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Flow Cytometry, a New Approach To Detect Anti-Live Trypomastigote Antibodies and Monitor the Efficacy of Specific Treatment in Human Chagas' Disease

OLINDO A. MARTINS-FILHO,[1,2] MARIA ELIZABETH S. PEREIRA,[1] JUÇARA F. CARVALHO,[1] JOAQUIM R. CANÇADO,[3] AND ZIGMAN BRENER[1]*

Centro de Pesquisas René Rachou, FIOCRUZ,[1] and Departamento de Bioquimica e Imunologia[2] and Faculdade de Medicina,[3] UFMG, Belo Horizonte, MG, Brazil

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Sera from patients chronically infected with *Trypanosoma cruzi* display antibodies that bind to epitopes of living trypomastigotes, known as lytic antibodies (LA), and are detected by a complement-mediated lysis test. Conventional serology antibodies (CSA) are also present in sera from patients with chronic infections but, in contrast to LA, are unable to recognize viable trypomastigotes. The presence of LA has been used as an important element in the criterion of cure in human Chagas' disease. Using flow cytometry technology, we introduced a new and sensitive immunomethod for the detection of anti-live trypomastigote membrane-bound antibodies. On the basis of serological tests (LA and CSA detection) and parasitological assays such as hemoculture (HE), patients were classified into the following groups: chronically infected untreated patients (NT) and treated not-cured patients (TNC), with positive HE and both LA and CSA in their sera; "dissociated" HE-negative patients (DIS), in whom LA was not detected whereas CSA were present; a group of cured HE-negative patients (CUR), who were both LA and CSA negative; and, as control, a group of non-chagasic individuals (NC). Sera from these patients were assayed by incubation with live bloodstream trypomastigotes, which were subsequently exposed to fluorescein isothiocyanate-conjugated anti-human immunoglobulin G. The parasites were then fixed, run in the cytometer, and identified on basis of their size and granularity gain adjustments. On the basis of experience with the complement-mediated lysis test, a level of 20% of parasites being fluorescein isothiocyanate fluorescence positive was used as a cutoff between effective and ineffective treatments. With this criterion, our results indicated that sera from NT and TNC patients were antibody positive whereas all sera from DIS, CUR, and NC patients did not contain membrane-bound antibodies. This new approach is a tool to easily identify anti-live *T. cruzi* membrane-bound antibodies that can be used to monitor the efficacy of Chagas' disease treatment.

Chagas' disease, an endemic infection caused by the protozoan parasite *Trypanosoma cruzi*, is a major public health problem in Latin America, where circa 16 million to 18 million people are already infected and 90 million are at risk of infection (16). Patients with chronic infection are affected by heart lesions and pathological dilatation of the digestive tract (megacolon and megaesophagus). Treatment with the nitroheterocyclic drugs, nifurtimox and benznidazole, cures about 50 to 70% of patients during the acute phase as evaluated by parasitological tests (hemoculture [HE] and xenodiagnosis) and conventional serology (hemagglutination and indirect immunofluorescence reaction [IIFR]) (5). During the lifelong chronic phase, the drugs cure only 7%; intriguingly, 20 to 25% of the treated patients show positive serology but repeated negative parasitological tests; the remaining uncured patients give positive results in all these tests (7).

Krettli and Brener (10) found that sera from patients with chronic disease contained two types of anti-parasite antibodies with different functional activities, namely, lytic antibodies (LA), which are associated with resistance in active ongoing *T. cruzi* infection and bind to live trypomastigotes as detected by complement-mediated lysis assay (CoML) (10, 12), and conventional serology antibodies (CSA), which are neither associated with host resistance nor detected by CoML but do recognize fixed *T. cruzi* developmental stages that are detected by IIFR and hemagglutination.

Longitudinal studies based on both the described serological and parasitological tests allowed us to classify patients subjected to specific treatment and controls into different groups according to the presence or absence of parasites in the blood, as detected by HE, as well as the presence or absence of LA, as detected by CoML. On this basis, individuals were placed in the following groups: chronically infected untreated patients (NT) and treated not-cured patients (TNC), with positive HE and both LA and CSA in their sera; "dissociated" HE-negative patients (DIS), in whom LA is not detected whereas CSA are present for long periods following treatment; and, finally, a group of cured HE-negative patients (CUR), who were both LA and CSA negative (4, 7, 11).

