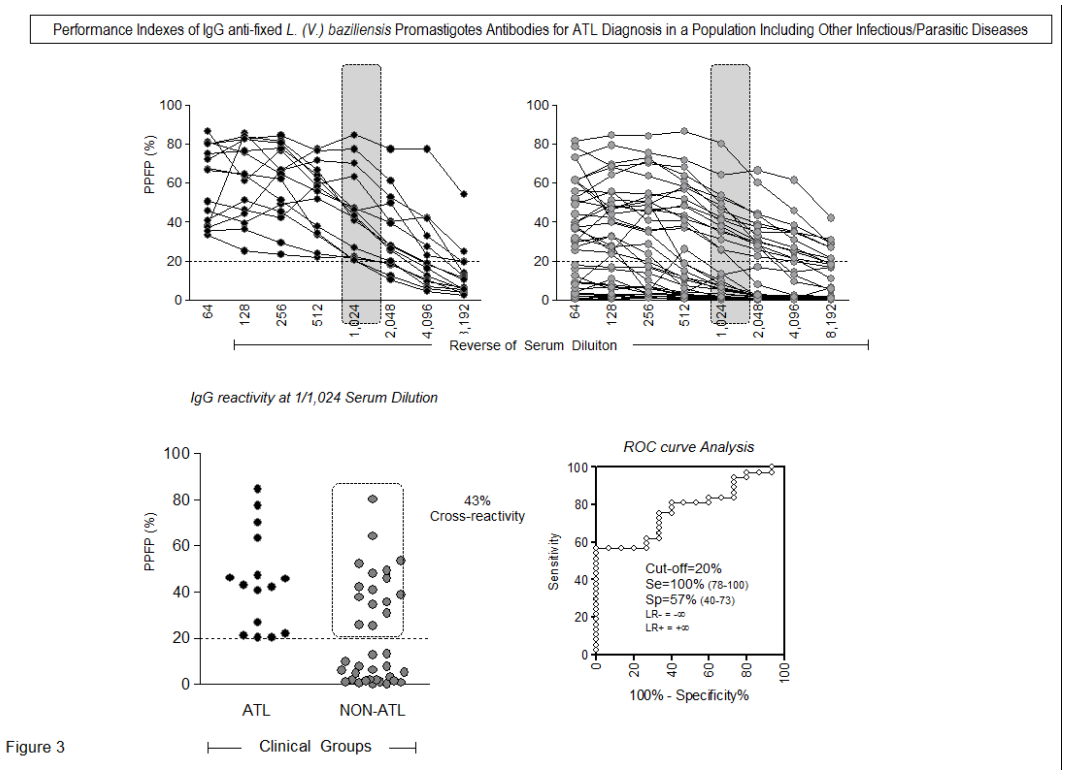

Figure 3: Titration curve average of IgG antibodies anti-fixed *Leishmania braziliensis* promastigotes from American tegumentary leishmaniasis patients (ATL ● n=15) and Non-ATL individuals (NI + CUR + VL + CH + HN + SPOR + TB ● n=37) serum samples **(A)**. The IgG antibodies anti-fixed *Leishmania braziliensis* of individual serum samples from ATL and Non-ATL groups at 1/1,024 dilution **(B).** ROC curve analyses confirmed the previously elected cut-off, and demonstrated performance indexes (Sensitivity-Se; Specificity-Sp; Area under the curve-AUC **(C)**. The results were expressed as percentage of positive fluorescent parasites (PPFP). The dotted line represents the cut-off between negative and positive results (cut-off = 20%).

3.4 Major infectious/parasitic diseases with cross reactivating in IgG anti-fixed *L. (V.) braziliensis* promastigotes antibody assay

Serum samples from 15 patients positive for ATL, 8 spontaneously cured (CUR) and 8 healthy individuals (NI) together with 21 sera samples for the differential diagnosis assessment including Visceral Leishmaniasis (VL=04), Chagas disease (CH=10), Hanseniasis (HN=05), Sporotrichosis (SPOR=01) and Tuberculosis (TB=01) were tested aiming the evaluation of the clinical value of the differential diagnosis in a population including other infectious/parasitic diseases (Figure 4A). The analysis of the 1/1,024 sera dilution (PPFP≥20% as cutoff) showed high cross reactivity from ATL samples when compared with VL (100%) and CH (100%) and HN (40%) with a great amount of false-positives (Figure 4). On the other hand, sera samples from SPOR and TB group did not show any cross reactivity with ATL group (Figure 4B).

4. DISCUSSION

ATL has been diagnosed microscopically by the identification of *Leishmania* parasites or amastigotes within macrophages of sample tissue. However, the presence of parasites depends on the duration of lesions, being fewer in number in chronic lesions (Andrade-Narvaez, 2005; Ameen, 2010). Although these tests are 100% specific, their sensitivity varies with the technician expertise. To maximize sensitivity and specificity, the following four investigations should ideally be performed: direct microscopy, histopathology, culture and PCR (Ameen, 2010). Therefore, serological tests are very important alternative for monitoring clinical cure, especially because the spontaneously cured patients, which presents decrease of the parasite load and of IgG antibody reactivity (Brito et al.,2001), can be identified.

The present work evaluated the detection of IgG antibodies anti-fixed *L. (V.) braziliensis* promastigote by flow cytometry to identify active infection, spontaneous cure and to proceed differential diagnosis of ATL. Several serological methods have been performed for diagnosis of ATL and for monitoring clinical cure through the decrease of parasite load after the healing of lesions (Romero et al., 2005; Mendonça et al., 2004). Previously, Romero et al (2005) demonstrated a decrease on the reactivity levels of IgG after specific treatment, showing that ELISA technique has a better performance compared to the IFA test. However,

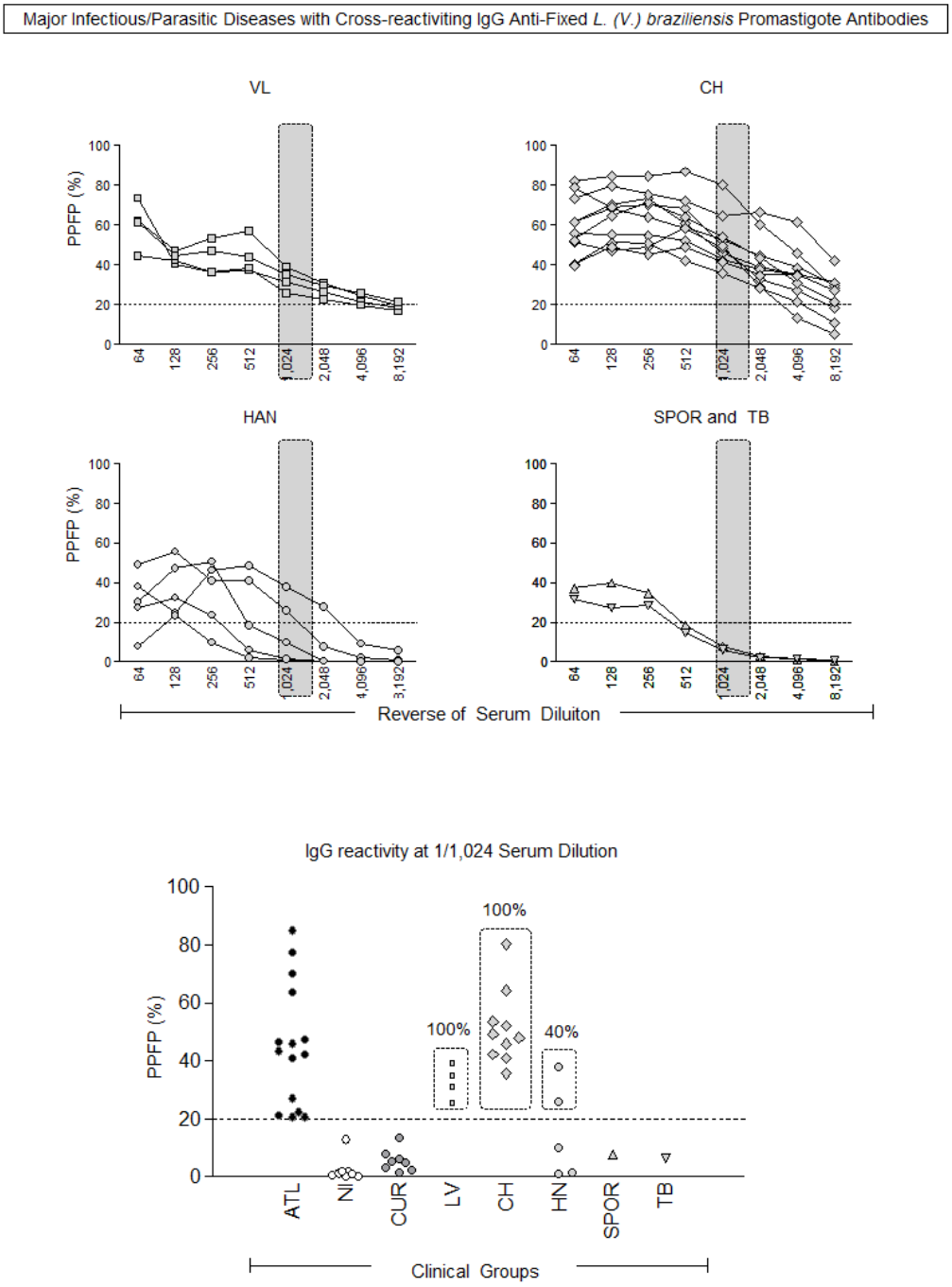

Figure 4

Figure 4. Titration curve average of IgG antibodies anti-fixed *Leishmania braziliensis* promastigotes from Visceral Leishmaniasis patients (VL ■ n=04); Chagas disease patients (CH ♦ n=10); Hanseniasis patients (HN ● n=05), Sporotrichosis patient (SPOR ▲n=01) and Tuberculosis patient (TB ▼n=01) serum samples **(A).** The IgG antibodies anti-fixed *Leishmania braziliensis* of individual serum samples from ATL, NI, CUR, VL, CH, HN, SPOR and TB groups at 1/1,024 dilution **(B)**. The results were expressed as percentage of positive fluorescent parasites (PPFP). The dotted line represents the cut-off between negative and positive results (cut-off = 20%).

the cure criterions for ATL remain controversial, since the persistence of positivity in conventional serological tests can be seen after spontaneous or therapeutic cure.

