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Parasitology

Evaluation of anti-lived and anti-fixed *Leishmania* (*Viannia*) *braziliensis* promastigote IgG antibodies detected by flow cytometry for diagnosis and post-therapeutic cure assessment in localized cutaneous leishmaniasis ☆

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ABSTRACT

This study aims to investigate a flow cytometry performance–based methodology to detect anti-live (FC-ALPA-IgG) and anti-fixed (FC-AFPA-IgG) *Leishmania* (*Viannia*) *braziliensis* promastigote IgG as a means to monitor post-therapeutic cure of patients with localized cutaneous leishmaniasis (LCL). Serum samples from 30 LCL patients infected with *L. (V.) braziliensis* were assayed, comparing the IgG reactivity before and after specific treatment with pentavalent antimonial. Reactivities were reported as the percentage of positive fluorescent parasites (PPFP), using a PPFP of 60% as a cut-off value. In the serum dilution of 1:1024, the positive percentage of LCL serum sample for FC-ALPA-IgG and FC-AFPA-IgG was 86% and 90%, respectively, before treatment. Analysis of ΔPPFP that represents the difference between PPFP after and before treatment appeared as a new approach to monitor post-therapeutic IgG reactivity in LCL. Our data support the perspective of using FC-ALPA and FC-AFPA as a useful serologic tool for diagnosis and for post-therapeutic follow-up of LCL patients.

1. Introduction

Leishmaniasis encompasses multiple clinical syndromes, most notably, visceral, cutaneous, and mucosal forms. Localized cutaneous leishmaniasis (LCL) is a disease associated with infections caused by several species of the genus *Leishmania* (Grimaldi and Tesh, 1993). Clinical manifestations depend on the parasite factors, the epidemiologic characteristic of the vector and the host genetic and immunologic constitution (Rogers et al., 2002). Cutaneous leishmaniasis is a serious public health problem and it is endemic to Brazil, particularly in the state of Pernambuco, where *Leishmania (Viannia) braziliensis* is known as the major circulating species (Brito et al., 2009).

At present, there is not a gold-standard test for cutaneous leishmaniasis and, frequently, a combination of different diagnosis techniques is needed to obtain more precise results. Thus, the diagnosis is performed by the association of clinical, epidemiologic, and laboratorial aspects. These techniques include amastigote identification through tissue immunocytochemical techniques, "imprints" (printing by biopsy apposition), in aspirated lesion and in histopathologic evaluation, besides the promastigote identification in vitro. The indirect immunofluorescence (IIF), enzyme-linked immunosorbent assay (ELISA), and Western blot, based on the presence of specific antibodies against parasite antigens, are the serologic methods used. Montenegro skin test is a late hypersensitivity test based on the immunity mediated by cells. At the same time, there has been a notable improvement in such techniques as polymerase chain reaction (PCR), real-time PCR, and flow cytometry, with the objective of increasing sensitivity and specificity (Vega-López, 2003).

The traditional laboratory methods have several limitations and present difficulties. Furthermore, immunoassays can show low antibody titers, due to a cross reactivity with *Trypanosoma cruzi* that depends on the antigen as well as the lack of well-standardized procedures used to detect the specific antibodies. Moreover, antibody production after treatment is not yet clear and the predictive value of lower or higher levels against specific antigenic fractions during follow-up is not well defined (Brito et al., 2001).

Chemotherapy treatment is based primarily on the administration of pentavalent antimonials. These antimonials are highly toxic

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difference between PPFP after treatment and PPFP before treatment, was used as a new approach to monitor post-therapeutic IgG reactivity in LCL as proposed by Lemos et al. (2007) to monitor patients with visceral leishmaniasis.

3. Results

3.1. Initial serum screening

In the first step of this study, we analyzed the anti–L. braziliensis IgG reactivity profile for live (FC-ALPA) and fixed (FC-AFPA) antigen preparations, aiming to characterize the titration curve and the serum dilution to differentiate LCL patients and noninfected individuals. Data analysis showed that both antigen preparations, FC-ALPA-IgG and FC-AFPA-IgG, have the same titration curves and patterns of reactivity (PPFP values). This analysis identified the specific serum dilution (1:1024 for FC-ALPA-IgG and FC-AFPA-IgG) that best segregates the PPFP values of LCL patients and noninfected individuals. As observed in Fig. 1, the results were classified as positive with PPFP 60% and as negative with PPFP 60%. This percentage was done according to Martins-Filho et al. (1995), from the analysis of antibody titration curves of individual serum expressed by the mean of PPFP values. The initial screening showed that sera were considered negative (PPFP 60%) when reacting in a dilution of 1:128 to 1:16,384 for FC-ALPA-IgG and 1:512 to 1:32,768 for FC-AFPA (Fig. 1). In view of these results, FC-ALPA-IgG and FC-AFPA-IgG were able to discriminate the IgG reactivities of patients compared to the control group.