There is evidence suggesting that LA is an immunoglobulin G (IgG) anti-*T. cruzi*-specific antigen (10). The *T. cruzi* epitope that elicits LA production was first identified by Martins et al. (13) as a 160-kDa glycoprotein, and it has been further isolated and purified by Norris et al. (14). This glycosylated protein is present in small amounts (the yield of purified protein is approximately 5 μg/10^{10} trypomastigotes); it easily breaks down, even at low temperatures, and is irregularly distributed on the parasite surface (14). The identification of membrane-bound antibodies on live trypomastigotes is an important problem. CoML, which involves the use of live trypomastigotes and optical microscopy, is highly inconvenient and has many limi-

* Corresponding author. Mailing address: Centro de Pesquisas René Rachou, FIOCRUZ, Av. Augusto de Lima, 1715, 30190-002—Belo Horizonte MG, Brazil. Phone: (55) (31) 295-3566. Fax: (55) (31) 295-3115.

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TABLE 1. Parasitological and serological tests to classify treated patients

Group	No. of individuals	Result of:		
		Serological test		Parasitological test (HE[a])
		CSA[b]	LA[c]	
NC	17	−	−	−
NT	12	+	+	+
TNC	9	+	+	+
DIS	12	+	−	−
CUR	4	−	−	−

[a] Results of serial HE, performed as described in Materials and Methods.
[b] Results obtained from IIFR (CSA).
[c] Results from CoML (LA).

tations. Here we report the application of FACScan analysis as a tool for detection of membrane-bound antibodies (MBA) to specifically monitor the efficacy of chemotherapy. For this, we examined sera from patients who had, for many years following treatment, been monitored for active infection and classified, as described above as to the efficacy of that treatment. This new approach is shown to be much more sensitive and accurate than CoML. The results obtained in this study, by the use of flow cytometry, reemphasize the correlation between the presence of MBA and ongoing infection. They also support previous suggestions that the presence of MBA is an important element in the criterion of effective treatment of *T. cruzi* infection and is a useful tool for investigation of basic aspects of immune system reactivity in Chagas' disease.

MATERIALS AND METHODS

Patients. The inclusion of all subjects in our investigation has the approval of the Ethical Committee from FIOCRUZ (Brazilian Health Ministry). The age range of the individuals included was 29 to 66 years old. All the patients had received a positive diagnosis for Chagas' disease and were subjected to specific treatment. The drugs used for the chemotherapy were tetrahydro-3-methyl-4-[(5-nitrofurfurylidene)amino]-2*H*-1,4-thiazine 1,1-dioxide (nifurtimox [Lampit]; Bayer) or *N*-benzyl-2-nitroimidazole-1-acetamide (benznidazole [Rochagan]; Roche). The compounds were given orally in daily doses of 8 to 10 mg of nifurtimox per kg and 5 to 7 mg of benznidazole per kg. A total of 54 individuals were previously monitored by routine laboratory tests for Chagas' disease (IIFR, CoML and HE). According to the results obtained in those tests and the criteria of Krettli and Brener (10) to evaluate the efficacy of chemotherapy, 37 patients were classified into four distinct groups, NT, TNC, DIS, and CUR (Table 1). A total of 17 non-chagasic individuals were included in this study as a negative control (NC) and were all laboratory members of René Rachou Research Center, FIOCRUZ, Belo Horizonte, Brazil. For our investigation, sera from the patients and non-chagasic individuals described above were used to evaluate the presence of anti-live *T. cruzi* antibodies by flow cytometry.

Parasites. Bloodstream forms of *T. cruzi* Y (15) were obtained as described by Krettli and Brener (10). Briefly, Swiss albino male mice, weighing 18 to 20 g, were used as a donors of *T. cruzi* bloodstream forms. The mice were exposed to a sublethal dose of X rays (600 to 800 rads) 24 h before the inoculation to avoid interference of murine Ig bound to the parasite surface. Bloodstream forms were obtained at the peak of parasitemia on day 7 of infection. The parasites were separated from blood cells by differential centrifugation and washed three times with RPMI 1640 protein supplemented with 10% fetal bovine serum, and the concentration was adjusted to 5×10^6 parasites per ml. Parasites were used on the day of isolation. A 100-μl volume of parasite suspension was preincubated with human complement at 37°C for 30 min to select parasite preparations without membrane-bound murine Igs.

