Morphometric changes in C57BL/6 mice retina infected by Toxoplasma gondii ME 49 strain

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HIGHLIGHTS

• Morphometric implications in T. gondii infection were evaluated in mice.
• Digital morphometry showed that infected mice have a thicker retina than control.
• The medians of retinal P/A in infected mice were significantly lower from uninfected.

GRAPHICAL ABSTRACT

ABSTRACT

This study evaluated the morphometric implications in C57BL/6 mouse retina infected by Toxoplasma gondii ME 49 strain. Twenty C57BL/6 female mice were divided into group 1 (n = 8, intraperitoneally infected with 30 cysts of T. gondii ME 49 strain) and group 2 (n = 12 non-infected controls). The eyes were enucleated on the 60th day after infection, fixed and processed for light microscopy. Changes in retinal thickness and in the perimeter/area ratio (P/A) of the retinal layers were analyzed by digital morphometry. We considered that P/A was the measurement of retinal architecture distortion induced by toxoplasmosis. This study considered the ganglion cells and nerve fiber layers as a monolayer, thus six layers of retina were evaluated: photoreceptors (PRL), outer nuclear (ONL), outer plexiform (OPL), inner nuclear (INL), inner plexiform (IPL) and ganglion cells/nerve fiber monolayer (GNL). Histological analysis of infected mouse retina showed inflammatory infiltrate, necrosis, glial reaction and distortion of the retina architecture. It also presented increased thickness (167.8 ± 24.9 µm versus 121.1 ± 15.4 µm, in controls) and increased retinal thickness within the retinitis foci (187.7 ± 16.6 µm versus 147.9 ± 12.2 µm out of the retinitis foci). A statistically significant difference in P/A was observed between infected and uninfected mouse retina. The same was observed in PRL, OPL, INL and GNL. Retinal morphometry may be used to demonstrate differences between infected and uninfected mouse retinas.

1. Introduction

Toxoplasma gondii, the causative agent of toxoplasmosis, is ubiquitously distributed and infects approximately 30% of the
human population worldwide (Tenter et al., 2000; Dalimi and Abdoli, 2012). In the United States and United Kingdom, it is estimated that 16–40% of the population is infected, whereas the estimated seroprevalence is 50–80% in Central America and Continental Europe (Hill and Dubey, 2002). In most immunocompetent individuals, infection with T. gondii is usually asymptomatic and self-limiting (Remington et al., 1995). Toxoplasmosis causes destructive inflammation that targets multiple organs, including the eyes. Only a few infected individuals develop ocular lesions or toxoplasmic retinochoroiditis (TR) (Holland, 2003; Butler et al., 2013). TR is the most common identifiable cause of posterior uveitis in many parts of the world (Henderly et al., 1987; Furtado et al., 2013). There is a wide variation in the clinical presentation of ocular toxoplasmosis, with some individuals presenting mild inflammation and others presenting multiple recurrences of severe uveitis, which can lead to loss of eyesight. Histopathological analyses usually show a mononuclear inflammatory infiltrate around the blood vessels and in the humor vitreous; alteration in the disposition of the retinal layers and edema, characterized by the increase of interstitial spaces, forming lacunae. These alterations take the form of retino-vitreal projections, characteristic of TR (Calabrese et al., 2008). Host genetics appears to be important in determining susceptibility and severity of infection, as shown by variable disease patterns in different inbred mouse strains upon infection (Suzuki et al., 1993; Vasconcelos-Santos, 2012). Moreover, disease severity is likely to be influenced by the genotype of the infecting parasite (Suzuki and Joh, 1994; Subauste et al., 2011). Several animal models (e.g. mouse, rabbit, and hamster) have been used to study ocular toxoplasmosis (Frenkel, 1955; Garweg et al., 1998; Pavesio et al., 1995; Tedesco et al., 2004). Most of the studies in ocular toxoplasmosis use the intraperitoneal (Pavesio et al., 1995) or intravitreal (Garweg et al., 1998; Tedesco et al., 2005) routes for parasite inoculation. Infection with T. gondii ME 49 strain by the intraperitoneal route leads to parasite dispersion causing an infection with low parasite loads in the eyes (Gormley et al., 1999). Experimental studies show differences in hosts, in parasite strains and in infection routes, which makes results difficult to compare. Even under the same experimental conditions, the descriptions of ocular toxoplasmosis histopathology may vary among researchers. Therefore, the aim of this study was to show that this descriptive heterogeneity decreases drastically with digital morphometry which provides objective (numerical) data and allows for a more accurate statistical analysis.

2. Materials and methods

2.1. Animals

Twenty C57BL/6 female mice at 6–8 weeks of age, weighing about 12–18 g were purchased from the Animal Facility of the Oswaldo Cruz Institute – FIOCRUZ.

2.2. Parasites

Tissue-derived cysts from T. gondii, ME 49 strain were obtained from the brains of previously infected C57BL/6 mice (Guimarães et al., 2003) and maintained by serial intraperitoneal passage in C57BL/6 mice.

2.3. Experimental schedule

Experiments were performed in accordance with the rules of the Ethics Committee in Animal Experimentation (CETEA) from the Federal University of Minas Gerais – UFMG, protocol no. 30/2008 and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were divided in two groups: group 1, eight mice that were intraperitoneally infected (Calabrese et al., 2008) with 30 cysts; group 2, twelve uninfected control mice. Sixty days after infection, animals were killed by CO2 inhalation and the eyes were enucleated and sectioned in the sagittal plane, dividing the eyes in two hemispheres, and processed routinely for paraffin-embedding and sectioning. Sections were obtained from the eyes and stained with hematoxylin and eosin.

2.4. Digital morphometric analysis

Digital morphometric analysis was performed according to Cagliari (1997). Histopathological images were digitalized in microscope Zeiss Axiohot 2. Changes in retinal thickness and in the retinal layers perimeter/area ratio (P/A) were analyzed with Kontron Elektronics, Carl Zeiss – KS 300, version 2.0, image analyzer. The retinal pigment epithelium was not included in these measurements. For the retinal thickness analysis, we performed three measurements: one central within the retinitis focus and the others on opposite sides, 50 μm away from the central measurement (Fig. 1A). Similarly, three measurements 50 μm distant from each other were taken in control retinas and in infected retinas out of the retinitis focus. For the P/A measurement, the contour of the neurosensory retina was manually delineated using the KS 300 software and the values of perimeter and area were obtained (Fig. 1B). We considered that P/A was a measurement of retinal architecture distortion induced by toxoplasmosis. In addition, this

Fig. 1. Retinal digital morphometry. (A) Retinal thickness measure in retinitis focus of C57BL/6 mice infected with T. gondii, ME 49 strain. White arrow points inflammatory reaction; (B) P/A measurements in retinitis focus of C57BL/6 mice infected with T. gondii, ME 49 strain. Asterisk (*) points distortion and white arrow outlined points initial fold formation.
study also evaluated six layers of the retina: photoreceptors layer (PRL), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL) and an inner monolayer composed by ganglion cells and nerve fiber layers (GNL). Three or more repetitions were done for each variable and the mean was obtained for each animal.

2.5. Statistical analysis

The experimental data was evaluated using the Tukey’s Studentized Range test for retinal thickness and retinal layer P/A because these variables had a Gaussian distribution, homocedasticity and low instability. The retinal P/A was evaluated using the nonparametric Mann–Whitney’s test because the distribution of data was not Gaussian, even after logarithmic, square root and arcsine attempts to adjust data. Because the retinal layers P/A showed normal distribution and homocedasticity, a randomized complete block split-plot with a 2 x 7 factorial arrangement was performed for data analysis. SAS (Statistical Analysis System, version 8.0, SAS Institute, Cary, NC, USA) was used for unpaired data analysis. p < 0.05 was considered statistically significant.

3. Results

Control eye sections showed a normal morphology of the retina. On the other hand, on the 60th day after parasite challenge, retinovitreal projections characteristic of TR were observed in all T. gondii-infected mice. Histological analysis of infected mouse retina showed inflammatory infiltrate, oedema and distortion of retina architecture (Fig. 1A and B). The statistics for retinal thickness are shown in Table 1. Retinal thickness did not vary significantly along the three measurement positions (central, left and right) in control (p = 0.9973) nor infected mice (p = 0.6699). Infected mice showed a thicker retina than uninfected animals (center: 167.8 ± 24.9 µm versus 121.1 ± 15.4 µm, p = 0.00002) as shown in Table 2.

Infected animals also showed a thicker retina within the retinitis foci than out of the retinitis foci (center: 187.7 ± 16.6 µm versus 147.9 ± 12.2 µm, p = 0.0001) as shown in Table 3. The medians of retinal P/A in infected mice were significantly lower from uninfected mice (0.0163 versus 0.0202, p = 0.0069).

As presented in Table 4, the P/A showed low instability in ONL, OPL and IPL in retinal layers. In contrast, PRL, INL and GNL presented P/A with medium instability. Differences between infected and uninfected mice were observed in PRL, OPL, INL and GNL, although, in theory, they (except the OPL) showed insufficient sample size (Table 5).