Several efforts have been made aiming to build up a more reliable serological approach to ATL diagnosis by using distinct antigen preparations to detect anti-*Leishmania* antibodies (Gonçalves et al., 2002; Celeste et al., 2004; Ryan et al., 2002; Rocha et al., 2006). The spontaneously cured patients (CUR) are the target of immunological research, possibly because they present a differential and more efficient protective response when compared with the ATL active infection cases (Snoek et al., 2009; Brito et al., 2001). Our results from CUR group showed a high performance of the method, presenting low reactivity of PPFP (PPFP<20%) similar with NI individuals. The infection by *L. (V.) braziliensis* tends to be difficult to heal and sometimes it can cause metastasis, involving mucous lesions on the nasopharynx (WHO, 2010). However, the spontaneous cure of the lesions suggests the development of an immune response capable to control the infection by *Leishmania* (Brito et al., 2001). Previous studies showed that spontaneously cured patients present low levels of IgG antibodies, which correlate with the decrease of parasite load. Thus, the low reactivity of antibodies showed by CUR patients in the present work suggests that our method can be useful to monitoring clinical cure. These findings are in agreement with other authors that also have shown that low levels of antibodies after therapy for ATL can indicate a successful cure (Lemos et al., 2007; Pissinate et al., 2008; Garcia et al., 2009).

It is important to point out that a differential diagnosis for ATL is essential and desirable, since some diseases such as hanseniasis, skin cancer, tuberculosis and cutaneous mycosis present similar clinical aspects with ATL, generating false-positive diagnosis. Besides that, Chagas disease (CH) and visceral leishmaniasis (VL), which present co-endemicity with ATL, are targets of cross reactivity (Goto and Lindoso, 2010; Reithinger et al 2007). Here, we report the applicability of flow cytometry for ATL diagnosis in a population including other infectious/parasitic diseases (VL and CH). Our data suggested that the new flow cytometry-based methodology has a good performance to identify active infection and spontaneous cure, however has high cross reactivity with VL, CH and HN, presenting great percentage of false-positive results.

These findings were already expected since some of the leishmanial antigens have common cross-reactive epitopes shared with other microorganisms particularly *Trypanosoma*, *Mycobacteria*, *Plasmodium* (Kar, 1995). Indeed, the use of whole promastigote as the source of antigens in the direct agglutination test (DAT) and immunofluorescent test (IFAT) gave cross reactions with the sera of leprosy, tuberculosis,

and African trypanosomiasis patients, representing for long a challenge to researchers (Celeste et al., 2004; Junqueira Pedras et al., 2003). Additionally, high frequency of cross reactivity was observed for sera from Chagas disease patients and with malaria patients, with both antigens, as previously reported for IgG and IgG3 (Luquetti, 1990, Marzochi, 1992, Teixeira and Vexanat, 1996).

The differential diagnosis, in order to reduce the disadvantage related to the occurrence of cross reactivity is highly relevant, mainly in regions where these parasites are co-endemic (Rocha et al., 2006). Several researchers have aimed to overcome the limitations of ATL serological diagnosis with alternative antigenic preparations to detect *Leishmania* antibodies (Pappas et al. 1983; Gonçalves et al. 2002; Celeste et al. 2004). The use of live promastigotes would represent a useful tool to increase specificity, since only external membrane epitopes are accessible for IgG binding, avoiding cross reactivity with intracellular components commonly distributed among trypanosomatidae (Andrade et al., 1988; Chiaramonte et al., 1996). On the other hand, the use of fixed promastigotes represents a practical way to store a massiveness amount of antigenic support that contributes to large scale production. Moreover, the risk of infection during manipulation of live parasites, further support the use of fixed preparations as antigen source for serological tests.

In summary, our data suggest the potential and applicability of the cytometry-based methodology for an alternative diagnosis for ATL active infection and follow-up cure monitoring of ATL patients but advise that adjustments in the methodology are still required in order to improve its performance for the differential diagnosis of ATL in co-endemic areas for other infectious/parasitic disease.

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6. CONCLUSÕES

I- A utilização de formas promastigotas vivas ou fixadas de *Leishmania Viannia braziliensis,* apresentou sensibilidade semelhante no estudo da LTA por citometria de fluxo, portanto aplicável como metodologia alternativa no diagnóstico da LTA.

II- A reatividade da *Leishmania (Viannia) braziliensis* no monitoramento da cura clínica dos pacientes após o tratamento por citometria de fluxo demonstrou ser mais específica utilizando parasitos vivos.

III- A análise comparativa dos valores de sensibilidade entre citometria de fluxo e imunofluorescência indireta, utilizando os pacientes AT, demonstrou que a citometria de fluxo foi positiva na presença da doença.

IV- A aplicabilidade da citometria de fluxo como critério de cura da LTA foi confirmada a partir do acompanhamento dos pacientes 1, 2 e 5 anos após o tratamento da LTA, onde se observou melhor ganho em especificidade da citometria de fluxo em relação a imunofluorescência indireta.

V- A porcentagem de parasitos fluorescentes positivos observadas nos pacientes curados espontaneamente localizou-se numa região de baixa reatividade em 100% dos pacientes, cofirmando a reprodutibilidade da citometria de fluxo na identificação de pacientes curados.

VI- O diagnóstico diferencial da LTA utilizando soros de pacientes com DC e LV demonstrou reação cruzada por citometria de fluxo. Porém, apresentou aplicabilidade com soros de pacientes com outras doenças dermatológicas, clinicamente semelhantes a LTA.

VII- Portanto, a presente tese demonstrou que os ensaios realizados utilizando anticorpo IgG detectados por *Leishmania (V.) braziliensis* através citometria de fluxo representam além de uma ferramenta alternativa para o diagnóstico da LTA, devido a reprodutibilidade e a possibilidade de identificar pacientes com a infecção e após cura clínica ou espontânea; mais também abrem perspectivas para a utilização no monitoramento e critério de cura da LTA.

7. PERSPECTIVAS

I- Avaliar a aplicabilidade da citometria de fluxo na identificação dos pacientes com doença ativa e após cura clínica por tratamento quimioeterápico ou espontânea com ênfase na pesquisa de isotipos IgG (IgG1, IgG2, IgG3, IgG4) anti-promastigotas vivas e fixadas de *Leishmania (Viannia) braziliensis*;

II- Analisar a curva de titulação dos anticorpos IgG por citometria de fluxo na identificação de pacientes 10 anos após o tratamento e a partir da curva ROC determinar os melhores valores de PPFP para o ensaio.

III- Avaliar a aplicabilidade da citometria de fluxo, utilizando-se diferentes proteínas recombinantes, como metodologia alternativa no diagnóstico e monitoramento pós-terapêutico de pacientes infectados com LTA.

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APÊNDICE A

Fundação Oswaldo Cruz, Ministério da Saúde

Centro de Pesquisas
AGGEU MAGALHÃES

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO – Grupo Paciente
Projeto: "Caracterização da resposta imune humoral em portadores de Leishmaniose Tegumentar Americana ativa e após cura clínica".

Eu, ..., concordo em participar voluntariamente neste projeto que será desenvolvido no Centro de Pesquisas Aggeu Magalhães da Fundação Oswaldo Cruz (CPqAM/FIOCRUZ). Fui informado que o objetivo principal do referido projeto é a investigação da defesa do organismo dos pacientes com leishmaniose tegumentar ativa, e após a cura clínica espontânea ou após tratamento quimioterápico.

Como faço parte do grupo de pacientes, serei submetido a coleta de 10 ml de sangue venoso antes e 10 ml de sangue venoso após a cura clínica espontânea ou após tratamento quimioterápico e a exames que incluirão a intradermoreação de Montenegro, pesquisa direta; punção aspirativa, biópsia da borda da lesão ativa, imunofluorescência indireta e PCR. Todas as informações e os detalhes dos exames que serão realizados serão previamente esclarecidos para mim. Além disso, também fui informado que receberei os resultados desses exames. Todo procedimento será realizado com material estéril descartável e por profissionais de saúde de reconhecida capacidade para executar os procedimentos. Fui informado que a coleta de sangue é um método invasivo, por isso pode causar desconforto no local, na hora da picada e que não devo friccionar (movimento com força) o local para não haver formação de hematoma, ou seja, pequena mancha roxa. O esquema terapêutico com Glucantime® (remédio utilizado para o tratamento) será composto por doses de 20 mg/Kg/dia através de injeções intramusculares em ciclos de vinte a trinta dias, sendo realizado no posto de saúde do município de Moreno por funcionários qualificados (médicos, enfermeiros ou auxiliares de enfermagem). Fui informado que esse remédio (Glucantime®) é o mais utilizado, promove cura da doença e pode ter efeitos colaterais como náuseas e indisposição (moleza). Fui informado que se ocorrer qualquer alteração em meu organismo, deverei procurar o médico do posto de saúde.Esse trabalho trará grande benefício, pois indicará se componentes do sistema de defesa do organismo poderão ser usadas como marcadores da resposta terapêutica e se outras células do sistema de defesa do organismo participam na evolução clínica de pacientes com leishmaniose tegumentar americana em Pernambuco. Antes de minha participação no referido projeto, fui incentivado a pedir esclarecimento adicional que julgasse necessário, esclarecida por um participante do projeto. Estou ciente que poderei recusar ou retirar meu consentimento, em qualquer momento da investigação, sem qualquer punição ou prejuízo. Autorizo a Fundação Oswaldo Cruz (CPqAM/FIOCRUZ) a utilização das informações obtidas através dos resultados dos procedimentos em reuniões, congressos, patentes e publicações científicas preservando, neste caso, a minha identidade. Autorizo, também que o CPqAM/FIOCRUZ poderá estocar amostra biológica para posteriores estudos. Fui informado que para usar a amostra biológica estocada, um pesquisador do projeto entrará em contato comigo.

Estou ciente que este documento é feito em duas vias, ficando uma em minha posse e a outra com a equipe do projeto e que todas e quaisquer dúvidas que eu venha a ter sobre o significado dos termos empregados no presente texto me serão completamente esclarecidos por um dos membros do projeto antes que eu assine este impresso.

_____ _____
Assinatura do paciente data

Endereço do sujeito da pesquisa: _____

_____ _____
Assinatura do médico responsável – CPqAM/FIOCRUZ data
Pesquisador responsável: Dra. Valéria Rêgo Alves Pereira. Telefone para contato: 81 21012500

Departamento de Imunologia, Centro de Pesquisas Aggeu Magalhães, FIOCRUZ, Av. Moraes Rego, s/n , Cidade Universitária, Cx. Postal 7472, CEP: 50670-420, Recife – PE, Brasil. Tel.: (081) 3301 2500; Fax: (081) 3453 2449; http://www.cpqam.fiocruz.