HE. To detect the presence of circulating parasites, serial HEs were performed as described by Chiari et al. (6) with some modifications. Briefly, 30 ml of heparinized blood from patients was centrifuged for 10 min at $900 \times g$ and 4°C. The pellet containing erythrocytes was washed in liver infusion tryptose medium (LIT), resuspended in 6 ml of LIT, and then divided among six tubes, each containing 3 ml of LIT. All cultures were maintained at 26 to 28°C and gently homogenized twice weekly, and aliquots were examined monthly up to 120 days for the presence of *T. cruzi* by optic microscopy.

IIFR. The IIFR, the World Health Organization standard test, is used in the diagnosis of Chagas' disease by measuring the presence of CSA in sera. The test

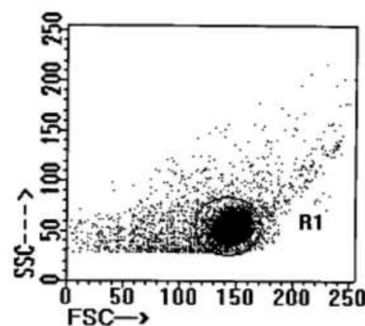

FIG. 1. Dot plot analysis of a representative bloodstream trypomastigote distribution on a graph of FSC (size) versus SSC (granularity). As observed, parasites assume a characteristic homogeneous distribution (R1) that allows selective analysis of trypomastigotes by creating a specific window over the parasite region. This profile was obtained by adjusting size and granularity gains with values of 10 and 225, respectively, on a logarithmic scale.

is carried out by incubating in vitro cultured epimastigotes, previously fixed on slides, in the presence of serial dilution of test sera at 37°C for 30 min. The slides are washed, and staining is performed with fluorescein isothiocyanate (FITC)-labelled anti-human IgG for 30 min at 37°C. The slides are washed again and analyzed for fluorescent staining under a UV-optic microscope.

CoML. CoML was carried out to detect the presence of LA. The test was performed as described by Krettli et al. (12), modified as follows. Briefly, bloodstream forms of *T. cruzi* were collected from X-ray-immunosuppressed Swiss mice, separated from blood elements by differential centrifugation, and adjusted to 6×10^6 trypomastigotes per ml. The parasite suspension was distributed in 5-ml Falcon tubes (50 μl per tube) and incubated at 37°C for 30 min in the presence of 50 μl of test sera. After incubation, tubes were placed on ice and fresh human serum from healthy volunteers was added (100 μl per tube) as the complement source. At that time, the original number of living trypomastigotes present in 10 μl-aliquots was counted in a hemocytometer under an optic microscope. The tubes were then reincubated at 37°C for 45 min, and 10 μl per tube was used to determine the number of remaining live parasites. For each assay, the formula to calculate the percent lysis was % lysis = [1 − (number of parasites after 45 min/original number of parasites)] × 100. Tests were considered positive when lysis was ≥20%. Nonspecific lysis percentages obtained with negative controls are always under 20%.

Flow cytometry. (i) Fluorescence immunoassay with living bloodstream trypomastigotes. The immunofluorescence reaction was carried out as described by Krettli and Brener (10), modified as described below. Briefly, 500,000 live trypomastigotes were incubated at 37°C for 30 min in the presence of different dilutions (1:2 to 1:1,000) of serum from all patients and controls selected for this study. After incubation with sera, parasites were washed once with phosphate-buffered saline (PBS) containing 10% fetal bovine serum and reincubated at 4°C for 60 min in the dark in the presence of FITC-conjugated anti-human IgG antibody preparation (Sigma immunochemical reagents). The FITC-conjugated antibody solution was diluted 400-fold with PBS containing 10% fetal bovine serum. Each assay included an internal control of nonspecific binding in which parasites, not exposed to human serum, were incubated with FITC-anti-human IgG (diluted 1:400). After being stained, labelled parasites were washed with PBS containing 10% fetal bovine serum and fixed on ice for 30 min with a BD FACS fix solution (10 g of paraformaldehyde per liter, 1% sodium cacodylate, 6.65 g of sodium chloride per liter, 0.01% sodium azide [pH 7.2]). Test tubes were then stored for up to 24 h before cytofluorometric acquisition and data analysis.