4. Discussion

Digital morphometry provided numerical data, thereby allowing for an accurate evaluation of changes in the retinal thickness and architecture induced by toxoplasmosis.

According to the literature (Calabrese et al., 2008), the ocular lesions observed in this study were classical and included a mononuclear inflammatory infiltrate around the blood vessels and in the humor vitreus, as well as an alteration in the disposition of the retinal layers and edema, characterized by the increase of interstitial spaces forming lacunae. These alterations took the form of vitreo-retinal projections, characteristic of TR.

Three positions of measures (central, left and right) were considered for obtaining retinal thickness data. Morphometrically, this was important in order to bypass the fact that we could not determine the center of a tridimensional structure (the retinitis focus) in a two-dimensional picture. As the retinal thickness did not show any difference along the three positions of measurement (central, left and right) in at least six different sections of the same eye and three different replicates of the same measurement, it is reasonable to infer that the retinal thickness does not vary significantly 50 µm either way, laterally or transversely. Therefore, even if the plane of the section is not necessarily in the center of the inflammatory focus, the measurement seems valuable.

The results showed increased retinal thickness in infected mice compared to controls. That was expected considering that swelling

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<td>Retinal thickness at various positions of measures in infected and uninfected mice.</td>
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*Means with the same letter are not significantly different.*
(tumor) is one of the four cardinal signs of inflammation as described by Celsus (Tracy, 2006). Nonetheless, histopathology showed edema in infected mouse retinas. Similarly, infected animals also showed a thicker retina within the retinitis foci than outside it. Such a result was not surprising because the histopathology showed a mononuclear inflammatory infiltrate plus edema in the retinitis foci. Thus, morphometry confirmed and quantified the histopathological data.

As defined before, this study considered P/A as a measurement of retinal distortion. The P/A effectively showed the changes in the architecture of infected mouse retinas. Several studies (Tedesco et al., 2004, 2005; Tracy, 2006; Goldenberg et al., 2013) have described the changes in retinal layers, especially those caused by edema in the PRL and plexiform layers, in addition to cellular infiltration and glial reaction in the inner layers. Our results showed significant differences in P/A for the PRL, OPL, INL and GNL of infected mice as compared to controls. Infected mice showed increased retinal thickness compared to uninfected controls. Within the infected eye, the retina was thicker within the retinitis foci than outside of the retinitis foci. Statistically significant differences were found in the P/A among infected and uninfected mouse retinas as well as in PRL, OPL, INL and GNL.

In conclusion, retinal digital morphometry, used here for the first time to access and demonstrate differences between infected and uninfected mouse retinas, shows that is possible to reduce the heterogeneity observed when other methods are used for evaluate morphometric implications in mouse retina infected by *T. gondii*.

**Acknowledgments**

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**References**


Increased tau phosphorylation and receptor for advanced glycation endproducts (RAGE) in the brain of mice infected with *Leishmania amazonensis*

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**A B S T R A C T**

Leishmaniasis is a parasitosis caused by several species of the genus *Leishmania*, an obligate intramacrophagic parasite. Although neurologic symptoms have been observed in human cases of leishmaniasis, the manifestation of neurodegenerative processes is poorly studied. The aim of the present work was to investigate if peripheral infection of BALB/c mice with *Leishmania amazonensis* affects tau phosphorylation and RAGE protein content in the brain, which represent biochemical markers of neurodegenerative processes observed in diseases with a pro-inflammatory component, including Alzheimer’s disease and Down syndrome. Four months after a single right hind footpad subcutaneous injection of *L. amazonensis*, the brain cortex of BALB/c mice was isolated. Western blot analysis indicated an increase in tau phosphorylation (Ser\textsuperscript{396}) and RAGE immunocompetent in infected animals. Brain tissue TNF-\textalpha, IL-1\textbeta, and IL-6 levels were not different from control animals; however, increased protein carbonylation, decreased IFN-\gamma levels and impairment in antioxidant defenses were detected. Systemic antioxidant treatment (NAC 20 mg/kg, i.p.) inhibited tau phosphorylation and recovered IFN-\gamma levels. These data, altogether, indicate an association between impaired redox state, tau phosphorylation and RAGE up-regulation in the brain cortex of animals infected with *L. amazonensis*. In this context, it is possible that neurologic symptoms associated to chronic leishmaniasis are associated to disruptions in the homeostasis of CNS proteins, such as tau and RAGE, as consequence of oxidative stress. This is the first demonstration of alterations in biochemical parameters of neurodegeneration in an experimental model of Leishmania infection.

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**1. Introduction**

Leishmaniasis is a parasitosis caused by several species of the genus *Leishmania*, an obligate intramacrophagic parasite. Endemic leishmaniasis transmission occurs in at least 98 countries (Alvar et al., 2012). There are three main human syndromes caused by *Leishmania*: cutaneous disease, the least severe form of disease; mucocutaneous disease, which can be due the extension or metastasis of local skin lesions; and visceral leishmaniasis, also known as Kala-azar, the most severe form (Desjeux, 2004). The outcome of each is determined by the species of infecting parasite and the genetic susceptibility of the host (McGwire and Satoskar, 2013). *Leishmania amazonensis* causes different diseases depending on the host and parasitic virulence factors (Souza et al., 2011). Commonly, *L. amazonensis* infection is more associated to cutaneous leishmaniasis (Murray et al., 2005), but parasites have been also isolated from patients with the entire spectrum of the disease, including localized and diffuse cutaneous lesions, mucosal and visceral leishmaniasis (Barral et al., 1991). Recently, *L. amazonensis* was also associated with disseminate cutaneous leishmaniasis, an intermediate clinical form (David and Craft, 2009).

Although neurologic symptoms have been largely observed in human cases of the disease, the manifestation of degenerative
processes associated with the central nervous system (CNS) in leishmaniasis is poorly studied. Cutaneous leishmaniasis is known to cause a peripheral neuropathy by direct or close parasite involvement with the nerve or nerve sheath (Petersen and Greenlee, 2011). In this form of the disease, parasites in the skin and draining lymph nodes are thought to cause an endocrine imbalance as a consequence of cytokines action on the CNS (de Moura et al., 2005). More recently, patients with cutaneous leishmaniasis were reported to exhibit an immune–endocrine imbalance with reduction of plasma levels of dehydroepiandrosterone-S, prolactin and testosterone (Baccan et al., 2011). Together with the observation that L. amazonensis may cross the blood–brain barrier and induce significant pathologic changes in the CNS (Abreu-Silva et al., 2003), these data indicate that relevant neurodegenerative processes may occur in the course of leishmaniasis.

The microtubule-stabilizing protein tau is mainly expressed in central nervous system (CNS) neurons and is essential for axon architecture and synaptic function. Initially, the aberrant hyperphosphorylation of tau was observed to be associated with the formation of neurotoxic histological structures in the CNS known as neurofibrillary tangles, characteristic of Alzheimer’s disease (AD) (Sonnen et al., 2008). Later, tau hyperphosphorylation was detected in at least twenty-two different CNS related pathologies, including prion diseases, amyotrophic lateral sclerosis and Down’s syndrome (Spikes-Jones et al., 2009). These conditions have been referred to as “taupathies”, in which tau aberrant phosphorylation is thought to exert a causative role in synapse impairment and progression of neuronal cell death. Although the molecular detailing of the steps coupling tau aberrant phosphorylation and neuronal death are currently not completely understood, it is clear that disruption of tau homeostasis is associated with an impairment of neural circuits and cognitive deficits (Gendron and Petrucelli, 2009).

The receptor for advanced glycation endproducts (RAGE) is a multiligand membrane receptor that exerts crucial roles in the development of chronic inflammatory processes (Srikanth et al., 2011). RAGE was initially observed to be associated with late diabetic complications, where the accumulation of advanced glycation endproducts (AGE) on the endothelial surface triggers its expression and activation. Further studies have characterized RAGE as a damage-associated molecular pattern (DAMP) receptor, as other molecules with pro-inflammatory and pro-apoptotic activities were observed to act as RAGE ligands (Coughlan et al., 2007). These include the extracellular forms of HMGB1 and members of the S100/calgranulin family, such as S100B and S100A7 (Bopp et al., 2008; Leclerc et al., 2007). RAGE activation induces the expression of pro-inflammatory cytokines and NADPH oxidase activation, which in turn stimulate reactive species (RS) production, causing oxidative damage to biomolecules and sustaining local inflammation and tissue damage (Maczurek et al., 2008). In the brain, a prominent role of RAGE in the contribution to neurodegenerative processes has been emerging since the earlier observations that the β-amyloid peptide function as a RAGE ligand (Arancio et al., 2004; Du Yan et al., 1997). Since then, extensive data have been indicating a key role for RAGE in the chronic pro-inflammatory axis responsible for the progression of neuronal death observed in AD (Yan et al., 2009). On the other hand, the involvement of RAGE in other neuroinflammatory states potentially associated with neurodegeneration is poorly studied. In the present work, we investigated the phosphorylation of tau and the modulation of the immunocompetence of RAGE in the brain cortex of BALB/c mice infected with L. amazonensis. We also analyzed oxidative stress and inflammatory parameters in order to study the possible relationship of RS production and inflammation in the modulation of neurodegeneration parameters in leishmaniasis.

