

## Immunoglobulin A Antibodies to *Trypanosoma cruzi* Antigens in Digestive Forms of Chagas' Disease

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In an attempt to find a serological marker for the diagnosis of chronic digestive forms of Chagas' disease, we compared amastigote and trypomastigote antigens obtained from immunosuppressed mice infected with *Trypanosoma cruzi* (Y strain) with conventional epimastigote antigens to search for immunoglobulin A (IgA) antibodies. A total of 255 serum samples from patients with acute and chronic (indeterminate, digestive, and cardiac) forms of Chagas' disease and with nonchagasic diseases and from healthy individuals were studied. Amastigote antigens proved to be the most adequate for our purpose, since IgA antibodies could be detected in 23 of 25 serum samples from patients with digestive forms, with relative indices of sensitivity, specificity, and efficiency of 0.920, 0.911, and 0.912, respectively. These antigens also showed high reactivity with IgA antibodies, with a geometric mean titer of 16,635 (12.7 log<sub>2</sub>). IgA antibodies were detected in 16 of 28 serum samples from patients with the acute form as well, but this clinical form is easily distinguished from the chronic form by the demonstration of IgM antibodies. Poor results were seen with trypomastigote and epimastigote antigens. The finding of IgA antibodies in about 20% of indeterminate forms and 20% of cardiac forms, although in low titers, requires further investigation to ascertain their role as an early signal of gastrointestinal lesions. In addition, the amastigote antigens described here seem more convenient for use in endemic areas than those obtained from cell cultures because of their lower cost.

In areas in which Chagas' disease is endemic, digestive forms either associated with or not associated with myocardopathies have been reported to occur in about 7 to 14% of the chronic cases (7, 17, 18). Radiologic diagnosis is of limited sensitivity, since it detects severe or moderate but not earlier phases of gastrointestinal dilatations. For this purpose, more sensitive methods are therefore required (18). Present serological tests, although very suitable for the diagnosis of *Trypanosoma cruzi* infections, provide no information on pathological differences among chronic forms of the disease (6, 13). In digestive forms, Ferreira et al. (11) found increased levels of total immunoglobulin A (IgA) in serum, but specific IgA antibodies could be detected in only about one-half of the cases by an immunofluorescence (IF) test with *T. cruzi* epimastigotes as antigens. Antigenic differences have been demonstrated between life cycle stages of *T. cruzi* (15). Trypomastigotes have been shown to be useful for the demonstration of lytic antibodies, which are considered markers of active infections (16). However, in an IF test, amastigotes have been found to be more reactive than other parasite stages (8, 10). We tried to better investigate IgA antibodies in digestive forms of Chagas' disease by using an IF test with *T. cruzi* amastigotes, trypomastigotes, and epimastigotes as antigens.

### MATERIALS AND METHODS

**Serum samples.** Serum samples were collected from 255 individuals, 135 of which had Chagas' disease, 70 of which had nonrelated infections or autoimmune diseases, and 50 of which were clinically healthy. Chagasic patients included 28 with acute (group 0), 56 with chronic indeterminate (group 1), 26 with cardiac (group 2), and 25 with digestive (group 3)

forms. All acute infections (group 0) were parasitologically confirmed by direct microscopic examinations of blood samples or xenodiagnosis. Of these cases, 7 patients were from an endemic area (J. C. Pinto Dias, Bambuí, Brazil), and the remaining 21 patients had acquired the disease via a blood transfusion in the city of São Paulo. Chronic cases were selected by one of us in the Hospital das Clínicas da Universidade Estadual de Campinas, Campinas, Brazil, and classified according to clinical, electrocardiographic, and radiological criteria. Group 1 included patients with no clinical signs or symptoms of the disease and a normal or only slightly abnormal electrocardiogram. Group 2 patients had cardiomyopathy with cardiac insufficiency, cardiomegaly, and an abnormal electrocardiogram. Group 3 included patients with radiographically detected digestive abnormalities. In 11 of the 25 cases in this group, cardiac disease was also present.

A total of 70 patients with nonrelated infections were studied: 10 with clinically active rubella; 10 with acute toxoplasmosis; 10 with malaria with patent parasitemia; 10 of different age groups and from areas not endemic for Chagas' disease with clinically active kala-azar; 10 with mucocutaneous leishmaniasis; 10 with recent syphilis; and 10 with connective tissue diseases, including 5 with antinuclear antibodies and 5 with high-titer rheumatoid factors. Of the clinically healthy individuals, 20 were selected in an area endemic for Chagas' disease, and 30 were blood bank donors. Negative serology (complement fixation, IF, and indirect hemagglutination [HA] tests) for Chagas' disease was seen in all nonchagasic individuals, except for a few of the patients with kala-azar. Patients with other diseases had positive results for their respective serodiagnoses.

