

High Resolution of *Trypanosoma cruzi* Amastigote Antigen in Serodiagnosis of Different Clinical Forms of Chagas' Disease

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The serodiagnosis of Chagas' disease, a highly prevalent disorder in South American countries, is usually made by the detection of antibodies to *Trypanosoma cruzi* epimastigote antigen. In this study, we assess the diagnostic performance of the immunofluorescence test with *T. cruzi* (Y strain) amastigote antigen from an LLC-MK₂-infected cell supernatant in comparison with a test with the conventional epimastigote antigen. A total of 238 serum samples from patients in the acute and chronic phases of the disease, with the chronic indeterminate, cardiac, and digestive forms, and from nonchagasic individuals were tested for the presence of immunoglobulin G (IgG), IgM, and IgA antibodies. The reactivity of the amastigote antigen in terms of geometric mean titers was 2 to 4 times higher than that of the epimastigote antigen. Clear-cut results were obtained with the amastigote antigen, with no overlapping of true and false positives. IgG antibodies to amastigotes were found in all patients with Chagas' disease, whereas all sera from nonchagasic patients were negative, except for those from patients with visceral leishmaniasis, in which 63% cross-reactivity was observed. IgM antibodies to amastigotes were detected in 100% of sera from patients with acute Chagas' disease and in 7.5% of sera from patients with chronic Chagas' disease, whereas IgA antibodies were found in 60% of sera from patients in the acute phase and in 33% of sera from patients in the chronic phase. Despite the cross-reactivity observed with sera from visceral leishmaniasis patients, the IgG immunofluorescence test with the amastigote antigen had the highest sensitivity, specificity, and efficiency. No relationship was observed between the class-specific antibodies or their titers and the clinical forms of patients in the chronic phase. Amastigotes from the cell culture supernatant proved to be useful as an alternative antigen to epimastigotes because of their high resolution in the serodiagnosis of Chagas' disease.

Chagas' disease is widespread in Central and South American countries, where it affects about 16 to 24 million people, and approximately 70 million are at risk of acquiring this disease (21). Acute chagasic manifestations often subside with the onset of immunity (6) and are followed by many years of a chronic asymptomatic infection. About 10 to 20% of chronically infected individuals may develop cardiomyopathy and/or megasyndrome of the digestive system (30). At present, *Trypanosoma cruzi* epimastigote forms are utilized as antigens for the serodiagnosis of Chagas' disease (13). Primavera et al. (29), reviewing the assessment of different trypanosome antigens for diagnostic purposes, found that epimastigotes have been extensively used (84%) in relation to use of trypomastigotes (9%) and amastigotes (7%). Nevertheless, the trypomastigote and amastigote antigens are thought to be more convenient, since they are predominant developmental forms in vertebrate hosts.

Although amastigote antigens from different in vitro (2, 14, 20, 25, 33) and in vivo (15, 29) sources have been reported to be more suitable for the serologic diagnosis of Chagas' disease, they present controversial results. These discrepant results may be caused in part by some antigenic differences between in vitro- and in vivo-derived amastigotes, as recently shown by Oliveira da Silva et al. (26) in an agglutination test with lectins.

However, authors (2, 14, 33) who tested amastigotes from infected tissue culture supernatants as antigens in serodiag-

nostic assays for Chagas' disease agree that specific antibodies exhibited greater reactivity with these intracellular parasite antigens compared with that of epimastigotes. In addition, they were more readily visualized in the indirect immunofluorescence (IIF) test. Araújo and Remington (3) and, more recently, Andrews et al. (1) have shown that this high reactivity is related to a double-band epitope with a 70,000 to 84,000 M_r range located on the surface of amastigote forms.

However, a better elucidation of whether serologic tests carried out with in vitro amastigote antigens may be more sensitive and specific than tests with epimastigote antigens is still needed.

Thus, in this study, we evaluated the diagnostic features of the immunofluorescence test with amastigote antigen to determine the presence of immunoglobulin G (IgG), IgM, and IgA antibodies in different clinical forms of Chagas' disease.