2. Materials and methods

2.1. Chemicals

Glycine, H₂O₂ (hydrogen peroxide), catalase (CAT, EC 1.11.1.6), superoxide dismutase (SOD, EC 1.15.1.1), thiobarbituric acid, epinephrine, AAPH (2,2′-azobis[2-methylpropionamidine]dihydrochloride), trichloroacetic acid (TCA), 2,4-dinitrophenylhydrazine (DNPH), 5,5′-dithiobinitrobenzoic acid (DTNB), Bile salts, sodium dodecyl sulfate, DNP polyclonal antibody and monoclonal TNF-α antibody were purchased from Sigma–Aldrich®(St. Louis, USA). Electrophoresis and immunoblot reagents were from Bio-Rad (Hercules, USA), GE Healthcare Brazilian Headquarter (São Paulo, Brazil) and Sigma–Aldrich®RAGE polyclonal antibody, phosphorylated tau polyclonal antibody, total tau polyclonal antibody, β-actin polyclonal antibody, IL-1β polyclonal antibody and anti-rabbit immunoglobulin linked to peroxidase were from Cell Signalling technology®(Beverly, USA). ELISA microplates were from Greiner Bio-One (Monroe, USA) and ELISA TMB spectrophotometric detection kit was from BD Biosciences (San Diego, USA). Immunoblot chemiluminescence detection was carried out with the West Pico detection kit from Thermo Scientific Pierce Protein Biology Products (Rockford, USA). MilliQ-purified H₂O was used for preparing solutions. All other reagents used in this study were of analytical or HPLC grade.

2.2. Ethics statement

All experimental procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publication number 80-23 revised 1996) and the Brazilian Society for Neuroscience and Behavior recommendations for animal care. The experimental protocols were approved by the Oswaldo Cruz Foundation Committee of Ethics for the Use of Animals (CEUA–Fiocruz) protocol number P-36/11-3.

2.3. Parasite strain and infection

L. amazonensis (MHOM/BR/75/JOSEFA) were maintained by regular passage in BALB/c mice. Amastigotes were purified from the footpad lesions of mice as previously described (Barbieri et al., 1993). Female BALB/c mice (6 weeks old) were obtained from Centro de Criação de Animais de Laboratório (CECAL)–Fiocruz, Rio de Janeiro, RJ, Brazil, and injected subcutaneously in the right hind footpad with 10⁵ amastigotes (Arrais-Silva et al., 2006). Four months post-infection the animals were euthanized, the brain cortex was removed and maintained in liquid nitrogen until the assays were performed. Additional groups of mice received a systemic antioxidiant therapy (N-acetylcysteine 20 mg/kg b.w., one daily injection, i.p.) for five consecutive days before euthanasia. During the post-infection period, animals show apathy and diminished eating behavior. To estimate parasite burden in the lesions, the entire infected footpads were removed and amastigotes were recovered from the lesions and counted. Inflammatory parameters in liver and serum (TNF-α and IL-1β) were measured to confirm systemic inflammation. In all assays five animals per group (n = 5) were utilized.

2.4. Antioxidant enzymes activities

Samples were homogenized in phosphate buffer (PB) 50 mM (KH₂PO₄ and K₂HPO₄, pH-7.4) and the protein content was determined by Bradford method (Bradford, 1976). Catalase (CAT; EC 1.11.1.6) activity was evaluated by following the rate of decrease in hydrogen peroxide (H₂O₂) absorbance in a spectrophotometer.
at 240 nm (Aebi, 1984). Results are expressed as units of CAT/mg of protein. The activity of superoxide dismutase (SOD; EC 1.15.1.1) was measured by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation in a sample buffer; adrenochrome formation was monitored at 480 nm for 10 min (32 °C) in a spectrophotometer (Misra and Fridovich, 1972). Results are expressed as units of SOD/mg of protein.

2.5. Oxidative damage to proteins (carbonyl)

As an index of protein oxidative damage, the carbonyl groups were determined as previously described (Levine et al., 1990). The homogenate were divided into two aliquots of 300 μL (1 mg of protein). Proteins were precipitated by the addition of 150 μL of 20% TCA for 5 min on ice and centrifuged at 4000 g for 5 min. The pellet was dissolved with 100 μL of sodium hydroxide (NaOH) (200 mM) and 100 μL of hydrochloric acid (HCl) (2 M) was added in blanks. DNPH (10 mM) was added for carbonyl groups derivatization. Samples were maintained for 30 min at room temperature. Proteins were precipitated with 20% TCA and washed three times with 500 μL of 1:1 ethanol:ethyl acetate with 15 min standing periods to remove the excess DNPH. Samples were dissolved in 200 μL of urea (8 M) pH 2.3, and the absorbance was read at 370 nm.

2.6. Western blot detection of dinitrophenyl (DNP)-labeled protein carbonyls

Tissue samples were homogenized in 1 volume of radio-immunoprecipitation assay (RIPA) buffer (20 mM Tris–HCl at pH 7.5, 150 mM NaCl, 1 mM Na2 EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na2VO4, 1 μg/mL leupeptin), centrifuged (14,000 g for 10 min at 4 °C) and the pellet proteins were quantified. Proteins were dissolved in 6% sodium dodecyl sulfate (SDS) and derivatized with an equal volume of DNPH 10 mM in 10% trifluoroacetic acid for 1 h (Shacter et al., 1994). Samples were subjected to SDS–polyacrylamide gel electrophoresis for Western blot detection of DNP-derivatized proteins with an antibody against DNP as described below.

2.7. Sulphydryl groups quantification

Oxidative status of thiol groups were assessed by quantification of total reduced sulphydryl (SH) groups in samples (Ellman, 1959). Briefly, for total SH content measurement, 60 μg sample aliquot was diluted in phosphate-buffered saline (PBS) (NaCl, Na2HPO4, KH2PO4), and 5,5’-dithiobis 2-nitrobenzoic acid (10 mM), and read in a spectrophotometer at 412 nm after 60 min of incubation in room temperature.

2.8. Index of lipid peroxidation (TBARS)

The quantification of thiobarbituric acid reactive substances (TBARS) was performed for evaluation of an index of liperoxidation, as previously described (Draper and Hadley, 1990). Brain cortex tissue was homogenized in ice-cold Tris–HCl 15 mM (pH 7.4) and reacted with an equal volume of 40% trichloroacetic acid (TCA), followed by centrifugation and addition of 0.67% TBA. Samples were then incubated at 100 °C for 25 min. After cooling, samples were centrifuged (750g/10 min) and supernatant absorbance was read at 535 nm.

2.9. Total reactive antioxidant potential (TRAP assay)

The total reactive antioxidant potential (TRAP) was used as an index of non-enzymatic antioxidant capacity. This assay is based on the quenching of peroxy radicals generated by AAPH (2,2 azobis[2-aminopropionitrile]) by antioxidants present in a given sample (Lissi et al., 1992). Briefly, a chemical system that generates peroxy radicals at a constant rate (an AAPH-containing buffer) is coupled to a luminescent reactant (luminol) which emits photons proportionally to its oxidation. The samples were homogenized with glycine buffer (pH 8.6). The reaction was initiated by injecting luminol to the 0.1 M glycine buffer containing AAPH that resulted in steady luminescence emission. Equal amounts of samples are then added to this reaction system, and the luminescence emission at the moment following this addition (t = 0) is recorded. This initial emission reflects the production of free radicals by AAPH at the first moment right after sample addition and is related to the endogenous oxidant state of the sample. Following incubation, the thermal decomposition of AAPH produces luminescence at a constant rate (“system”), and the presence of free radical scavengers in the added sample will decrease this rate according to its content of non-enzymatic antioxidants. We followed TRAP luminescence emission for 80 min and calculated the area under the curve (AUC) relative to the system without samples (which was considered as 100% of luminescence emission at all time points). The addition of the homogenate samples decreases or facilitates the luminescence emission proportionally to its redox state. The luminescence emission was recorded in a MicroBeta luminoscence counter (Perkin Elmer, USA).

2.10. TNF-α, IL-1β, IFN-γ, IL-6 and nitrotyrosine levels (ELISA)

TNF-α, IL-1β, IFN-γ, IL-6 and nitrotyrosine were quantified by indirect ELISA. Brain cortex homogenate was placed in ELISA plates. After 24 h incubation, plates were washed three times with Tween–Tris buffered saline (TTBS, 100 mM Tris–HCl, pH 7.5, containing 0.9% NaCl, and 0.1% Tween-20). Subsequently, 200 μL of anti-TNF-α, anti-IL-1β, anti-IFN-γ, anti-IL-6 or anti-nitrotyrosine (1:1000) were added and incubation was carried for 24 h at 4 °C. The plates were washed three times with TTBS and incubated with rabbit or mouse IgG peroxidase-linked secondary antibody (1:1000) for 2 h. After washing the plate three times with TTBS, 200 μL of substrate solution (TMB spectrophotometric ELISA detection kit) were added to each well and incubated for 15 min. The reaction was terminated with 50 μL/well of 12 M sulfuric acid stopping reagent and the plate read at 450 nm.

