SPECIFIC AND SENSITIVE IMMUNOLOGICAL DIAGNOSIS OF CHAGAS' DISEASE BY COMPETITIVE ANTIBODY ENZYME IMMUNOASSAY USING A TRYPSANOSOMA CRUZI-SPECIFIC MONOCLONAL ANTIBODY

J. L. LEMESRE,* D. AFCHAIN,* O. OROZCO,* M. LOYENS,* F. S. BRENIERE,** P. DESJEUX,** Y. CARLIER,† U. MARTIN,‡ J. A. NOGUEIRA-QUEIROZ,§ D. LE RAY,** AND A. CAFRON*

*Centre d’Immunologie et de Biologie Parasitaire, Institut Pasteur, 15 rue Camille Guérin, 59019 Lille Cedex, France. **Instituto Boliviano de Biologia de Altura, La Paz, Bolivia. †Laboratoire de Parasitologie, Faculté de Médecine, ULB, Bruxelles, Belgium. §Centro de Investigaciones Sobre Enfermedades Nacionales, Santa Fe, Argentina. ‡Nucleo de Medicina Tropical, Fortaleza, Brazil, and \*Institut de Médecine Tropicale, Anvers, Belgium

Abstract. Coexistence of Chagas’ disease with leishmaniasis and T. rangeli infection in endemic areas and cross-reactivity between corresponding etiological agents can confuse the immunodiagnosis of Chagas’ disease. A discriminative serological test could therefore represent a major advance in specific immunodiagnosis. A competitive antibody enzyme immunoassay against a component 5-enriched preparation, using a T. cruzi species-specific monoclonal antibody has allowed development of a specific serodiagnosis of Chagas’ disease with high sensitivity (96.6% in undetermined and chronic phases of infection). This test can differentiate Chagas’ disease from other cross-reacting parasitic diseases in areas where concomitant infections are unknown or suspected.

The parasitic protozoan Trypanosoma cruzi is the causative agent of Chagas’ disease (American trypanosomiasis). At least 24 million people in Central and South America are estimated to be infected with T. cruzi.1

In the initial acute phase of Chagas’ disease, lethal in about 10% of cases, trypanosomiasis can usually be detected in the circulating blood. In the undetermined and chronic phases of infection, parasitemia is very low and immunosero-diagnosis is required.

In vast areas of Central and South America, cutaneous, mucocutaneous2-3 or visceral leishmaniasis4 and T. rangeli infections5-7 are associated with T. cruzi infection. Due to a variable degree of cross-reactivity between the corresponding etiological agents8-10 the precise diagnosis of Chagas’ disease using classical serological tests is not effective in areas where these diseases are coendemic.

Attempts have been made to overcome such cross-reactivity using amastigote or trypomastigote forms of T. cruzi.11-12 “Live” T. cruzi antigen,13 human sera absorption,14 a monospecific serum anti-T. cruzi component 5 to identify specific antibodies,15 and defined T. cruzi-specific antigens.16,17 The World Health Organization has emphasized the need for a discriminative serological test ensuring the specific diagnosis of South American trypanosomiasis.

In previous studies, a T. cruzi-specific component with regard to other Trypanosomatidae (T. rangeli, L. donovani, L. mexicana, L. braziliensis)5-13 so called “5,” was identified. Moreover, anti-component 5 precipitating antibodies frequently were found in sera from patients chronically infected with T. cruzi.15,18,19 In recent papers,20-22 we reported the production of murine monoclonal antibodies directed against component 5 of T. cruzi and the characterization of target antigens corresponding to the 72 Kd glycoprotein and its maturation products (51 Kd, 43 Kd and 24 Kd).

In the present work, we describe a simple, highly specific and sensitive serological test for Chagas’ disease by competitive (antibody) enzyme immunoassay (CEIA). It uses a 24 Kd-enriched fraction as antigen and a species-specific anti-T. cruzi monoclonal antibody. The detection of specific antibodies in the sera of chagasic patients reacts highly specific and sensitive serological test for Chagas’ disease. A competitive antibody enzyme immunoassay (CEIA) using a T. cruzi species-specific monoclonal antibody has allowed development of a specific serodiagnosis of Chagas’ disease with high sensitivity (96.6% in undetermined and chronic phases of infection). This test can differentiate Chagas’ disease from other cross-reacting parasitic diseases in areas where concomitant infections are unknown or suspected.

Accepted 1 July 1985.