APÊNDICE B

Fundação Oswaldo Cruz, Ministério da Saúde

Centro de Pesquisas
AGGEU MAGALHÃES

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO – Grupo Paciente menor (15 a 18 anos)
Projeto: "Caracterização da resposta imune humoral em portadores de Leishmaniose Tegumentar Americana ativa e após cura clínica".

Eu, ..., concordo que meu filho ..... participe voluntariamente neste projeto que será desenvolvido no Centro de Pesquisas Aggeu Magalhães da Fundação Oswaldo Cruz (CPqAM/FIOCRUZ). Fui informado que o objetivo principal do referido projeto é a investigação da defesa do organismo dos pacientes com leishmaniose tegumentar ativa, e após a cura clínica espontânea ou após tratamento quimioterápico. Como meu filho (a) faz parte do grupo de pacientes, será submetido a coleta de 10 ml de sangue venoso antes e 10 ml de sangue venoso após a cura clínica espontânea ou após tratamento quimioterápico e a exames que incluirão a intradermoreação de Montenegro, pesquisa direta; punção aspirativa, biópsia da borda da lesão ativa, imunofluorescência indireta e PCR. Todas as informações e os detalhes dos exames que serão realizados serão previamente esclarecidos para mim que sou responsável pelo menor. Além disso, também fui informado que como responsável pelo menor receberei os resultados desses exames. Todo procedimento será realizado com material estéril descartável e por profissionais de saúde de reconhecida capacidade para executar os procedimentos. Fui informado como responsável pelo menor que a coleta de sangue é um método invasivo, por isso pode causar desconforto no local, na hora da picada e que ele não deve friccionar o local para não haver formação de hematoma, ou seja, pequena mancha roxa. O esquema terapêutico com Glucantime® (remédio utilizado para o tratamento) será composto por doses de 20 mg/Kg/dia através de injeções intramusculares em ciclos de vinte a trinta dias, sendo realizado no posto de saúde do município de Moreno por funcionários qualificados (médicos, enfermeiros ou auxiliares de enfermagem). Fui informado que esse remédio (Glucantime®) é o mais utilizado, promove cura da doença e pode ter efeitos colaterais como náuseas e indisposição (moleza). Fui informado como responsável pelo menor que se ocorrer qualquer alteração no organismo do menor que sou responsável, deverei procurar o médico do posto de saúde. Esse trabalho trará grande benefício, pois indicará se componentes do sistema de defesa do organismo poderão ser usadas como marcadores da resposta terapêutica e se outras células do sistema de defesa do organismo participam na evolução clínica de pacientes com leishmaniose tegumentar americana em Pernambuco. Antes da participação do menor que sou responsável no referido projeto, fui incentivado a pedir esclarecimento adicional que julgasse necessário, esclarecida por um participante do projeto. Estou ciente que o menor que sou responsável poderá recusar ou retirar o consentimento, em qualquer momento da investigação, sem qualquer punição ou prejuízo. Como responsável pelo menor, autorizo a Fundação Oswaldo Cruz (CPqAM/FIOCRUZ) a utilização das informações obtidas através dos resultados dos procedimentos em reuniões, congressos, patentes e publicações científicas preservando, neste caso, a identidade do menor que sou responsável. Como responsável pelo menor, também autorizo que o CPqAM/FIOCRUZ poderá estocar amostra biológica para posteriores estudos. Como responsável pelo menor, fui informado que para usar a amostra biológica estocada, um pesquisador do projeto entrará em contato comigo. Como responsável pelo menor, estou ciente que este documento é feito em duas vias, ficando uma em minha posse e a outra com a equipe do projeto e que todas e quaisquer dúvidas que eu venha a ter como responsável pelo menor sobre o significado dos termos empregados no presente texto me serão completamente esclarecidos por um dos membros do projeto antes que eu assine este impresso.

_____ _____
Assinatura do responsável pelo menor data

Endereço do sujeito da esquisa:_____

_____ _____
Assinatura do médico responsável – CPqAM/FIOCRUZ data
Pesquisador responsável: Dra. Valéria Rêgo Alves Pereira. Telefone para contato: 81 21012500

Departamento de Imunologia, Centro de Pesquisas Aggeu Magalhães, FIOCRUZ, Av. Moraes Rego, s/n , Cidade Universitária, Cx. Postal 7472, CEP: 50670-420, Recife – PE, Brasil. Tel.: (081) 3301 2500; Fax: (081) 3453 2449; http://www.cpqam.fiocruz.

APÊNDICE C

Fundação Oswaldo Cruz, Ministério da Saúde

Centro de Pesquisas
AGGEU MAGALHÃES

FIOCRUZ

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO – Grupo Controle
Projeto: "Caracterização da resposta imune humoral em portadores de Leishmaniose Tegumentar Americana ativa e após cura clínica".

Eu, ... concordo em participar voluntariamente neste projeto que será desenvolvido no Centro de Pesquisas Aggeu Magalhães da Fundação Oswaldo Cruz (CPqAM/FIOCRUZ). O objetivo principal do referido projeto é a investigação da defesa do organismo dos pacientes com leishmaniose tegumentar ativa, e após a cura clínica espontânea ou após tratamento quimioterápico. Eu farei parte do grupo controle, ou seja, grupo de indivíduos que não apresentam a doença e que servirão de comparação com os indivíduos doentes. Serei submetido a uma única coleta de 10 ml sangue venoso.

Todo procedimento será realizado com material estéril descartável e por profissionais de saúde de reconhecida capacidade para executar os procedimentos. Fui informado que a coleta de sangue é um método invasivo, por isso pode causar desconforto no local, na hora da picada e que não devo friccionar (movimento com força) o local para não haver formação de hematoma, ou seja, pequena mancha roxa. Esse trabalho trará grande benefício, pois indicará se componentes do sistema de defesa do organismo poderão ser usadas como marcadores da resposta terapêutica e se outras células do sistema de defesa do organismo participam na evolução clínica de pacientes com leishmaniose tegumentar americana em Pernambuco. Como faço parte do grupo controle não serei submetido a nenhum tratamento com Glucantime® (remédio utilizado no tratamento da leishmaniose tegumentar americana). Fui informado que esse remédio (Glucantime®) é o mais utilizado, promove cura da doença e pode ter efeitos colaterais como náuseas e indisposição (moleza).

Antes de minha participação no referido projeto, fui incentivado a pedir esclarecimento adicional que julgasse necessário, esclarecido por um participante do projeto, sobretudo em relação a importância do grupo controle. Estou ciente que poderei recusar ou retirar meu consentimento, em qualquer momento da investigação, sem qualquer punição ou prejuízo. Autorizo a Fundação Oswaldo Cruz (CPqAM/FIOCRUZ) a utilização das informações obtidas através dos resultados dos procedimentos em reuniões, congressos, patentes e publicações científicas preservando, neste caso, a minha identidade. Autorizo, também que o CPqAM/FIOCRUZ poderá estocar amostra biológica para posteriores estudos. Fui informado que para usar a amostra biológica estocada, um pesquisador do projeto entrará em contato comigo.

Estou ciente que este documento é feito em duas vias, ficando uma em minha posse e a outra com a equipe do projeto e que todas e quaisquer dúvidas que eu venha a ter sobre o significado dos termos empregados no presente texto me serão completamente esclarecidos por um dos membros do projeto antes que eu assine este impresso.

_____ _____
Assinatura do voluntário data

Endereço do sujeito da pesquisa:_____

_____

_____ _____
Assinatura do médico responsável – CPqAM/FIOCRUZ data

Pesquisador responsável: Dra. Valéria Rêgo Alves Pereira. Telefone para contato: 81 21012500

_____

Departamento de Imunologia, Centro de Pesquisas Aggeu Magalhães, FIOCRUZ, Av. Moraes Rego, s/n , Cidade Universitária, Cx. Postal 7472, CEP: 50670-420, Recife – PE, Brasil.

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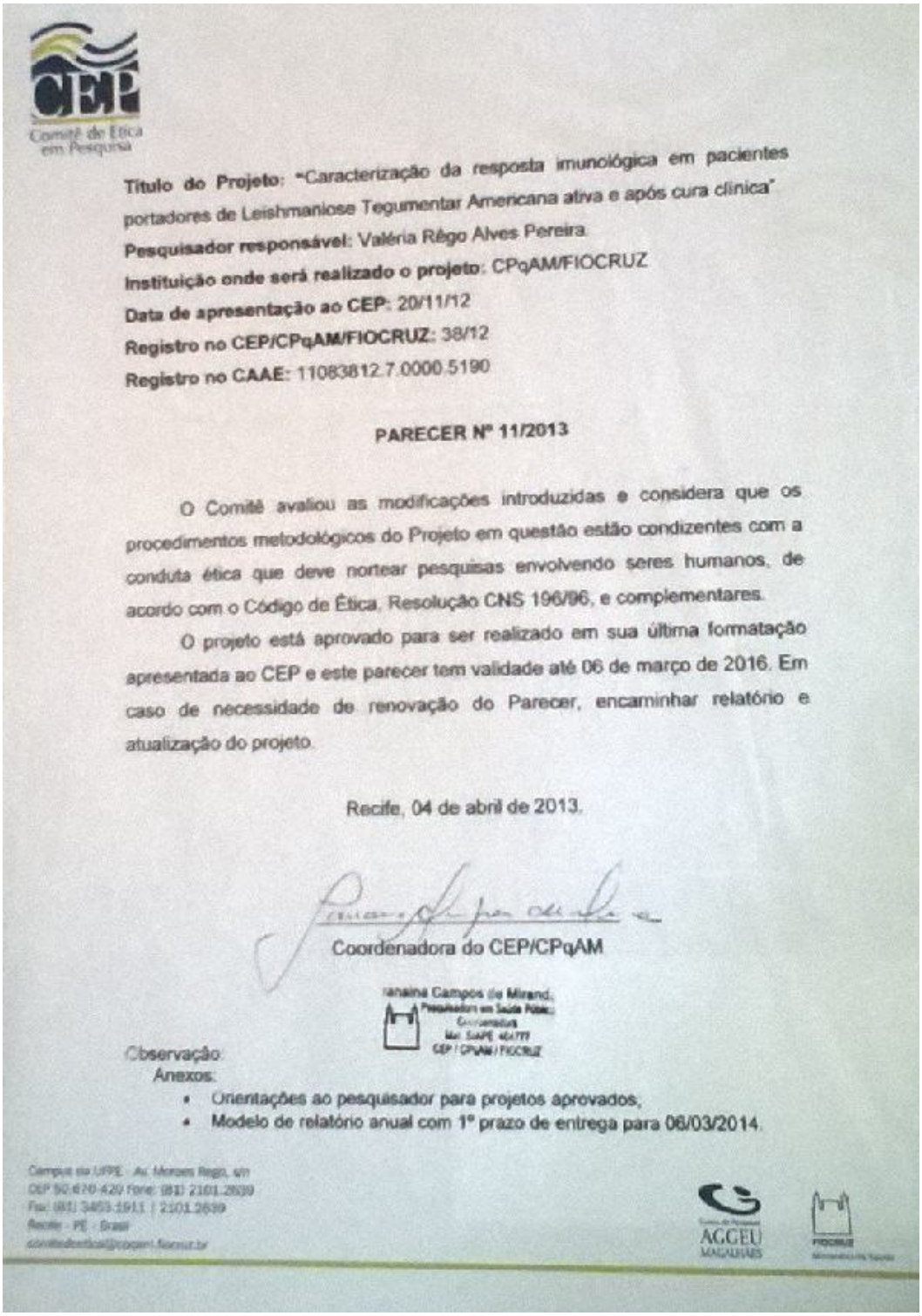
ANEXO 1

