Age Modifies the Immunologic Response and Clinical Presentation of American Tegumentary Leishmaniasis

Augusto M. Carvalho, Camila F. Amorim, Juliana L. S. Barbosa, Alexsandro S. Lago, and Edgar M. Carvalho*

Serviço de Imunologia, Hospital Universitário Prof. Edgard Santos, Universidade Federal da Bahia, Salvador, Bahia, Brazil;
Instituto Nacional de Ciência e Tecnologia em Doenças Tropicais (INCT-DT), CNPq/MCT, Salvador, Bahia, Brazil;
Centro de Pesquisa Gonçalo Moniz, Fundação Oswaldo Cruz FIOCRUZ-Salvador, Bahia, Brazil

Abstract. *Leishmania (Viannia) braziliensis* is the main causal agent of American tegumentary leishmaniasis (ATL) that may present as cutaneous, mucosal, or disseminated cutaneous leishmaniasis. The disease is highly prevalent in young males and there is a lack of studies of ATL in the elderly. Herein, we compared clinical manifestations, immunologic response, and response to antimony therapy between patients > 60 years of age ($N = 58$) and patients who were 21–30 years of age ($N = 187$). The study was performed in Corte de Pedra, Bahia, Brazil, a well-known area of *L. braziliensis* transmission. Cytokine production by cultured peripheral blood mononuclear cells stimulated with soluble *Leishmania* antigen was performed. Elderly subjects more frequently had a previous history of cutaneous leishmaniasis, large lesions, or mucosal leishmaniasis, and they were less likely to have lymphadenopathy. There was no difference regarding gender and response to therapy. Peripheral blood mononuclear cells from elderly subjects produced a similar amount of tumor necrosis factor than young patients but they produced less interferon-gamma and more interleukin-10 than young subjects. We concluded that elderly patients with cutaneous leishmaniasis should be searched for mucosal or disseminated leishmaniasis. The decreased interferon-gamma production and increase in interleukin-10 observed in elderly patients may contribute to parasite persistence and *L. braziliensis* infection dissemination.

INTRODUCTION

Leishmaniasis are a group of infectious diseases with several clinical forms caused by protozoa of *Leishmania* genus.[1] The disease is subdivided into visceral leishmaniasis, which is associated with parasites in the spleen, bone marrow, and liver and tegumentary leishmaniasis characterized by skin and mucosal involvement. *Leishmania (Viannia) braziliensis* is the most important causal agent of leishmaniasis in Brazil and causes three clinical forms of the tegumentary disease: cutaneous leishmaniasis (CL), mucosal leishmaniasis (ML), and disseminated cutaneous leishmaniasis (DL).[2] In Brazil, more than 5,000 new cases of CL were reported in 2012.[3] Cutaneous leishmaniasis is characterized by a well-limited ulcer with raised borders. Mucosal leishmaniasis is observed in about 3% of patients with CL and presents mainly with nasal disease in patients with concomitant or a previous cutaneous ulcer.[4] Disseminated cutaneous leishmaniasis is defined by the presence of more than 10 cutaneous lesions in at least two parts of the body. Usually patients present at the same time with acneiform, papular, and ulcerated lesions.[5] Furthermore, it has been reported that up to 17% of residents from *L. braziliensis*-endemic areas have a positive delayed-type hypersensitivity skin test with *Leishmania* antigens or produce in vitro interferon-gamma (IFNγ) in cultures stimulated with soluble *Leishmania* antigens (SLA) but do not have evidence of clinical manifestation of the disease.[6] These individuals are considered as having a subclinical *L. braziliensis* infection.

Few studies have evaluated the clinical behavior of American tegumentary leishmaniasis (ATL) in elderly patients. In 2012, a study in Brazil evaluated clinical and epidemiological changes that occurred in a cohort with 20 years follow-up, to describe changes in the pattern of disease and risk factors of infection.[2]