2.11. Immunoblot detection of phosphorylated tau and RAGE

To perform immunoblot experiments, the tissue was homogenated with 1X RIPA buffer, centrifuged (10,000g for 5 min at 4 °C) and the pellet proteins were measured by the Bradford method (Bradford, 1976). Laemmli-sample buffer (62.5 mM Tris–HCl, pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol) was added to complete volume according the protein content of each sample and equal amounts of cell protein (30 μg/well) were fractionated by SDS-PAGE and electro-blotted onto nitrocellulose membranes with Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell, Bio-Rad (Hercules, CA, USA). Protein loading and electro-blotting efficiency were verified through Ponceau S staining, and the membrane was washed with Tween–Tris buffered saline (Tris 100 mM, pH 7.5, 0.9% NaCl and 0.1% Tween-20). Membranes were incubated 20 min at room temperature in SNAP i.d.® 2.0 Protein Detection System Merck Millipore (Billerica, MA, USA) with each primary antibody (anti-RAGE, anti-phospho-tau, anti-tau, anti-β-actin – 1:500 dilution range each) and then washed with TTBS. Anti-rabbit
or mouse IgG peroxidase-linked secondary antibody (1:5000 dilution range) was incubated with membranes for additional 20 min in SNAP l.d. system (Millipore, Billerica, MA, USA), washed again and the immunoreactivity was detected by enhanced chemiluminescence using Supersignal West Pico Chemiluminescent kit from Thermo Scientific (Luminol/Enhancer and Stable Peroxide Buffer). Densitometric analysis of the films was performed with Image J software. Blots were developed to be linear in the range used for densitometry.

2.12. Statistical analysis

Statistical analysis was performed with GraphPad 5.0 software. Student’s t test (two-tailed) was applied for simple comparisons between control and infected animals in each assay. For comparison of multiple means, ANOVA with Tukey’s post hoc was performed. The results of measurements were expressed as mean ± standard error of the mean (SEM). Differences were considered significant when p < 0.05. The tissue protein content was measured by Bradford method for data normalization in all assays (Bradford, 1976).

3. Results

Tau may be phosphorylated by diverse protein kinases at approximately 25 different sites (Wang et al., 2013). However, phosphorylation of tau on Ser396 is one of the earliest events leading to neurofibrillary tangles formation in AD and Down syndrome and it was suggested to play a key role in the formation of paired helical filaments, the major component of neurofibrillary tangles (Mondragon-Rodriguez et al., 2014). We evaluated the content of phosphorylated and total tau isoforms in the brain cortex of mice infected by L. amazonensis by Western blot. An increase in the content of phospho-tau (Ser396) was detected, but the content of total tau was not changed (Fig. 1A). This result indicates that tau phosphorylation in infected animals is increased by a mechanism that is not associated with modulation of tau expression.

We next evaluated the immunocountent of RAGE in these samples of brain cortex. In animals infected by L. amazonensis, the total content of RAGE was significantly increased compared to control animals (Fig. 1B). In AD, both tau aberrant phosphorylation and RAGE up-regulation are strongly implicated in the molecular mechanisms underlying the progression of neuronal death (Li et al., 2012; Yan et al., 2009). RAGE has been suggested to be the key component of the chronic pro-inflammatory axis responsible for microglia activation and reactive species production that ultimately leads to neuronal cell death in AD and also in other neurodegenerative processes (Maczuk et al., 2008). For this reason, we next sought to evaluate parameters of inflammation and oxidative/nitrosative stress in the brain cortex of mice infected with L. amazonensis.

The levels of TNF-α and IL-1β were evaluated by ELISA. These cytokines are considered markers of acute pro-inflammatory activity and have been associated with modulation of tau phosphorylation and RAGE expression in some neuropathological states (Krstic et al., 2012; Roe et al., 2011). Although an increase in TNF-α levels was detected in other organs, such as liver (data not shown), no significant changes in the levels of both TNF-α and IL-1β were detected in brain cortex of infected animals (Fig. 2A and B). Thus, our results indicate that tau phosphorylation and RAGE upregulation are not associated with TNF-α and IL-1β in leishmaniasis.

We next evaluated parameters of oxidative and nitrosative damage in biomolecules of brain cortex samples from mice infected with L. amazonensis. We quantified levels of nitrotyrosine, a marker of peroxynitrite-mediated protein nitration, in brain cortex samples by ELISA. Besides, the quantification of free (reduced) thiol groups indicates the redox state of proteins and peptides containing sulphhydryl groups that may undergo oxidation or reduction and form disulfide bonds. No changes in the levels of nitrotyrosine and free thiol groups were observed between control and infected animals (Fig. 3A and B). On the other hand, the levels of TBARS were significantly increased in infected animals, suggesting an elevated status of brain lipid peroxidation (Fig. 3C). Besides, the Western blot analysis of DNP-reactive proteins indicated that protein carbonylation in infected animals was increased (Fig. 3D), which was also confirmed by quantification of carbonyl groups in the samples (Fig. 3E).

The antioxidant status of brain cortex samples was also analyzed. The activities of the antioxidant enzymes CAT and SOD were measured. A decrease in CAT activity was observed in infected animals, indicating impaired H2O2 detoxification capacity in brain cortex (Fig. 4A). SOD activity did not differ between control and infected animals (Fig. 4B). Also, the analysis of the non-enzymatic antioxidant status by the TRAP assay showed that mice infected by L. amazonensis had a decreased status in non-enzymatic antioxidant defense, indicating a diminished antioxidant capacity compared to control animals (Fig. 4C and D).

Finally, to verify whether tau phosphorylation was associated to the elevated status of oxidative stress in the brain cortex, mice infected with L. amazonensis were subjected to a daily i.p.

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**Fig. 1.** Tau phosphorylation and RAGE content in brain cortex of mice infected with L. amazonensis. Four months after infection, brain cortex tissue from infected and control animals was removed and proteins were subjected to SDS–PAGE/Western blot analysis. (A) Representative immunoblots of tau phosphorylated at Ser396 (p-tau Ser396, upper panel) and total tau (lower panel) from four control and four infected animals. Bar graph corresponds to mean ± SEM quantification values of the p-tau/total tau ratio from all samples. (B) Representative immunoblots of RAGE content from four control and four infected animals (upper panel). Lower panel corresponds to β-actin immunocountent used as control for protein constitutive expression. Bar graph corresponds to mean ± SEM quantification values of the RAGE/β-actin ratio from all samples. Values for p depicted were obtained by applying two-tailed student’s t test.
administration with the antioxidant NAC (20 mg/kg) for five consecutive days before euthanasia. Infected animals that received NAC had decreased levels of phosphorylated tau compared to infected animals that did not receive antioxidant therapy (Fig. 5A and B), indicating a role for reactive species in tau phosphorylation. Additionally, we compared the levels of IFN-$\gamma$ and IL-6, plus TNF-$\alpha$ and IL-1$\beta$, between control and infected animals receiving NAC. The levels of IFN-$\gamma$ were decreased in infected animals, while the infected animals that received NAC recovered IFN-$\gamma$ levels to control values (Fig. 5C), which indicates a role for oxidative stress in the modulation of IFN-$\gamma$ in the brain cortex of animals infected with $L.\ amazonensis$. We did not observe any effect on IL-6 levels at any group of animals (Fig. 5D). Besides, infected animals receiving NAC presented decreased TNF-$\alpha$ levels compared to infected animals that did not receive antioxidant therapy (Fig. 5E). No variations in IL-1$\beta$ levels were observed in all groups (Fig. 5F).

4. Discussion

Oxidative stress is an important factor in the course of Leishmania infection. $L.\ amazonensis$ was reported to contain a cluster of genes expressing META1 and META2 proteins, which were found to be responsible for resistance of the parasite against heat shock and oxidative stress (Ramos et al., 2011). These characteristics probably evolved as a parasite’s strategy of resistance against the oxidizing agents generated by inflammatory cells aimed to kill intracellular and extracellular pathogens. However, depending on
the parasite’s ability to cope with the oxidative stress generated by the host, the inflammatory response may be further intensified in order to kill more resistant micro-organisms. The intensification of the production of oxidizing agents during this response may affect cells and tissues of the host, thus contributing to further development of the disease. The observation of oxidative damage to proteins in the CNS of mice infected with \textit{L. amazonensis} reported here may bring new information for the understanding of neurological symptoms in cutaneous leishmaniasis and related parasitic infections. In previous studies, alterations in the redox state of liver were observed only in visceral leishmaniasis caused by infection of hamsters with \textit{Leishmania chagasi} (Oliveira and Cecchin, 2000).