PARECER DO COMITÊ DE ÉTICA EM PESQUISA



CEP
Comitê de Ética em Pesquisa

Título do Projeto: "Caracterização da resposta imunológica em pacientes portadores de Leishmaniose Tegumentar Americana ativa e após cura clínica"
Pesquisador responsável: Valéria Rêgo Alves Pereira
Instituição onde será realizado o projeto: CPqAM/FIOCRUZ
Data de apresentação ao CEP: 20/11/12
Registro no CEP/CPqAM/FIOCRUZ: 38/12
Registro no CAAE: 11083812.7.0000.5190

PARECER Nº 11/2013

O Comitê avaliou as modificações introduzidas e considera que os procedimentos metodológicos do Projeto em questão estão condizentes com a conduta ética que deve nortear pesquisas envolvendo seres humanos, de acordo com o Código de Ética, Resolução CNS 196/96, e complementares.

O projeto está aprovado para ser realizado em sua última formatação apresentada ao CEP e este parecer tem validade até 06 de março de 2016. Em caso de necessidade de renovação do Parecer, encaminhar relatório e atualização do projeto.

Recife, 04 de abril de 2013.

Coordenadora do CEP/CPqAM

Observação:
Anexos:
- Orientações ao pesquisador para projetos aprovados,
- Modelo de relatório anual com 1º prazo de entrega para 06/03/2014.

Campus da UFPE - Av. Moraes Rego, s/n
CEP 50.670-420 Fone: (81) 2101.2639
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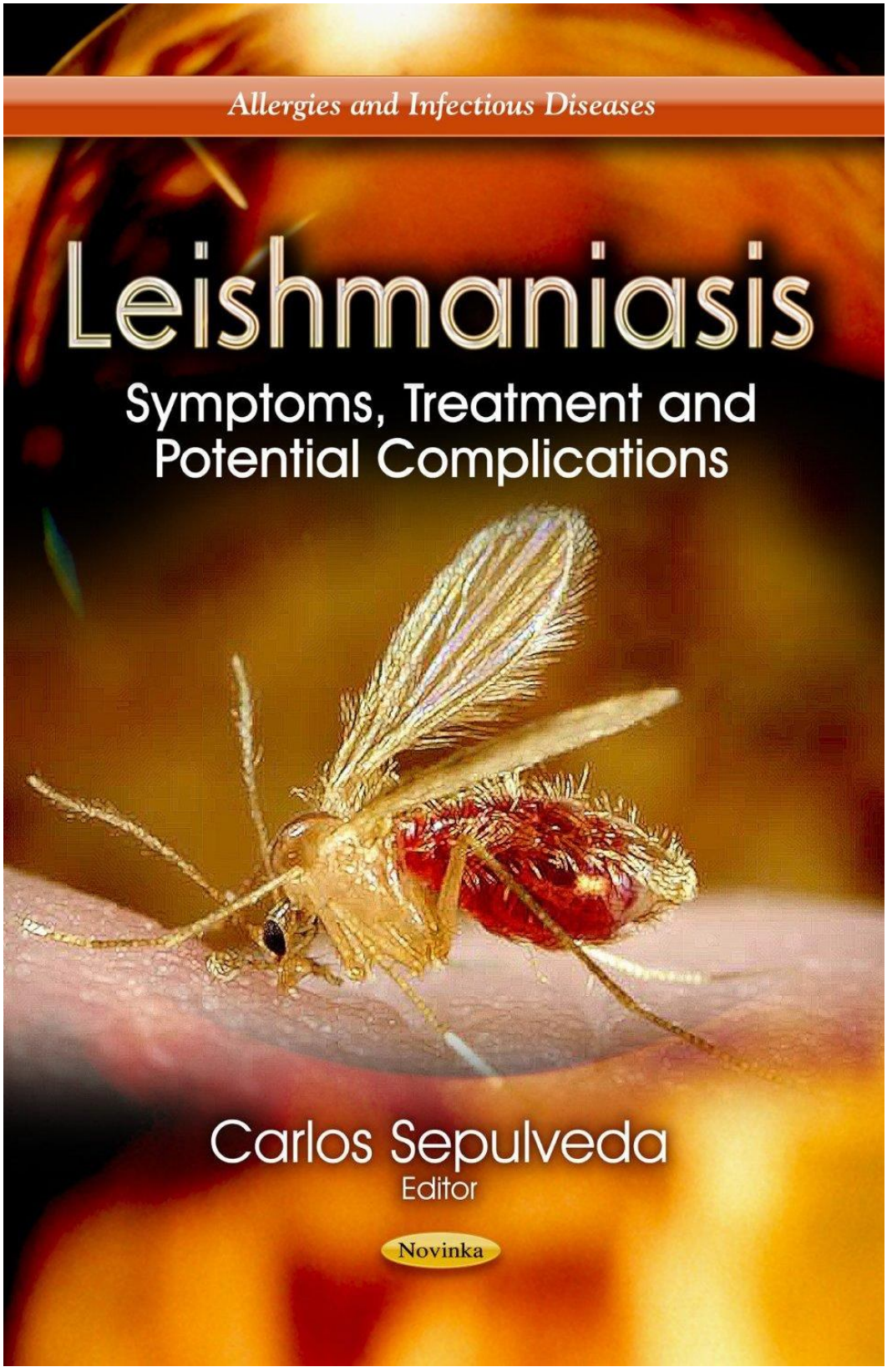
Recife - PE - Brasil
comitedeetica@cpqam.fiocruz.br

AGGEU MAGALHÃES
FIOCRUZ

ANEXO 2

CAPÍTULO DE LIVRO – Immunity against Leishmaniasis



Chapter

LEISHMANIASIS: SYMPTOMS, TREATMENT AND POTENTIAL COMPLICATIONS. IMMUNITY AGAINST LEISHMANIASIS

Marina de Assis Souza[1], Maria Carolina Accioly Brelaz de Castro[1], Andresa Pereira de Oliveira[1], Beatriz Coutinho de Oliveira[1], Amanda Ferreira de Almeida[1], Thays Miranda de Almeida[1] and Valéria Rêgo Alves Pereira[1]•

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ABSTRACT

Leishmaniasis is an anthropozoonotic, vectorially transmitted disease, which is caused by different *Leishmania* species. It is estimated that 350 million people worldwide are at risk of acquiring the disease, which has an annual incidence of 2 million cases. Under the influence of characteristics of the vector, vertebrate host and parasite, leishmaniasis can appear in the cutaneous (localized, disseminated and diffuse), mucocutaneous and visceral forms. In all clinical manifestations, the immune response plays an important role, contributing to the clinical cure or disease progression. Components of innate and acquired immunity act dynamically attempting to control the infection, so the host can achieve clinical cure.

Considering these aspects, this chapter describes the functions of some important elements in innate and acquired responses against *Leishmania* (i. e. chemokines, co stimulatory molecules, receptors, cytokines and cells) in the different clinical forms of leishmaniasis.

Keywords: leishmaniasis, innate immune response, cellular immune response.

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1. INTRODUCTION

Leishmaniasis is an anthropozoonotic, vectorially transmitted disease, which is caused by different *Leishmania* species. It is estimated that 350 million people worldwide are at risk of acquiring the disease, which has an annual incidence of 2 million cases. Depending on some features of the parasite, vector and the vertebrate host, including immunological state, the development of the disease can happen under a spectrum of clinical forms [1]. Localized cutaneous leishmaniasis is the most frequent outcome, being characterized by the presence of one or more ulcerated lesions which tend to self-healing. In rare cases, the lesions can be numerous due to multiple sand-fly bites or parasite dissemination by blood [2]. In diffuse leishmaniasis, there are several popular or nodular lesions throughout the body surface that can persist indefinitely. The mucocutaneous form is the most aggressive, presenting infiltrative lesions, with ulceration and tissue destruction in the nasal cavity, pharynx and larynx [3]. The appearance of different clinical manifestations is influenced by the host immune response. Thus, the presence of immune effector cells such as macrophages, natural killer cels, CD4+ and CD8+ T cells, cytokines, effector molecules and specific antibodies are critical components to the control of leishmaniasis [4,5,6]. Considering these aspects, this chapter describes the functions of some important elements in innate and acquired responses against *Leishmania* in the different clinical forms of leishmaniasis.

2. INNATE IMMUNE RESPONSE

Innate responses develop after the initial sensing of invading microbes, leading to the production of effector molecules that contribute to contain initial infection and to mount the subsequent adaptive immune response [5,7]. There is growing evidence that the innate immune response mechanisms are also important to the antiparasitic response and infection control[4,7]. We will discuss the aspects of the innate immune response in Leishmaniasis with more details below.

2.1. Contributing Cells

Leishmania life cycle inside the host is dependent upon internalization by phagocytic cells either resident or recruited to the wound site [7]. *Leishmania* spp. has been considered an obligate intracellular pathogen of macrophages, but the parasite also has adapted to live within different host cells than those previously described [8,9].