MATERIALS AND METHODS

Antigen. *T. cruzi* amastigote forms were harvested from a continuous LLC-MK₂ (rhesus monkey kidney) monolayered cell culture maintained in 199 medium supplemented with 2% fetal calf serum at 37°C as described by Umezawa et al. (32). On the 8th to 9th day after cell infection, amastigotes were collected from the supernatant and washed three times in 10 mM phosphate-buffered saline (PBS; pH 7.2) by centrifugation at 3,500 × g for 15 min at 4°C. The final suspension, having less than 5% trypomastigotes as contam-

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inants, was fixed with 2% formalin in PBS. Epimastigote forms were obtained in LIT medium as originally described (7). The parasites were washed three times in PBS and fixed with 2% formalin in PBS.

Serum samples. Sera were collected from 238 chagasic and nonchagasic patients with confirmed clinical and serologic diagnoses.

The sera from chagasic patients in the acute phase were collected from 10 patients 1 to 2 months after infection, as described elsewhere (31). Acute-phase patients were diagnosed on the basis of clinical symptoms and positivity for anti-*T. cruzi* IgM antibodies and xenodiagnosis (31).

The 120 chagasic patients in the chronic phase were classified according to clinical data obtained by electrocardiography and radiology (30) as follows: (i) indeterminate form ($n = 50$), patients without clinical symptoms and with normal electrocardiograms; (ii) cardiac form ($n = 50$), patients presenting cardiomyopathy associated with cardiac failure, cardiomegaly, and normal electrocardiograms; and (iii) digestive form ($n = 20$), patients with digestive symptoms or radiographic abnormalities but with a normal electrocardiogram. The sera were previously submitted to the complement fixation and passive hemagglutination tests in the Epidemiology Laboratory of SESI, São Paulo, Brazil (data not shown).

The nonchagasic patients ($n = 108$) included the following: (i) healthy controls ($n = 71$) and (ii) patients with unrelated infections ($n = 37$; 10 with syphilis, 9 with malaria, 5 with connective tissue disease with high anti-nuclear antibody titers, 5 with rheumatoid arthritis, and 8 with active visceral leishmaniasis). The samples were collected at Fundação Hemocentro de São Paulo and at Institut Adolfo Lutz, where the patients with no evidence of Chagas' disease were serologically diagnosed.

All sera were stored at -20°C after inactivation at 56°C for 30 min.

IIF test. The IIF test was carried out as previously described (8). The sera were serially diluted in PBS from 1:5 to 1:10,240. Fluorescein-labeled specific anti-human IgG and IgA (affinity purified, γ and α chain specific; Biolab Diagnostica SA, RJ) and IgM (affinity purified, μ chain specific; Hyland Division of Travenol Laboratories, Inc.) were diluted to 1:900, 1:100, and 1:100, respectively, in Evans blue-PBS. To avoid rheumatoid factor interference in the IgM IIF assay, all sera (diluted 1:5) were absorbed with aggregated human gamma globulin (10% [vol/vol]) (10). The same positive and negative controls were always included in the tests.

Statistical analysis. The sensitivity, specificity, and efficiency of the IIF with amastigote and epimastigote antigens were calculated as described by Galen and Gambino (19). The agreement kappa index (KI) (18), its statistical analysis (24), and the KI rank (17) were estimated. In the analysis among groups and within groups, the titers were transformed to \log_{10} (negative values were considered equal to 1) and computed by one-way analysis of variance with 95% binomial confidence intervals, and this analysis was confirmed by the Kruskal-Wallis rank test (22). To verify the association between tests, the data were analyzed by the Spearman rank correlation (23).

RESULTS

The amastigote antigen used here displayed considerably stronger fluorescence staining intensity than the epimastigote antigen, as illustrated in Fig. 1. The conventional

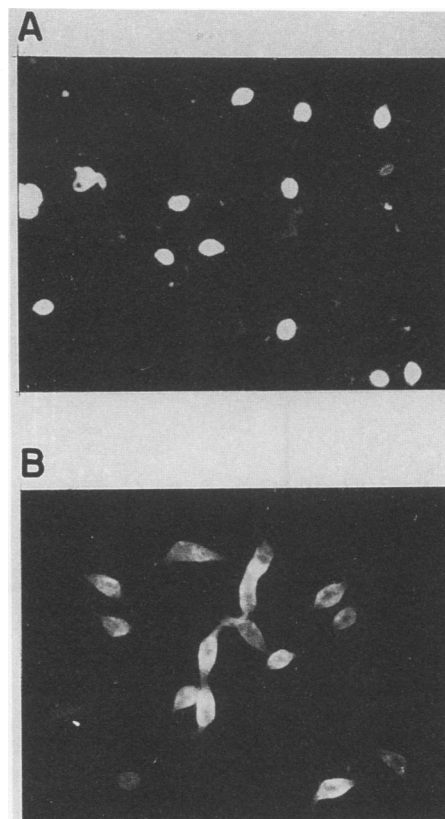


FIG. 1. Comparison of the reactivities of amastigote (A) and epimastigote (B) forms of *T. cruzi* in the IIF test.