Oxidative stress in patients and experimental models of leishmaniasis is generally analyzed in the context of infected cells or focusing on the microenvironment (tissue) of the injury. Granulomatous inflammatory reaction is associated with the presence of amastigotes within macrophages, reflecting in the function of organs such as liver, spleen, lymph nodes and bone marrow. Cells of the mononuclear phagocyte system are present in these organs (Baneth and Aroch, 2008; Engwerda et al., 2004). In a previous study conducted with the same animal model and infection protocol used here, it was observed that BALB/c mice infected with \textit{L. amazonensis} displayed inflammatory infiltrates of mononuclear cells and neutrophils without parasites in the meninges and the presence of macrophages containing parasites in the cerebral parenchyma (Abreu-Silva et al., 2003). However, as observed in studies with human subjects, the mere presence of parasites in skin and draining lymph nodes (as in localized cutaneous leishmaniosis) affects several neuroendocrine axes, and these effects are believed to emerge as consequences of the actions of pro-inflammatory factors released by peripheral tissues on the CNS (Baccan et al., 2011).

Inflammation and oxidative stress are important components in several neurodegenerative conditions. In AD and related tauopathies, tau phosphorylation and RAGE up-regulation are unequivocally stimulated. These characteristics are strongly believed to be associated with the activation of ROS production caused by factors that sustain a chronic state of local inflammatory activation in CNS. However, we did not observe any changes in the levels of TNF-\(\alpha\), IL-1\(\beta\) and IL-6 in the brain cortex of infected animals. These cytokines are associated with acute inflammatory responses, while RAGE is considered a marker of chronic pro-inflammatory development (Ibrahim et al., 2013). TNF-\(\alpha\) promotes the induction of IL-6 and IL-1\(\beta\), and high levels of these cytokines in macrophages of rats acutely infected with \textit{Leishmania braziliensis} have been previously reported (Brelaz-de-Castro et al., 2012). However, in leishmaniasis, the action of these cytokines is more pronounced at either acute phases or advanced stages of the chronic disease (Oliveira et al., 2014). On the other hand, the levels of IFN-\(\gamma\) were decreased in the brain of infected animals, and the antioxidant therapy with NAC recovered IFN-\(\gamma\) status to control levels. IFN-\(\gamma\) is associated to the Th1 response, which is a necessary step of an adequate immune response to control the parasite. A Th1 predominant response is considered a good prognosis for control of the infection with most species of \textit{Leishmania}, while Th2-predominant response is associated to the evolution of the disease (Pereira and Alves, 2008). Nonetheless, infections caused by \textit{L. amazonensis} were observed to downregulate IFN-\(\gamma\) levels in lymph node cells to a greater extent compared to other \textit{Leishmania} species (Maioli et al., 2004), and this was suggested to be part of a specific mechanism by which the parasite modulates the host immune system, as INF-\(\gamma\)-mediated induction of macrophage activation is essential for control of the parasite (Alexander and Bryson, 2005). Inhibition of IFN-\(\gamma\) production in leishmaniasis is directly associated to increased lesion size. The shift between Th1 and Th2 responses is influenced in different ways by the parasite at earlier and later stages of the disease, in order to couple the host immune response with the progression of the parasite’s cycle and its change from amastigote to promastigote forms (Pereira and Alves, 2008).

Interestingly, infected mice that received NAC presented decreased TNF-\(\alpha\) levels in the brain cortex compared to infected animals that were not treated with NAC. Variations in the levels of this cytokine were not observed between control and infected animals, but the antioxidant treatment was able to reduce this pro-inflammatory mediator in the brain of both control and infected mice. At systemic level, an increase in IL-4 production caused \textit{L. amazonensis} promotes a Th2 response and downregulates
IFN-$\gamma$ production. These effects are believed to be directly related to the decrease of macrophage activation and TNF-$\alpha$ production associated to the progression of leishmaniasis, as these events would be a consequence of the shift from Th1 to Th2 response caused by the parasite (Pereira and Alves, 2008). Thus, it is surprising that TNF-$\alpha$ levels are not decreased along with IFN-$\gamma$ in infected animals. However, the changes in the levels of these cytokines caused by NAC treatment suggest that redox-dependent mechanisms are a key factor in the regulation of pro-inflammatory mediators at CNS level in leishmaniasis. It is possible that the pro-oxidant environment in the brain cortex of infected animals is responsible for maintaining TNF-$\alpha$ levels unaltered compared to control animals, instead of an expected decrease in the levels of this cytokine, since Th1 response is supposed to be suppressed.

In AD, accumulated evidence shows that stimulation of the amyloidogenic pathway results in the activation of innate immune system mainly by pattern recognition receptors (PRRs), including RAGE (Salminen et al., 2009). In the amyloidogenic pathway, beta-amyloid peptide release in CNS stimulates oligomers and fibril accumulation (early steps of amyloid plaques formation), which may act as danger-associated molecular patterns (DAMPs). DAMPs stimulate the up-regulation and activation of PRRs such as Toll-like receptors, NOD-like receptors and RAGE (Maczurek et al., 2008; Salminen et al., 2009). RAGE activation, in turn, triggers a wide array of cellular responses relevant to neurodegenerative progression in AD, such as the transcriptional activation of NF-kB-regulated genes (which include several genes associated with pro-inflammatory activation, oxidative stress and cell survival-associated responses), MAPK activation (which regulates both cell survival/death responses as well as inflammatory activation) and NADPH oxidase activation (which leads to microglia recruitment/activation, increased RS production and modulation of redox-sensitive protein kinases and transcription factors) (Arancio et al., 2004; Kojro and Postina, 2009; Maczurek et al., 2008; Sims et al., 2010; Yan et al., 2009).

Oxidative stress-related protein carbonylation, nitrotyrosination and thiol oxidation favor the formation of intra- and intermolecular protein cross-links, which leads to conformational changes increasing hydrophobicity and aggregation. These oxidative alterations favor the formation of protein cross-links, inducing generalized cellular dysfunction (Halliwell, 2006). Although we did not detect changes in thiol oxidation or nitrotyrosine formation...
here, protein carbonylation was significantly increased in infected animals. This oxidative modification is responsible for the impairment in several protein functions by causing disruption of proteosomic-mediated protein turnover and generation of aberrant conformations (Kastle and Grune, 2011). We also observed that the non-enzymatic antioxidant potential in the brain cortex of infected animals was decreased, suggesting a state of enhanced RS production. These observations, altogether, strongly suggest that the increase in tau phosphorylation and RAGE protein content in the brain cortex of animals infected with *L. amazonensis* is associated with a state of oxidative stress in the CNS caused by the infection. It is possible brain oxidative stress may be a sustained response to acute pro-inflammatory activation induced in early stages of the infection, contributing later to the evolution of the chronic disease. Also, as previously stated, it is important to note that even when the presence of the parasite within the CNS is not certain, *Leishmania* spp. infection have been reported by different works to exert neurologic effects as consequence of systemic alterations originated by local inflammatory responses in organs such as liver and spleen (Melo et al., 2013; Petersen and Greenlee, 2011). In human patients with localized cutaneous leishmaniasis, plasma levels of cortisol, estradiol or prolactin positively correlated with at least one clinical parameter of the disease (lesion size, dose used to reach cure and time to cure), indicating a reflect in neuroendocrine regulation (Baccan et al., 2011). It is possible that such modifications may contribute to brain oxidative stress, since neuroendocrine hormones may exert significant changes in the redox state of the CNS (Mancini et al., 2010). It is reasonable to suggest, based on our present data, that the increase in tau phosphorylation and RAGE immunoreactivity is a consequence of the oxidative stress in brain cortex. Tau phosphorylation and RAGE expression have been previously observed to be regulated by pro-inflammatory cytokines, including TNF-α and IL-1 (Li et al., 2003; Shi et al., 2011). We observed that levels of TNF-α in infected animals treated with NAC are decreased compared to infected animals that were not treated with NAC. These data indicate that a participation of TNF-α in the induction of tau aberrant phosphorylation may not be completely ruled out. On the other hand, we must consider that the decrease in IFN-γ levels could be involved in this effect, as the levels of this cytokine were recovered in infected animals subjected to antioxidant treatment. The inhibitory effect of NAC treatment on tau phosphorylation was very clear in our experimental model. These data, altogether with the effects observed other cytokines with NAC treatment, strongly suggests that aberrant tau phosphorylation is caused primarily by a redox-dependent mechanism in the brain of animals infected with *L. amazonensis*.