3.2. Performance of anti-live and fixed L. (V.) braziliensis IgG reactivity to identify active LCL

In this step, we evaluated the test performance to identify patients with active LCL. Thus, sera were separated and reactivity was determined with a dilution of 1:1024 and a cut-off value of PPFP 60% (Fig. 1). Using this approach, the sensitivity of FC-ALPA-IgG and FC-AFPA-IgG was 86% and 90%, respectively. The specificity was 78%, showing a cross-reactivity of 22% for both parasite preparations (live and fixed) (Fig. 2). The results obtained in this evaluation showed that, despite the cross-reactivity observed for NI samples, FC-ALPA-IgG and FC-AFPA-IgG have an important value in identifying LCL cases.

Additionally, we analyzed the applicability of FC-ALPA-IgG and FC-AFPA-IgG to monitor the post-therapeutic cure of LCL. The analysis of PPFP values performed after treatment did not demonstrate the applicability of this parameter for post-therapeutic cure assessment at a serum dilution of 1:1024. No significant changes in the PPFP values at serum dilution 1:1,024 were observed after treatment showed reactivity less than 60% to FC-ALPA-IgG and FC-AFPA-IgG (Fig. 3).

Further comparative analysis used the PPFP values of paired samples evaluated before and after treatment along the titration curves (1:2048 to 1:16,384 for FC-ALPA-IgG and 1:2048 to 1:32,678 for FC-AFPA-IgG). Our data demonstrated that only FC-ALPA-IgG led to differential reactivity when comparing the mean PPFP values observed before and after treatment (Fig. 4, asterisks).

3.3. Introducing PPFP as a new strategy applied to post-therapeutic cure assessment in LCL

As the analysis of PPFP values performed after treatment did not demonstrate any applicability for post-therapeutic cure assessment at a sera dilution of 1:1024, we searched for a new tool to comparatively analyze the IgG reactivity before and after treatment.

The proposed strategy was to analyze the differential PPFP reactivity detected by paired samples (delta reactivity [Δ]). In the present study, we used anti-Leishmania IgG reactivity ΔPPFP which represents the difference between PPFP after treatment and PPFP before treatment ($\Delta PPFP = PPFP_{AT} \quad PPFP_{BT}$). Initially, we evaluated the ΔPPFP values throughout the FC-ALPA-IgG and FC-AFPA-IgG titration curves, aiming to identify the serum dilution ranges of 1:2048 to 1:16,384 for FC-ALPA-IgG and 1:2048 to 1:32,678 for FC-AFPA-IgG that represent the highest differential reactivities (Fig. 5). As shown in Fig. 5, the FC-ALPA-IgG at a sera dilution of 1:8192 showed that 81% of the treated patients displayed negative ΔPPFP values, demonstrating a decrease in the IgG reactivity after treatment. Moreover, the FC-AFPA-IgG at a sera dilution of 1:4096 was able to identify 61% of patients with negative ΔPPFP values. Consequently, these dilutions were the best choices to analyze the ΔPPFP values for FC-ALPA-IgG and FC-AFPA-IgG, respectively.

