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High Specificity of *Trypanosoma cruzi* Epimastigote Ribonucleoprotein as Antigen in Serodiagnosis of Chagas' Disease

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We assessed the performance of an enzyme-linked immunosorbent assay (ELISA) with the *Trypanosoma cruzi* epimastigote ribosomal fraction (Tulahuen and Y strains) in order to improve the diagnostic specificity of the test. A total of 100 serum samples from patients with chronic Chagas' disease from Brazil and Argentina were studied. Sera from 116 patients, without Chagas' disease, including 10 with active mucocutaneous leishmaniasis and 20 with visceral leishmaniasis, were used as controls. Immunoglobulin G (IgG) antibodies against the ribosomal fraction (ribonucleoproteins [RNPs]) in the ELISA were found in 97% of samples from patients with Chagas' disease. A total of 99% of the sera from patients without the disease were negative, including sera from patients with mucocutaneous and visceral leishmaniases. The distribution of IgG isotypes in randomly chosen serum samples was determined by ELISA; IgG1 and IgG3 were predominant (100% exhibited IgG1 and 85% exhibited IgG3, and 50% also presented the IgG2 isotype. The distribution of the IgG subclasses was confirmed by the Western blot (immunoblot) technique. When total IgG was assayed by Western blot assay, no correlation was found between the pattern of serum reactivity and the clinical features of the patients with Chagas' disease. Therefore, no typical profile of polypeptide recognition could be associated with any clinical form of Chagas' disease (cardiomyopathy or megaviscera). Our results showed that sera from patients with Chagas' disease react with ribosomal antigens and display a typical profile of IgG isotypes (IgG1 plus IgG3). The RNP ELISA seems to have improved specificity compared with those of routine techniques such as the indirect immunofluorescence assay and hemagglutination because it better discriminates between patients with Chagas' disease and patients without the disease. Since sera from patients with leishmaniasis failed to show cross-reactivity with this antigen, the ELISA seems useful for detecting Chagas' disease as well as confirming the nature of sera, when it is doubtful whether the patient has Chagas' disease, by the isotype distribution of IgG.

Chagas' disease, whose etiological agent is the hemoflagellate *Trypanosoma cruzi*, is a tropical parasitosis that affects a large part of the Latin American population. In many areas its geographical distribution overlaps that of leishmaniasis. Because *T. cruzi* shares antigenic determinants with members of the *Leishmania* genus, a reliable diagnosis of Chagas' disease is difficult to make where the two organisms are overlapping.

There have been several attempts to use different parasitic forms and subcellular fractions as well as recombinant proteins in serological assays for the detection of *T. cruzi*-specific antibodies (6, 8, 22, 23). Among studies directed to finding a serological marker of chronic Chagas' disease, a λ*gt*11-cloned parasite peptide (JL5) was characterized as the C-terminal portion of a *T. cruzi* ribosomal P protein and was reported to be related to heart damage (17).

On the other hand, Skeiky et al. (27) suggested that *T. cruzi* P proteins might contribute to the development of autoreactive antibodies in patients with Chagas' disease and that the appearance of anti-P autoantibodies may be explained by a similar mechanism both in patients with Chagas' disease and in patients with systemic lupus erythematosus (SLE). One of us has previously demonstrated that *T. cruzi* ribosomal antigens react specifically with sera from *T. cruzi*-infected rabbits and mice (20, 28), and recently, Bonfa et al. (3) described in patients with chronic Chagas' disease an antibody that cross-reacts with human and *T. cruzi* ribosomal proteins.

In the present study, we used an enzyme-linked immunosorbent assay (ELISA) to evaluate the usefulness of ribonucleoproteins (RNPs) from *T. cruzi* epimastigotes in order to detect antibodies in sera from Argentine and Brazilian patients with Chagas' disease. Immunoglobulin G (IgG) subclasses were determined for the purpose of discriminating the profile of Chagas' disease from the non-Chagas' disease profile, while Western blotting (immunoblotting) was performed to characterize the ribosomal antigens recognized by the sera from patients with Chagas' disease.