This study included 1,209 patients diagnosed with ATL, and the analyses showed that 11.2% of this population was > 50 years of age. Age was associated with mucosal and disseminated cutaneous leishmaniasis.[2] Furthermore, most adults > 37 years of age had lesion duration > 60 days, whereas most patients < 14 years of age had an illness duration between 0 and 30 days.[2] Recently, Oliveira and others[7] evaluated the nutritional status of adult and elderly patients with ATL in a longitudinal study involving 68 patients > 20 years of age, and they found that low body weight and mucosal disease were directly associated with age. Another prospective study showed a positive correlation between CL ulcer size and age in patients with CL caused by *L. braziliensis* infection.[8] Moreover, age has been an important factor regarding side effects caused by antimony therapy.[9,10]

Epidemiologic studies have pointed out that ATL occur predominantly in young subjects, a population that is more exposed to sand fly bites.[4] However, cohort studies have shown an increasing number of elderly present with CL[2,4] and it is well known that ML and DL occur more in adults than in children.[5,11] As there is a lack of studies of leishmaniasis in the elderly, we compared clinical, epidemiological, and immunologic features of ATL in elderly subjects with the same features observed in young subjects. We found that elderly subjects with ATL had a longer illness duration, more CL scars, more ML, larger lesions, and less lymphadenopathy. These patients also presented with an increase in interleukin (IL-10) and decrease in IFNγ productions in cell cultures stimulated with SLA, which may explain the clinical peculiarities observed in elderly subjects.

MATERIALS AND METHODS

Patients and study area. This study included a retrospective component regarding epidemiologic and clinical features and a cross-sectional immunological study. It was conducted in Corte de Pedra, an area with a high transmission of *L. braziliensis*, localized in the southeast region of the state of Bahia, Brazil. Corte de Pedra Health Post is the reference center for diagnosis

*Address correspondence to Edgar M. Carvalho, Serviço de Imunologia, Hospital Universitário Prof. Edgard Santos, 5 andar, Rua João das Botas s/n, Canela 40110-160, Salvador-Bahia, Brazil. E-mail: imuno@ufba.br

and treatment of ATL for an area of 8,000 km^2 and clinical, epidemiological, and immunological studies have been performed in this area for over 30 years. All patients admitted to the clinic are evaluated by a physical examination by a physician, and those suspected of having leishmaniasis are tested for previous contact with *Leishmania* by a *Leishmania* skin test (LST). Confirmation of the diagnosis is performed by isolation of the parasite in cultures of aspirated lesions, histopathologic analysis, or by polymerase chain reaction (PCR) of biopsied tissue, as previously described.[12] In a limited number of patients, in vitro immunologic studies to determine production of cytokines (IFNγ, tumor necrosis factor [TNF], and IL-10) were performed. The diagnosis of CL is made based on the presence of a typical skin ulcer associated with parasite isolation, documentation of amastigotes in tissue or a positive PCR in biopsied tissue. The DL is defined by the presence of 10 or more acneiform, papular, and ulcerated lesions in at least two parts of the body.[5] The diagnosis of ML is performed based on visualization of a typical ML lesion plus isolation of parasites by culture or a positive PCR. All patients diagnosed with leishmaniasis are treated with a 20 days course of intravenous pentavalent antimony (15–20 mg/Kg/day) for CL and 30 days for ML and DL. Patients are evaluated every 30 days for response to therapy and failure is defined as persistence of active lesions 90 days after therapy. Those that remain with active lesions after 90 days receive a second course of antimony or intravenous amphotericin B.

Study design. During the year of 2012 a total of 1,248 cases of ATL were seen at the Health Post. All patients with age equal or higher than 60 years ($N = 58$) and with age between 21 and 30 years ($N = 187$) were selected to participate in the study and their charts were reviewed. No personally identifiable data beyond the study subject's clinic file number was recorded. The informed consent was obtained from all participants of this study, and it was approved by the ethical committee by the Federal University of Bahia.

Soluble *Leishmania* antigen. The SLA was prepared as previously described,[13] and used at a concentration of 5 µg/mL to stimulate mononuclear cells cultures. For LST, 0.1 mL (25 µg) of *L. braziliensis* antigen was inoculated in the forearm of individuals and induration was measured 48 hours post inoculation. A positive LST was considered when the induration was > 5 mm.