Serum samples were obtained from venous blood and kept at -20°C until use.

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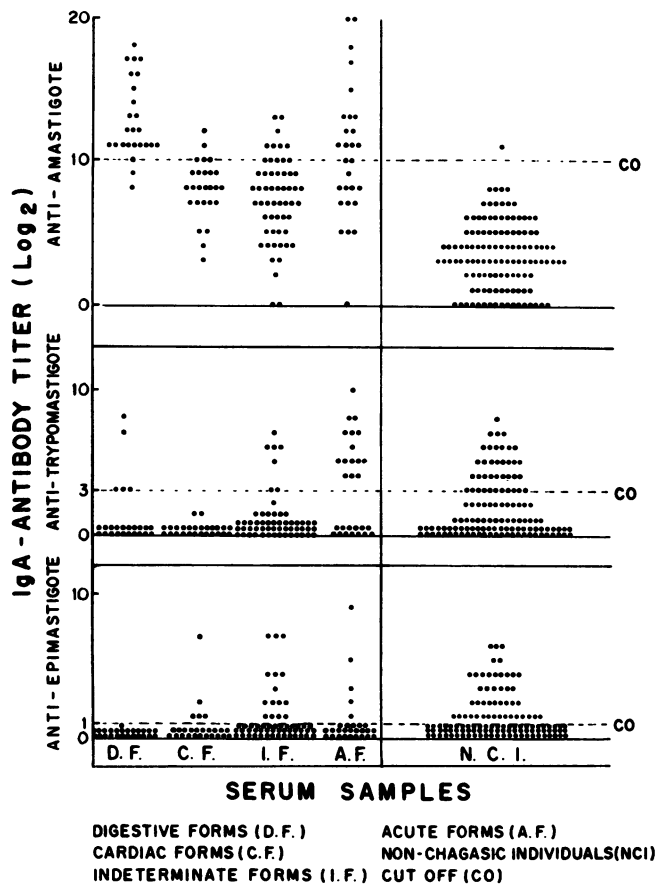


FIG. 1. Distribution of IgA antibody titers for epimastigote, trypomastigote, and amastigote *T. cruzi* antigens in chagasic and nonchagasic patients.

**Serological tests for Chagas' disease.** All sera were assayed by complement fixation, IF for IgG antibody, and indirect HA tests for Chagas' disease as described previously (5). Serum samples from chagasic patients were also tested by an enzyme-linked immunosorbent assay for IgG antibodies (23). Also, IF for IgM antibody and polysaccharide HA tests were performed to detect IgM antibodies in the acute cases (14). To avoid interference of rheumatoid factors in the IgM IF test, we pretreated serum samples which were positive in a rheumatoid factor latex agglutination test (Behringwerke A.G.) with aggregated human gamma globulin (22). In the IF tests, an anti-IgA,  $\alpha$ -chain-specific conjugate was used. To ensure the specificity of results with amastigote antigens, we retested positive sera in the IF test after they were absorbed with sheep erythrocytes and mouse liver and kidney powders.

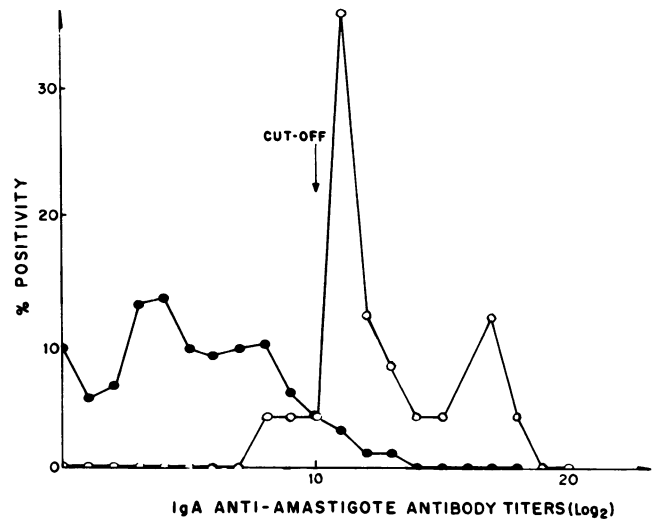


FIG. 2. Frequency, determined by the IF test, of anti-amastigote IgA antibody titers in patients with digestive forms of Chagas' disease (O) and in patients with other chronic forms of Chagas' disease, patients with unrelated pathologies, and healthy individuals (●).