MATERIALS AND METHODS

Serum samples. Two hundred sixteen serum samples were collected: 100 were from patients with a confirmed clinical and serological diagnoses of Chagas' disease and the rest were from patients without the disease. The 100 serum samples from patients with chronic Chagas' disease were collected from Argentines (n = 52) and Brazilians (n = 48) living in areas where the disease is not endemic. These patients were classified according to clinical data obtained by electrocardiography and radiology as follows: (i) indeterminate form (n = 49), i.e., patients without clinical symptoms and with normal electrocardiograms; (ii) cardiac form (n = 43), i.e., patients presenting cardiomyopathy associated with heart failure, cardiomegaly, and/or abnormal electrocardiograms; and (iii) diges-

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tive form ($n = 8$), i.e., patients with digestive symptoms or radiographic abnormalities but with a normal electrocardiogram. Sera were previously subjected to passive hemagglutination (HA), ELISA with total epimastigote extract (6), and indirect immunofluorescence (IIF) and were proved to be positive by all three tests, which were performed at the Tropical Medicine Institute, São Paulo, Brazil.

Patients without Chagas' disease ($n = 116$) included 76 patients with unrelated diseases (5 with other cardiomyopathies, 5 with rheumatoid arthritis, 8 with SLE, 6 with syphilis, 8 with toxoplasmosis, 4 with rheumatic fever, 10 with malaria [half caused by *Plasmodium falciparum* and half caused by *Plasmodium vivax*], 20 with active visceral leishmaniasis, and 10 with mucocutaneous leishmaniasis). The remaining 40 subjects were healthy controls.

Serum samples were stored at −20°C after heating at 56°C for 30 min.

Antigen. RNPs were prepared as described by Sherton and Wool (25) and Sosa Miatello and Fiorotto (28). The *T. cruzi* epimastigote forms of the Tulahuen (29) and Y (26) strains were obtained from biphasic medium (13) and from LIT medium (4), respectively. Parasites obtained during the exponential growth phase were washed twice (by centrifugation at 5,000 × *g* for 10 min at 4°C) in 30 mM Tris-HCl (pH 7.0)–100 mM KCl–5 mM $MgCl_2$–3 mM $CaCl_2$ and were spun at 14,000 × *g* for 30 min at 4°C. The pellet was disrupted in a Potter-Elvejham glass Teflon homogenizer with a hypotonic solution: 10 mM Tris-HCl (pH 7.6), 10 mM NaCl, 9 mM $MgCl_2$, and 2 mM phenylmethylsulfonyl fluoride (PMSF). This suspension was centrifuged (13,300 × *g* for 30 min at 4°C), and after adding 250 mM sucrose, 10 mM $MgCl_2$, and 240 mM KCl, the supernatant was spun at 106,000 × *g* for 2 h at 4°C. The pellet was treated with detergents (10% sodium deoxycholate, 10% Tween 80 in 10 mM $MgCl_2$) and was centrifuged at 13,300 × *g* for 15 min at 4°C, and the supernatant was layered onto 500 mM sucrose and centrifuged (145,000 × *g* for 90 min at 4°C). Ribosomal particles (RNPs) were obtained from the pellet resuspended in 20 mM Tris-HCl (pH 7.4), 60 mM KCl, and 10 mM $MgCl_2$; after protein quantification (21) small aliquots of the RNPs were stored at −70°C.

ELISA. Polystyrene microtiter plates (Costar, Cambridge, Mass.) were coated at 4°C with 10 μg of RNP antigen (Tulahuen strain) per ml in 100 mM carbonate-bicarbonate buffer (pH 9.5). The subsequent steps of the ELISA protocol were performed as described by Voller et al. (31).

Twofold serial dilutions of sera (1:50 to 1:3,200) were assayed. Peroxidase-conjugated goat serum anti-human IgG (γ chain specific; Biosys Co., Compiégne, France) was used as the secondary antibody. The enzyme substrate *o*-phenylenediamine (Sigma Co., St. Louis, Mo.), 0.05% H_2O_2, and 4 N HCl were used to complete the reaction.

The test result was read at 490 nm in an automatic ELISA reader (Dynatech MR 5000).