Neutrophils rapidly infiltrate the skin after *Leishmania* spp. infection, in cutaneous and visceral leishmaniasis, and are present in early lesions being the most immediate responders [9,10]. Both host protective and disease promoting roles for neutrophils have been reported. The protective role of neutrophils is associated with rapid recruitment to sites of tissue damage and pathogen entry, and the subsequent clearance of these recruited neutrophils by macrophage/monocyte populations [10,11]. Active neutrophils kill promastigotes via reactive oxygen and reactive nitrogen species as wells as neutrophils extracellular traps [4,9,11]. However neutrophils are short-lived and undergo apoptosis, and when their corpses are

phagocytosed by macrophages it allows silent entry of the parasites into macrophages through direct ingestion of the parasite or through ingestion of parasites that hide outside the dead neutrophils [4,9,12]. These apoptotic neutrophils at infection site may also suppress macrophages functions with the release of anti-inflammatory cytokines such as TGF-β and can cause immune mediated tissue pathology [4,8,9,10,12]. Passage through neutrophils is believed to be temporary, a way of camouflage. Parasites usually can establish infections in macrophages, differentiating into amastigotes that replicate inside parasitophorous vacuole [7,11]. However reports showed that in human visceral leishmaniasis neutrophils can harbor parasites during active disease [11]. *Leishmania* amastigotes, the intracellular form of the parasite, are able to multiply within macrophages, dendritic cells (DC) and neutrophils [13]. However, it is within mononuclear phagocytes that there is the best evidence for replication and long-term survival of *Leishmania* spp [8]. The resolution of infection with *Leishmania* is associated with presentation of *Leishmania* antigens by macrophages and dendritic cells (DCs) and priming of CD4+ and CD8+ T lymphocytes. Ultimately, induction of nitric oxide synthase (iNOS) and interferon-gamma (IFN-γ) leads to nitric oxide (NO) production, reactive oxygen species (ROS), and parasite killing by macrophages [4]. The central irony of leishmaniasis is that the macrophage is both the principal immune effector cell charged with killing *Leishmania* amastigotes and also the principal site of parasite proliferation and dissemination [4]. A complex network of immune cells within the skin—dendritic cells, macrophages and Langerhans cells—have a prominent role in cutaneous leishmaniasis, as a bridge from innate to adaptive immune responses [12,13,14]. DCs not only play a key role in the development of a protective immune response to *Leishmania*, but also act as a host cell for the parasites [15]. Resident dermal macrophages are also rapidly infected, and they become the dominant infected population after 24 hours allowing differentiation, growth of *Leishmania spp* [8,11]. These antigen presenting cells engage pathogens and then acquire a mature phenotype, increase their expression of co-stimulatory molecules and then travel along lymphatics to the nearest lymph node, where T cell responses are developed to control infection. In general, accumulation of DCs bearing protein antigen in lymph nodes is found to peak around 24h after inoculation [12,13,14]. Together with phagocytes, NK cells represent the first line of defense against pathogens, working by two principal mechanisms: cytolytic destruction of infected cells and secretion of proinflammatory cytokines. K cells can be identified at the site of infection as early as 24 hours after *Leishmania* infection [14]. In patients, the amount of NK cells and activity has mainly been related with protection against or healing of disease, and reports from patients with active leishmaniasis (cutaneous and visceral) show that they have a reduction in the frequency of peripheral NK cells [11]. The activation of NK cells in visceral and most likely also in cutaneous leishmaniasis results from the intimate interaction of these cells with dendritic cells, which are triggered by *Leishmania* parasites for the production of IL-12 in a TLR 9 dependent fashion [5].

2.2. Chemokines

Chemokines and chemokine receptors have been shown to have different roles in determining the outcome of leishmaniasis. Chemokines are chemotactic cytokines that coordinate recruitment of leukocytes involved in homeostasis as well as in innate and

adaptive immune responses [6,14]. Infection with *Leishmania* induces the expression of a number of chemokine genes in the host. This could potentially be beneficial to the parasite through recruitment of host cells it can infect, survive in and proliferate. In addition to mediating cellular recruitment, chemokines can activate various cell populations, participate in cell mediated immunity and possess anti-leishmanial properties, having roles in adaptive immunity, in macrophage activation and parasite killing [6,14].

Chemokines produced at the site of an infection are critical in determining the composition of infiltrating cells and defining the eventual outcome of the disease [14]. Patients with visceral leishmaniasis show elevated concentrations of CXCL9 and CXCL10 in their serum during active infection and it has been suggested that these chemokines along with IFN-γ play an important immunopathogenic role in the disease [6]. In localized cutaneous leishmaniasis (LCL) a Th1 chemokine profile is observed in the lesions, consisting of CCL2 (monocyte chemotactic protein-1), CXCL9 and CXCL10- associated with a concentrated dermal infiltrate comprising of macrophages and large numbers of CD4 positive cells. In contrast, the chemokine profile of lesions of chronic diffuse cutaneous leishmaniasis (DCL) is Th2 associated, dominated by the expression of CCL3 (macrophage inflammatory protein 1-α), and the dermal infiltrate is more diffuse with fewer CD4 positive cells [9,16].

2.3. Effector Molecules

The key antileishmanial effector molecules in experimental cutaneous and visceral leishmaniasis are reactive nitrogen intermediates (NO and NO-derived metabolites) and reactive oxygen intermediates ($O2^-$ and subsequent metabolites) [5]. While the production of NO is required for the leishmanicidal activity against *L.major* and *L. braziliensis* in the skin of infected mice, it is dispensable in the spleen and mildly important in the lymph node [7].

The entry of *Leishmania* parasite into host macrophages results in the onset of respiratory burst, characterized by the increased production of reactive oxygen species (ROS), like superoxide (O2 -) and hydrogen peroxide (H2O2), which is required for the killing of the parasites. These O2 - are generated by activities of a multi component enzyme complex i.e., nicotinamide adenine dinucleotide phosphate (NADPH)/NADH oxidase. Moreover, in later stages of infection, reactive nitrogen intermediates (RNI) like nitric oxide groups (NOg) are also produced by the activity of inducible nitric oxide (iNOS), which further contribute to innate immunity and parasitic elimination. However, in leishmanial infections, the microbicidal activities of macrophage are severely hampered, leading to the survival and proliferation of parasites inside the macrophages [19].

2.4. Toll-Like Receptors (TLRs)

The TLR family is highly relevant to immunity against *Leishmania* and other parasites, as they recognize pathogen-associated molecules and participate in innate responses to infections [4,7,19,22]. TLR activation induces innate responses in multiple ways, leading to the production of effector molecules such as nitric oxide, inflammatory cytokines, chemokines, and other anti-microbial products that can directly destroy the pathogens. TLRs are known to participate in the control of *Leishmania* infection by inducing Th1 responses. A

few *Leishmania*-derived molecules have been reported to activate TLRs, and the majority of the studies to date focused on the activation of TLR2, TLR4, and TLR9 [7,13,19,22].

Evidences indicate that TLR4 contributes most significantly to control the growth of *Leishmania* spp. in both phases of the immune response. The TLR4 has been found to be a strong regulator of inducible nitric oxide synthase (iNOS, a marker of innate immunity) leading to the death of parasites. In addition to TLR4, TLR2 and 9 have been detected in the skin of patients with cutaneous leishmaniasis [22].

Lipophosphoglycans (LPG) on the *Leishmania* cell surface have been implicated as agonists of TLR 2, 3, 6 and have also been associated with NK cell activation in *L. major* infection [4]. Purified *L. major* lipophosphoglycan induced the upregulation and stimulation of TLR2 on human NK cells, with additional enhancement of TNF-α and IFN-γ. LPGs of *L. major*, *L. mexicana*, *L.aethiopica*, and *L. tropica* were defined as TLR2 ligands in studies using murine macrophages, although the stimulation with *L. tropica* LPG was only marginal. More recently, it was shown that LPG stimulates cytokine production by human peripheral blood mononuclear cells via TLR2 as well. Those findings assign a protective role for TLR2 which seems required to mount an effective Th1 response [7].

2.5. Complement System

The complement system is a complex set of serum proteins that forms a controlled sequence for the generation of activated molecules. The role of the activated molecules is to increase inflammatory reactions mediated by antibodies.

In addition, generation of the membrane attack complex C5b–C9 leads to the lysis of "unwanted" cells. The complement receptor system is directed against mediators generated by the host right after parasite contact. In *Leishmania* infections the parasites interact with serum and activate complement in both the classical and the alternative pathways. Opsonization of *Leishmania* promastigotes with complement is fast, with lysis by the membrane attack complex beginning seconds after serum contact, resulting in the elimination of more than 90% of the inoculated parasites within a few minutes [13].

2.6. Modulation of Infection in Innate Immune Response

Leishmania parasites are capable of using different components of the host defense innate mechanisms to avoid their elimination from the host before an infection is established. Some of the parasites surface molecules are capable of activating the complement system, resulting in the binding of C3bi and C3b to the surface of the parasite. *Leishmania* parasites smartly use this opsonization to escape from the hostile environment by promoting phagocytosis via complement receptors in cells such as in macrophages, neutrophils and erythrocytes [9,11,13]. They can also entry macrophages using the engagement of non-triggering receptors such the phosphatidyl serine (PS) receptor. *Leishmania* can also evade effector mechanisms of the immune system by direct inhibiting macrophage function through interference with NFB transcription and IL-12 production, disturbing macrophage phagosomal maturation and killing functions.