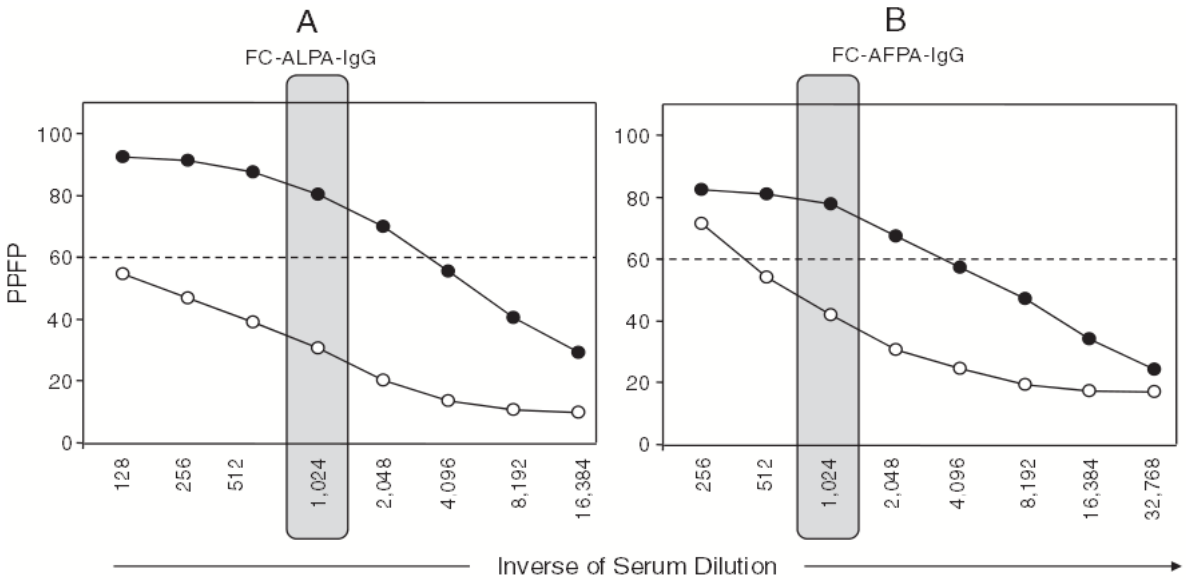

Fig. 1. Anti-live FC-ALPA-IgG (A) and anti-fixed FC-AFPA-IgG (B) *L. (V.) braziliensis* IgG reactivity in serum samples from patients with localized cutaneous leishmaniasis (LCL = ●) and from noninfected individuals (NI = ○). The results are expressed as mean percentage of positive fluorescent parasites (PPFP) at sera dilutions of 1:128 to 1:16,384 for FC-ALPA-IgG and 1:256 to 1:32,768 for FC-AFPA-IgG. The rectangles represent the selected serum dilution of the higher segregation range between patients and negative control (1:1024 for FC-ALPA-IgG and FC-AFPA-IgG).

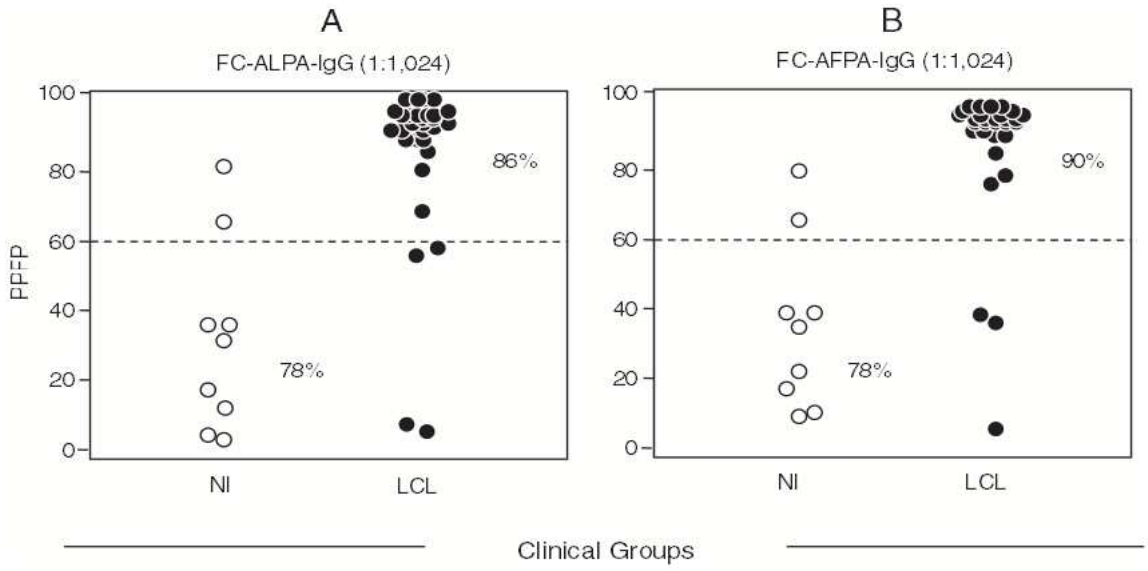

Fig. 2. Anti-live FC-ALPA-IgG (A) and anti-fixed FC-AFPA-IgG (B) *L. (V.) braziliensis* IgG reactivity in serum samples from patients with LCL (●) and from NI (○). The results are expressed as individual PPFP at a serum dilution of 1:1024. The dashed line represents the cut-off between negative and positive results.