The analysis of IgG subclasses in randomly selected sera from patients with Chagas' disease ($n = 21$) and those without the disease ($n = 9$) was carried out by ELISA with the RNP antigen at 20 μg/ml, and the sera were diluted 1:8 to 1:256 following essentially the same protocol described above (7). Mouse monoclonal antibodies specific for each human IgG isotype (Sigma) were used in the following working dilutions: anti-IgG1 (clone HP-6001), 1:3,000; anti-IgG2 (clone HP-6002), 1:2,000; anti-IgG3 (clone HP-6050), 1:5,000; anti-IgG4 (clone HP-6023), 1:2,000. These four monoclonal antibodies have been recently evaluated for their specificities by using a wide range of immunological techniques in a collaborative study sponsored by the International Union of Immunological Societies and the World Health Organization (15). Working dilutions, resulting in equivalent sensitivities for all monoclonal antibodies, were determined by the manufacturer in enzyme immunoassays with freshly prepared solutions of each human IgG subclass of 1 μg/ml.

The antigen-antibody reaction was detected with a peroxidase-conjugated sheep anti-mouse IgG (Sigma) at a 1:400 dilution.

The cutoff value was determined as the mean value of optical densities from control sera plus 3 standard deviations.

IIF assay. The IIF assay was carried out as described previously (5) with formalin-treated Y strain epimastigotes. Sera were diluted 1:10 to 1:1,280, and fluorescein-labeled anti-human IgG (affinity-purified γ chain specific; Sigma) was used at a 1:400 dilution.

HA test. The HA test was carried out with Chagas-HAI Immunoserum kit (Polychaco S.A.I.C., Buenos Aires, Argentina) according to the instructions of the manufacturer by using sera at a 1:20 dilution in order to confirm the serological diagnosis of Chagas' disease.

Western blot analysis. RNP (90 μg) was resuspended in electrophoresis sample buffer (16) containing 1 mM N_2-*p*-tosyl-L-lysine chloromethyl ketone and 1 mM PMSF, heated for 5 min at 100°C, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% gel). Samples were transferred to nitrocellulose membranes (pore size, 0.45 μm; Bio-Rad, Richmond, Calif.) as described by Towbin et al. (30). The nitrocellulose strips were then saturated and incubated with sera from patients with Chagas' disease or those without the disease. The sera were diluted 1:250. Immunocomplexes were detected after the addition of peroxidase anti-human IgG (Sigma) and 4-chloro-1-naphthol as substrate. The molecular mass markers (Sigma) were β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), albumin (66 kDa), and ovalbumin (45 kDa).

IgG subclasses in randomly selected sera from patients with Chagas' disease ($n = 20$) and those without the disease ($n = 20$) were evaluated by Western blotting as described above. The human anti-IgG subclasses used were from mouse monoclonal antibodies (Sigma) and included anti-IgG1 (clone HP6001; 1:3,000), anti-IgG2 (clone HP6002; 1:1,000), anti-IgG3 (clone HP6050; 1:2,500), and anti-IgG4 (clone HP6023; 1:1,000) at the indicated dilutions. The antigen-antibody reaction was developed with peroxidase-conjugated sheep anti-mouse IgG (Sigma). Finally, H_2O_2 and 4-chloro-1-naphthol were added to complete the reaction.

Statistical analysis. The specificity, sensitivity, and efficiency of the RNP ELISA and the IIF test were calculated as described by Galen and Gambino (12). The kappa index measuring the degree of true agreement was estimated as described by Fleiss (11).

TABLE 1. Positivity in the RNP ELISA, IIF test, and HA test for IgG in serum samples from patients with Chagas' disease and patients without the disease

Group and form of disease or disease	No. of patients	No. (%) of positive serum samples		
		RNP ELISA	IIF test	HA test
Chagasic patients	100	97 (97)	100 (100)	98 (98)
Indeterminate	49	46 (94)	49 (100)	48 (98)
Cardiac	43	43 (100)	43 (100)	42 (98)
Digestive	8	8 (100)	8 (100)	8 (100)
Nonchagasic patients	116	1 (0.9)	22 (18.9)	8 (6.9)
Healthy	40	0 (0)	1 (2.5)	1 (2.5)
Cardiomiopathy	5	0 (0)	1 (2.5)	0 (0)
SLE	8	0 (0)	0 (0)	0 (0)
Rheumatoid arthritis	5	0 (0)	0 (0)	0 (0)
Syphilis	6	0 (0)	0 (0)	0 (0)
Malaria	10	0 (0)	0 (0)	0 (0)
Rheumatic fever	4	0 (0)	0 (0)	0 (0)
Toxoplasmosis	8	0 (0)	0 (0)	0 (0)
Leishmaniasis	30	1 (5)	20 (67)	7 (23)
Visceral	20	1 (5)	15 (75)	4 (20)
Mucocutaneous	10	0 (0)	5 (50)	3 (30)