**Parasite antigens.** Parasite antigens were prepared from various *T. cruzi* (Y strain) life cycle stages. Epimastigote antigens were obtained as described previously (4). Bloodstream trypomastigotes were collected from immunosuppressed mice (9) inoculated with  $10^5$  trypomastigotes 4 h after injection of the immunosuppressor drug. Blood was taken on day 7 postinfection, at the peak of parasitemia, by heart puncture, defibrinated with glass beads, centrifuged at  $1,000 \times g$  for 1 min at room temperature, and left for 10 min at  $37^\circ\text{C}$  before removal of the supernatant plasma. This plasma, rich in parasites, was then centrifuged at  $1,000 \times g$  for 15 min at  $4^\circ\text{C}$ . Sedimented trypomastigotes were washed twice in medium 199 (GIBCO Laboratories) with 1% bovine serum albumin (fraction V; Sigma Chemical Co.). Parasites were then processed as described for epimastigotes (4). Intracellular amastigotes were taken from the spleen and liver of infected mice, purified by two-step discontinuous gradient centrifugation as described previously (1), and processed as described for epimastigotes (4).

## RESULTS

Serum samples from chagasic patients were all reactive in the enzyme-linked immunosorbent assay, and most were reactive in the three other conventional tests, IgG IF, HA, and complement fixation, or at least in two of these. Samples from patients with other diseases were nonreactive, except for five cases of kala-azar. The 28 serum samples from

TABLE 1. IgA IF test results with *T. cruzi* amastigote, trypomastigote, or epimastigote antigens in individuals with chronic digestive forms (DF) of Chagas' disease and in nonchagasic individuals (NCI)

Antigen	Sensitivity <sup>a</sup>	Specificity <sup>a</sup>	Efficiency <sup>a</sup>	Cutoff titer (log <sub>2</sub> )	Log <sub>2</sub> GMT $\pm$ SD for:	
					DF	NCI
Amastigote	0.920	0.911	0.912	10	12.7 $\pm$ 3.8	5.1 $\pm$ 3.0
Trypomastigote	0.200	0.777	0.714	3	1.4 $\pm$ 2.0	1.6 $\pm$ 1.8
Epimastigote	0.000	0.678	0.604	1	0.0	1.2 $\pm$ 1.5

<sup>a</sup> Reported as relative indices.

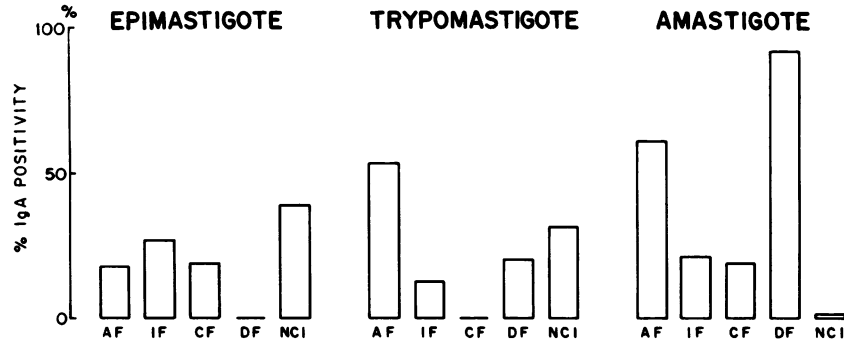


FIG. 3. Positive IgA IF tests with *T. cruzi* epimastigote, trypomastigote, or amastigote antigens in individuals with acute (AF), indeterminate (IF), cardiac (CF), and digestive (DF) forms of Chagas' disease and in nonchagasic individuals (NCI).

patients with acute Chagas' disease were reactive in the IgM IF and polysaccharide HA tests. The results of the IF for IgA antibody tests with epimastigotes, trypomastigotes, or amastigotes are shown in Fig. 1.

Our purpose was to define an immunological marker for the digestive forms of Chagas' disease. Cutoff titers (Fig. 1) were established for each test to separate, with the highest possible efficiency, patients with digestive forms from patients with all other chronic forms of Chagas' disease, other infections, and no infections (normal individuals). Patients with acute forms were not included in either group since, although they have IgA-reactive antibodies, they can be distinguished from patients with other forms of Chagas' disease because they have anti-*T. cruzi* IgM antibodies in their sera. Figure 2 depicts the frequency of titers for both groups in the IgA IF test with amastigotes. The best cutoff observed between the left curve corresponding to the digestive forms and the right curve corresponding to all other forms except for acute forms is also shown.