Cell culture and cytokine production. Peripheral blood mononuclear cells (PBMC) were separated from heparinized venous blood by Ficoll-Hypaque gradient centrifugation. After washing three times in 0.9% NaCl, cells were resuspended in RPMI 1640 culture medium (Gibco BRL, Grand Island, NY) supplemented with 10% human AB serum, 100 IU/mL of penicillin, and 100 µg/mL of streptomycin. Cells were adjusted to 3×10^6 cells/mL, plated in 24-well plates (Falcon, Becton Dickinson, Lincoln Park, NJ), and stimulated with SLA (5 µg/mL) or phytohemagglutinin (PHA). After incubation for 72 hours at 37°C and 5% CO$_2$, supernatants were collected and stored at −20°C. The levels of IFNγ, TNF, and IL-10 were measured by the enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN) sandwich method and the results expressed as pg/mL.

Statistical analysis. As the sample did not present a normal distribution, statistical analysis was performed with nonparametric tests. Age data are presented as the mean ± SD and the other continuous variables are presented as median and range. Categorical data were compared using the χ^2 test or Fisher's exact test and for continuous variables the Mann-Whitney test was used. The Mann-Whitney was also used to assess differences between cytokines levels among the two groups and a P value of < 0.05 was considered statistically significant.

RESULTS

Demographic and epidemiological features in 58 elderly and 187 young subjects are shown in Table 1. The mean age and standard deviation was 70 ± 8.6 years in a group of elderly individuals and 25 ± 2.9 years among the young subjects. Overall, there was a predominance of male individuals in both elderly and young groups (56.8% and 67.3%, respectively). Regarding occupation, although the most frequent activity in young individuals was agriculture, the majority of elderly patients were retired. There were no differences between the area of LST in the elderly group, 255.5 mm^2 (56–800 mm^2), and in the younger group, 229.5 mm^2 (36–930 mm^2) ($P = 0.3$).

The clinical aspects of elderly and young subjects with ATL are shown in Table 2. The duration of illness was determined by the time between the beginning of the symptoms and diagnosis of ATL; this was higher among elderly than younger patients. Scars of leishmaniasis usually remain for life and are characterized by well-delineated lesions in subjects who reported a previous diagnosis of leishmaniasis or had an ulcer characteristic of CL that took more than 60 days to heal. There were more scars and history of leishmaniasis in the elderly than the young subjects. Among the 10 elderly with a scar typical of leishmaniasis, 7 (70%) had a previous history of therapy for leishmaniasis. In three patients a self-healing lesion was reported. Elderly patients displayed larger cutaneous lesions than young subjects, and only elderly subjects developed ML.

Lymphadenopathy is an early sign of CL and it was documented more in young than in elderly subjects ($P = 0.0003$). The absence of lymphadenopathy was documented in up to 50% of the elderly patients. We compared the illness duration and lesion size in patients with or without lymphadenopathy and did not find a statistical difference ($P > 0.05$, data not shown). There was no difference in the number of cutaneous lesions between both groups and in the frequency of the lesions above the belt.

Regarding therapy, no statistically significant differences were found in the frequency of patients who failed to respond to antimony therapy or in the time between initiation of therapy and cure.

TABLE 1

Demographic, *Leishmania* skin test, and epidemiological features in elderly and young subjects with tegumentary leishmaniasis

Variables	Elderly subjects	Young subjects	P value
Age (years)	70.1 ± 8.6	25.3 ± 2.9	< 0.0001*
Gender (male)	33 (56.8%)	126 (67.3%)	0.1†
Weight (Kg)	58.5 (29–87)	64 (42–111)	< 0.0001*
Occupation			< 0.0001‡
Retired	47 (81.1%)	0 (0%)	
Agriculture	11 (18.9%)	140 (74.8%)	
Others	0 (0%)	47 (25.2%)	
Skin test area (mm^2)	255.5 (56–800)	229.5 (36–930)	0.3*

Continuous variables are represented by median (range).
*Mann Whitney test.
†Fisher's exact test.
‡Chi-square test.