(ii) FACScan data storage and analysis. Flow-cytometric measurements were performed on a Becton Dickinson FACScan interfaced to a digital Micro HP 9153C. The Consort 30 software package was used in both data storage and analysis. Stained parasites were run in the cytometer, and 10,000 events per sample were counted. Trypomastigotes were identified on the basis of their specific forward (FSC) and side (SSC) light-scattering proprieties. Following FSC (size) and SSC (granularity) gain adjustments, parasites were found by assuming a characteristic FSC × SSC dot plot distribution (Fig. 1). Trypomastigotes were selected by gating on the FSC × SSC dot plot representation. The relative FITC fluorescence intensity of each event was analyzed with a single histogram representation. A marker was set up on the histogram representation of the FITC-conjugated internal control and used in all data analysis reported here to determine, for each serum sample, the relative percentage of positive fluorescent parasites (PPFP).

Statistical analysis. The statistical studies were performed with the Minitab 9.2 software package. One-way analysis of variance was used for the comparative study between groups. The McNemar Test was used to compare the flow cytometry (PPFP) and the CoML (percent lysis) results.

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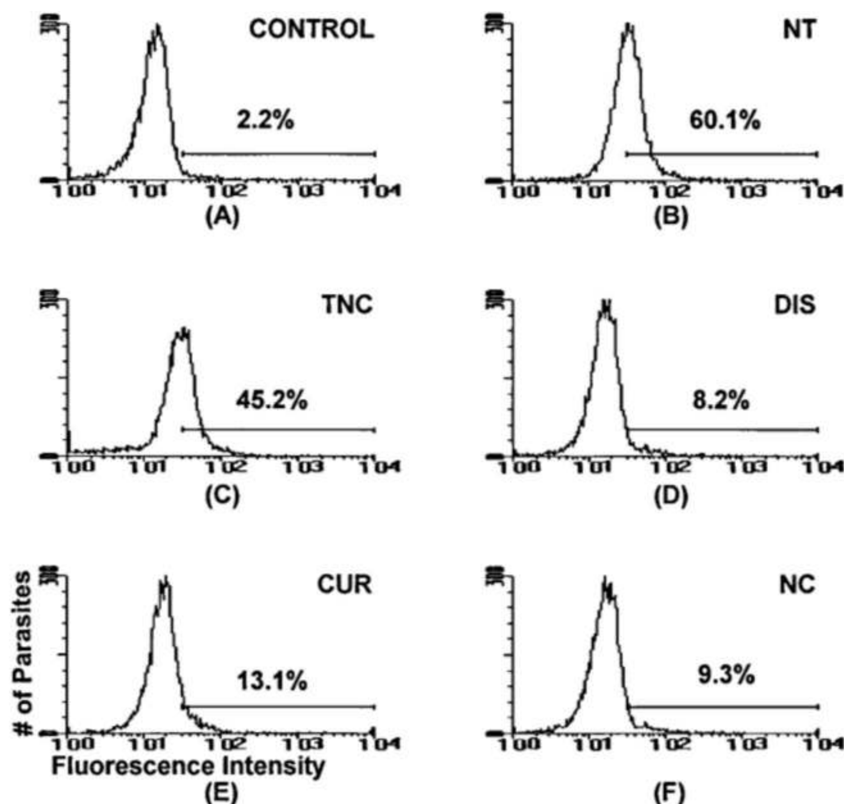

FIG. 2. FL1 single-histogram spectra representing the mean PPFP for nonspecific binding (A) and for each group of patients analyzed (B to F). (B to F) Histograms representing positive parasite spectra obtained by testing 1:256-diluted sera from NT, TNC, DIS, CUR, and NC groups. As observed, the mean PPFP profiles, obtained with sera from NT and TNC patients, who have LA in their sera, were higher than those observed with sera from DIS, CUR, and NC patients, who do not have LA in their sera. These results support previous suggestions that the presence of MBA is an important element in the criterion of effective treatment of *T. cruzi* infection.