After setting the specific serum dilutions to monitor the ΔPPFP values, we investigated whether the time after treatment would interfere in the performance of this new strategy of post-therapeutic follow-up. For this purpose, the treated patients were categorized into 3 subgroups based on the time (months) after treatment (mAT) when they were evaluated: 1–3 mAT, 4–7 mAT, and 12–24 mAT. Data analysis was performed after the establishment of a gray zone corresponding to the first quartile of the ΔPPFP range (cut-off edge of 25%, considering PPFP values from 0 to 100%) according to Lemos et al. (2007). We believe that the use of this gray zone would give further strength to data interpretation since it would avoid interference regarding the possible intrinsic flow cytometry measurement variability. Our data demonstrated that 75% of the patients evaluated at 1–3 mAT showed negative ΔPPFP values detected by FC-ALPA-IgG, whereas 25% of them showed negative ΔPPFP values detected by FC-AFPA-IgG. Patients evaluated at 4–7 mAT displayed 43% and 29% of negative ΔPPFP values detected by FC-ALPA-IgG and FC-AFPA-IgG, respectively. When ΔPPFP reactivity was evaluated at 12–24 mAT, 58% and 33% of the patients presented negative results in FC-ALPA-IgG and FC-AFPA-IgG, respectively (Fig. 6).

4. Discussion

One of the major limitations in the use of serologic approaches in the diagnosis and cure assessment of cutaneous leishmaniasis is the scarcity of sensitive methods to discriminate the IgG reactivity during active infection and the residual serologic reactivity after effective treatment. In general, the diagnosis and the cure criterion are based on clinical and epidemiologic findings, and complete therapeutic effectiveness is considered when complete lesion healing is observed. Nevertheless, this is an unsatisfactory criterion, as lesion reactivation may occur even after treatment and complete healing of the initial

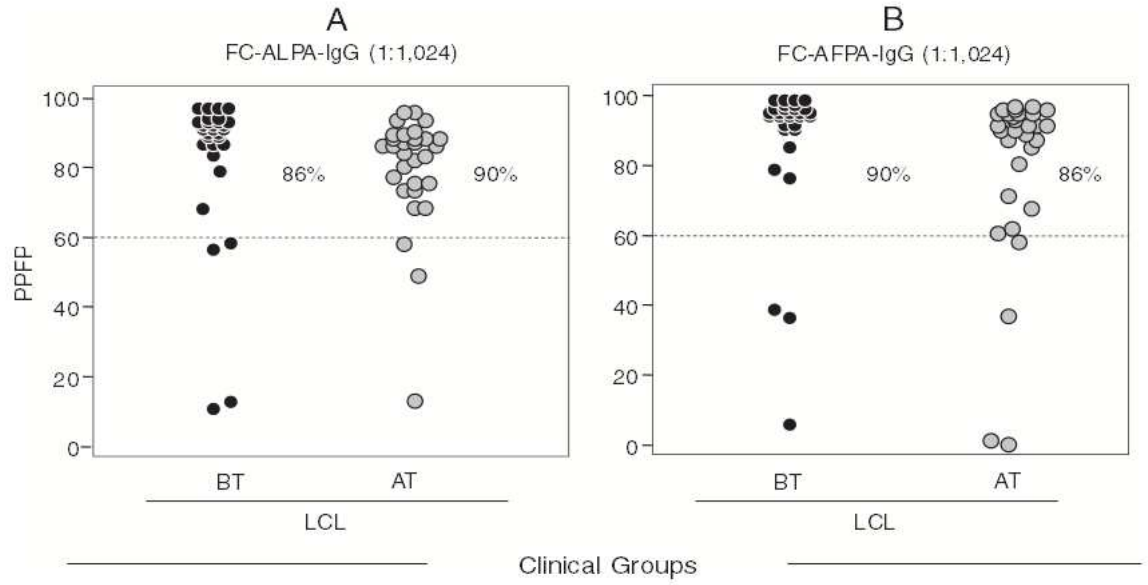

Fig. 3. Comparative reactivity of anti-live FC-ALPA-IgG (A) and anti-fixed FC-AFPA-IgG (B) *L. (V.) braziliensis* IgG reactivity in serum samples from patients with LCL before treatment (BT = ●) and after treatment (AT = ◎). The results are expressed as individual PPFP at a serum dilution of 1:1024. The dashed line represents the cut-off between negative and positive results.