epimastigote IIF (IIF-E) was utilized as a reference test.

The individual results obtained with amastigote IIF (IIF-A) and IIF-E for the detection of class-specific antibodies in the different groups are illustrated in Fig. 2. Analysis of variance by ranks showed that the titers of IgG antibodies from chagasic patients were significantly different from those of nonchagasic individuals in the IIF-A and IIF-E tests (see Fig. 2 and 4) (obtained $F = 1.94$; $P < 0.0001$).

The geometric mean titers (GMT) of IgG antibodies found by IIF-A and IIF-E in the acute chagasic group differed statistically from those of the cardiac and indeterminate chronic groups but not from those of the chronic digestive group (Fig. 2).

The GMT of IgG antibodies detected by IIF-A in the chronic chagasic groups was 1,220, a value 4.3 times higher than the 282 GMT value provided by IIF-E. In the acute chagasic group, the GMT of IgG, IgM, and IgA antibodies detected by IIF-A were 6.9, 3.5, and 2.1 times higher, respectively, than the GMT given by IIF-E.

The higher reactivity of IgG antibodies to amastigotes than to epimastigotes is illustrated in Fig. 3 in terms of ratios between their titers. Only 3% of the serum samples from chagasic patients showed anti-amastigote and anti-epimastigote antibody titer agreement, while in the remaining 97%, the anti-amastigote antibodies presented 2- to 32-times-higher titers than anti-epimastigote antibodies.

The cutoff titers for each IIF assay were selected to provide the highest diagnostic efficiency (1.000), allowing a better discrimination between chagasic and nonchagasic

