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Research paper

Anti-*Leishmania chagasi* immunoglobulin G3 detected by flow cytometry for early cure assessment in American visceral leishmaniasis

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ABSTRACT

We have previously reported a novel flow cytometric based methodology to access the reactivity of seric anti-live (FC-ALPA) and fixed (FC-AFPA) *L. chagasi* IgG antibodies applicable for cure assessment after specific therapy of VL. Both, FC-ALPA-IgG and FC-AFPA-IgG are promising targets to be used for early cure assessment. However, our finding suggested that further refinements were still required to improve the performance of FC-AFPA IgG for early cure assessment in VL. In the present investigation, we have established and evaluated the performance of FC-AFPA-IgG1/IgG2/IgG3/IgG4 aiming to increase the performance index of the previously reported for FC-AFPA-IgG. The data was expressed as percentage of fluorescent positive parasites after incubation of pre-fixed *L. chagasi* promastigotes with the test sera samples and addition of second-step FITC-labeled anti-human IgG subclasses conjugates. The analysis of anti-*L. chagasi* IgG reactivity in polled sera samples from VL patients demonstrated that, before the etiological treatment, the IgG subclass profile was characterized by IgG1>IgG3 with the absence of IgG2 and IgG4 at the specific sera dilution tested. Following the establishment of specific PPFP cut-off-edges to segregate negative and positive results (PPFP of 50% for FC-AFPA-IgG1 and PPFP of 40% for FC-AFPA-IgG3), the analysis of IgG1 and IgG3 reactivity demonstrated good performance for early cure assessment in VL. The analysis of individual samples indicated that despite at 2 mAT, most treated VL patients (81%) still displayed positive results in FC-AFPA-IGg1 analysis, an increased fraction of treated patients (76%) presented negative in FC-AFPA-IgG1 analysis at 6 mAT. Interestingly, the data from FC-AFPA-IgG3 demonstrated an outstanding performance of this method to early cure assessment in VL with increased frequency of treated patients displaying negative results at 2 mAT (90.5%) as well as at 6 mAT (95.2%). The analysis of likelihood ratio (LR) further confirmed the remarkable performance of FC-AFPA-IgG3 as an early complementary biomarker useful to monitor the post-therapeutic cure in human VL.

1. Introduction

Visceral leishmaniasis (VL) is a worldwide, disseminated intracellular infection mainly caused by parasites pertaining to the *Leishmania donovani* complex including *L. donovani*, *L. infantum* and *L. chagasi*. Active VL is characterized by fever, weight loss, anemia and hepatosplenomegaly, and is usually fatal without specific treatment (Ravindran et al., 2004). To date, the definition of clinical cure is mostly based on clinical improvement denoted by vanishing of fever, reduction of spleen and liver size and normalization of hematological scores. As definitive cure assessment based on clinical findings requires one year follow-up to exclude the possibility of disease

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relapse, the search for alternative methods to be used for early cure assessment still represents a broad field for investigation.

Although, positive parasitological tests indicate the persistence of circulating parasites and represent a marker of therapy failure, these tools are invasive and therefore not practical for clinical monitoring of the therapeutic response. On the other hand, most types of conventional serological methods evaluating the presence of specific antibodies remain positive for long periods after treatment. Thus, one of the major challenges has been the establishment of laboratorial tools to assess treatment effectiveness. In this context, many studies have proposed different antigen preparations and methodologies and evaluated their performance as laboratorial tools to monitor post-chemotherapy effectiveness in VL (Guimarães et al., 1990; Zijlstra et al., 1998; da Matta et al., 2000; Barbosa-de-Deus et al., 2002; Braz et al., 2002; Carvalho et al., 2003).

Our group has previously reported the applicability of immunofluorescence assay based on flow cytometry for detection of anti-live and fixed *L. chagasi* IgG antibodies and evaluated its performance for cure assessment after specific therapy in human VL (Lemos et al., 2007). Our data showed that live and fixed parasites are equally efficient antigen preparations to use for cure assessment at twelve months after treatment. However, for early cure assessment at two and six months, live promastigotes showed better performance. Considering the difficulty of working with live parasites, herein, we evaluated IgG subclasses using fixed promastigotes of *L. chagasi* as antigen in order to increase the performance of immufluorescence by flow cytometry for monitoring the efficacy of treatment in human VL.