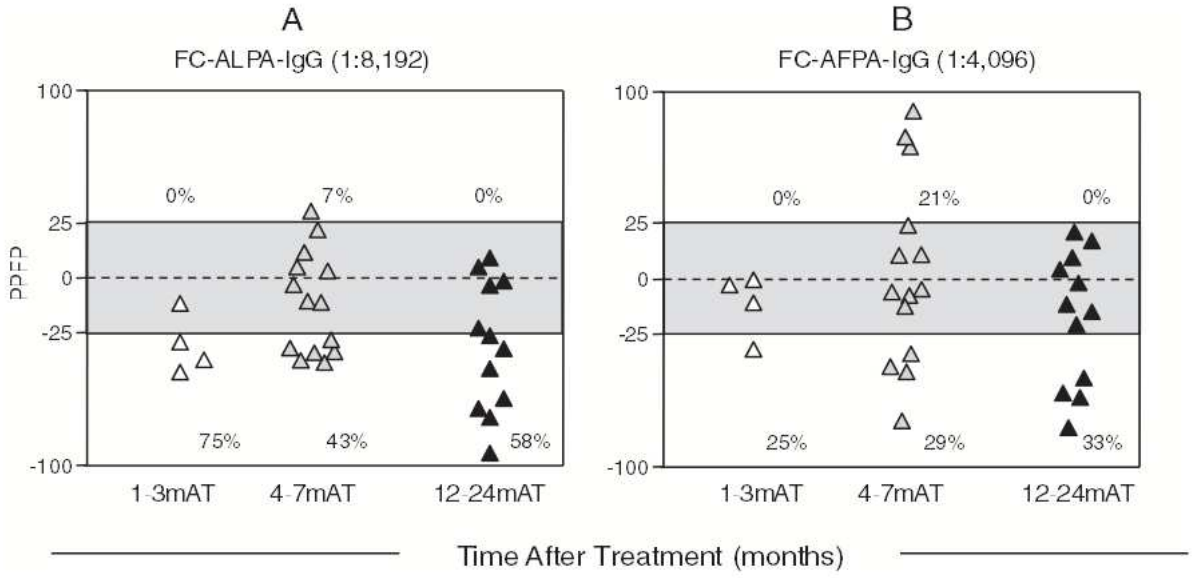

Fig. 6. Differential anti-*L. (V.) braziliensis* IgG reactivities of paired samples detected by FC-ALPA-IgG (A) and FC-AFPA-IgG (B) defined as differential percentage of fluorescent positive parasites (ΔPPFP) for pairs of samples from LCL patients evaluated before and after treatment. Patients were categorized into 3 groups based on the time (months) after treatment (mAT) and referred as 1–3 mAT (Δ), 4–7 mAT (▲), and 12–24 mAT (▲). The results are expressed as ΔPPFP for each pair of samples. Data analysis was performed after the establishment of a gray zone (the gray rectangle) corresponding to the first quartile of the ΔPPFP range (cut-off of 25%).

preparation is the major concern about its use in clinical laboratory practice. On the other hand, although the use of fixed promastigotes represents a feasible way to produce and store a bulk amount of pre-fixed antigen that contributes to large-scale production, it would contribute to the development of a larger amount of intracytoplasmic epitopes and complicate the observation of minor changes in the serologic reactivity following therapeutic intervention (Pissinate et al., 2008).

Another important aspect of the serologic methods applied to the cutaneous leishmaniasis diagnosis is the choice of antigen sources, which still represents a relevant obstacle. When total promastigotes are used as antigen, it is common to find false-positive reactions due to cross-reactions with other diseases. It is important to investigate alternative preparations to detect Leishmania antibodies (Celeste et al., 2004; Gonçalves et al., 2002). In the present investigation, we have chosen the L. (V.) braziliensis promastigotes as the antigenic source to access IgG reactivity in LCL patients. Although this species is very difficult to grow in vitro, requiring the use of axenic cultures, complex medium composition and fine pH and temperature control (Lemesre et al., 1988), L. (V.) braziliensis is the most important LCL causative agent in Brazil and especially in the Brazilian state of Pernambuco.