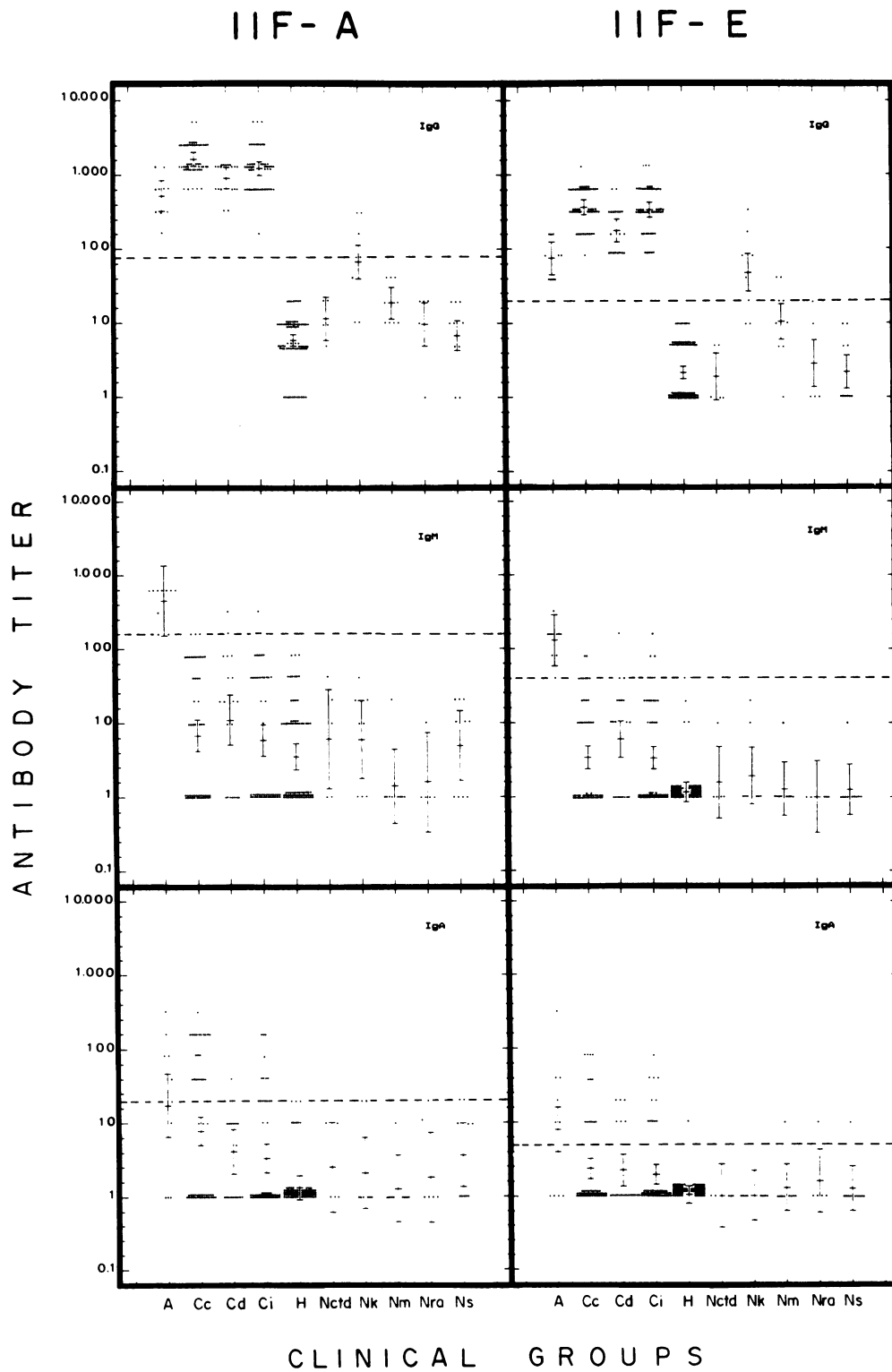


FIG. 2. Results of the IIF test obtained in the study of 130 sera from Chagas' disease patients (A, acute; Cc, chronic cardiac; Cd, chronic digestive; Ci, chronic indeterminate) and 108 sera from nonchagasic patients (H, healthy individuals; Nctd, connective tissue disease; Nk, visceral leishmaniasis; Nm, malaria; Nra, rheumatoid arthritis; Ns, syphilis) in the search for IgG, IgM, and IgA antibodies. Bars represent the intervals of GMT, and the dashed lines represent the cutoff of the test.

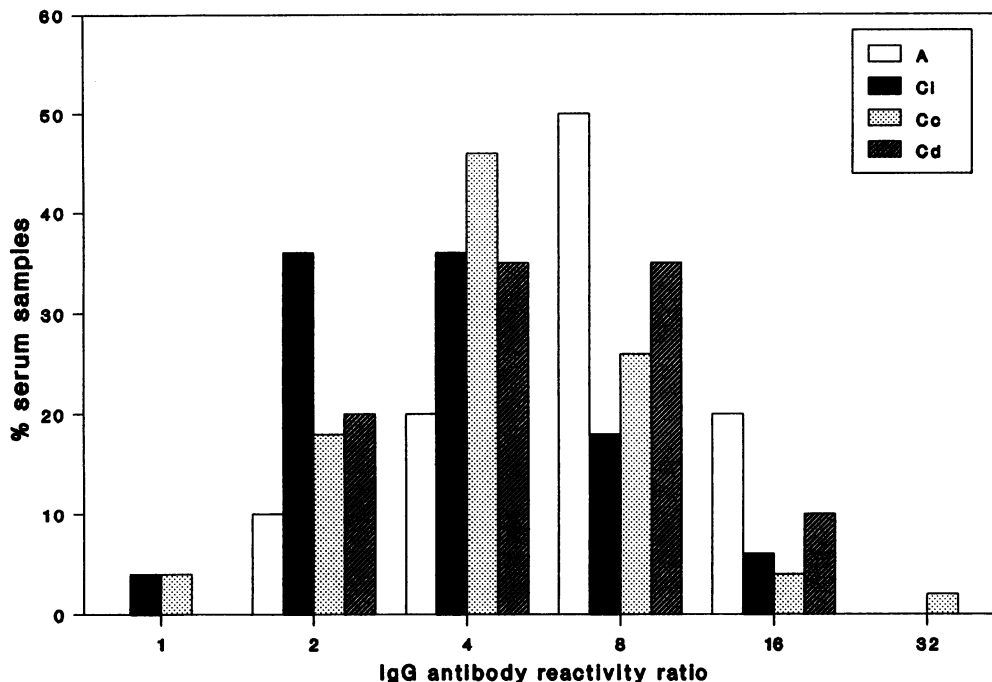


FIG. 3. Frequency of the reactivity ratio between IgG anti-amastigote and anti-epimastigote antibody titers in chagasic patients. A, acute; Ci, chronic indeterminate; Cc, chronic cardiac; Cd, chronic digestive.