Several evidence associates increased RS production and oxidative damage to biomolecules to both tau aberrant phosphorylation and RAGE up-regulation/activation (de Bittencourt Pasquali et al., 2013; Mondragon-Rodriguez et al., 2014, 2013). In AD, there is a clear role of oxidative stress in the progression of neurodegeneration, and both tau phosphorylation and aggregation are involved in RS-dependent neuronal death in the course of the disease (Schmitt et al., 2012). RAGE up-regulation in response to oxidative stress was observed in different diseases where increased RS production is a characteristic, including diabetes, atherosclerosis and AD (Guo et al., 2008; Kojro and Postina, 2009). In AD, RAGE is believed to contribute in maintaining an elevated state of RS production by stimulating NADPH oxidase (Kojro and Postina, 2009), enhance tau phosphorylation via the ERK1/2-GSK3β pathway (Barroso et al., 2013; Li et al., 2012) and amyloid translocation through blood–brain barrier (Candela et al., 2010). In this context, it is possible that neurologic symptoms associated with chronic leishmaniasis are related to disruptions in the homeostasis of CNS proteins, such as tau and RAGE, as consequence of oxidative stress which in turn may be originated initially by systemic infection. This is the first demonstration of alterations in biochemical parameters of neurodegeneration in an experimental model of *Leishmania* infection. The relationship between the regulation of RAGE expression and function in the course of leishmaniasis and its relationship with modulation of Th1/Th2 responses arises from this work as an interesting issue to be addressed in future studies. This was an exploratory study and, as such, more detailed evaluations aiming to understand mechanistic relationships or propose intervention procedures could not be performed at this stage. Therefore, it is important to point some limitations inherent to this study. First, although data presented here strongly indicate an association of oxidative stress with modulation of tau phosphorylation and RAGE in leishmaniasis, this relationship would only be fully established if administration of antioxidants is able to reverse the effects of *L. amazonensis* infection on these parameters. Also, we did not explore the full complexity of the inflammatory response, as we focused on biochemical parameters more commonly observed in inflammatory diseases of the CNS and in neurodegenerative conditions. Thus, the absence of increase in TNF-α and IL-1β levels do not necessarily mean absence of brain inflammation. A detailed study focused on a longitudinal evaluation of neurochemical, neurodegenerative and pro/anti-inflammatory parameters (including a wider range of Th1/Th2 mediators) must be performed for a full comprehension of the mechanisms involved in the etiology of CNS damage in leishmaniasis. Nonetheless, this is the first observation of classic neurodegenerative parameters in this disease, which is not only clinically relevant as it also opens perspectives of new mechanistic and intervention studies.

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**References**


Original article

The concurrent occurrence of Leishmania chagasi infection and childhood acute leukemia in Brazil

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Objective: This study investigated the co-existence of Leishmania chagasi infection and childhood leukemia in patients naïve to treatment; this has serious clinical and epidemiological implications.

Methods: The seroprevalence of L. chagasi antibodies prior to any treatment was investigated in children with clinical features of acute leukemia. Serological tests were performed in 470 samples drawn from under 14-year-old children from different regions of Brazil with clinical suspicion of acute leukemia. Acute leukemia subtypes were characterized by immunophenotyping using flow cytometry. Morphological analyses of bone marrow aspirates were systematically performed to visualize blast cells and/or the formation of L. chagasi amastigotes. Data analysis used a standard univariate procedure and the Pearson’s chi-square test.

Results: The plasma of 437 children (93%) displayed antibodies against L. chagasi by indirect immunofluorescence assay and enzyme-linked immunosorbent assay tests. Of the 437 patients diagnosed from 2002 to 2006, 254 had acute lymphoblastic leukemia, 92 had acute myeloid leukemia, and 91 did not have acute leukemia. The seroprevalence of L. chagasi antibodies according to the indirect immunofluorescence assay test (22.5%) was similar in children with or without acute leukemia (p-value = 0.76). The co-existence of visceral leishmaniasis and acute leukemia was confirmed in 24 children. The overall survival of these children was poor with a high death rate during the first year of leukemia treatment.

Conclusion: In the differential diagnosis of childhood leukemia, visceral leishmaniasis should be considered as a potential concurrent disease in regions where L. chagasi is endemic.

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Introduction

Acute leukemia (AL) is the most common childhood malignancy. It is recognized as a disease with heterogeneous biological characteristics. Great progress has been made toward a cure and understanding the pathogenesis of AL. An international survey of data that compared the relative frequencies of the different AL subtypes has demonstrated consistent frequencies among groups stratified according to age, gender, ethnicity, and social conditions. To clarify the etiology of childhood leukemia, epidemiological studies have attempted to gain some understanding about the different rates of acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) in association with genetics, infections, and other environmental factors. A recent population-based study of childhood leukemia demonstrated that substantial regional differences exist in the incidence of AL in Brazil, which warrants further studies. These differences may be related to the underreporting of AL cases in some less-developed areas. In this context, visceral leishmaniasis (VL) or Kala-zar, a tropical disease caused by the intracellular protozoan parasite, Leishmania infantum (syn. L. chagasi), should be considered clinically as a co-morbid disease that can complicate the diagnosis of AL. The signs and symptoms of VL are very similar to those of some childhood types of AL. Affected children present splenomegaly, anemia, neutropenia, thrombocytopenia, and/or increased abnormal lymphocyte counts. Coagulation abnormalities have also been found in patients, often associated with disseminated intravascular coagulopathy. Additionally, atypical cells and unusual blasts may be observed in bone marrow aspirates of patients with VL. Thus, it is important to include VL in the differential diagnosis of AL in endemic areas.

In Brazil, VL frequently occurs in remote locations and endemic areas, and for the general pediatrician, AL is not the first disease to be investigated. Furthermore, VL treatment is often performed on the basis of clinical suspicion, because it is associated with high mortality in the absence of treatment. The final diagnosis of AL is hampered by the complexity of clinical diagnoses, for instance, infections can stimulate the hypothalamic–pituitary–adrenal axis, leading to increased plasma cortisol levels that are sufficiently high to eliminate clonal leukemic cells, thereby delaying decisions. In essence, AL and VL are serious diseases that require a rapid, proper diagnosis and adequate treatment to reduce childhood mortality. The present study investigated a series of samples from patients suspected of childhood leukemia at diagnosis, for the presence of L. chagasi antibodies and evaluated how the relationship between these two severe diseases can affect children.

Methods

Subjects

Serum samples from 785 children were selected for this study. The study population was enrolled throughout a multidisciplinary project that had been ongoing in the Pediatric Hematology-Oncology Program of the Research Center at the Instituto Nacional de Cáncer (INCA), Rio de Janeiro, Brazil. Bone marrow (BM) aspirations and peripheral blood (PB) samples were sent for immunophenotyping-genotyping for a study on acute childhood leukemia during the period of 2001–2007. Complete epidemiological data have been described in detail elsewhere. Biological samples (BM and PB) were first evaluated to determine the morphological characteristics of lymphoid and myeloid blast cells. Then, an algorithm of immuno-molecular testing was performed: (i) morphological characteristics of lymphoid and myeloid cells according to standard criteria, (ii) immunophenotyping of BM aspirates; (iii) DNA index (only in ALL) and (iv) identification of abnormal fusion genes according to leukemia subtypes. The panel of monoclonal antibodies (MoAb) recommended by the European Group for the Immunological Characterization of Leukemias was applied to isolated mononuclear cells and analyzed by flow cytometry. Briefly, the combination of fluorochrome-labeled MoAbs was used in triplet and/or quadruple staining experiments, using fluorescein isothiocyanate (FITC), phycoerythrin (PE) and PE-cyanine 5 (PECy5) and/or APC fluorochrome conjugates in each tube. Cell samples were analyzed by flow cytometry using a FACSCalibur device (Becton, Dickinson and Company, CA, USA) with the CellQuest and Paint-a-Gate computer programs.

1. Intracytoplasmatic – CD79b and/or CD22FITC/CD3PE/CD45PECy5 or APC; TdTFITC/aMPOPE/CD33/CD13 PEcy5/CD45APC as initial screening;
2. Membrane surface according to screening results – if B-cell markers (CD79/CD22/TdT+) were positive, then CD10FITC/CD19PE/CD45PECy5, CD34FITC/CD38PE/CD45PECy5, CD58FITC/CD10PE/CD19PECy5/CD45APC, SmIgFITC/CD20PE/CD19 PEcy5 and CD4FITC/CD8PE/CD3PEcy5/CD45APC were performed. If T-cell markers (cD3/TdT+) were positive, then CD7FITC/CD33/CD13PE/CD45PECy5, CD34FITC/CD1aPE/CD45PECy5 and CD4FITC/CD8PE/CD3 PEcy5/CD45APC were performed. Finally, a panel for anti-myeloid antigen cells was tested when myeloid morphology and/or intracytoplasmatic CD13/aMPO+ were predominant. This panel consisted of CD34FITC/CD38PE/CD7PECy5/CD45APC, CD64FITC/CD14PE/CD33PECy5/CD45APC and CD15FITC/HLA-DRPE/CD7PECy5/CD45APC.

Cell surface antigens were considered positive when 20% or more cells showed fluorescence intensity greater than the negative control in the gate for CD45low cells, while the cutoff for the cytoplasmic antigen aMPO was 10% in the gate for CD45low cells. Cases with unusual positive markers were tested twice.

AL types were classified as B cell precursor ALL (Bcp-ALL), pro-B-cell, common B, and pre-B ALL; B-ALL; T-ALL, and AML accordingly. Subsequently, RNA was processed for c-DNA; MLL-AR4, TEL-AML1, E2A-PBX1 and BCR-ABL1 were performed in the Bcp-ALL samples, whereas, the SIL-TAL1 fusion and HOX11L2 were performed in T-ALLs as has been described elsewhere.

Patients with diagnoses that excluded ALL or AML and other malignant diseases were designated to the ‘Non-leukemic Group’.
Exclusion criteria for the serological analysis were samples from children that presented with malignancies other than AL, children who had been submitted to treatment for malignancies or those with well-documented VL treatment.