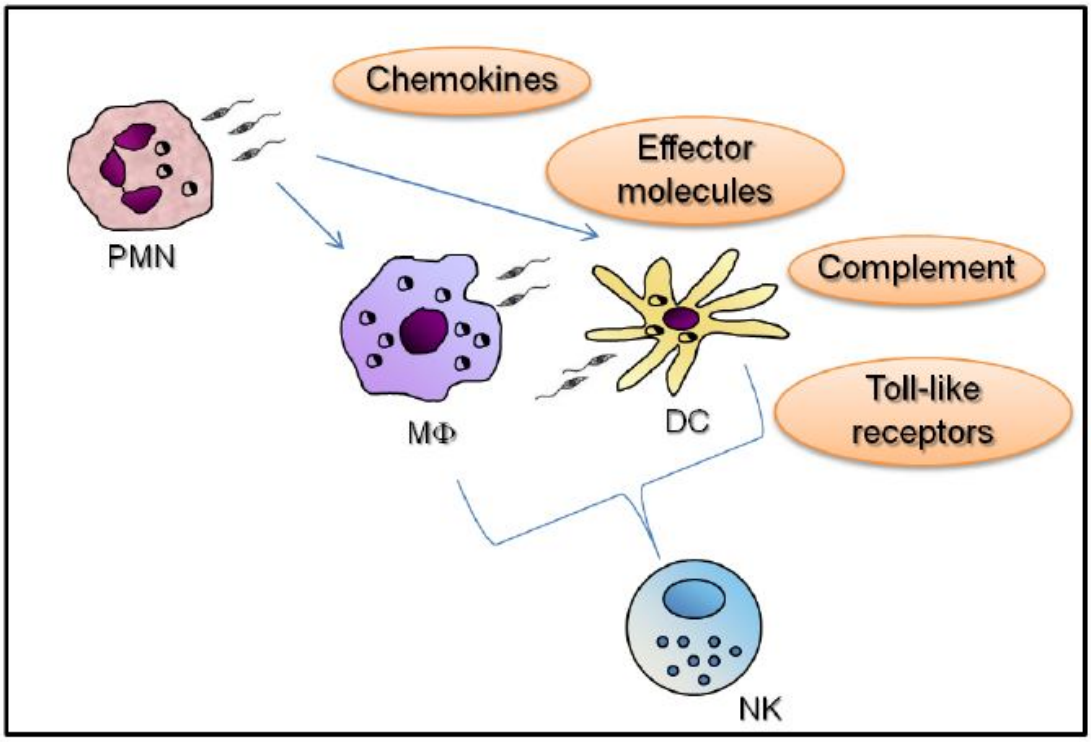

Figure 1. The main components of the innate immune response against Leishmaniasis include some important cells and chemical mediators. Neutrophils are the most immediate responders, which rapidly infiltrate skin after parasite infection. However, it is within mononuclear phagocytes that there is the best evidence for replication and long-term survival of *Leishmania* spp.. Ironically, the macrophage is both the principal immune effector cell charged with killing amastigotes and also the main site of parasite proliferation and dissemination. Within the skin, DCs not only play a key role in the development of a protective immune response to *Leishmania*, but also act as a host cell for the parasites. Together with phagocytes, NK cells represent the first line of defense against pathogens, making cytolytic destruction of infected cells and secreting inflammatory cytokines. The composition of inflitrating cells and the definition of the eventual outcome of the disease are critically determined by chemokines produced at the site of infection. Also, depending on the site of infection, antileishmanial effector molecules play an important role in innate immunity. Composed by a complex set of serum proteins, the complement system is activated in both the classical and alternative ways in leishmaniasis. However, *Leishmania* parasites are capable of using different components of innate defense to avoid their elimination from the host before an infection is established.

They can additionally down regulate MHC class II expression; promote the production of regulatory cytokines like IL-10 and TGFβ and can inhibit dendritic cell maturation and chemotaxis [4,11,15].

3. Acquired Immune Response

In human and experimental leishmaniasis, immunity is predominantly mediated by T lymphocytes. T cells play a major role in generating specific and memory T-cells responses to intracellular parasitic infection and these have been extensively characterized in *Leishmania* infection [25]. In addition, T lymphocytes play critical role in shaping the host immune

response by secreting cytokines, which may act both synergistically and antagonistically through complex signaling pathways to direct both protective and non-protective immunities against intracellular parasites [24].

Although the immune response induced by infection with *Leishmania* has been the subject of many investigations, the mechanisms that underlie host resistance and pathogenesis in leishmaniasis are not entirely understood. During the late 80s and early 90s, the discovery of two distinct subpopulations of CD4+ T helper cells based on their cytokine production, Th1 and Th2 [26], finally explained resistance and susceptibility to *L. major* in the murine model [27].

Early studies using mouse models of experimental cutaneous leishmaniasis (CL) have revealed a clear dichotomy between Th1-associated cytokines mediating protection and Th2-associated cytokines mediating susceptibility [23,24,28,31]. Failure to mount an efficient anti-*Leishmania* Th1 response was shown to cause progressive disease and absence of lesion resolution [29,31]. In resistant C57BL/6 mice, resolution of the disease is mediated as a consequence of IFN-γ release by Th1 cells and upregulation of NO in macrophages the harbor parasites [30,31]. Conversely, persistence of lesions in BALB/c mice is due to Th2 –type $CD4^+$ T cell differentiation and production of IL-4, which suppresses macrophage activation, resulting in parasite survival [29,31]. On the other hand, during VL, Th2 response and cytokines such as IL-4 and IL-13 seem to be necessary for immunity and efficient response to antileishmanial chemotherapy [31,32].

In the murine model of *L. major* infection, the predominant $CD4^+$ T cell subpopulation resulting from infection greatly influences the outcome of disease [34,35,36]. Interleukin-12 (IL-12) produced by macrophages and dendritics cells and interferon-gamma (IFN–γ) produced by natural killer cells (NK), and previously activated T cells, promote the development of Th1 cells, whereas IL-4 induces the development of Th2 cells. The Th1 subpopulation, important for induction of leishmaniasis resistance, produce IFN-γ and tumor necrosis factor – alpha (TNF-α) which play an important role in cellular immune responses against intracellular pathogens by activating macrophages for intracellular killing of pathogens[36,37]. On the other hand, Th2 cells produce IL-4, IL-5, IL-10, IL-13 and TGF-β, and are associated with leishmaniasis susceptibility in *L. major* infection murine models [36,38,39,40].

Most data point to the fact that same or similar Th1 dependent mechanisms are involved in control of human disease. Self-healing forms of leishmaniasis and cure of VL is typically accompanied by parasites specific proliferation and IFN-γ production. Human macrophages are activated to kill intracellular parasites by IFN-γ and exogenous IFN-γ can promote cure of human CL [41,45]. Though Th2 responses can act in favor of the parasite, polarized Th2 response has never been able to explain non-curative or visceralizing human disease. Th2 independent disease progression is also supported by studies on non-healing disease in Th1 phenotypic B6 mice [42,45]. In this context it can also be noted that in patients with VL the effect of IFN-γ administration was limited [43,45] and in human LC, IFN-γ production by CD4+ cells, alone, in response to *Leishmania* antigens is not predictive of protection or disease development [44,45]. This indicates that other mechanisms acting in synergy with IFN-γ or counteracting the effects of IFN-γ as important. Thus, the Th1/Th2 dichotomy as an indicator of resistance and susceptibility might be a generalization and is far more complex than what we currently know and understand [31,45].

Of particular interest in this context is the differentiation of Naïve $CD4^+$ Th cells into various effector lineages orchestrating different immune responses. Naïve $CD4^+$ Th cells can differentiate into IFN-γ producing Th1 cells; into Th2 cell secreting IL-4, IL-5, IL-13, and IL-10; or into the recently described Th17 cells. In addition, Naïve $CD4^+$ Th cells can differentiate into IL-10-secreting regulatory T cells like regulatory type 1 T cells, IL-10, and TGF-β producing Th3 cells or into Foxp3-expressing regulatory T cells [46]. Some cytokines are described in the following section.

3.1. Th1 and Th2 Cytokines

To control leishmaniasis infections, activation of macrophages dependent of CD4+ T cell, IFN-γ and tumor necrosis factor (TNF) are usually required. These effector molecules and cells are typically present in a cell mediated immune response. This leads to a (post)transcriptional upregulation of antimicrobial effector mechanisms, including the acidification of the phagolysosomes and the expression of inducible nitric oxide synthase [18,24]. TNF-α is a key cytokine mediating T cell-mediated inflammation. It is involved in leukocyte recruitment by increasing expression of adhesion molecules on vascular endothelium and increasing angiogenesis. Although TNF-α promotes increased macrophage activation, and contributes to control of *Leishmania* parasites, deleterious consequences of excessive TNF-α production have been reported. The high levels of TNF-α and IFN-γ secreted by mononuclear cells from these patients is positively correlated with lesion size and the use of drugs that down modulate production of TNF-α in combination with antimony increases the rate of healing and allows the cure of refractory cases of mucosal and cutaneous disease [15].

The main biological role of IFN-γ is to activate macrophages, inducing iNOS expression and NO production. This contributes to increase the microbicidal activity of these cells and therefore helps in the elimination of parasites and in the resolution of *Leishmania* infection [5,24]. IFN-γ biological effects can be associated with the activation of STAT1 transcription factors. STAT1/IFN-γ signaling pathway stimulates the expression T-bet, a transcription factor associated with the Th1 profile. STAT1 and T-bet are considered crucial to host protection against *Leishmania* infection in mice, since they are necessary to mount an efficient Th1 immune response [24].

Type I interferons *α* and *β* (IFN-α/β) are proinflammatory cytokines that are able to activate and phosphorylate STAT1 and STAT2. Their functions in innate and acquired immunity to bacterial and parasitic infections are shown in some studies. IFN-*α/β* can act as early regulators of the innate response to infection and are essential for initiating the expression of nitric oxide synthase type 2 (NOS2) and the production of NO. IFN*α/β* play a critical role in the innate immune response to CL infection by mediating events involved in parasite repression, IFN-γ expression, and cytotoxic NK cells activity- all through NOS2. IFN- *α/β* rather than IFN-γ was shown to account for the initial induction of iNOS in the skin and lymph node at day 1 of infection with *L. major*. The task of STAT2 in VL is essentially unknown [5,24]. Known as a proinflammatory cytokine, IL-12 is a heterodimer composed of two subunits, p35 and p40 and is produced primarily by macrophages and dendritic cells (DCs) in response to microbial pathogens. IL-12 functions as the main physiological inducer of gamma interferon (IFN- γ) by activated T cells and promotes Th1-type CD4+ T cell

differentiation, and therefore is a key cytokine for the generation of protective immunity in response to *Leishmania* infection. The specific cellular effects of IL-12 are due to the activation of Janus kinase (JAK)-STAT pathways, primarily to the activation of the specific transcription factor, STAT4. In activated T cells and NK cells, STAT4 functions to induce IFN-γ production in response to IL-12 signaling [5,24].

IL-10, an anti-inflammatory cytokine, is produced by a variety of cells, such as T cells, monocytes, macrophages, DCs, and B cells. Many other cells can produce IL-10, but its main role seems to be on macrophages and DCs, having a part as an anti-immune and anti-inflammatory cytokine. IL-10 inhibits the production of the proinflammatory cytokines IL-1, IL-6, IL-12, and tumor necrosis factor (TNF), preventing the development of a Th1 profile associated with a protective immunity during *Leishmania* infection. IL-10 also promotes the development of a humoral immune response, with the production of antibodies, which aids parasite entry into host cells. Studies demonstrated that IL-10 is a master cytokine in cutaneous and visceral leishmaniasis that is critical for the initial survival and long-term persistence of *Leishmania* parasites in both human and experimental models. Because IL-10 can act as an inhibitor of IFN-γ induced NO synthesis, it is likely that the antagonistic effects of IL-10 are related to its ability to suppress NO production, a critical component for parasite elimination [18,24]. IL-4 is an important cytokine that has been shown to deactivate macrophages and to regulate the induction of Type-2 [20,21]. Furthermore, IL-4 inhibits the responsiveness of CD4+ T cells to IL-12, due to its down regulatory effects on the expression of the IL-12 receptor b2-subunit and also inhibits the deviation of CD4+ T cells towards Th1 cells by modulation of the regulatory function of the transcription factor T-bet [20,21]. Moreover, macrophage activation by IL-4 induces a pathway of arginine metabolism toward arginase with production of polyamines that enhance *Leishmania* growth [21]. Since IL-4 has been shown to suppress macrophages and Th1 cells and enhances *Leishmania* growth, it is conceivable that the host ability in production of this cytokine may determine the susceptibility to CL. This hypothesis is supported by recent report on the association of IL-4 gene polymorphisms with susceptibility to visceral leishmaniasis [21].