infections. The cutoff titers chosen for IIF-A for the detection of IgG, IgM, and IgA antibodies are indicated in Fig. 2.

Under the present conditions, the IIF-A assay demonstrated better diagnostic resolution, since the nonchagasic patients displayed antibody titers remarkably different from those of the chagasic group when visceral leishmaniasis patients were excluded compared with those of the IIF-E assay in which some antibody titers overlapped (Fig. 4).

The values of sensitivity, specificity, efficiency, agreement KI, KI rank, and the statistical validity of KI values are expressed in terms of the obtained Z (Z_o), which permitted a comparison with the critical Z (1.96) at the 95% confidence interval (see Table 2).

The diagnostic performance of IIF-A and IIF-E for the detection of IgG antibodies did not differ statistically, with their KIs ranking as almost perfect. However, the values obtained for IgM and IgA in the IIF tests showed no statistical significance, since the number of serum samples from patients with acute chagasic infection was small ($n = 10$), in contrast to that of chronic chagasic plus nonchagasic patients ($n = 228$).

IgG, IgM, and IgA antibodies were found in chagasic patients, and in some nonchagasic patients these antibodies were also detected by the IIF-A and IIF-E tests, as shown in Table 1.

In all sera from chagasic patients, IgG antibodies were detected by IIF-A and IIF-E. The IgM antibodies were found in all sera from patients in the acute phase by both tests, but these antibodies were also detected in a small proportion of sera from patients in the chronic phase, i.e., 7.5% by IIF-A and 15% by IIF-E. IgA antibodies were detected in 60% of sera from patients with acute chagasic infection by both tests, whereas in 33% of sera from patients in the chronic phase, IgA antibodies were also detected by IIF-A, and IgA antibodies were detected in 25% of sera by IIF-E.

In nonchagasic patients, only 4.6% of sera were positive in the IIF-A for IgG antibodies, corresponding to the patients with visceral leishmaniasis, against 10.2% positive for the IIF-E which comprised the patients with visceral leishmaniasis, malaria, and rheumatoid arthritis. Nonspecific IgM antibodies were not detected by IIF-A or IIF-E, but IgA antibodies were detected in 7.4% of sera by IIF-A and in 3.7% of sera by IIF-E.

The Spearman correlation coefficient between titers of IgG antibodies against amastigotes and epimastigotes was high, $r = 0.89$ ($P < 0.0001$), in contrast to those found for IgM and IgA antibody titers, $r = 0.72$ ($P < 0.0001$) and $r = 0.50$ ($P < 0.0001$), respectively, which were lower.

DISCUSSION

In this report, we demonstrated the high resolution of in vitro-derived amastigotes in comparison with conventionally utilized epimastigotes as antigens in the IIF test. The ability of the IIF-A test to detect IgG, IgM, and IgA antibodies was verified in chagasic patients with the acute and chronic forms of the disease (indeterminate, cardiac, and digestive) and in a control group comprising clinically healthy individuals and patients with unrelated diseases, including visceral leishmaniasis.

Corroborating previous data (2, 14, 33), a higher antigenicity of amastigotes was observed, and the parasite displayed a sharp and strong membrane fluorescence, in contrast to epimastigotes which revealed less-pronounced fluorescence staining.

Thus, amastigotes seem to quantitatively expose more epitopes on their membrane than epimastigotes, in addition to possible qualitative antigenic differences (Fig. 1).