TABLE 2
Clinical aspects from elderly and young subjects with tegumentary leishmaniasis

Variables	Elderly subjects ($N = 58$)	Young subjects ($N = 187$)	P value
Duration of the illness (weeks)	5 (2–28)	4 (1–24)	0.003*
Scars of CL	10 (17.2%)	5 (2.6%)	0.0003†
ML	4 (6.8%)	0 (0%)	0.002†
DL	3 (5.1%)	2 (1%)	0.08†
Lymphadenopathy	29 (50%)	142 (75.9%)	0.0003†
Number of lesions			1†
1	38 (70.3)‡	131 (70.8)§	
>1	16 (29.7)‡	54 (29.2)§	
Lesion size	290 (6–5250)	144 (4–2000)	0.002*
Lesion site (below the waist)	38 (70.3%)‡	119 (65%)¶	0.5†
Failure to antimony therapy	20 (34.5%)	69 (36.9%)	0.8†
Time between treatment and cure (days)	92 (38–365)	92 (28–400)	0.4*

Continuous variables are represented by median (range).
*Mann Whitney test.
†Fisher's exact test.
‡Total $N = 54$.
§Total $N = 185$.
¶Total $N = 183$.
CL = cutaneous leishmaniasis; ML = mucosal leishmaniasis; DL = disseminated cutaneous leishmaniasis.

The cytokine profile (IFNγ, TNF, and IL-10) in supernatants of PBMC stimulated with SLA and the IFNγ production in cultures stimulated with PHA are shown in Figure 1. Young patients exhibited higher levels of IFNγ 4,470 pg/mL (434–12,560 pg/mL) than elderly subjects 1,093 pg/mL (64–1,894 pg/mL) when stimulated with SLA (Figure 1A). There was no difference in TNF production between both groups (Figure 1B) and IL-10 levels were significantly higher in elderly patients (median of 143 pg/mL, ranging between 17 and 1,282 pg/mL) compared with young individuals (mean 1 pg/mL, range 0–87 pg/mL) upon SLA stimulation (Figure 1C). There was no difference in IFNγ produced by PBMCs among both groups after PHA stimulation (Figure 1D).
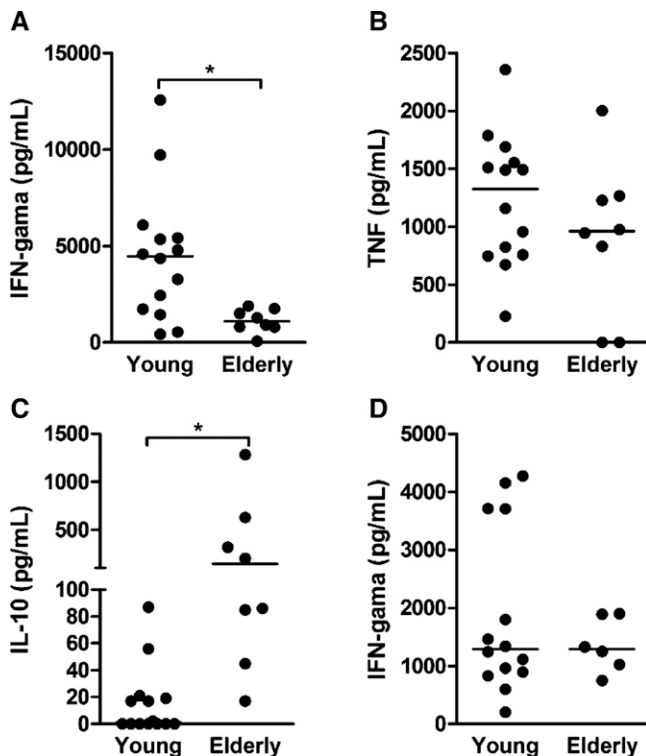

FIGURE 1. Cytokine production in elderly and young subjects with American tegumentary leishmaniasis. Peripheral blood mononuclear cells (PBMCs) from young subjects ($N = 14$ of 187) or elderly patients ($N = 8$ of 58) were cultured in presence of soluble *Leishmania* antigens (SLA) (5 μg) or phytohemagglutinin (PHA) for 72 hours and cytokine levels in culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA). Interferon-gamma (IFNγ) (**A**), tumor necrosis factor (TNF) (**B**), interleukin (IL-10) (**C**) levels after SLA stimulation. The IFNγ levels after PHA stimulation (**D**). Horizontal bars indicate median levels. * $P < 0.05$.