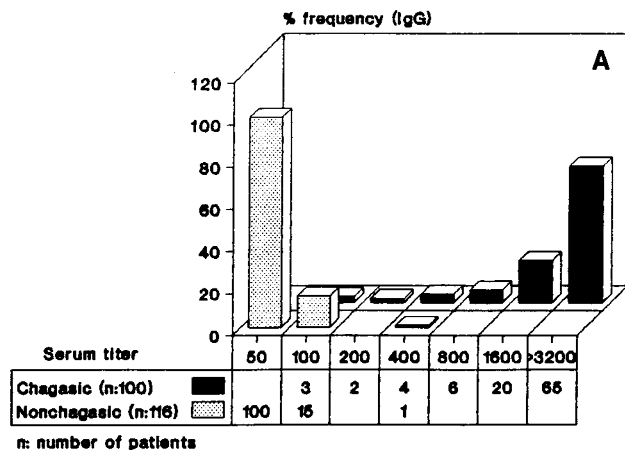
RESULTS

Cutoff values for sera from patients with Chagas' disease were a 1:200 dilution for the RNP ELISA and a 1:40 dilution for the IIF test when the tests were carried out with total IgG.

For the group with Chagas' disease, positivity values were 97% for the RNP ELISA, 100% for the IIF test, and 98% for the HA test. For the control group comprising clinically healthy individuals and patients with unrelated diseases, positivity values were 0.9% for the RNP ELISA (corresponding to a single patient with visceral leishmaniasis), 18.9% for the IIF test (corresponding to 20 patients with leishmaniasis, 1 with cardiomyopathy, and 1 healthy subject), and 6.9% for the HA test (corresponding to 7 patients with leishmaniasis) (Table 1).

The sensitivities, specificities, and efficiencies of the RNP ELISA were 0.97, 0.99, and 0.98, respectively. Those for the IIF test were 1.00, 0.81, and 0.90, respectively. The cutoff for the RNP ELISA + 3 standard deviations (0.116) was a 1:100 serum dilution. The cutoff for the IIF test was a 1:40 serum dilution. The kappa index for the RNP ELISA was calculated to be 0.95. In our population, the RNP ELISA provided better diagnostic resolution, since the antibody titers in the group with Chagas' disease differed remarkably from those in the group without the disease, whereas the IIF test rendered overlapping antibody titers for some patients with leishmaniasis (Fig. 1 and Table 1). RNP ELISA positivity closely agreed with data from immunoblotting of RNP in which several polypeptides were recognized by sera from patients with Chagas' disease (26 to 80 kDa). However, sera from two Argentine patients proved to be reactive for one or two polypeptides in the immunoblot (data not shown).

Although the individual patterns of reactivity of sera to RNP



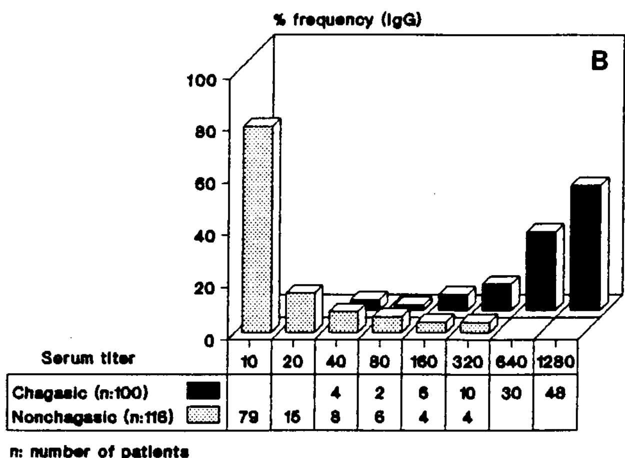


FIG. 1. Frequency (%) of IgG titers in the group of patients with Chagas' disease (n = 100; chagasic ■) and those without the disease (n = 116; ▨) by the RNP ELISA (A) and the IIF test (B).

antigens were different, a doublet of apparently 60 kDa was recognized by all sera from patients with Chagas' disease from Brazil (Fig. 2, lanes 1 to 6) and Argentina (Fig. 2, lanes 7 to 12).