RESULTS

PPFP. Individual serum samples were analyzed for the presence of MBA. FACScan data analysis showed that for 10,000 events acquired, an average of 7,000 to 8,000 trypomastigotes could be selected and gated for further analysis of their fluorescence parameter (FL1). Nonspecific binding was monitored by incubation of live parasites with FITC-conjugated anti-IgG only. On the basis of the control histogram representing the positive fluorescent parasite spectrum (Fig. 2A), a control marker of up to 4% of parasites that were fluorescence positive was set up. For each test serum, PPFP was evaluated in the respective FL1 single-histogram spectrum in relation to the same control marker. By serum titration, it was verified that PPFP extended sensitivity up to a 1:2,000 dilution (data not shown). We selected a 1:256 dilution as the maximal PPFP, to avoid parasite agglutinations which would interfere with our data analysis. Figure 2B to F shows histograms representing positive parasite spectra obtained by testing 1:256-diluted sera from the NT, TNC, DIS, CUR, and NC groups of individuals, respectively.

Comparative analysis of PPFP between patient groups. PPFP in serum samples from 47 patients with Chagas' disease, previously classified in terms of the efficacy of their treatments, and in serum samples from 17 noninfected individuals was determined by flow cytometry. PPFP obtained with sera from NT and TNC patients ranged from 32.5 to 93.3% and from 9.4 to 74.5% of gated trypomastigotes, respectively. Analysis of sera from DIS, CUR, and NC patients revealed that PPFP ranged from 4.8 to 17.0%, 5.5 to 18.2%, and 4.2 to 17.1% of gated trypomastigotes, respectively.

Figure 3 shows overlay FL1 histograms representing the pattern of fluorescent parasite distribution obtained with sera from treated patients. Each profile presented in Fig. 3 was from a serum sample in which the PPFP value was very close to the mean of the PPFP of the group. The histogram pattern of the serum sample from the DIS patient was closely related to the one of the serum sample from the CUR patient, and they overlapped in the negative region of the graph. The histogram pattern of the serum sample from the TNC patient was distinct from the other two, showing minimal overlapping, with the peak being located in the positive region of the graph (Fig. 3).

Initially, 1 of 12 DIS individuals gave 26% PPFP (Fig. 4A). Sera from this DIS patient and from a TNC patient as control (Fig. 4C) were absorbed in a SYNSORB 115 column before being used to detect MBA. In the absorbed sera, the PPFP in the serum from the DIS patient changed to 13.8% (Fig. 4B) whereas the PPFP in the serum from the TNC patient did not change (Fig. 4D).

Parallel study of PPFP and percent lysis. Figure 5 is a scatter graph showing the results obtained when the same sera were tested in both the PPFP and percent lysis assays. For comparative purposes, the PPFP and percent lysis results for each individual serum sample are indicated by a connecting line. On the basis of experience with CoML, the FACScan data analysis of serum samples from 54 individuals was performed

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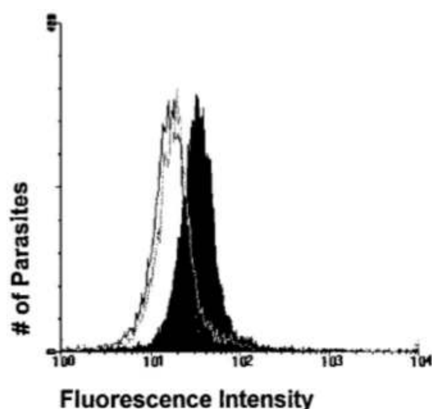

FIG. 3. FL1 histograms representing the mean pattern of fluorescent parasite distribution obtained with sera from treated patients. The histogram profile of serum from a DIS patient totally overlaps the profile of serum from a CUR patient, whereas serum from a TNC patient differs from the others, showing minimal overlapping and with the peak located in the positive region of the graph. According to the data, sera from patients with active infection following specific treatment could be distinguished from both DIS and CUR patients. These results reemphasize that MBA searching is a useful tool to monitor the effectiveness of treatment of Chagas' disease.

by establishing 20% PPFP as the cutoff between effective and ineffective treatment (Fig. 5).