DISCUSSION

Epidemiologic and clinical studies of ATL have shown a higher prevalence of the disease among young males than older individuals but recently an increasing number of elderly subjects with ATL has been observed.[2] However, there is little information on the disease presentation in older patients. Herein, we documented that there are significant differences in the epidemiologic, clinical, and immunologic features of ATL among elderly compared with young patients. Specifically, elderly subjects had less agriculture activity, more scars of CL, less lymphadenopathy, and larger ulcer size, and the only subjects with ML in this study were the elderly. Immunologic responses were also different between elderly and young patients; older individuals produced less IFNγ and more IL-10 than young patients.

The predominant activity in the endemic area of Corte de Pedra is agriculture, which would put subjects at a higher risk of exposure to infected phlebotomine bites. The observation that ATL occurs frequently in patients who are retired indicates there are changes in the pattern of transmission of the infection, with acquisition of leishmaniasis occurring in the peri-domicile or intra-domicile environments. This observation is consistent with the documented observation of *Lutzomyia intermedia* and *Lutzomyia whitmani* infected with *L. braziliensis* in peri-domiciliary areas.[14]

There were marked differences between the clinical presentation of the disease in the elderly as compared with young patients. Exuberant lymphadenopathy is one of the first signs of CL as a result of *L. braziliensis* and indicates the presence of an immunological response to the parasite.[15] Mice that have surgically removed popliteal lymph nodes, which are subsequently infected in the footpad with *L. amazonensis* disseminated *Leishmania* infection, whereas intact mice contain the disease locally, indicating a role for local T cell responses in the control of parasite multiplication and dissemination.[16] Herein,

lymphadenopathy was observed less frequently among the elderly than young subjects, and these patients also had a more severe disease based on the size of the lesion and development of ML. As lymph node enlargement is observed early in the infection and disappears over the course of the disease, one possibility is that elderly subjects develop infrequently because they were diagnosed later in the course of infection. Arguing against this possibility, the duration of the disease did not differ among elderly patients with or without lymph nodes enlargement. Furthermore, the immune response documented in young patients compared with elderly subjects showed the latter had lower antigen-induced IFNγ production in PBMC stimulated with SLA. Therefore, it seems unlikely that the absence or an early disappearance of lymphadenopathy would explain the decrease in the type 1 immune response observed in older patients.

Evidence of delayed-type hypersensitivity to *Leishmania* antigen and a previous history of leishmaniasis are associated with a lowered risk of developing CL as a result of *Leishmania major*.[17] It is also uncommon for patients to develop more than one episode of leishmaniasis. For example, in a previous study we showed that only 5.2% of patients with CL had a previous history of the disease.[2] However, in this study we showed the presence of CL scar and a history of previous therapy for leishmaniasis were more frequent among elderly than young subjects. Because elderly subjects have more time in the endemic area the explanation could be trivial. Alternatively, elderly subjects may have impaired protective immune responses, which allows reinfection. Nevertheless, immune factors leading to protection against *L. braziliensis* infection are not entirely known, and as a result we could not determine whether these immunologic responses are lacking in the elderly. It is known that elderly individuals display impaired T cell immune responses and this likely contributes to the frequency of respiratory syncytial virus reinfection.[18] In fact, immunosenescence is an important factor predisposing to infection in elderly individuals.[19] Consistently, we documented in 8 of 58 elderly patients who had been studied immunologically, a lower type 1 immune response and an increase in IL-10 production upon stimulation with SLA.

Although we focus on the host, both host and parasite factors participate in the pathogenesis of ATL. The outcome of *L. braziliensis* in Corte de Pedra is polymorphic and there is an association between DL and genotypic characteristics of *L. braziliensis* isolates.[20,21] Parasites isolated from DL cases also induce a poorer immune response than isolates of CL patients.[22] Therefore, it cannot be ruled out that parasite factors also contribute to the differences observed in the disease in elderly patients. Nonetheless, we documented herein that at least some host immune factors may contribute to the infection outcome in elderly patients.