No correlation was found between the clinical phases of chronic Chagas' disease and the pattern of serum reactivity by Western blotting. Regarding the group of individuals without Chagas' disease (Fig. 2, lanes 13 to 25), sera from patients with leishmaniasis (Fig. 2, lanes 20 to 25) failed to recognize the specific polypeptides, with one exception. The serum of a single patient with visceral leishmaniasis who lived in an area where Chagas' disease is endemic showed a pattern characteristic of Chagas' disease in the immunoblot with RNP (data not shown). Considering the epidemiological risk, further studies are required to confirm whether this patient is also infected with *T. cruzi.*

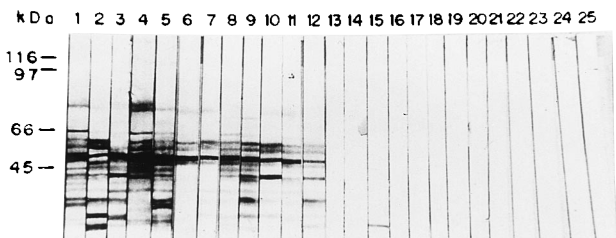


FIG. 2. IgG immunoblot analysis of sera from 12 patients with Chagas' disease (Argentines, lanes 1 to 6; Brazilians, lanes 7 to 12) and from 13 individuals without the disease (healthy individuals and individuals with other diseases, lanes 13 to 19; individuals with leishmaniasis, lanes 20 to 25). Molecular mass markers are given on the left.

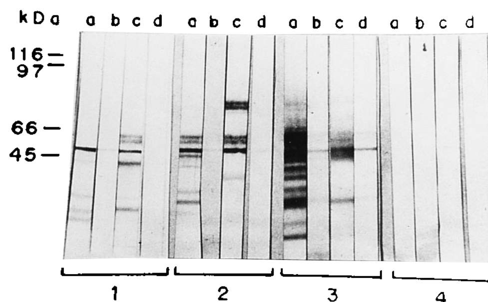


FIG. 3. Distribution patterns of IgG isotypes by immunoblot analysis of three serum samples from patients with Chagas' disease (group 1, indeterminate form; groups 2 and 3, cardiac form) and one serum sample from a patient with leishmaniasis (group 4). Each serum sample was assayed for IgG1 (lanes a), IgG2 (lanes b), IgG3 (lanes c), and IgG4 (lanes d). Molecular mass markers are given on the left.

The specific humoral responses of the IgG subclasses to the RNP antigen were assayed by ELISA by using sera from 21 patients with Chagas' disease and 9 patients without the disease; a clear predominance of IgG1 (100%) and IgG3 (85%) was shown, although IgG2 was also found in 50% of patients with Chagas' disease. This isotypic profile was confirmed by Western blotting. Several bands whose molecular masses ranged from 20 to 70 kDa were detected in sera from 20 patients with Chagas' disease (Fig. 3).

DISCUSSION

The diagnosis of Chagas' disease poses a veritable challenge because of the overlapping geographical distribution of both *T. cruzi* and *Leishmania* sp. infections (6, 22). We reported here on an ELISA in which the ribosomal fraction (RNP) of *T. cruzi* was used as antigen to detect specific antibodies against the parasite in sera from patients with the chronic form of the disease. This test was chosen because the technique is used in most diagnostic laboratories.