Serologic assays and diagnosis of visceral leishmaniasis

Serum and plasma samples were screened for reactions to different antigens related to VL using an indirect immunofluorescence antibody assay (IFA) and the enzyme-linked immunosorbent assay (ELISA) (both kits from Bio-Manguinhos/Oswaldo Cruz Foundation, Rio de Janeiro, Brazil). IFA is indicated for the diagnosis of VL by the Health Ministry in Brazil. ELISA screening tests were performed according to de Assis et al. Briefly, soluble antigens of L. chagasi and the recombinant K39 were immobilized to solid-phase wells for 16–18 h at room temperature. Unbound antigens were removed, and the wells were blocked. A peroxidase-conjugated anti-human IgG secondary antibody was used to detect antibody binding. The reaction was blocked using 50 µL 8 M sulfuric acid, and was analyzed with a BioRad-Benchmark Microplate Reader equipped with a 490 nm filter. Specimens were considered positive when titers were at or above 1:80 and negative when titers were less than 1:40; results that fell between these levels were considered indeterminate.

BM smears were evaluated by optic microscopy to search for the presence of amastigotes within histiocytes and neutrophils.

Statistical analysis

Patient characteristics (age, skin color, Brazilian region of residence, and AL sub-type) and serological results were analyzed with the standard univariate procedure. The Pearson chi-square test was used to compare the frequency of serological positivity between different groups (e.g., the AL Group vs. the Non-leukemia Group, different Brazilian regions, different age strata); the Fisher exact test was used when a cell count of less than five was expected. p-values less than 0.05 were considered statistically significant. All statistical analyses were performed with the IBM SPSS Statistics package, version 18.0 (Chicago, IL, USA).

Due to the lack of consensus between ELISA and IFA results, the percentage of agreement and Cohen’s Kappa statistic were calculated with a standard formula. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were used to evaluate the performance of the ELISA test. Sensitivity was calculated with the formula: TP/(TP + FN); where TP represents the true positive results based on IFA results (recommended as reference), and FN represents the false negative results. Specificity was calculated with the formula: TN/(TN + FP); where TN represents the true negative and FP represents the false positive results. The concordance between the IFA and ELISA tests was calculated. Quality control criteria regarding concordance values were: ≤40%, fair; 40.1–79.9%, moderate; 80–89.9%, substantial; and ≥90%, almost perfect.

Ethical considerations

The Ethics and Scientific Committees of the Instituto Nacional de Cancer – INCA, Rio de Janeiro, approved the study (CEP#070/07: O papel das infecções e da resposta imune desregulada na etiologia das leucemias da infância analisado através da assinatura genética). Written, informed consent was obtained from mothers or relatives responsible for the enrolled children prior to AL treatment.

Results

For the present study, records of demographic characteristics, disease signs and symptoms noted at the time of sample collection, diagnostic procedures, and clinical follow-ups were examined. Data from 752 out of 785 (95.8%) children who had undergone serological tests are shown in Table 1. The patients were identified from pediatric cancer centers located in different Brazilian states, including Rio de Janeiro, São Paulo (Southeastern region); Bahia, Paraíba, (Northeastern region); Mato Grosso, Mato Grosso do Sul, Federal District (Central-western region); and Paraná, Rio Grande do Sul (Southern region). Thirty-three cases were excluded because children had undergone previous chemotherapy and/or VL treatment, and/or they lacked biological samples for double-checking the serological tests. For 115 patients, morphology, immunophenotyping, and clinical follow-ups ruled out the diagnosis of AL, and they were designated the Non-leukemic Group. There were 637 patients with ALL; of these, 391 (61.5%) had Bcp–ALL, 84 (13.2%) had T–ALL, and 153 (24.1%) had AML. All children were under 14 years old at diagnosis, with a range of four months to 12.3 years. There was a predominance of children between two to five years of age (40.3%), and from the Northeastern region (61.6%).

To examine whether a possible bias was introduced in selecting study patients, the clinical characteristics of patients with and without AL in this study were compared (Table 2). No statistical difference was found between groups in respect to gender, age, skin color/ethnicity (not shown), or AL subtypes. However, the variability found might be due to missing values from the number of valid observations in the input data set of the Non-leukemia Group.

All samples were first tested with ELISA to determine the presence of L. chagasi. Fifty-four had positive results; of these, 39 samples were confirmed positive by IFA. Similarly, the 381 samples that were negative in the ELISA test were also tested by IFA. Of these, the IFA identified 56 positive and 325 negative cases. IFA was determined as the gold standard for seroprevalence. The observed agreement between ELISA and IFA was 83.1% and the observed unweighted Kappa statistic was 0.42 (95% confidence interval [95% CI]: 31.2–52.6). The percentage of agreement was considered ‘moderate’. Regarding the performance of the ELISA, its sensitivity was found to be 41.1% (95% CI: 36.1–51.6%), specificity was 94.8% (95% CI: 91.8–96.9%), the positive predictive value was 68.4% (95% CI: 54.8–80.1%) and negative predictive value was 85.3% (95% CI: 81.3–88.7%). Therefore, cases diagnosed as IFA seropositive have a 41.1% chance of being ELISA positive too (sensitivity); likewise, ELISA positive have a 68.4% chance of being IFA positive (specificity).
Table 1 – Demographic and serological characteristics of patients with and without leukemia in Brazil.

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Total n (%)</th>
<th>All leukemia n (%)</th>
<th>ALL n (%)</th>
<th>AML n (%)</th>
<th>Non-leukemia n (%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤1</td>
<td>66 (8.8)</td>
<td>51 (8.0)</td>
<td>38 (8.0)</td>
<td>13 (8.0)</td>
<td>15 (13.0)</td>
<td>0.001</td>
</tr>
<tr>
<td>2–5</td>
<td>303 (40.3)</td>
<td>246 (38.6)</td>
<td>197 (41.5)</td>
<td>49 (30.2)</td>
<td>77 (49.6)</td>
<td></td>
</tr>
<tr>
<td>6–10</td>
<td>208 (27.7)</td>
<td>188 (29.5)</td>
<td>141 (29.7)</td>
<td>47 (29.0)</td>
<td>20 (17.4)</td>
<td></td>
</tr>
<tr>
<td>&gt;11</td>
<td>175 (23.3)</td>
<td>152 (23.9)</td>
<td>99 (20.8)</td>
<td>53 (32.7)</td>
<td>23 (20.0)</td>
<td></td>
</tr>
</tbody>
</table>

- Geographical regions
  - Northeast: 463 (6.1) 380 (59.7) 275 (57.9) 105 (64.8) 83 (72.2) 0.59
  - Southeast/South: 196 (26.1) 175 (27.5) 135 (28.4) 40 (24.7) 21 (18.3)
  - Central-west: 93 (12.4) 82 (12.9) 65 (13.7) 17 (10.5) 11 (9.6)

- Seroprevalence of L. chagasi:
  - Overall: 95 (21.7) 75 (21.5) 65 (25.6) 10 (10.5) 20 (22.5) 0.01
  - Total: 752 (100) 637 (100) 475 (100) 162 (100) 115 (100)

All: acute lymphoblastic leukemia; AML: acute myeloid leukemia.
* Indirect immunofluorescence antibody test positive to Leishmania chagasi.

Based on the IFA results, the prevalence of L. chagasi antibodies was 21.7% of the entire cohort, 25.6% among ALL cases, and 10.5% among AML cases; the seroprevalence in the Non-leukemic Group was 22.5% (p-value = 0.01). The seroprevalence was also analyzed according to Brazilian region (Table 3). The prevalence was lowest in the southeastern and central-western regions. The prevalence ranged from 7.1% for AML cases and 16.7% for non-leukemia cases in the southeastern region to 12.9% for AML cases and 30.0% in ALL cases in the northeastern regions (p-value = 0.01).

Based on symptoms, clinical signs, and antibody positivity in both serological tests, the diagnosis of VL was confirmed in 20 children (without leukemia) and additionally, 24 children (19 ALL and 5 AML) had high L. chagasi antibody titers by ELISA and positive IFA. Most of these 24 children presented symptoms characterized by fever, anorexia, and weight loss. Clinical examinations showed that the majority of patients demonstrated pallor, splenomegaly, hepatomegaly, and lymphomegaly. Laboratory analyses showed that all of these patients demonstrated anemia and thrombocytopenia. Leukopenia was found in four cases, normal leukocyte levels were found in seven cases, and elevated white blood cell counts were found in 15 cases (data not shown). Amastigotes were found in five cases (four patients with AML and one patient with Bcp-ALL); BM hypocellularity and an increased number of histiocytes were observed in five patients with ALL. The co-existence of AL and VL was strongly correlated in these cases. Overall survival of these children was poor; ten patients died before the first year of treatment. A one-year follow up showed persistent BM eosinophilia in one child.