The immune response in CL is complex in that either a lack of a type 1 immune response, or an exaggerated inflammatory response can contribute to the pathogenesis of the disease.[8,23–25] During the early stages of the infection, before the appearance of an ulcer, there is a down modulation of IFNγ mediated by IL-10.[26] There is also evidence that IL-10 plays a major role in the pathogenesis of the disease.[27,28] The IL-10 is the most important regulatory cytokine in leishmaniasis responsible for downregulating the proinflammatory effects of IFNγ and TNF. The IL-10 may also facilitate parasite growth and replication by decreasing the ability of macrophages to kill *Leishmania*.[29–31] Alternatively, IL-10 may decrease the exaggerated inflammatory response observed in CL that has been associated with ulcer development.[23,24] Therefore, it is an imbalance between pro-inflammatory and regulatory cytokines that leads to ATL pathogenesis.[23,32,33] Herein, we showed that elderly subjects have a decrease in the antigen-response of IFNγ, and thus lack the main cytokine responsible for macrophage activation and killing. Furthermore, we also observed an increase in IL-10 that may facilitate parasite growth. Therefore, it is possible that these alterations may explain the recurrence of enhanced susceptibility to new *Leishmania* infections in elderly patients, and their large lesions and parasite dissemination.

Because this was a retrospective, cross-sectional study with only a few patients studied immunologically, definitive conclusions about the observed differences in clinical features between young and elderly subjects cannot be drawn. However, several findings of this study are highly relevant and potentially useful for the management of CL in the elderly. For instance, mucosal disease should be specifically examined and considered in all elderly patients with ATL. The presence of larger lesions, occurrence of parasite dissemination, and the decreasing type 1 immune responses in elderly subjects might suggest that these patients could require larger doses of leishmanicidal drugs to prevent parasite dissemination and recurrence of leishmaniasis.

Authors' addresses: Augusto M. Carvalho, Camila F. Amorim, Juliana L. S. Barbosa, and Alexsandro S. Lago, Serviço de Imunologia, Hospital Universitário Prof. Edgard Santos, Universidade Federal da Bahia, Salvador, Bahia, Brazil, E-mails: augustomarcelino1@hotmail.com, camilafarias112@gmail.com, julianalbarbosa@hotmail.com, and alex-lago@hotmail.com. Edgar M. Carvalho, Serviço de Imunologia, Hospital Universitário Prof. Edgard Santos, Universidade Federal da Bahia, Salvador, Bahia, Brazil, and Instituto Nacional de Ciência e Tecnologia em Doenças Tropicais (INCT-DT), CNPq/MCT, Salvador, Bahia, Brazil, E-mail: imuno@ufba.br.

REFERENCES

1. Grimaldi G Jr, Tesh RB, 1993. Leishmaniasis of the New World: current concepts and implications for future research. *Clin Microbiol Rev 6:* 230–250.

2. Jirmanus L, Glesby MJ, Guimarães LH, Lago E, Rosa ME, Machado PR, Carvalho EM, 2012. Epidemiological and clinical changes in American tegumentary leishmaniasis in an area of *Leishmania* (*Viannia*) *braziliensis* transmission over a 20-year period. *Am J Trop Med Hyg 86:* 426–433.

3. World Health Organization, 2012. *Control of Neglected Tropical Disease (NTD)*. Available at: http://www.who.int/leishmaniasis/en/. Accessed December 9, 2014.

4. Jones TC, Johnson WD Jr, Barretto AC, Lago E, Badaro R, Cerf B, Reed SG, Netto EM, Tada MS, Franca TF, Wiese K, Golightly L, Fikrig E, Costa JML, Cuba CC, Marsden PD, 1987. Epidemiology of American cutaneous leishmaniasis due to *Leishmania braziliensis braziliensis*. *J Infect Dis 156:* 73–83.

5. Turetz ML, Machado PR, Ko AI, Alves F, Bittencourt A, Almeida RP, Mobashery N, Johnson WD Jr, Carvalho EM, 2002. Disseminated leishmaniasis: a new and emerging form

of leishmaniasis observed in northeastern Brazil. *J Infect Dis 186:* 1829–1834.

6. Follador I, Araújo C, Bacellar O, Araújo CB, Carvalho LP, Almeida RP, Carvalho EM, 2002. Epidemiologic and immunologic findings for the subclinical form of *Leishmania braziliensis* infection. *Clin Infect Dis 34:* e54–e58.

7. Oliveira AG, Brito PD, Schubach AO, Oliveira RV, Saheki MN, Lyra MR, Salgueiro MM, Terceiro BF, Pimentel MI, Vasconcellos EC, Valete-Rosalino CM, 2013. Influence of the nutritional status in the clinical and therapeutical evolution in adults and elderly with American tegumentary leishmaniasis. *Acta Trop 128:* 36–40.

