Preclinical diagnosis of American visceral leishmaniasis during early onset of human *Leishmania (L.) infantum chagasi*-infection

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American visceral leishmaniasis (AVL) is an infectious disease, often with long-duration evolution, caused by *Leishmania (L.) infantum chagasi*. However, although the disease is considered the major clinical manifestation of the link between *L. (L.) i. chagasi* and the human immune response, we have recently identified five clinical-immunological profiles of infection in the Brazilian Amazon: three asymptomatic (Asymptomatic Infection — AI, Sub-clinical Resistant Infection — SRI, and Indeterminate Initial Infection — III), and two symptomatic ones [Symptomatic Infection — SI (=AVL) and Sub-clinical Oligosymptomatic Infection — SOI]. We confirm here the preclinical diagnosis of AVL through the IgM-antibody response in a case of an early infection (profile III) that evolved to the full disease after 6 weeks.

Keywords: *Leishmania (L.) infantum chagasi*, Human indeterminate initial infection, IgM-antibody response, Preclinical diagnosis, American visceral leishmaniasis

Introduction

American visceral leishmaniasis (AVL) is an infectious disease, often with long-duration evolution, caused by the protozoan parasite *Leishmania (L.) infantum chagasi*,¹ characterized by fever, hepatosplenomegaly, hypergammaglobulinemia, and severe progressive wasting. The term ‘visceral leishmaniasis’ refers to the viscerotropism of the parasite (mainly to the liver, spleen, and bone marrow) during the evolution of the infection. Most AVL cases (~70%) occur in young children (less than 5 years old), as they are immunologically more vulnerable. Once the disease becomes established, it can be fatal if a diagnosis is delayed and/or effective treatment is not available.²

Although AVL is considered the major clinical manifestation of the link between *L. (L.) i. chagasi* and the human immune response, recent studies carried out in Pará State in the Brazilian Amazon have demonstrated the capacity to diagnose asymptomatic or symptomatic infections in endemic areas

of this disease based on the combined use of semi-quantitative delayed-type hypersensitivity (DTH) and indirect fluorescent antibody (IFAT-IgG) tests.^{3,4} The high specificity of this diagnostic approach using species-specific *L. (L.) i. chagasi*-antigens [promastigotes for DTH and amastigotes for IFAT-(IgG)], linked to the clinical status of the infected individuals, has allowed the identification of five different clinical-immunological profiles: (1) Asymptomatic Infection (AI) (DTH^{+/++++}, IFAT⁻); (2) Symptomatic Infection (SI = AVL); (3) Sub-clinical Oligosymptomatic Infection (SOI), both with the same immune response profile (DTH⁻, IFAT^{+/++++}); (4) Sub-clinical Resistant Infection (SRI) (DTH^{+/++++}, IFAT^{+/++}); and (5) Indeterminate Initial Infection (III) (DTH⁻, IFAT^{+/++}). This diagnostic approach confirmed three previously established profiles (AI, SI = AVL and SOI)⁵ as well as two new ones (SRI and III) that represent new infection stages.⁶

The AI profile is characterized by a positive DTH response and the absence of an IgG-antibody response, which are both strongly linked to genetic resistance to infection.⁷ The SI profile (=AVL), in contrast, is associated with genetic susceptibility to

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Figure 1 Dynamics of the clinical and immunological evolution of human *L. (L.) i. chagasi*-infection in the Brazilian Amazon region. IFAT: indirect fluorescent antibody test (IgG); IFAT⁺⁺⁺⁺: 5120–10240 (IgG); IFAT⁺⁺⁺: 1280–2560 (IgG); IFAT⁺⁺: 320–640 (IgG); IFAT⁺: 80–160 (IgG); IFAT⁻: negative reaction; LST: leishmanin skin test; LST⁺⁺⁺⁺: exacerbate reaction (≥ 16 mm); LST⁺⁺⁺: strong reaction (13–15 mm); LST⁺⁺: moderate reaction (9–12 mm); LST⁺: weak reaction (5–8 mm); LST⁻: negative reaction; AI: Asymptomatic Infection; SI: Symptomatic Infection (=AVL); SOI: Sub-clinical Oligosymptomatic Infection; SRI: Sub-clinical Resistant Infection; III: Indeterminate Initial Infection.