Copyright © 1986 by The American Society of Tropical Medicine and Hygiene.
SPEcific anti-T. cruzi monoclonal antibody, allows the detection of anti-component 5 antibodies in the sera of chagasic patients, and can differentiate Chagas’ disease from leishmaniasis.

MATERIALS AND METHODS

Antigen preparation

Trypanosoma cruzi (Tehuantepec strain) epimastigotes cultured in GLSH medium as previously described were collected by centrifugation at 400 x g for 15 min at 4°C and washed four times with Hank’s balanced salt solution. Six grams (wet weight) of epimastigotes frozen in 100 ml 0.1% NaCl were disintegrated by four passages through an hydraulic press (X press, Bio-LKB) at 18,000 psi. It was then centrifuged at 26,000 x g for 1 hr at 4°C. The supernatant was dialyzed against distilled water for 24 hr at 4°C, lyophilized and used as a total soluble extract of T. cruzi (TSE). Twenty mg of TSE were resuspended in 4 ml distilled water and added with an equal volume of chloroform/methanol solution (2:1). The mixture was shaken and centrifuged at 1,000 x g for 30 min at 4°C. The aqueous phase was collected and extracted twice in the same way. Organic solvents were evaporated and the remaining aqueous fraction was precipitated by the addition of 3 volumes of ethanol for 4 hr at -20°C. After centrifugation, the precipitate was washed with ethanol, dried, and resuspended in 2 ml distilled water. The solution was centrifuged, dialyzed against distilled water for 24 hr at 4°C and lyophilized. This successive extraction by chloroform/methanol and ethanol precipitation created a component 5-enriched preparation (C5-EP) as verified by immunoelectrophoresis results of mouse sera immunized with this fraction.

Monoclonal antibody (Mab)

A murine IgG, Mab (11-180-30) produced in our laboratory has been demonstrated to recognize the component 5 corresponding to the 72 Kd glycoprotein and its maturation products (51 Kd, 43 Kd and 24 Kd). Ascites were produced in BALB/c mice and immunoglobulins were isolated from the ascitic fluid by precipitation with 50% saturated ammonium sulfate. The pellet was dissolved in 0.5% NaCl and dialyzed overnight. The solution was then submitted to ion exchange chromatography on a DEAE-Trisacryl column. The purified Mab was labeled with alkaline phosphatase (grade I from calf intestine, Boehringer) by the one-step glutaraldehyde method previously described.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Total soluble extract and C5-EP were dissolved in 40 μl of sodium dodecyl sulfate (SDS)-containing slab gel buffer with 5% β-mercaptoethanol and boiled for 3 min. Insoluble material was removed by centrifugation at 2,000 x g for 10 min before samples were applied to the gel. SDS-PAGE was carried out on vertical slab gels according to the method of Laemmli using 10% polyacrylamide gel. After electrophoresis the gel was stained with Coomassie blue, destained and then dried under vacuum.

Competitive antibody enzyme immunoassay (CEIA)

Polypropylene beads (6.5 mm) were incubated overnight at room temperature by gentle agitation in 0.015 M carbonate-0.035 M bicarbonate buffer (pH 9.6) containing the antigen (see Results for concentrations). After washing three times in PBS-Tween (0.01 M phosphate buffer, pH 7.2, 0.1% Tween 20), the beads were incubated with PBS + 0.1% bovine serum albumin for 2 hr and washed twice in PBS-Tween, then once in phosphate buffered saline (PBS). CEIA was performed in disposable polystyrene tubes. Coated beads were incubated for 3 hr at 37°C in a mixture of 100 μl diluted Mab labeled with alkaline phosphatase, 100 μl diluted human serum sample and 150 μl PBS-Tween. Tubes were emptied by suction and beads were washed three times in PBS-Tween and transferred to another tube: the amount of enzyme fixed to the beads was determined using 300 μl of enzyme substrate (1 mg ml 4-nitrophenyl phosphatase in 0.5 M Na2CO3, 0.001 M MgCl2 buffer, pH 10.4). After 1 hr incubation at 37°C, further reaction was stopped by addition of 300 μl 2 N NaOH and the resulting yellow color was measured in a spectrophotometer at 405 nm. The CEIA test involved the inhibition of binding of
Human sera

The control group was comprised of 50 healthy patients with a negative serology for *T. cruzi*, including 20 Europeans, 20 Bolivians and 10 Argentinians.