Table 1 indicates sensitivity, specificity, and efficiency indices for the selected cutoffs. As shown in Fig. 1 and 3,

antiamastigote IgA antibodies in the digestive forms had a geometric mean titer (GMT) of 16,635 and titers of 2,560 or higher in 23 of 25 cases. In contrast, GMTs were 394 and 597, respectively, for the indeterminate and cardiac forms, both of which were represented by titers of 2,560 or higher in only 20% of cases. In the group of nonchagasic individuals, 99.2% had negative results and a GMT of 86.

In the IgA IF tests with epimastigotes and trypomastigotes, much lower titers were seen, and no significant differences were observed among individuals with the different forms of Chagas' disease and nonchagasic individuals. Table 2 indicates the reactivities seen for serum samples from patients in the several groups studied in relation to the established cutoffs.

DISCUSSION

The data presented here show a close positive correlation between the digestive forms of chronic Chagas' disease and high titers of anti-amastigote IgA antibodies in serum. Titers between 2,560 and 655,360 were seen in 23 of 25 cases, with a GMT of 16,635.

In patients with digestive tract involvement, the finding of IgA antibodies in serum seems to be significant and might constitute an early signal of active mucosal lesions of, for example, mucocutaneous leishmaniasis (12, 21) and mycoses with mucous membrane or respiratory tract implications (3). However, specific IgA antibodies in serum are not observed in advanced cases of mucocutaneous leishmaniasis with a long history of destructive lesions (12). The persistence of IgA antibodies in the digestive forms of chronic Chagas' disease is thus an unexpected finding. Although present in lower titers, anti-amastigote IgA antibodies were also observed in significant titers in about 20% of patients with indeterminate forms. These patients should be closely monitored to verify whether the disease will progress with the onset of digestive disturbances. For the cardiac form, about 20% of the patients were also positive for anti-amastigote IgA antibodies; a few already had digestive impairment.

Amastigote antigens proved to be much more sensitive for revealing IgA antibodies than the other *T. cruzi* antigens. Amastigotes from *T. cruzi*-infected mice were not only easier to obtain but also had a higher reactivity than those from cell cultures (2). In tests with the former, serum samples were previously absorbed with mouse liver and kidney powders to avoid possible interference by animal tissue components.

The presence of IgA antibodies in the acute phase of Chagas' disease, as observed here, was an expected finding, since these class-specific antibodies have pathogenic signifi-

TABLE 2. Positive IgA IF tests with amastigote, trypomastigote, or epimastigote antigens in serum samples from chagasic and nonchagasic patients

Diagnosis	No. of serum samples	No. of positive results with:		
		Amastigotes	Trypomastigotes	Epimastigotes
<b>Chagas' disease</b>				
Acute	28	16	15	5
Chronic				
Digestive	25	23	5	0
Cardiac	26	5	0	5
Indeterminate	56	12	7	15
<b>Other diseases</b>				
Rubella	10	0	2	0
Toxoplasmosis	10	0	2	1
Malaria	10	0	0	0
Kala-azar	10	0	0	1
Mucocutaneous leishmaniasis	10	0	7	0
Syphilis	10	0	9	10
Connective tissue diseases	10	0	3	4
None (normal individuals)	50	1	15	29

icance and diagnostic value in several nonrelated acute infections (12, 19, 20). The results shown in Table 2 suggest that IgA antibodies in acute Chagas' disease differ in specificity from IgA antibodies related to the digestive forms. The former antibodies reacted in a similar way to both amastigotes and trypomastigotes, while the latter had a much higher reactivity with amastigotes. Such aspects are now under investigation with the help of Western blotting (immunoblotting) techniques. The high cross-reactivity of IgA antibodies from patients with syphilis or mucocutaneous leishmaniasis against trypomastigote and epimastigote antigens might be explained by ubiquitous antigenic determinants common to *Treponema pallidum* and *Leishmania* sp. amastigotes but absent from *T. cruzi* amastigotes.

Confirmation of the development of digestive impairment in patients with the indeterminate forms by a positive anti-amastigote IgA test may provide a valuable alternative and serve as an early aid in the diagnosis of digestive forms of Chagas' disease, usually limited to radiologic procedures.

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