infection, with a strong inhibition of DTH and a high expression of the IgG-antibody response. SOI and SRI profiles represent borderline genetic expressions of susceptibility and resistance respectively — the former being characterized by mild clinical signs of susceptibility (such as fever, asthenia, cutaneous pallor, and spleen enlargement), but with spontaneous clinical resolution of the infection after a 2-month period;⁸ the latter is characterized by an asymptomatic profile, and represents an evolved stage towards the AI resistant profile. The asymptomatic III profile represents the earliest stage of the infection (which is not well defined from an immunogenetic point of view), with the capacity to evolve to either a resistant AI profile or a susceptible SI profile (=AVL) depending on the genetic background of the infected individual — although passing first through the SRI and SOI profiles respectively (Fig. 1). We undertook two prospective studies in the Brazilian Amazon focusing on the dynamics of the clinical and immunological evolution of infection, and estimate that 1–3% of the cases of an III profile will evolve to classical AVL.^{3,4}

In the present communication, we confirm the preclinical diagnosis of AVL by a very early IgM-antibody response to *L. (L.) i. chagasi*-infection (III

profile) in a 3 year-old girl (who 6 weeks later developed the disease) and correlate it with expressive IL-6 serum level.

Case Presentation

The case of an III profile that later developed to classical AVL involved a 3-year-old girl living in the peri-urban area of Redenção City (8°2'35"S: 50°1'22"W) in southern Pará State, Brazil. This city is a recently established focus of AVL, with the first cases of this disease being recorded only about 5 years ago. Tragically, what led us to visit this young girl (and seven other members of her household) was the death of one of her sisters due to acute AVL (July 2013), as confirmed by clinical signs and/or symptoms and specific serology (IFAT-IgG); the sister who died was 2 years old. At the time of our first visit (18 March 2014), we did not detect any clinical signs of AVL and/or symptoms in any of the household members, but nonetheless carried out specific DTH and IFAT-IgG assays among those eight individuals. Two were found to have anti-*L. (L.) i. chagasi* IgG-antibody responses [the young girl (with a serological titer of 640), and her grandmother (320)], although neither showed any reactivity to DHT — which gave them the status of the III

profile (Table 1). The other six individuals did not show any reactivity to IFAT-IgG or DTH. Accordingly, we also performed an IFAT-IgM assay on the two antibody responding patients, but only the young girl showed an anti-*L. (L.) i. chagasi* IgM-antibody response (with a serological titer of 1280). We then proceeded with flow cytometry serum cytokine analyses for IL-4, IL-6, IL-10, and TNF-alpha using a BD™ system Cytometric Bead Array Human inflammation Cytokine kit (BD Biosciences, San Diego, CA, USA) in a FACS CantoII — which revealed expressive IL-6 serum levels (828.64 fg/ml) in comparison to those of IL-4 (5.06 fg/ml), IL-10 (454.76 fg/ml), and TNF-alpha (208.49 fg/ml). The young girl was then monitored on a weekly basis by a health-care agent from the Redenção City Health Care Division to detect any clinical signs and/or symptoms of AVL. After a 6-week period, we were notified that the child had had a fever with chills for 5 days, as well as asthenia, weakness, and a loss of appetite. Another IFAT-IgG assay was then performed that revealed a quick up-tick of her serological titer to 10240, confirming the preclinical diagnosis of AVL that had been predicted by the presence of a straight-forward anti-*L. (L.) i. chagasi* IgM-antibody response. Additional laboratory analyses indicated pancytopenia (2 250 000/mm³ red blood cells, hemoglobin 8.6 g/dl, 1980/mm³ white blood cells, and 43 000/mm³ platelets), and conversion of the albumin (2.3 g/dl) and globulin (6.2 g/dl) ratios. Additionally, a physical examination of the patient confirmed the enlargement of her spleen (~3 cm below the left costal border). After this diagnosis of AVL, the girl was hospitalized and received antimony therapy (20 mg/Sb^v/kg) for 20 days, which resulted in

the successful remission of all clinical signs and symptoms of the disease.