2. Materials and methods

2.1. Serum samples

As part of ongoing evaluation studies focusing the epidemiology and chemotherapy studies in VL, serum were obtained from 21 patients with amastigote positive bone marrow aspirates and stored at −20 °C. The patients included in this study ranged from 6 months to 10 years old. Patients were enrolled at University Hospital in Montes Claros, Minas Gerais, Brazil. For each patient, a medical history was obtained and a complete physical examination performed. Treatment of patients was carried out with 1.0 mg/kg/day of amphotericin B, during 14 days and no parasites were detected in bone marrow aspirate collected in the end of treatment. They were followed for twelve months and considered cured during this period. For post-treatment evaluations the blood samples were drawn before, 2, 6 and 12 months following the beginning of treatment. Twenty non-infected individuals were included in this study as negative control (NI). The serum samples were inactivated by heating for 30 min at 56 °C and kept at −20 °C until use. The inactivated sera were diluted in 0.15 M phosphate-buffered saline (PBS), pH7.2, containing 10% heat-inactivated fetal bovine serum (FBS) and used to evaluate the presence of anti-*L. chagasi* antibodies by flow cytometry.

Approvals for testing were obtained through the Ethical Committee of Universidade Estadual de Montes Claros. Informed consent was obtained from parents or legal guardians of minors.

2.2. Parasite preparation

L. chagasi promastigotes forms (MHOM/BR/74/PP75) were grown in liver infusion tryptose medium (LIT) at 25 ± 1 °C. After serial passages *in vitro*, the parasites at stationary-phase were transferred to 50 mL polypropylene tubes and homogenized in a low-rotation vortex to dissolve clumps. The parasite suspension was then submitted to a differential centrifugation at 25 °C, 100 g for 10 min to remove contaminants such as erythrocytes and clusters of parasites in the pellet. Prior to recovery of the single parasite suspension, the supernatant was left to rest for 10 min at room temperature. The supernatant containing most of the parasites was transferred to another 50 mL polypropylene tube and spin down at 4 °C, 1000 g for 10 min. The pellet was washed twice by centrifugation at 4 °C, 1000 g for 10 min, using 0.15 M phosphate-buffered saline, pH7.2, containing 10% heat-inactivated fetal bovine serum (PBS-10% FBS) and the parasites were immediately re-suspended in equal volume of PBS and FACS fix solution (per liter, 10 g of paraformaldehyde, 10.2 g of sodium cacodylate and 6.65 of sodium chloride, pH 7.2; Sigma Chemical Corp., St Louis, Mo). After 24 h, the parasites were washed and stored at 4 °C until use. The suspension of parasites was adjusted to 5.0×10^6/mL and used for flow cytometric immunofluorescence assay.

2.3. Immunofluorescence by flow cytometry

The detection of anti-fixed *L. chagasi* promastigotes antibodies by flow cytometry (FC-AFPA) was performed as described by Lemos et al. 2007. Briefly, 250,000 fixed parasites/well were incubated at 37 °C for 30 min in the presence of 50 μL of different serum dilutions in PBS-3% FBS. After incubation with sera, parasites were washed twice with 150 μL of PBS-3% FBS (4 °C, 1000 × g, for 10 min). The parasites were re-incubated in the dark, at 37 °C for 30 min in the presence of 50 μL of biotin-conjugated anti-human IgG subclass (Sigma Chemical Corp., St. Louis, MO) diluted in PBS-3% FBS — IgG1 (1:16,000), IgG2, IgG3 and IgG4 (1:500). After a second wash procedure, the biotin-labeled parasites were re-incubated at 37 °C for 30 min, in the presence of 20 μL of streptavidin–phycoerythrin (SAPE) diluted 1:400. The parasites were washed twice with PBS-3% FBS and fixed with 200 μL of FACS fix solution (per liter, 10 g of paraformaldehyde, 10.2 g of sodium cacodylate and 6.65 of sodium chloride, pH 7.2; Sigma Chemical Corp., St Louis, Mo) and maintained at least for 30 min, at 4 °C in the dark before flow cytometric analysis.

An internal control, in which the parasites were incubated in the absence of human serum, however in the presence of secondary reagents, was included to monitor unspecific reactions. In all batches of experiments, positive and negative VL control samples were included.

2.4. Data acquisition and analysis

Flow-cytometric measurements were performed on a Becton Dickinson FACSort interfaced to an Apple Quadra FACStation. The Cell-Quest software package was used for data acquisition and storage for following analysis. Stained parasites were run in the cytometer, and 5000 events per sample were acquired. Promastigotes were identified based