3.2. Regulatory T Cells

To achieve cure in Leishmaniasis, the infected host must develop an immune response capable of eliminating the parasite, but harmless to itself. This balance is given by regulatory T cells, which exhibit two well-defined subpopulations: naturally occurring CD4+CD25+ Tregs, which originate in the thymus during ontogeny, and inducible Tregs, which develop in the periphery from conventional CD4+ T cells [46]. The first subpopulation of Tregs was initially described as a population of CD4+ T cells that prevent the expansion of self-reactive lymphocytes and, therefore, autoimmune disease in mice [47]. This population can be defined by their constitutive expression of the IL-2 receptor α chain (CD25), the cytotoxic T lymphocyte antigen (CTLA4), the TNF receptor family member GITR (glucocorticoid-induced TNF-receptor-related protein), and the a chain of the αεβ7 integrin (CD103) [48]. However, expression of these molecules is not specific to Tregs. In contrast, the forkhead/winged helix transcription factor Foxp3 is thought to program the development and function of Tregs and is specifically expressed in natural Tregs in mice, as well as in $CD25^-$ T cells with regulatory activity [49,50,51].

Cells with regulatory functions have been frequently described in *Leishmania* infections, and the existence of concomitant immunity is discussed [52,53,54]. This phenomenon consists in the long-term persistence of pathogens in a host that is also able to maintain strong resistance to reinfection. In the murine model of infection with *L. major*, $CD4^+CD25^+$ T cells accumulate in the dermis, where they suppress – by both interleukin-10-dependent and interleukin-10-independent mechanisms – the ability of $CD4^+CD25^-$ effector T cells to eliminate the parasite from the site. The sterilizing immunity achieved in mice with impaired IL-10 activity is followed by the loss of immunity to reinfection, indicating that the equilibrium established between effector and regulatory T cells in sites of chronic infection might reflect both parasite and host survival strategies [53].

Regarding the experimental infection with *Leishmania (Viannia) braziliensis*, a Treg activity has also been related. $CD4^+CD25^+$ cells expressing GITR, CD103 and Foxp3 were detected throughout the duration of clinical disease both at the ear and in draining lymph nodes of infected mice. In both sites, they were capable of suppress $CD4^+CD25^-$ proliferation. Interestingly, in the outcome of a reinfection, parasites were mainly detected in the LN draining the primary infection site where a high frequency of $CD4^+IFN\text{-}\gamma^+$ T cells was also present. Thus, in this model, Tregs are present in healed mice but this population does not compromise an effective immune response upon reinfection with *L. braziliensis* [54].

Suppression of T cell response is thought to be involved in the pathogenesis of human leishmaniasis. In patients with CL caused by *L. braziliensis*, a frequency of CD4+CD25+ cells was observed in the skin lesions, along with expression of CTLA-4 and GITR markers and secretion of IL-10 and TGF-β. Moreover, CD4+CD25+ T cells in peripheral blood (PB) from the same patients exhibited higher levels of CTLA-4 than healthy individuals[55]. Because CTLA-4 is highly expressed on Treg cells [56,57], and because it is supposed that this molecule plays an important role in their suppressor function[57], it is possible that the suppressor activity of CD4+CD25+ T cells was increased in the patients with CL.

A similar immune regulation in human visceral leishmaniasis is observed. The presence of CD4+CD25+ in the bone marrow, one of the disease sites, and the production of IL- 10 by these lymphocytes may inhibit T cell activation in IL-10 dependent manner [58]. In contrast, CD4+CD25+ lymphocytes did not accumulate in and were not a major source of IL-10 in the spleen, and their removal did not rescue antigen-specific interferon-γ responses. Thus, in different sites the regulation of immune response may be performed by different T cell subpopulations, once IL-10 is secreted in the spleen by CD25-Foxp3- T cells [59].

It is also interesting to investigate whether there is an influence of mechanisms of immune regulation on the response to chemotherapy. The analysis of the frequency of CD25+ cells in PB from patients with active and cured CL showed a higher presence of cells expressing this marker after treatment. Thus, CD25+ T cell expansion, presented by patients, may be due the role of these cells in the modulation of an exacerbated response by effector T cells, and maintenance of a small number of parasites in the localized lesion as an antigenic stimulus to prevent reinfection [60]. Among all the data obtained so far, immune regulation seems to happen as a way to maintain a homeostatic environment to allow the achievement of clinical cure by the host and the parasite persistence. Nevertheless, conclusive role of Treg cells in suppression of immunity in patients and its consequences is yet to be well defined.

3.3. Th17 Responses

Similar to the Th1 and Th2 subsets, the Th17 subset is orchestrated by specific cytokines and transcription factors [61]. The Th17 response has been studied since 1995, when it was found that T helper cells can produce IL-17 under stimulation with specific antigens [62]. Nowadays, it is known that the production of Th17 specific cytokines is present in allergy and inflammatory diseases [63,64]. However, these inflammatory mediators can orchestrate protective responses to several agents, as it is shown in *M. tuberculosis* and *T. cruzi* infections[65,66].

The Th17 response is activated by a combination of the cytokines IL-6 and TGF-β, and the transcription factors RORγt, RORα and Stat3 are essential for Th17 commitment [60,67]. IL-6 plays an important role in the differentiation of the Th17 subset, since TGF- β can also induce Foxp3, a transcription factor required for the generation of regulatory T (Treg) cells, and the presence of IL-6 suppresses the induction of Foxp3 [67].

Th17 cells produce cytokines such as IL-17A, IL-17F, IL-22, IL-21 and IL-23, which promote Th17 responses functionality. The cytokine IL-27, on the other hand, is the main negative regulator of the Th17 response, despite its structure's similarity to IL-6 [60]. Research over the role of these cytokines in many infections is under constant development. The research of the influence of Th17 cells in leishmaniasis is primordial to understand the mechanisms related to protective or damaging immune responses in this disease. In the next section, some features of these cytokines are described.

3.3.1. IL-17

The IL-17 cytokines include a family of six members (IL-17A-F), of which at least two of them exhibit potent proinflammatory properties: IL-17A (also known as CTLA-8) and IL17-F, which seem to have similar functions. IL-17B and IL-17C are members of the family whose cellular sources are unknown at this point, and whose biology seems unrelated to IL-17A. IL-17D and IL-17E (alternative names: IL-27 and IL-25), in turn, are the two members of the IL-17 family with lowest homology to IL-17A. None of the last is produced by Th17 cells, and they exert a negative control on the Th17 subset development[60]. In this chapter, we will refer to IL-17A as IL-17.

By signaling through the receptor IL-17RA, IL-17 can induce the production of different kinds of proteins, many of them related to inflammation, including chemokines (CXCL-1, CXCL-2, CXCL-8-10, CCL-2, CCL-20), cytokines (IL-6, TNFα, G-CSF, GM-CSF), proteins of the acute phase response, tissue remodeling factors (MMP1, MMP3, MMP9, MMP13, TIMP2), and anti-microbial products (β-defensins, mucins, calgranulins) [60]. The role of IL-17 in immune responses is being widely studied. It is known that IL-17 is a potent activator of neutrophils. Increased levels of this cytokine are responsible for neutrophil immigration, most likely via CXCL2, whereas IFN-γ is responsible for activating macrophages to kill intracellular pathogens [68]. IL-17 seems to have a role in the protective immunity against many bacterial and fungi infections, as in the case of *Klebsiella pneumoniae*, *Mycobacterium tuberculosis*, *Candida albicans* and *Aspergillus fumigate* infections [69,70]. IL-17 could also be defensive against some parasites, as in the infection with the protozoan *Toxoplasma gondii* [71]. Also, IL-17 production appears to be downstream of IL-1αβ in several pathological conditions. DC derived IL-1 is important for efficient Th1 induction in leishmaniasis [68].

Whilst in some models IL-17 and IL-23 seem to have a protective role on the outcome of the infection, as in the case of extracellular pathogens (e.g., *Klebsiella pneumonia* bacteria, *Toxoplasma gondii* parasites and *Cryptococcus neoformans* fungi) [72], in *Schistosoma mansoni* infections, increased levels of IL-23 and IL-17 are associated with disease exacerbation [73].

As to leishmaniasis, Kostka et al (2009) reported that BALB/c mice produced increased levels of IL-17 after infection with *L. major* and that IL-17-deficient (IL-17$^{-/-}$) BALB/c mice exhibited dramatically attenuated disease despite typical Th2 development. They also demonstrate that elevated levels of IL-17A in BALB/c mice were associated with increased production of IL-23, but not IL-6 and TGF-β1, by infected DC.

In humans, studies have shown that IL-17 is present at the initial phase of the immune response in the cutaneous forms of leishmaniasis [68,74,75], leading to the conclusion that this cytokine could be injurious for the disease resolution. On the other hand, Novoa et al (2011) observed an increase in IL-17 levels in individuals with subclinical ACL, in comparison to patients with active lesions, concluding that this cytokine presents a protective part in the immune response. Pitta et al (2009) have also shown that *L. donovani*, a visceral leishmaniasis agent, strongly induces IL-17 and IL-22 production in PBMCs of healthy individuals, suggesting that these cytokines can present a protective role in *Leishmania* infections.

3.3.2. IL-21

Although IL-21 does not look like an essential factor for Th17 lineage commitment, it is able to induce IL-17 expression in collaboration with TGF-β even in the absence of IL-6. Furthermore, generation of Th17 cells is attenuated by blocking IL-21, and loss of its expression, or its receptor, results in defective Th17 differentiation. Similar to IL-6, IL-21 inhibits Foxp3 expression induced by TGF-β. IL-21 is produced by Th17 cells under IL-6 induction and autocrinally induces its own synthesis and the expression of IL-23R to allow IL-23 responsiveness [68].