The *T. cruzi*-infected group included 15 Argentinian children (1–10 years) with evident acute phase Chagas’ disease (Romani sign, general edema, presence of blood parasites) and 89 Bolivian patients with chronic phase Chagas’ disease and a positive serology for *T. cruzi* (immunofluorescence, complement fixation test, immunoelectrophoresis and ELISA). They lived in lowland areas of Bolivia where leishmaniasis has never been found.

The third group was infected by other Trypanosomatidae, including *Leishmania braziliensis* (15 Bolivian patients with evident lesions of mucocutaneous leishmaniasis and a negative serology for *T. cruzi* who lived in areas where Chagas’ disease had never been found), *L. tropica* (6 African patients with evident cutaneous leishmaniasis), *L. donovani* (14 Mediterranean patients with visceral leishmaniasis), and *T. gambiae* (11 African patients with sleeping sickness).

A fourth group was infected by other protozoa including *Toxoplasma gondii* (14 European patients), *Plasmodium* (10 African patients), and *Entamoeba histolytica* (3 African patients).

The fifth group was comprised of patients living in areas with possible mixed infection: 40 Brazilians clinically and serologically confirmed as being infected with *Schistosoma mansoni* and 47 Bolivian patients with clinical and serological confirmation of *L. braziliensis* infection, living in the Yungas Valley.

**RESULTS**

**Composition of the C1-EP antigen**

The C1-EP was analyzed by SDS-PAGE followed by Coomassie blue staining. Figure 1 shows a comparison between protein patterns of the *T. cruzi* total soluble extract (TSE) (500 µg, lane B) and the C1-EP (50 µg, lane A). More than 20 peptide chains could be clearly distinguished in the TSE pattern and a major band of 24,000 daltons with some minor contaminating proteins were identified in the C1-EP profile. Approximately, 2.5 mg of C1-EP was obtained from 20 mg of TSE.

**ALKALIN PHOSPHATASE-LABELED MAB**

alkalin phosphatase-labeled Mab by sera from *T. cruzi*-infected patients; low extinction values indicate the presence of anti-component 5 antibodies in human serum. A total of 1,750 sera can be tested using 2.5 mg of C1-EP and 3.5 mg of purified Mab.

**FIGURE 1.** Protein patterns after Coomassie blue staining of 500 µg of total soluble extract of *T. cruzi* (B) and 50 µg of the component 5-enriched preparation (A) by SDS-PAGE. The positions of migration of the marker proteins (M, × 10⁻⁴) are indicated.
SPECIFIC IMMUNOLOGICAL DIAGNOSIS OF CHAGAS' DISEASE

Figure 2. Direct binding assays of various dilutions (■ 1/50, ● 1/100, ★ 1/200, ▲ 1/400) of the conjugate (alkaline phosphatase labeled Mab) to polypropylene beads coated with different concentrations of T. cruzi total soluble extract (A) or component 5-enriched preparation (B).

TSE and C₅-EP in CEIA

Various dilutions of the conjugate (alkaline phosphatase-labeled Mab) and different concentrations of TSE and C₅-EP were used in preliminary studies. The highest range of optical density (OD) values was obtained with 1/100 diluted conjugate (Fig. 2A and B). The optimal antigen concentrations were 50 µg/ml of TSE (Fig. 2A) and 5 µg/ml of C₅-EP (Fig. 2B).

In these conditions, CEIA test was performed with sera from T. cruzi-infected patients and from controls. In both cases, optimal sensitivity of the test was achieved using the 1/5 diluted serum (Fig. 3A and B), which obtained a 2.3 higher difference in OD values between positive and negative sera with C₅-EP than with TSE. This increase in the sensitivity of the test was used in all subsequent studies, using the 5 µg/ml C₅-EP coating.

Sensitivity and specificity of CEIA

Figure 4 shows the CEIA OD values in the control group (0.92 ± 0.17) significantly higher than those of the acute (0.50 ± 0.30, P < 0.001) and those with the chronic phase (0.19 ± 0.14, P < 0.001). There was a clear cut-off point be-
between chagasic and non-chagasic patients using the OD 0.58 [calculated as \( m \) (control group) - 2 SD; i.e., 0.92 - 2 \( \times (0.17) = 0.58 \). The sensitivity of CEIA positive detection in relation to the cut-off value was 66.7% in acute infection and attained 96.6% in the chronic phase of Chagas' disease.

As shown in Figure 4, CEIA OD values in sera from patients with other Trypanosomatidae or protozoal infections were between 0.6 and 1.3 and showed a significant difference from those of chronic Chagas' disease.