8. Oliveira F, Bafica A, Rosato AB, Favali CB, Costa JM, Cafe V, Barral-Netto M, Barral A, 2012. Lesion size correlates with *Leishmania* antigen-stimulated TNF-levels in human cutaneous leishmaniasis. *Am J Trop Med Hyg 85:* 70–73.

9. Araujo-Melo M, Meneses AM, Schubach AO, Moreira JS, Conceição-Silva F, Salgueiro MM, Pimentel MI, Araújo-Silva M, Oliveira RV, Carmo CN, Valete-Rosalino CM, 2010. Risk factors associated with dizziness during treatment of mucosal leishmaniasis with meglumine antimoniate: 16-year retrospective study of cases from Rio de Janeiro, Brazil. *J Laryngol Otol 124:* 1056–1060.

10. Diniz DS, Costa AS, Escalda PM, 2012. The effect of age on the frequency of adverse reactions caused by antimony in the treatment of American tegumentary leishmaniasis in Governador Valadares, State of Minas Gerais, Brazil. *Rev Soc Bras Med Trop 45:* 597–600.

11. Machado-Coelho GL, Caiaffa WT, Genaro O, Magalhães PA, Mayrink W, 2005. Risk factors for mucosal manifestation of American cutaneous leishmaniasis. *Trans R Soc Trop Med Hyg 99:* 55–61.

12. Weirather JL, Jeronimo SM, Gautam S, Sundar S, Kang M, Kurtz MA, Haque R, Schriefer A, Talhari S, Carvalho EM, Donelson JE, Wilson ME, 2011. Serial quantitative PCR assay for detection, species discrimination, and quantification of *Leishmania* spp. in human samples. *J Clin Microbiol 49:* 3892–3904.

13. Reed SG, Badaró R, Masur H, Carvalho EM, Lorenco R, Lisboa A, Teixeira R, Johnson WD Jr, Jones TC, 1986. Selection of a skin test antigen for American visceral leishmaniasis. *Am J Trop Med Hyg 35:* 49–85.

14. Miranda JC, Reis E, Schriefer A, Goncalves M, Reis MG, Carvalho L, Fernandes O, Barral-Netto M, Barral A, 2002. Frequency of infection of *Lutzomyia phlebotomines* with *Leishmania braziliensis* in a Brazilian endemic area as assessed by pinpoint capture and polymerase chain reaction. *Mem Inst Oswaldo Cruz 97:* 185–188.

15. Barral A, Guerreiro J, Bomfim G, Correia D, Barral-Netto M, Carvalho EM, 1995. Lymphadenopathy as the first sign of human cutaneous infection by *Leishmania braziliensis*. *Am J Trop Med Hyg 53:* 256–259.

16. Reed SG, Andrade ZA, Roters SB, Inverso JA, Sadigursky M, 1986. *Leishmania mexicana amazonensis* infections in 'resistant' inbred mice following removal of the draining lymph node. *Clin Exp Immunol 64:* 8–19.

17. Ben Salah A, Louzir H, Chlif S, Mokni M, Zaatour A, Raouene M, Ismail RB, Dellagi K, 2005. The predictive validity of naturally acquired delayed-type hypersensitivity to leishmanin in resistance to *Leishmania major*-associated cutaneous leishmaniasis. *J Infect Dis 192:* 1981–1987.

18. Cusi MG, Martorelli B, Di Genova G, Terrosi C, Campoccia G, Correale P, 2010. Age related changes in T cell mediated immune response and effector memory to Respiratory Syncytial Virus (RSV) in healthy subjects. *Immun Ageing 7:* 14.

19. Gavazzi G, Krause KH, 2002. Ageing and infection. *Lancet Infect Dis 2:* 659–666.

20. Schriefer A, Schriefer AL, Góes-Neto A, Guimarães LH, Carvalho LP, Almeida RP, Machado PR, Lessa HA, de Jesus AR, Riley LW, Carvalho EM, 2004. Multiclonal *Leishmania braziliensis* population structure and its clinical implication in a region of endemicity for American tegumentary leishmaniasis. *Infect Immun 72:* 508–514.