Comparisons between groups showed that the DIS (mean PPFP, 8.2%) and CUR (mean PPFP, 13.1%) groups were not significantly different from each other ($P > 0.05$) or from the NC group (mean PPFP, 9.3%; $P = 0.431$). We could not find significant differences between the NT (mean PPFP, 60.1%) and TNC (mean PPFP, 45.2%) patient groups ($P = 0.168$). However, we did detect significant differences between groups in which treatment had been effective (DIS and CUR) and

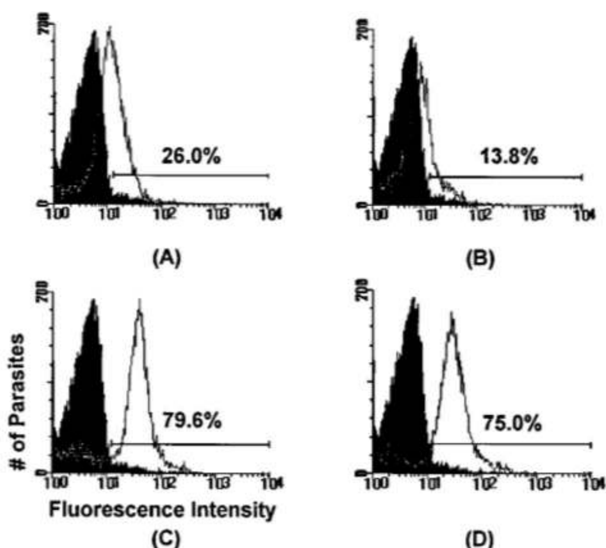

FIG. 4. Histograms representing PPFP before (A and C) and after (B and D) the SYNSORB 115 absorbing assay. Each panel presents the results of an overlay FL1 histogram by using the test serum (blank area) and the FITC-conjugated anti-human IgG control (solid area). The PPFP of a serum sample from a DIS patient (A) changed after the use of a SYNSORB 115 column (B), whereas the PPFP of a serum sample from an NT patient (C) did not change (D). This result confirms the expectation that detection of MBA in that particular serum sample from a DIS patient was due to cross-reactive galactosyl-protein epitopes, as previously reported (6), and can be abolished by absorption.

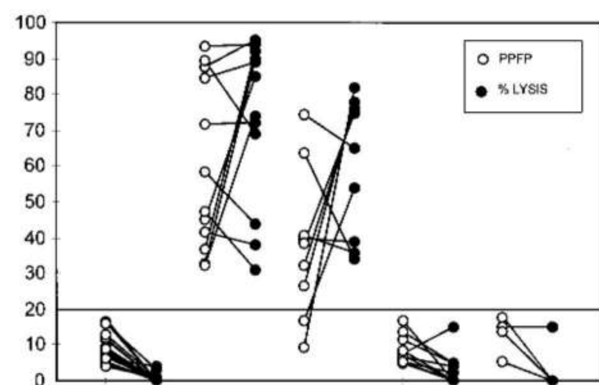

FIG. 5. Scatter graphs showing PPFP and percent lysis ranges for the groups analyzed. For comparative analysis, the PPFP and percent lysis for each serum sample tested are indicated by connecting lines. Using 20% as a cutoff between effective and ineffective treatment, we identified that all NT patients could be distinguished from both DIS and CUR patients as well as from NC individuals. However, two TNC patients fell below the cutoff.

patient groups which were either untreated or treated but not cured (NT and TNC) ($P < 0.001$).

DISCUSSION

Several clinical analyses of the efficacy of Chagas' disease treatment with nitroheterocyclic drugs have been carried out in different parts of South America. Studies concerned with the establishment of a cure criterion following specific treatment remain inconclusive. In this context, a 10-year follow-up study has been recently reported (7). This study showed that after specific treatment, some patients are regularly negative in CoML assays but only a small percentage of treated patients presented negative results in CSA detection. Galvão et al. (7) argued that repeatedly negative CoML and HE tests after chemotherapy indicate successful cure of *T. cruzi* infections, despite continuing positive results obtained by conventional serology. The reason for the continuing presence of CSA in the serum of cured patients is still an open question. However, a number of hypotheses have been put forward to explain this, such as the presence of residual parasite antigens following treatment (3), the influence of parasite mimic epitopes expressed by anti-idiotypic antibodies (9), and the cross-reactivity of anti-galactosyl α(1-3) galactose antibodies with *T. cruzi* epitopes (8).