Furthermore, IL-21 has been recently proven to induce IL-10 production under stimulation with *L. donovani* antigens. It is also known to critically regulate Ig production, and could be a contributing factor to the high titers of anti-leishmanial Abs in VL patients [76].

3.3.3. IL-22

IL-22 is also produced by Th17 cells, and to a lesser extent by Th1 and NK cells, and is involved in immunity at the epithelium and mucosal surfaces [77,78]. The functional IL-22 receptor is expressed on hepatocytes, keratinocytes, and fibroblasts. IL-22 increases the production of proinflammatory molecules, such as the S-100A proteins and CXCL5. IL-17 and IL-22 synergistically increase the production of antimicrobial peptides, such as β-defensins, by epithelial cells [68,78].

Both IL-17 and IL-22 have been shown to increase protection against certain bacterial and fungal pathogens in experimental models [78]. As to protozoans, Pitta et al (2009) stated that IL-17 and IL-22 are the cytokines most strongly associated with protection in the visceral forms of leishmaniasis.

These cytokines may contribute to protective immunity to *L. donovani* in several ways. Studies using animal models suggest that neutrophils could be involved in controlling the

Leishmania infection through the generation of skin and liver granulomas that form around *Leishmania* at early stages of infection. Furthermore, IL-22 is involved in epithelial repair and liver protection in chronic infections. Both the increases in epithelial protective barrier function and the recruitment of inflammatory cells, including neutrophils, to the skin and liver, could contribute to protection against *L. donovani* [78].

3.3.4. IL-23

The function of IL-23 in promoting Th17 cell expansion or survival has been proposed. A recent report suggests that IL-23 maintains the Th17 phenotype without affecting proliferation or survival. On the other hand, IL-23 has been demonstrated to maintain the pathogenic Th17 functions compared with culture under TGF-β and IL-6, depending on IL-10 production by Th17 cells [60].

Recent studies have also implicated IL-23 and IL-17 in immunity against extracellular pathogens, as bacteria (*Klebsiella pneumoniae*), *Toxoplasma gondii* parasites and fungi (*Cryptococcus neoformans*). In *Schistosoma mansoni* infections, increased levels of IL-23 and IL-17 are associated with disease exacerbation[72]. Kosksta et al (2009) suggests that DC-derived IL-23, in addition to IL-1β and IL-12p80, can contribute to disease susceptibility in BALB/c mice infected with *Leishmania* parasites.

3.3.5. IL-27

IL-27 is one of the main negative regulators of Th17 development. This cytokine is structurally related to IL-6, but has many different actions. Research studies show a damaging role of IL-27 on IL-17 producer cells. These studies conclude that the absence of IL-27 signaling exacerbates chronic inflammation in correlation with increased number of Th17 cells.

Moreover, IL-27 is able to promote IL-10 production, another negative player in the network of Th17 activity regulation [60].

Novoa (2009) reported a higher expression of mRNA for IL-27 *ex vivo* or in cultures stimulated with soluble *Leishmania* antigen in patients with active lesions compared to individuals with subclinical disease. Ansari et al (2011) also associated active visceral leishmaniasis with elevated levels of IL-27 in plasma and IL-27 mRNA in spleen.

IL-27 produced by macrophages, along with IL-21 from T cell sources, are suggested to be disease-promoting cytokines in visceral leishmaniasis by virtue of their roles in promoting the differentiation and expansion of Ag-specific, IL-10–producing T cells.

The studies support the notion that IL-27 is a key instructional cytokine involved in regulating the balance between immunity and pathology in human visceral leishmaniasis [76].

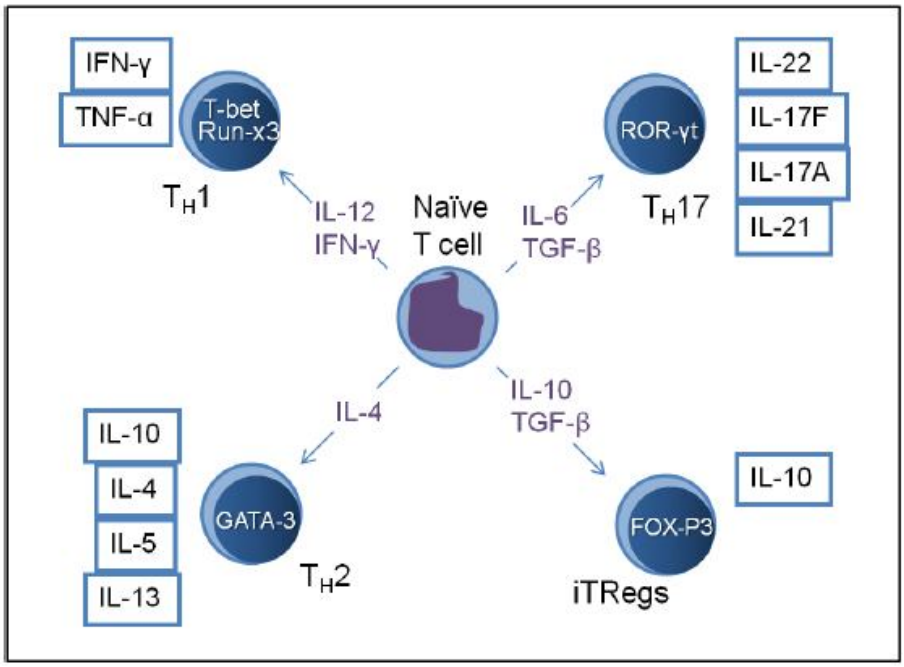

Figure 2. To all of the clinical forms of Leishmaniasis, the adaptive immune response is mainly executed by $CD4^+$ T-cells, through cytokine production. The development of a T cell profile may vary according the cytokine microenvironment. The presence of IFN-γ and IL-12 will contribute to set a Th1 profile, initially beneficial to the host, but harmful if exacerbated expressed. Th2 cells will dominate under influence of IL-4, which contributes to parasite growth and development of the disease. Cytokines such as IL-17A, IL17F and IL22 will be mainly secreted by Th17 cells, which develop in the presence of IL-6 and TGF-β. IL-27 works as a down-regulator of the profile. TGF-β is also required to the development of a regulatory response, mediated by IL-10 secretion by $Foxp3^+$ induced regulatory T cells.

3.4. Humoral Response

The infection by *Leishmania* in humans is characterized by the appearance of anti-leishmanial antibodies in the patients' serum. In respect of the humoral immune response, a successively high titer of specific antibodies can be observed in Localized ATL, Mucocutaneous Leishmaniasis (MCL) and Diffuse ATL. An exceptionally high titer of antibodies against *Leishmania* antigens can be detected in the most severe form of the disease, Visceral Leishmaniasis (VL), as a consequence of polyclonal activation of B cells, resultant of the presence of large numbers of parasites in the bone marrow and spleen [79].

To evaluate the humoral immune response on Leishmaniasis, works have shown the role of the immunoglobulins on immunopathological mechanisms which are involved in the resistance and/or pathogenesis of the infection [80,81,82]. In some studies the presence of antibodies against *Leishmania braziliensis* in the sera of infected patients is still unclear but these antibodies have been monitored and they are utilized for diagnosis and prognosis of ATL [84,85]. Contrastingly, strong anti-leishmanial antibody titers are as well documented in VL [86,87].

However, it has been shown that the class IgG not only offers protection against this intracellular parasite, but indeed, it contributes to the progression of the infection. Previous analysis of *Leishmania* antigen-specific immunoglobulin isotypes and IgG subclasses in VL patients' sera has shown that elevated levels of IgG, IgM, IgE and IgG subclasses were lasting [79]. This is due to differential patterns of immunoglobulin isotypes observed during the disease progression. Drug resistance and cure were specific for antigens of *Leishmania donovani*. IgG subclass analysis has revealed expression of all the subclasses, with a prevalence of IgG1 during the disease [87], nevertheless, some studies have shown the advantage of using specific subclass antibodies for the diagnosis of VL [79,88,89].

Although studies have been evaluating the humoral immune response on ATL, the role of specific antibodies on the immunity against *Leishmania* is still not completely clear. On Cutaneous Leishmaniasis (CL) and Mucocutaneous Leishmaniasis (MCL), the cellular immunity and the prevalence of the isotypes IgG1, IgG2 and IgG3 have been associated with the Th1 response; on the other hand, the Th2 profile has been related to Diffuse Cutaneous Leishmaniasis (DCL), with the presence of IgG4. Studies lead the attention to the correlation of the subclasses of IgG with the clinical manifestations of ATL. Therefore, high levels of the isotypes IgG1, IgG2 and IgG3 and low levels or absence of the IgG4 isotype can be detected in the sera of patients with CL. In patients with MCL, there are high levels of IgG1 while the levels of IgG2, IgG3 and IgG4 are similar to the findings on the sera of patients with CL. The levels of IgG4 in patients with DL are highly elevated, as the level of IgG1 and IgG2 are similar to the patients with CL and MCL. Studies show that all specific isotypes anti-*Leishmania,* except for IgD, are detected in the sera of patients with ATL. There are high levels of IgE in patients with more time of disease evolution and high levels of IgA in patients with MCL [82].

The intensity of the antibody response appears to reflect both the parasite load and the chronicity of the infection and it also can be observed high titers of antibodies in all clinical manifestations of ATL [90]. Studies with immunological and serological methods which are available to the research of antibodies in ATL, showed controversial results due to its low sensibility and specificity [91,92]. However, studies have shown the advantages of using specific antibodies in the diagnosis of VL [79,88,89].

4. Final Considerations

Classically, *Leishmania* infections can induce the host to mount an immune response, which is characterized by the enrichment of T CD4+ cells, with Th1 or Th2 cytokines profile. Although this definition exists, the complexity in host-parasite interaction has promoted the investigation of other response profiles, in which cytokines, molecules and mediators take part. These may contribute favorably or not in the evolution of the different clinical forms in leishmaniasis. The scientific community has evaluated distinct cell subtypes, such as regulatory T cells, that accumulate in the lesion site and also acts mediating immune response through effector cells. Recently the Th17 profile was evidenced, and it was firstly related to the pathogenesis of chronic inflammatory disease or autoimmunity. Furthermore, the involvement of antibodies in diagnostic evaluations and as a criterion of cure must be considered.

In the balance between cure and progress of the disease, studies have shown that regardless of the cell and or molecules of a given profile, none is sufficient to act independently in the immune response. Thus, the balance between the innate and adaptive immune system and the parasite evasion mechanisms is critical for the decision if disease is observed and if (lifelong) immunity develops [13].

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