**Discussion**

This unique study tested the co-existence of VL and childhood AL in patients, prior to any chemotherapeutic treatment. The association between VL and chronic leukemia in adulthood has been reported previously. In addition, anecdotal studies have described cases of VL that occurred during the treatment of childhood ALL.7,17–20 The co-existence of these two diseases is clinically relevant, because they have serious clinical and epidemiological consequences in childhood. Among these are: (i) VL can mimic AL (or vice versa) in young children; (ii) L. chagasi can infect children that have ALL or AML (as a co-morbidity); and (iii) L. chagasi may play a role as a risk factor in the hematological malignancy process.

To appraise these points, it is first mandatory to discuss the wide prevalence of the two entities. The incidences of both diseases in the population depend on their recognition and notification. As pointed out by de Assis et al.,7 the clinical diagnosis of VL may be inaccurate, because its clinical presentation shares some common features with critical diseases. The laboratory diagnosis of L. chagasi infection remains complex and, until recently, there was no consensus gold standard; thus, VL treatment is frequently based on clinical suspicion.5,21 Despite this, about 3500 cases of VL are registered in Brazil every year. Generally, the disease is associated with poor living conditions. Nevertheless, a shift has been observed toward an increase of cases in urban areas; thus, L. chagasi infections have become an important medical problem in different areas of Brazil.22,23 Brazilian national initiatives have given rise to referral centers that provide oncological care for childhood leukemia in different regions. This has created particularly favorable conditions for exploring the natural history of childhood leukemia in Brazil.11,24 The present study indicated that the majority of oncological clinics located in areas with endemic L. chagasi infections continue to have difficulties in making a firm diagnosis of VL. The clinical manifestations of VL, such as fever, anemia, splenomegaly, lymphadenopathy, thrombocytopenia, and myelodysplasia features can lead to a misdiagnosis of AL, due to overlapping clinical criteria. In the present series of cases, 91 children were evaluated for AL and excluded as non-leukemic patients. These patients had reactive serology for L. chagasi; thus, according to Brazilian
Table 2 – Clinical, laboratorial characterization of the cohort of acute leukemia cases and non-leukemic children in Brazil.

<table>
<thead>
<tr>
<th>Clinical and laboratorial variables</th>
<th>Total (n = 752)</th>
<th>ALL (n = 475)</th>
<th>AML (n = 162)</th>
<th>Non-leukemia* (n = 115)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (× 10^9/L) – median (interquartile range)</td>
<td>17,400 (5000–64,100)</td>
<td>20,000 (5760–71,150)</td>
<td>24,000 (7300–86,500)</td>
<td>5520 (2900–16,000)</td>
</tr>
<tr>
<td>Hemoglobin (g/dL) – median (interquartile range)</td>
<td>7.5 (5.5–9.3)</td>
<td>7.4 (5.4–9.3)</td>
<td>7.4 (6.1–8.9)</td>
<td>8.1 (5.5–10.3)</td>
</tr>
<tr>
<td>Platelets (× 10^9/L) – median (interquartile range)</td>
<td>50,000 (24,750–105,500)</td>
<td>49,500 (26,250–99,750)</td>
<td>42,000 (20,000–88,000)</td>
<td>92,000 (27,000–238,25)</td>
</tr>
<tr>
<td><strong>ALL subtypes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-ALL</td>
<td>391 (61.5)</td>
<td>391 (61.5)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>T-ALL</td>
<td>84 (13.2)</td>
<td>84 (13.2)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>AML</td>
<td>–</td>
<td>–</td>
<td>153 (24.1)</td>
<td>–</td>
</tr>
<tr>
<td><strong>Initial findings</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatomegaly</td>
<td>453 (72.6)</td>
<td>275 (76.0)</td>
<td>117 (75.0)</td>
<td>61 (57.5)</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td>356 (57.0)</td>
<td>238 (65.4)</td>
<td>77 (49.7)</td>
<td>41 (38.7)</td>
</tr>
<tr>
<td>Enlarged lymph nodes</td>
<td>300 (54.2)</td>
<td>213 (65.9)</td>
<td>46 (33.6)</td>
<td>41 (43.6)</td>
</tr>
</tbody>
</table>

ALL: acute lymphoblastic leukemia; AML: acute myeloid leukemia; WBC: white blood cells count.

* Designated as Non-leukemic Group due to absence of laboratorial criteria for acute leukemia.

b Due to missing values, the number of observations used for an individual table analysis can differ from the number of valid observations in the input data set; the concomitant visceral leishmaniasis patients are included.

Table 3 – Serological analyses for detecting *Leishmania chagasi* according to major geographical regions in Brazil.

<table>
<thead>
<tr>
<th>Serological test</th>
<th>Northeast (n = 265)</th>
<th>Southeast (n = 144)</th>
<th>Central-west (n = 29)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ALL (n = 140)</td>
<td>AML (n = 62)</td>
<td>Non-leukemia (n = 63)</td>
</tr>
<tr>
<td>ELISA – n (%)</td>
<td>24 (17.1)</td>
<td>11 (17.7)</td>
<td>12 (19.0)</td>
</tr>
<tr>
<td>IFA – n (%)</td>
<td>42 (50.0)</td>
<td>8 (12.9)</td>
<td>16 (25.4)</td>
</tr>
</tbody>
</table>

ALL: acute lymphoblastic leukemia; AML: acute myeloid leukemia; ELISA: enzyme-linked immunosorbent assay; IFA: indirect immunofluorescence antibody test.
Public Healthcare System guidelines, they were treated for VL. Some of the most striking features of this study is (1) the extremely high prevalence of exposure to *L. chagasi* at a young age (as low as one-year old); (2) the lack of values of an ELISA screening test that showed a sensitivity of only 41.1%; (3) the presentation of AL in many cases as hypoplastic marrow thereby misleading the diagnosis.

Our results could not elucidate the question of whether the development of VL would be facilitated in children with AL due to the immunocompromised status of these patients. However, we did find that 24 children with ALL or AML had concomitantly high serological *L. chagasi* titers. Immundeficiency is often found in patients with leukemia; this condition increases vulnerability to opportunistic diseases. Our results did not distinguish whether *L. chagasi* infections occurred before or after the onset of leukemia. However, the detection of low levels of *L. chagasi* antibodies in patients with ALL suggested that a significant fraction of children with AL in Brazil had had contact with *L. chagasi*. It has been shown that antibody titers decline sharply over time, with or without VL treatment. Therefore, it is possible that some children in our study had been exposed to leishmania infections.

A very speculative topic is whether an *L. chagasi* infection might be a risk factor for the malignancy process. One potential role of either a leishmania infection or VL treatment might be mutational hits that would facilitate the development of AL. This notion is biologically plausible and should be explored further with the appropriate study design and other refined methods. The *L. chagasi* infection promotes lymphoid differentiation and proliferation, which disturbs the immunological framework, or the balance between Th1 and Th2 functional cells. It has been postulated that perturbations of the Th1/Th2 balance may contribute to an increasing incidence of serious childhood diseases, such as asthma, diabetes, and AL.

The present study demonstrates that children affected by VL harbored 25–35% of immature cells, characterized by immature B-cell precursor markers and dysplastic BM morphology features. This could mean that *L. chagasi* infections might stimulate the immune system with increased B-cell precursors and induce susceptibility to somatic mutations.

Another important issue is the impact of VL treatment with highly active, toxic drugs in young children. Sodium stibogluconate (SAG), pentamidine isethionate, paramomycin, sitamaquine, imidazole derivatives, alkyphosphocholine analogs, amphotericin B, and lipid-associated amphotericin B are the drug choices for the treatment of VL. Most of these drugs can potently damage DNA. The precise mechanism of action of some of these drugs is not fully known however, SAGs have been shown to inhibit glycolytic enzymes and fatty acid oxidation in leishmania amastigotes. It is reasonable to postulate that first, the *L. chagasi* infection greatly stimulates the immune system by increasing the production of B-cell precursors, and then, the addition of DNA-damaging drugs (to treat the *L. chagasi* infection) would hit genes related to the pathogenesis of AL. These hypotheses could be tested in prospective epidemiological studies that included long-term follow-ups of children affected by *L. chagasi* infections.

This study had some limitations that should be considered when interpreting the results. First, the relatively small number of subjects included may not have been representative of the populations in the central-western, southern, and northeastern regions of Brazil. A convenience sample was used, but it may have introduced a potential selection bias, which would limit the generalizability of the results. The gold standard for diagnosing VL is parasitological identification; however, in this study, the prevalence of *L. chagasi* was calculated based on the IPA test. This approach may have reduced the accuracy of the diagnoses.

**Conclusions**

In summary, our findings suggest that VL is a prevalent disease in children from endemic areas, and it is important to include VL in the differential diagnosis of ALL and AML. Therefore, we strongly recommend that, in areas endemic for VL, the algorithm of diagnostic tests to identify AL should include serological tests for *L. chagasi*. Furthermore, we recommend applying the predictive models suggested by de Assis et al. Finally, VL can occur as a concomitant disease that could lead to poor outcomes in treating childhood leukemias. This study shows that the prevalence of concurrent *L. chagasi* and AL is not negligible in Brazil. We also show that both diseases can be detected before administering treatment. These findings should provide a basis for developing safer means of treating these diseases in the future.

**Conflicts of interest**

The authors declare no conflicts of interest.

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