A well-recognized feature of the immune response to parasitic infections, including *T. cruzi*, is the production of specific antibodies involved in both resistance to the parasite (10) and the morbidity associated with the chronic phase of infection (9). Because LA but not CSA are associated with active infection (10) and because LA disappear from the serum of some patients after specific chemotherapy, assaying for the presence of LA should be a useful method of monitoring the efficacy of treatment of Chagas' disease.

We report here a new approach to clearly identifying the binding of antibodies to live parasites and easily establishing a relationship between either the presence or absence of these antibodies in human serum and the effectiveness of treatment. We have designated these anti-live trypomastigote antibodies MBA to avoid confusion with LA detected in the CoML.

Our results show, for the first time, the applicability of flow cytometry to the investigation of anti-live trypomastigote antibody binding. Using this method, we examined sera from noninfected individuals and patients who had been followed up

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TABLE 2. Comparative study between flow cytometry and CoML methods[a]

PPFP result	% Lysis result	
	+	−
+	19	0
−	2	33

[a] The statistical McNemar test was used.

after treatment, classified into NC, NT, TNC, DIS, and CUR groups on the basis of CSA and LA detection and serial hemocultures (Table 1).

The CoML and flow cytometry techniques were compared by using the McNemar statistical test, and the results are shown in Table 2. Despite the variability found in the percentages (PPFP versus percent lysis), the 20% cutoff between positive and negative assays led to a 95% coincident classification in relation to efficacy of treatment ($P = 0.5$).

We attributed the nonspecific binding found in one serum sample from a DIS patient to the presence of natural anti-Gal antibodies, which, as previously demonstrated (8), are able to bind to the surface of trypomastigotes. Recent papers (1, 2) show that anti-α-Gal antibodies present in patients with chronic Chagas' disease are similar but not identical to the natural anti-Gal antibodies present in humans and Old World monkeys. Thus, in some treated DIS patients, natural anti-Gal antibodies may persist whereas the anti-Gal antibodies induced by *T. cruzi* structures disappear. Taken together, the PPFP (26%) detected could be due to cross-reactive galactosyl-protein epitopes. We eliminated this nonspecific binding by using a SYNSORB 115 column, thus demonstrating that absorbed serum did not bind to live trypomastigotes (Fig. 4B). We were also able to show that SYNSORB 115 treatment has no influence on MBA detected in one serum sample from an NT patient (Fig. 4C and D).

In this work, we analyzed 54 serum samples, comparing percent lysis with PPFP (Fig. 5). The sensitivity of flow cytometry was much higher than that of CoML, as shown by the optimum dilutions used, 1:256 and 1:4, respectively. Also, it is important to consider the capacity of flow cytometry, which counts 8,000 parasites per assay, improving confidence in the percentage data. According to flow cytometer analysis, sera from patients with active infection (NT and TNC) could indeed be distinguished from sera from both DIS and CUR patients as well as from NC individuals. However, for some patients, CoML gave a higher percentage of parasites lysed than did PPFP (Fig. 5). This led to two TNC patients falling below the cutoff level in the PPFP. This difference, in some cases, could be because the two assays have different reaction times. That is, the binding of complement-fixing antibody could be a faster process than the 60-min incubation period used for the FITC-conjugated antibodies. This would mean that the PPFP test is more adversely affected by parasite membrane shedding. We are now testing to see if shorter incubation times at lower temperatures will resolve this question.

By comparative analysis of PPFP with sera from NT and TNC patients, we found that TNC patients have lower levels of MBA, although this was not statistically significant (Fig. 5). This phenomenon might be a reflection of these patients entering the DIS group after different periods. We will be able to test this possibility easily by monitoring these patients following specific chemotherapy.

The results presented here could be used to predict efficacy of treatment and are strongly correlated with the classification based on CoML (Fig. 5). This significant correlation between the new immunoassay and CoML indicates that the new assay might be a useful indicator of the efficacy of specific treatment of Chagas' disease. In conclusion, we suggest that flow cytometry can be a powerful method for linking MBA to efficacy of treatment in the context of Chagas' disease. In addition, these findings strongly support previous suggestions that negative results in LA detection tests are an important diagnostic marker for assessing the efficacy of specific therapy in human *T. cruzi* infection.

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