The fluorescence pattern of amastigotes from LLC-MK₂ culture cells was similar to that of amastigotes from LIT liquid culture medium obtained by Andrews et al. (1). In a

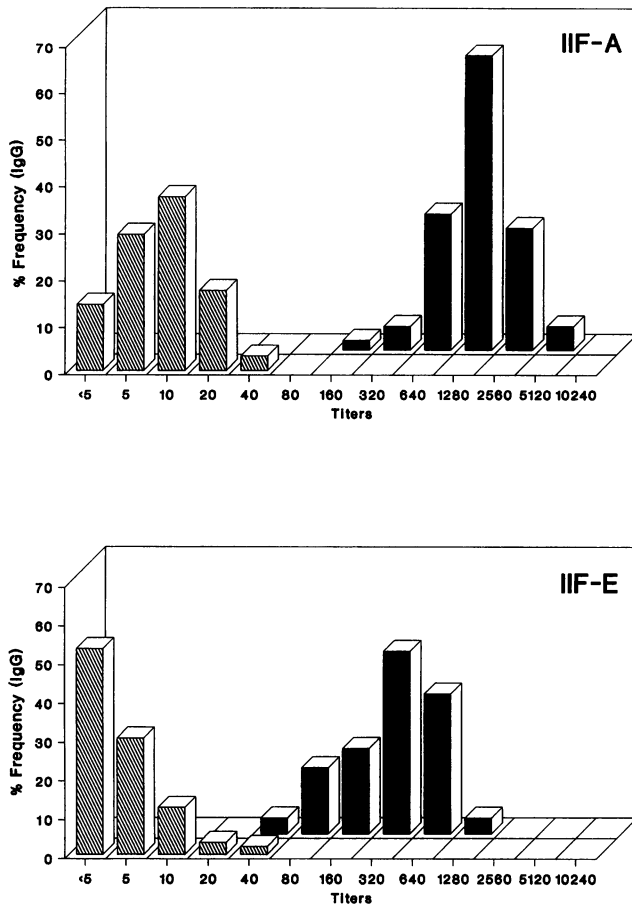


FIG. 4. Frequency of IgG titers in the groups of chagasic ($n = 130$; ■) and nonchagasic ($n = 100$; ▨) patients determined by IIF-A and IIF-E, excluding visceral leishmaniasis patients.

preliminary study by our group, the similarity of the two types of amastigotes could be verified on the basis of the same reactivity displayed by a monoclonal antibody (2C2) produced against an epitope of LIT-derived amastigote (data

not shown). However, the absence of cross-reactivity observed by Andrews et al. (1) in a few leishmaniasis cases needs to be confirmed, taking into account that this cross-reactivity with leishmaniasis is partial, as observed by us (Table 2) as well as by Primavera et al. (29).

In all patients, IgG antibodies were detected by IIF-A, showing no cross-reactivity with unrelated diseases, except visceral leishmaniasis. Cerisola et al. (14) and Araújo and Guptill (2) also found maximum sensitivity and specificity, although they worked with a smaller number of unspecified clinical forms of Chagas' disease and did not include leishmaniasis in their control groups.

Nevertheless, the antigenicity of amastigotes provided by the LLC-MK₂ cell culture differs from that of amastigotes from HeLa cells (14) and of amastigotes obtained from L929 cells (2), if we consider the interactions of these antigens with IgG antibodies. For instance, the GMT of antibodies against the amastigotes studied here was 1,220, and the GMT values calculated from available data provided by those authors (2, 14) were 4,945 and 640, respectively.

The reactivity of IgG antibodies to amastigotes from LLC-MK₂ cells was found (Fig. 3) to be similar to that reported by Cerisola et al. (14) in a study of amastigotes from HeLa cells.

Our GMT data for IgG antibodies are also close to those obtained with amastigotes from an in vivo system consisting of skeletal muscle sections from *T. cruzi*-infected mice, which were 3 to 4 times higher than those of anti-epimastigote antigens (15). However, the data reported by Araújo et al. (GMT = 640 [2]), who worked with amastigotes from L929 cells, do not agree with our results.

Thus, the microantigenic diversity of in vitro-derived amastigotes seems to be related to *T. cruzi* strain, host cell line, or supplemented medium, as is also the case for some bacterial models (5).

The most interesting feature of IIF-A is the absence of titer overlap, which permits the distinction of chagasic patients from nonchagasic patients (Fig. 4). This higher diagnostic resolution of the IIF-A in relation to IIF-E, as well as to other serologic tests with epimastigote antigens (12, 16), is significant. This type of serologic profile which gives high diagnostic resolution is usually seen when monoclonal or recombinant antigens are used in the tests.