Discussion

The definition of the clinical-immunological profiles of human *L. (L.) i. chagasi*-infections has recently been debated in terms of the validity of very early identifications of the III profile, as this asymptomatic profile was originally based on its clinical and immunological evolution without, however, showing IgM-antibody responses against infection.^{3,4} We therefore evaluated the anti-*L. (L.) i. chagasi* IgM-antibody responses in symptomatic (SI profile) and asymptomatic (III profile) infections in the Brazilian Amazon, using the IFAT-IgM assay to confirm the nature of the III profile.⁹ To that end, 14 serum samples of the III profile and 22 of the SI profile (=AVL) (seven during the first 2 months of disease evolution, and 15 after more than 2 months of evolution) were examined. The results were 100% negative in all 14 serum samples of the III profile, but 100% positive in the seven serum samples of the SI profile during the first 2 months of disease and 26.7% positive (4/15) after more than 2 months — resulting in 50% (11/22) overall IgM-antibody reactivity in the SI profile (=AVL). IgM-antibody responses of Sudanese and Indian kala-azar patients have likewise been observed in acute cases of that disease.^{10,11} However, considering that none of the 14 cases of the III profile evolved to classical AVL, we conclude that those individuals must have been genetically resistant to infection, as most of them (11–78.5%) had reverted to a DTH⁺ reaction within a 6- to 12-month period, and their IL-10 serum levels were similar to those of individuals with resistant AI and SRI profiles.¹² These findings could at least partially explain why the IgM-antibody responses were 100% negative in all 14 serum samples of the III profile.

As such, our findings indicate that IgM-antibody responses will only be seen when a susceptible asymptomatic infection (III profile) evolves to a symptomatic one (SI profile) — when the infection is able to escape macrophage microbicidal action and amplify the parasite load on the mononuclear phagocyte system — thus providing an antigenic stimulus capable of promoting an IgM-antibody response. This information appears to be crucial to our understanding of the immunopathogenic role of IgM-antibody responses during the course of infection.

We confirm here for the first time in Brazil the preclinical diagnosis of AVL in a case demonstrating the III profile with a strong anti-*L. (L.) i. chagasi* IgM-antibody response (1280 serological titer). It seems reasonable to assume that preclinical IgM-antibody responses in cases showing the III profile may function as immunological marker of AVL

Table 1 Characteristics of the case in which an III profile developed to AVL (as well as those of seven other household members) and the results of the DTH and IFAT-(IgG/IgM) assays used to diagnosis human *Leishmania (L.) infantum chagasi* infections, in Redenção City, Pará State, Brazil

Cases	Gender	Age	DTH	IFAT	
				IgG	IgM
1	Female	55	Neg	320	Neg
2	Male	24	Neg	Neg	...
3	Female	24	Neg	Neg	...
4	Male	22	Neg	Neg	...
5	Female	19	Neg	Neg	...
6	Male	10	Neg	Neg	...
7	Female	04	Neg	Neg	...
8	Female*	03	Neg	640/10 240†	1280

Note: DTH, delayed-type hypersensitivity; IFAT, indirect fluorescent antibody test; IgG, immunoglobulin G; IgM, immunoglobulin M.

*The case of the III profile who developed to AVL.

†The second IFAT-IgG assay was performed 6 weeks after the first one.

evolution, and also represent a strong evidence of the early stage of that profile.

Additionally, considering that there is currently no efficient vaccine available in Brazil that can protect humans against *L. (L.) i. chagasi*-infections, this diagnostic approach may prove useful for improving AVL control by allowing monitoring of human infections and identifying those cases of the III profile with a propensity for evolving towards full AVL. The preclinical diagnosis of AVL in cases of the appearance of the III profile could help prevent the severe morbidity of this disease and the requirement for long-duration treatments.

Finally, it should be stressed that the expressive IL-6 (828.64 fg/ml) serum level observed in the present case of the III profile (as compared with those of other cytokines analyzed, such as IL-4 (5.06 fg/ml), IL-10 (454.76 fg/ml), and TNF-alpha (208.49 fg/ml), provides evidence for associating IL-6 with AVL development^{12,13} and with severe manifestations of this disease.¹⁴ As such, our findings also indicate expressive IL-6 serum levels with the entire clinical-immunological spectrum of human *L. (L.) i. chagasi* infection, ranging from the earliest asymptomatic III profile to the extremely symptomatic SI profile (=AVL).

Disclaimer Statements

Contributors LVRL, MBC, PKR, TVS, and FTS: experiments design and development; CMCG, MDL, CEPC, and FTS: data analysis; LVRL, MBC, PKR, TVS, and FTS: manuscript writing; CMCG, MDL, CEPC, and FTS: manuscript revision; MDL, CEPC, and FTS: getting financial support. All authors read and approved the final version of the manuscript.

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