A diagnostic method based on flow cytometry to detect anti-live L. (V.) braziliensis antibodies has been described by Rocha et al. (2002, 2006). They demonstrated 96% sensitivity for FC-ALPA-IgG L. braziliensis in active patients. Using fixed L. amazonensis promastigotes, Pissinate et al. (2008) showed a good performance of FC-AFPA-IgG in the serologic diagnosis of LCL. However, they found cross-reactivity with other co-endemic diseases, like trypanosomatidae infections. Our data demonstrated that both methods display low specificity and still require methodological adjustments in order to improve their performance as a confirmatory diagnostic tool. In fact, Rocha et al. (2002) and Pissinate et al. (2008) have already demonstrated that the occurrence of false-positive results in FC-ALPA-IgG and FC-AFPA-IgG in endemic areas mostly related to the cross-reactivity of sera samples from patients with Chagas disease and visceral leishmaniasis, co-endemic diseases generally observed in areas of prevalent cutaneous leishmaniasis.

In our study, we found that FC-ALPA-IgG using L. (V.) braziliensis displayed 86% sensitivity, whereas FC-AFPA-IgG showed 90% sensitivity for the diagnosis of LCL. The difference obtained could be explained by the heterogeneity of Leishmania (V.) spp in Brazil. Brito et al. (2009) demonstrated the presence of 10 circulating zymodemes in the well-defined "Zona da Mata" region of Pernambuco. The heterogeneity observed among L. (V.) braziliensis parasites from this region is noteworthy, particularly in contrast to the homogeneity of parasites isolated from other regions of Brazil (Brandão-Filho et al., 2003; Brito et al., 1993; Cupolillo et al., 2003).

The results obtained showed that both techniques (FC-ALPA-IgG and FC-AFPA-IgG) are useful for the serodiagnosis of LCL as compared to the conventional immunofluorescence assay. Although FC-AFPA-IgG displays a slightly higher sensitivity in the diagnosis of LCL, the FC-ALPA-IgG seems to be more reliable for cure monitoring, being able to identify more differences between IgG reactivity before and after treatment when assessed by ΔPPFP. We found 81% of the treated patients with negative ΔPPFP results for FC-ALPA-IgG compared to 61% with negative ΔPPFP for FC-AFPA-IgG. These results show that FC-ALPA-IgG represents better performance than FC-AFPA-IgG for post-therapeutic monitoring of LCL patients. We have a general belief that FC-ALPA-IgG represents better performance than FC-AFPA-IgG as previous studies of our group have demonstrated a real advantage of using live instead of fixed parasites in serologic approaches applied to the diagnosis and cure assessment of human protozoa (Martins-Filho et al., 1995, 2002; Pissinate et al., 2008; Vitelli-Avelar et al., 2007). In fact, the use of live promastigotes seems to represent a better tool to achieve better performance in serologic approaches since in this antigenic preparation only the outer membrane epitopes are available for IgG binding, in contrast to the fixed antigenic preparation in which the cytoplasmic antigens are also available for IgG recognition. The use of a selected set of outer membrane antigens is a good strategy to work with a more restricted IgG repertoire that would potentially find slight differences resulting from the loss of B-cell clones early after effective etiologic treatment.

We have further investigated whether the performance would be influenced if the test was performed at different times after treatment. For this purpose, serum samples collected after treatment were segregated into 3 groups referred to as 1–3 mAT, 4–7 mAT, and 12–24 mAT. The FC-ALPA-IgG and FC-AFPA-IgG were assayed, and the ΔPPFP values were generated to monitor seroreactivity at different times

following treatment. Our findings showed an overall low performance of ΔPPFP to demonstrate differential reactivity according to the time after treatment. However, FC-ALPA-IgG still demonstrated better performance as compared to FC-AFPA-IgG, leading to higher frequency of cases with negative ΔPPFP. It is important to mention that the low performance of ΔPPFP to detect differential reactivity in this cross-sectional investigation should not be considered the end point of using this parameter for cure assessment in LCL, since this approach would be better evaluated in a longitudinal investigation in order to generate more accurate data for cure assessment in LCL.

Although most flow cytometry–based methods still represent higher cost compared to conventional methods, such as immunosorbent and immunofluorescence assay, the possibility of working with a microplate serologic approach has reduced the final cost of a given test. Moreover, at the present time, several clinical laboratories in developing countries are considering the acquisition of flow cytometers. Therefore, in the near future, the implementation of new flow cytometry-based tests will became routine as will the interchange between research centers and clinical laboratories. In our experience, flow cytometry–based serologic approaches present good reproducibility and outstanding concordance among independent analysts (Garcia et al., 2009).

In conclusion, our data suggested that the new flow cytometry–based methodology has promising potential to identify active LCL clinical cases in patients. Further longitudinal studies are currently under investigation in order to better characterize the approach to for monitoring post-therapeutic cure as well as to obtain the clinical values of this new approach and to validate its use in medical studies.

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