**Detection of Chagas' disease in areas with possible mixed infections**

In the two groups of Brazilian and Bolivian sera with possible mixed infections, CEIA detected anti-*T. cruzi*-specific antibodies in 3 and 15 sera samples, respectively (Fig. 5), giving evidence of Chagas' infection in 7.5% of visceral leishmaniasis cases and in 31.4% of leishmaniasis cases.

**DISCUSSION**

In areas where mucocutaneous and/or visceral leishmaniasis are coendemic with Chagas' disease, the classic serological tests do not allow a differential immunodiagnosis. Consequently, various methods have been proposed to increase the specificity of the serodiagnosis of *T. cruzi* infection.\(^{11,12}\) But the increase in specificity with these tests is often at the expense of efficiency and sensitivity.\(^{13}\) In the present investigation, we propose a specific and sensitive enzyme immunoassay using a Mab that provides a potential
serodiagnosis for Chagas' disease, particularly in areas with concomitant infections.

Previous studies, using immunoelectrophoresis \(^{14}\) and a microdouble diffusion test, \(^ {15}\) have demonstrated the high frequency of anti-component 5 antibodies in sera of patients with Chagas' disease. More recently, murine Mabs (II-190/30, III-160/18 and I-35/67) have been produced against component 5 \(^ {20,21}\) which recognized a glycoprotein of 72 Kd (GP72) and its maturation products (51, 43 and 24 Kd). These molecules are exclusively expressed in epimastigote and amastigote forms of \(T. cruzi\) \(^ {21}\) and not on other trypanosomatid parasites of humans (\(Leishmania\) and \(T. rangeli\)). \(^ {1} \) Preliminary results \(^ {21}\) using Mab II-190/30 in a competitive radioimmunoassay suggest its potential value for specific serodiagnosis of Chagas' disease. Thus, the use of a species-specific Mab in a competitive enzyme immunoassay allows precise immunodiagnosis. \(^ {21}\) It possesses all the advantages of constant specificity and reproducibility of a monoclonal reagent. Finally, this enzyme immunoassay is sensitive, requires low reagent consumption (microassays) and can be automated for large scale applications. \(^ {26,27}\)

Recently, two sensitive and specific tests using purified antigens of \(T. cruzi\) (25 Kd and 90 Kd) have been developed. \(^ {16,17}\) However, they are very expensive and require large quantities of purified antigens, precluding a large field application. The present simple chemical extraction procedure from a total hydrosoluble extract of \(T. cruzi\) can isolate a measurable quantity of 24 Kd-enriched fraction. The 24 Kd antigen, one of the maturation products of the GP72, \(^ {21,22}\) seems to be produced by a proteolytic degradation occurring during extraction.

No false positive reactions were observed with the CEIA test using sera from healthy individuals as well as from patients with other parasitic infections. This was due to the specificity of the component 5 of \(T. cruzi\) with regard to other Trypanosomatidae as previously demonstrated. \(^ {6} \) However, further evaluations of the usefulness of this test are required in areas where \(T. rangeli\) infections are associated with Chagas' disease.

More than 95% of the patients with undetermined and chronic phases of Chagas' disease presented specific high levels of anti-component 5 antibodies. This high sensitivity was not only dependent on the monoclonal reagent but also on the use of a component 5-enriched fraction

![Figure 5. Detection of mixed infections in two groups of sera samples from South American patients (S. mans. = Brazilian patients with schistosomiasis; L. braz. = Bolivian patients with mucocutaneous leishmaniasis).](image)

In fact, when a crude extract of \(T. cruzi\) was used, the sensitivity of the CEIA test was lower.

On the other hand, only 66.7% of the patients with acute Chagas' disease showed anti-component 5 antibodies. Several hypotheses can account for these results: a) the low level of anti-component 5 antibodies; b) the eventual weak affinity of corresponding IgG or c) the presence of IgM antibodies and/or immune complexes in sera of acute cases.

The level of associated Chagas' disease detected in endemic areas (schistosomiasis and leishmaniasis endemic areas, respectively) demonstrates that the CEIA test provides an extremely precise diagnosis of Chagas' disease. Indeed, it is very important to obtain such confirmation of \(T. cruzi\) infection, since the therapeutic management of leishmaniasis and Chagas' disease are very different.

In view of its simplicity, specificity and sensitivity, the CEIA test is recommended for a spec-
cific diagnosis and for large screening of unde-
terminate and chronic phases of Chagas' disease,
especially in areas where several parasitic dis-
cases are coendemic.

ACKNOWLEDGMENTS

This investigation received financial support
from the UNDP/World Bank/WHO Special Programme
for Research and Training in Tropical Diseases.