The RNP ELISA showed good sensitivity and specificity. The kappa index value of 0.95 indicates that the RNP fraction is an excellent diagnostic antigen since the World Health Organization recommended a value of greater than 0.80 for the diagnosis of Chagas' disease (23). Indeed, 97% of sera from patients with different clinical forms of leishmaniasis failed to react with RNP antigens, with all but one serum specimen showing optical densities below the cutoff value. Serum from a single patient with visceral leishmaniasis proved to be reactive by the RNP ELISA, while 75% of sera from patients with visceral leishmaniasis as well as 50% of sera from patients with mucocutaneous leishmaniasis were IIF test positive. The latter test confirms the results previously described by Matsumoto et al. (22). On the other hand, when sera were assayed by ELISA with a total extract of epimastigotes as antigen (6), the sensitivities and specificities were similar to those obtained by the IIF test (data not shown). These results suggest that purified RNP antigens may be used for differential serodiagnosis in geographical areas where Chagas' disease and leishmaniasis overlap.

The marked predominance of IgG1 and IgG3 in most *T.*

cruzi-infected humans was in agreement with the information presented in previous reports showing that the response developed by patients against the whole parasite and cytosol acidic antigens of *T. cruzi* exhibits the same isotypic profile (7, 24). Because no control sera displayed a similar distribution of IgG isotypes, such a finding suggests that the anti-RNP response may play a leading role in the humoral anti-*T. cruzi* immunoresponse. Although it has been reported that the response mounted against a synthetic peptide (R 13) representing the C-terminal residues of a cloned peptide from a *T. cruzi* ribosomal P protein was restricted to the IgG1 isotype, it would seem that the anti-R 13 response represents a component of the anti-RNP response (17–19).

Patients with SLE can develop an anti-ribosomal P protein response; however, its prevalence has been reported to reach 12% (1). Because they show typical IgG1 and IgG2 profiles (2), the RNP ELISA could discriminate between sera from patients with Chagas' disease and these doubtful sera by the isotypic distribution of IgG antibodies.

On the basis of our overall results, the profile of the IgG subclasses in the anti-RNP response for larger populations may well afford a useful clue to confirming the nature of sera when it is doubtful whether the patient has Chagas' disease.

In other protozoan infections such as malaria, different patterns of IgG subclasses according to geographical location have been observed (9, 10). In the present study, no difference in the isotypic profile was found between Argentine and Brazilian patients, thus ruling out any population difference in the response against the *T. cruzi* RNP antigen.

The subclass distributions of RNP-specific antibodies indicate that antibodies play an immunological role in the course of infection since IgG1 and IgG3 represent the major complement-fixing isotypes with a tendency for cell binding through their Fc receptors.

In order to determine the polypeptides recognized by each IgG isotype, a Western blot was performed. Despite great variability in the pattern of reactivity among sera from patients with Chagas' disease all presented IgG1 and IgG3 isotypes, while none of the sera from subjects without Chagas' disease showed this profile. Moreover, the serum sample from a single Argentine patient with leishmaniasis displayed the IgG1 isotype alone, thus confirming that the patient did not have Chagas' disease.

In the present study, we also used immunoblotting to characterize the RNP polypeptides recognized by specific antibodies of patients with Chagas' disease. A large number of polypeptides were detected by such sera, although some variability in the pattern of reactivity was found. However, a doublet of apparently 60 kDa and a certain distribution of low-molecular-mass bands were invariably observed, regardless of the clinical status of the patients with chronic Chagas' disease. Israelski et al. (14) also reported that the pattern of recognition varied throughout the course of infection. Although the heterogeneity of different parasite strains might be responsible for the recognition of a wide range of peptides, our results disclosed that antibodies in sera from patients with Chagas' disease bound to peptides of similar molecular mass in the ribosomal fractions of epimastigotes of two different strains, Y and Tulahuen, isolated from separate geographical areas, Brazil and Chile, respectively. Since these strains showed similar biological behaviors, further studies with strains with different tropisms should be carried out.

We conclude that the methodology described here allows for patients with Chagas' disease to be discriminated from patients without the disease, since sera from patients with other infections or autoimmune diseases failed to cross-react with the RNP antigen. Despite the lack of correlation between the clinical forms of disease among patients with Chagas' disease and the RNP ELISA or Western blot results, this approach may be useful both for detecting Chagas' disease and confirming the nature of doubtful sera by using the isotypic profiles of IgG subclasses.

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