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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Keywords: Visceral leishmaniasis, Brazil, Lymphoid leukemia, Myeloid leukemia, Acute leukemia.
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 hypothalamus–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 Cell-Quest and Paint-a-Gate computer programs.

1. Intracytoplasmatic – CD79b and/or CD22FITC/CD33PE/CD45PECy5 or APC, TdTFITC/aMPOPE/CD33/CD13PECy5/CD45APC as initial screening;
2. Membrane surface according to screening results – if B-cell markers (CD79b/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 (cCD3/TdT+) were positive, then CD7FITC/CD33/13PE/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/HLADRPE/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-AF4, TEL-AML1, E2A-PBX1 and BCR-ABL1 were performed in the Bcp-ALL samples, whereas, the SLI-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 BioManguinhos/Oswaldo Cruz Foundation, Rio de Janeiro, Brazil). IFA is indicated for the diagnosis of VL by the Health Ministry in Brazil.\(^7\) ELISA screening tests were performed according to de Assis et al.\(^5\) 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 8M sulfuric acid, and was analyzed with a BioRad Benchmark Microplate Reader equipped with a 490 nm filter. Specimens were considered positive when titer were at or above 1:80 and negative when titer 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.\(^9\,11\)

**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 AL; 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 (specificity); likewise, ELISA positive have a 68.4% chance of being IFA positive (specificity).
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 hypopcellularity and an increased number of histiocytes were observed in five patients with AML. 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. 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 comorbidity); 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., 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 consensual gold standard; thus, VL treatment is frequently based on clinical suspicion. 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. 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. 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.

| Clinical and laboratorial variables | Total (n = 752) | ALL (n = 475) | AML (n = 162) | Non-leukemia* (n = 115) |
|------------------------------------|----------------|--------------|---------------|------------------------|
| WBC (×10^9/L) – median (interquartile range) | 17,400 (5000–64,100) | 20,000 (5760–71,150) | 24,000 (7300–86,500) | 5520 (2900–16,000) |
| Hemoglobin (g/dL) – median (interquartile range) | 7.5 (5.5–9.3) | 7.4 (5.4–9.3) | 7.4 (6.1–8.9) | 8.1 (5.5–10.3) |
| Platelets (×10^9/L) – median (interquartile range) | 50,000 (24,750–105,500) | 49,500 (26,250–99,750) | 42,000 (20,000–88,000) | 92,000 (27,000–238,25) |
| AL subtypesb – n (%) | | | | |
| Bcp-ALL | 391 (61.5) | 391 (61.5) | – | – |
| T-ALL | 84 (13.2) | 84 (13.2) | – | – |
| AML | – | – | 133 (24.1) | – |
| Initial findingsb – n (%) | | | | |
| Hepatomegaly | 453 (72.6) | 275 (76.0) | 117 (75.0) | 61 (57.5) |
| Splenomegaly | 356 (57.0) | 238 (65.4) | 77 (49.7) | 41 (38.7) |
| Enlarged lymph nodes | 300 (54.2) | 213 (65.9) | 46 (33.6) | 41 (43.6) |

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.

| Serological test | Northeast (n = 265) | Southeast (n = 144) | Central-west (n = 29) |
|------------------|---------------------|---------------------|----------------------|
|                  | ALL (n = 140)       | AML (n = 62)        | Non-leukemia (n = 63) |
|                  | ALL (n = 98)        | AML (n = 28)        | Non-leukemia (n = 18) |
|                  | ALL (n = 16)        | AML (n = 5)         | Non-leukemia (n = 8)  |
| ELISA – n (%)    | 24 (17.1)           | 11 (17.7)           | 12 (19.0)            |
| IFA – n (%)      | 42 (30.0)           | 8 (12.9)            | 16 (25.4)            |
|                  | 6 (6.1)             | 1 (3.6)             | 1 (5.6)              |
|                  | 19 (19.4)           | 2 (7.1)             | 3 (16.7)             |
|                  | 0                   | 1 (20.0)            | 1 (12.5)             |
|                  | 4 (25.0)            | 0                   | 1 (12.5)             |

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. Immunodeficiency 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, alkylphosphocholine 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 northern 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 IFA 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.

Acknowledgments

The authors would like to thank the pediatricians that provided clinical data in this study, particularly Dr. Lilian Maria Burlacchini Carvalho (Clinica Onco-Bahia), Dr Rosania Maria Baseggi (Hospital Regional Rosa Pedrossian – CETHOI, Campo Grande, Mato Grosso do Sul), and Dr Flavia Pimenta (Hospital Napoléao Laureano, João Pessoa-Paraíba). We are also grateful to Mariana Sant’Anna and Synara No Cordeiro for technical support in the serological and immunophenotypic tests. This work was supported in part by a grant-in-aid from CNPq (No. No. 302423/2010-9); FAPERJ E-26/101.562/2010; E026-110.712/2012.

The English language was polished by San Francisco Edit (kretchmer@sfedit.net http://www.sfedit.net).

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