The authors wish to thank D. Henry and J. L.
Neyrinck for their excellent technical assistance
and C. Colson and M. F. Massard for their secret-
arial help.

We are also grateful to Drs. F. Darcy and F.
Santero for invaluable help in correction of this
manuscript.

REFERENCES

letter, 18: 7.
2. Walton, B. C., Chinel, L. V., and Egusa, O. E.,
1973. Onset of espondia after many years of
occult infection with Leishmania amazonensis.
A propos de 113 cas de leishmaniose cutanée et
cutanéo-muqueuse observés en Bolivie. Etude
4. Desjeux, P., Aranda, E., Allaga, O., and Molli-
 nedo, S., 1983. Human visceral leishmaniasis
in Bolivia: First proven case from “Los Jungas.”
5. Sousa, O. E., and Johnson, C. M., 1971. Fre-
quency and distribution of Trypanosoma cruzi
and Trypanosoma rangeli in the Republic of
soma (Herpetosoma) rangeli. Pages 339–403 in W. 11.
R. Lumsden and D. A. Evans, eds., Biology of
7. Anthony, R. L., Johnson, C. M., and Sousa, O. E.,
1979. Use of micro-ELISA for quantifying anti-
body to Trypanosoma cruzi and Trypanosoma
8. Afchain, D., Le Ray, D., Fruit, J., and Capron, A.,
1979. Antigenic make-up of Trypanosoma cruzi
culture forms: Identification of a specific com-
1980. Diferencias y similitudes antigenicas entre
Trypanosoma rangeli y Trypanosoma cruzi.
Medicina (Buenos Aires), 40: 43–49.
10. Anthony, R. L., Cody, T. S., and Constantine, N.
T., 1981. Antigenic differentiation of Trypano-
soma cruzi and Trypanosoma rangeli by means
of monoclonal hybridoma antibodies. Am. J.
11. Cerisola, J. A., Alvarez, M., Bock, M., and Weg-
from amastigotes of Trypanosoma cruzi and an
antigen from epimastigotes for the diagnosis of
Chagas’ disease by the indirect immunofluores-
162–166.
of cell culture with epimastigote antigens of
26: 47–57.
13. Guimarães, M. C., Celeste, B. J., Ayres, E. C.,
Minoe, J. R., and Diniz, J. M. P., 1981. Im-
munoenzymatic assay (ELISA) in mucocuta-
neo-leishmaniasis, kala-azar and Chagas’ dis-
ease: An epimastigote Trypanosoma cruzi antigen
able to distinguish anti-Trypanosoma and anti-
30: 942–947.
reactivity in fluorescence tests for Trypanosoma
and Leishmania antibodies. A simple inhibition
procedure to ensure specific results. Am. J. Trop.
15. Brienère, F. S., Carrasco, R., Molinédo, S., Le-
mesle, J. L., Desjeux, P., Afchain, D., and Car-
lier, Y., 1986. Specific immunodiagnosis of
Chagas’ disease: Immunodiffusion test using a
specific serum anti-Trypanosoma cruzi com-
16. Schechter, I., Rodrigues, M. M., Andrede Alves,
C., de Sousa, W., Previtali, J. O., and Mendonça-
Previtali, L. 1983. Trypanosoma cruzi: Descrip-
tion of a highly purified surface antigen de-
fined by human antibodies. J. Immunol., 131:
972–976.
17. Schechter, M., Voller, A., Marínkelle, C. J., Flint,
J. E., Guhl, F., and Miles, M. A., 1983. purified
Trypanosoma cruzi specific glycoprotein for dis-
criminative serological diagnosis of South
American trypanosomiasis (Chagas’ disease).
anticorps précipitants dans la leishmaniose amé-
147.
19. Brienère, F. S., Carrasco, R., Miguel, A., Lemesle,
J. L., and Carlier, Y., 1985. Comparison of im-
munological tests for serodiagnosis of Chagas’
(In press.)
20. Orozco, O., Afchain, D., Rodríguez, C., Oválaque,
G., Loyensa, M., and Capron, A., 1982. Pro-
duction d’un anti-corps monoclonal anti-anti-
gène 5 de Trypanosoma cruzi. C. R. Acad. Sc.
21. Orozco, O., Afchain, D., Dissou, C., Rodríguez,
C., Oválaque, G., Lemesle, J. L., Loyens, M., and
Capron, A., 1984. Different monoclonal anti-
obodies against the component 5 specific for
30: 560–568.