TABLE 1. Positive reactivity and GMT for anti-*T. cruzi* antibodies

Patient status and diagnosis (<i>n</i>)	% Positive reaction ^a (GMT) for anti- <i>T. cruzi</i> antibodies					
	IIF-A			IIF-E		
	IgG	IgM	IgA	IgG	IgM	IgA
Chagasic (130)						
Acute (10)	100 (520)	100 (452)	60 (17)	100 (75)	100 (130)	60 (8)
Chronic (120)						
Indeterminate (50)	100 (1,228)	8 (6)	30 (3)	100 (338)	14 (3)	22 (2)
Cardiac (50)	100 (1,620)	6 (7)	42 (8)	100 (367)	16 (3)	26 (2)
Digestive (20)	100 (905)	10 (11)	20 (4)	100 (177)	15 (6)	30 (2)
Nonchagasic (108)						
Healthy individuals (71)	0 (6)	0 (3)	4 (1)	0 (2)	1 (1)	1 (1)
Connective tissue disease (5)	0 (11)	0 (6)	0 (2)	0 (2)	0 (1)	0 (1)
Malaria (9)	0 (18)	0 (1)	0 (1)	44 (10)	0 (1)	11 (1)
Rheumatoid arthritis (5)	0 (9)	0 (1)	20 (2)	20 (3)	0 (1)	20 (1)
Syphilis (10)	0 (7)	0 (5)	20 (4)	0 (2)	0 (1)	10 (1)
Visceral leishmaniasis (8)	63 (67)	0 (6)	25 (2)	75 (47)	0 (2)	0 (1)

^a Percentage of positive cases.

TABLE 2. Diagnostic performance of IIF-A and IIF-E tests for the detection of antibody isotypes

Test and antibody	Diagnosis	Sensitivity	Specificity	Efficiency	Agreement (KI) ^a	KI rank	
IIF-A	IgG	Chagasic × nonchagasic					
		Excluding VL ^b	1.000	1.000	1.000	1.000	Almost perfect
	Including VL	1.000	0.953	0.979	0.957	Almost perfect	
	IgM	Acute chagasic × chronic and nonchagasic	1.000	0.960	0.962	0.672	Substantial
IgA	Acute chagasic × chronic and nonchagasic	0.600	0.789	0.782	0.125	Poor	
IIF-E	IgG	Chagasic × nonchagasic					
		Excluding VL	1.000	0.950	0.978	0.956	Almost perfect
	Including VL	1.000	0.898	0.954	0.906	Almost perfect	
	IgM	Acute chagasic × chronic and nonchagasic	1.000	0.916	0.920	0.480	Moderate
	IgA	Acute chagasic × chronic and nonchagasic	0.600	0.850	0.840	0.185	Poor

^a Z obtained was significant (>1.96 for 0.05 confidence limit).

^b VL, visceral leishmaniasis.

IgM antibodies were also detected by IIF-E in all patients with acute Chagas' disease, confirming previous data (9, 29), in spite of the small number of sera utilized here.

As for IgA antibodies to amastigote, approximately 60% of the acute infections showed high titers, although the positive correlation with this clinical form of the disease was not statistically significant. This observation agrees with that reported by Primavera et al. (28), who did not observe a significant correlation between IgA antibodies against in vivo-derived amastigotes and the acute phase of Chagas' disease. It is relevant to emphasize that these authors also found a positive correlation between IgA antibodies and chronic digestive forms of Chagas' disease by using the same in vivo-derived amastigotes. Our results, however, demonstrated no such correlation, suggesting that amastigotes from in vitro systems are quite different from those obtained from infected animals.

Aspects related to the identification of specific LLC-MK₂ cell-derived amastigote antigens are now under investigation by Western blotting (immunoblotting) and other immunochemical techniques, as are studies to determine the possible involvement of carbohydrate cross-reactive epitopes, suggested by Avila et al. (4), and studies of cross-absorption with *Leishmania* spp., as described by Camargo and Rebonato (11).

The present data indicate that amastigotes could be helpful as an alternative antigen to conventional epimastigotes for screening chagasic infection because of advantages provided by amastigotes, such as higher antigenicity and better diagnostic resolution.

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