21. Queiroz A, Sousa R, Heine C, Cardoso M, Guimarães LH, Machado PR, Carvalho EM, Riley LW, Wilson ME, Schriefer A, 2012. Association between an emerging disseminated form of leishmaniasis and *Leishmania (Viannia) braziliensis* strain polymorphisms. *J Clin Microbiol 50:* 4028–4034.

22. Leopoldo PT, Machado PR, Almeida RP, Schriefer A, Giudice A, de Jesus AR, Ho JL, Guimarães LH, Bacellar O, Carvalho EM, 2006. Differential effects of antigens from *L. braziliensis* isolates from disseminated and cutaneous leishmaniasis on in vitro cytokine production. *BMC Infect Dis 6:* 75.

23. Bacellar O, Lessa H, Schriefer A, Machado P, Ribeiro de Jesus A, Dutra WO, Gollob KJ, Carvalho EM, 2002. Up-regulation of Th1-type responses in mucosal leishmaniasis patients. *Infect Immun 70:* 6734–6740.

24. Antonelli LR, Dutra WO, Almeida RP, Bacellar O, Carvalho EM, Gollob KJ, 2005. Activated inflammatory T cells correlate with lesion size in human cutaneous leishmaniasis. *Immunol Lett 101:* 226–230.

25. Machado PR, Lessa H, Lessa M, Guimarães LH, Bang H, Ho JL, Carvalho EM, 2007. Oral pentoxifylline combined with pentavalent antimony: a randomized trial for mucosal leishmaniasis. *Clin Infect Dis 44:* 788–793.

26. Rocha PN, Almeida RP, Bacellar O, de Jesus AR, Filho DC, Filho AC, Barral A, Coffman RL, Carvalho EM, 1999. Downregulation of Th1 type of response in early human American cutaneous leishmaniasis. *J Infect Dis 180:* 1731–1734.

27. de Assis Souza M, de Castro MC, de Oliveira AP, de Almeida AF, de Almeida TM, Reis LC, Medeiros ÂC, de Brito ME, Pereira VR, 2013. Cytokines and NO in American tegumentary leishmaniasis patients: profiles in active disease, after therapy and in self-healed individuals. *Microb Pathog 57:* 27–32.

28. Salhi A, Rodrigues V Jr, Santoro F, Dessein H, Romano A, Castellano LR, Sertorio M, Rafati S, Chevillard C, Prata A, Alcaïs A, Argiro L, Dessein A, 2008. Immunological and genetic evidence for a crucial role of IL-10 in cutaneous lesions in humans infected with *Leishmania braziliensis*. *J Immunol 180:* 6139–6148.

29. Vouldoukis I, Bécherel PA, Riveros-Moreno V, Arock M, da Silva O, Debré P, Mazier D, Mossalayi MD, 1997. Interleukin-10 and interleukin-4 inhibit intracellular killing of *Leishmania infantum* and *Leishmania major* by human macrophages by decreasing nitric oxide generation. *Eur J Immunol 27:* 860–865.

30. Vieth M, Will A, Schröppel K, Röllinghoff M, Gessner A, 1994. Interleukin-10 inhibits antimicrobial activity against *Leishmania major* in murine macrophages. *Scand J Immunol 40:* 403–409.

31. Scott P, 2003. Development and regulation of cell-mediated immunity in experimental leishmaniasis. *Immunol Res 27:* 489–498.

32. Faria DR, Gollob KJ, Barbosa J Jr, Schriefer A, Machado PR, Lessa H, Carvalho LP, Romano-Silva MA, de Jesus AR, Carvalho EM, Dutra WO, 2005. Decreased in situ expression of interleukin-10 receptor is correlated with the exacerbated inflammatory and cytotoxic responses observed in mucosal leishmaniasis. *Infect Immun 73:* 7853–7859.

33. Gomes-Silva A, de Cássia Bittar R, Dos Santos Nogueira R, Amato VS, da Silva Mattos M, Oliveira-Neto MP, Coutinho SG, Da-Cruz AM, 2007. Can interferon-gamma and interleukin-10 balance be associated with severity of human *Leishmania (Viannia) braziliensis* infection? *Clin Exp Immunol 149:* 440–444.