

Serodiagnosis of American Trypanosomiasis by Using Nonpathogenic Trypanosomatid Antigen

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***Crithidia luciliae*, a nonpathogenic trypanosomatid, could provide a good alternative source of antigen for serodiagnosis of Chagas' disease. An enzyme-linked immunosorbent assay showed 100% sensitivity and 83% specificity when 91 human serum samples from Chagas' disease patients and 127 human serum samples from people suffering from toxoplasmosis (21 samples), leishmaniasis (32 samples), systemic rheumatic diseases (33 samples), and heart diseases (41 samples) were tested simultaneously with *Trypanosoma cruzi* and *C. luciliae* crude extracts. By Western blotting, an immunodominant band (30 kDa) was recognized by chagasic sera on the *C. luciliae* crude extract; specificity reached 97% with respect to this protein band. The carbohydrate moieties were not antigenic.**

Seroimmunologic tests are paramount for the diagnosis of chronic Chagas' disease, when parasitemia is low and sporadic parasitism and tissue parasitism are almost impossible to demonstrate. Current serologic assays have limited value, and because of problems with low specificity and two different assays with a single sample, increasing the diagnostic yield has been recommended (18). The cultured form of *Trypanosoma cruzi* (epimastigote) is generally used as a source of antigen, because it is cultured easily and a fairly good amount of antigen is obtained. Although the trypomastigote or amastigote parasite phase produces antigens with better sensitivity and specificity in clinical tests, there are difficulties linked to infectiousness and low yield (5, 6). Purified or recombinant moieties have also been used for diagnostic purposes; however, either these products are not available in most clinical diagnostic laboratories, or their performance has not fulfilled expectations (10). Therefore, crude extracts of cultured epimastigotes are the current source of antigen for seroimmunologic assays in the clinical laboratory (13, 16).

Over the course of many years, a search for an alternate source of antigen has been undertaken. During the 1970s, insect trypanomatid parasites, which are not pathogenic to human beings, were assayed, and a very high sensitivity (up to 98% by indirect immunofluorescence was reported) (9, 17).

In this study, we report the use of chaenomastigote or semimastigote (1) forms of *Crithidia luciliae*, a monoxenous trypanosomatid parasite of insects of various orders (Diptera, Hemiptera, Hymenoptera, and Lepidoptera), as an alternative source for antigen in clinical studies aimed at recognizing human serum antibodies specific for *T. cruzi*.

We compared results with *T. cruzi* antigen used as a reference to results with *C. luciliae* crude antigen. Enzyme-linked immunosorbent assays (ELISAs) and immunoelectric transference (Western blotting [WB]) (14) were applied to sera from people with or without chronic Chagas' disease.

Parasites (either *T. cruzi* or *C. luciliae*) were cultured in a brain heart infusion medium with 10% fetal calf serum, harvested at the log phase of growth, centrifuged at $1,800 \times g$ 4°C,

extensively washed with phosphate-buffered saline (PBS), and sonicated at maximum output (100 W) under protease inhibition. After centrifugation at $12,000 \times g$ for 30 min at 4°C, the clear supernatant was collected, the protein concentration was adjusted to 1 mg/ml, and the samples were aliquoted and frozen until used (12).

For ELISA, polystyrene wells (Dynatech) were sensitized with antigen (1 µg/well) in carbonate buffer and blocked with PBS-Tween 20 (0.01%) containing bovine serum albumin (1%). Human serum diluted to 1:400 was incubated and washed; a second antibody, peroxidase-labeled antihuman immunoglobulin G (IgG), was added. Color was developed by orthophenylenediamine and H₂O₂. A micro-ELISA reader at 490 nm was used to quantify results (11).

A WB assay was performed by electrophoresis of extracts on a polyacrylamide gel (10%). Total protein (150 µg) was run and transferred to nitrocellulose paper sheets, blocked with PBS-Tween 20 (0.1%) containing 3% bovine serum albumin; human serum diluted to 1:1,000 in the same buffer was added to react. Finally, antihuman IgG (Fc specific) peroxidase conjugate reacted, and the color reaction was developed with 4-chloronaphthol and H₂O₂ (14).

We tested 39 Brazilian serum samples and 52 Mexican serum samples from patients with a definitive diagnosis of chronic Chagas' disease on epidemiologic, clinical, and serologic bases (a total of 91 positive serum samples) (7, 8). A total of 127 serum samples from subjects with a clinical diagnosis of toxoplasmosis, leishmaniasis, systemic rheumatic disease, or heart disease were included as a comparative group.

The ELISA was always positive when the chagasic sera were assayed. In contrast, only 3 of 127 serum samples from the comparative group gave a positive result when *T. cruzi* antigen was used for plate sensitization. When *Crithidia* antigen was used instead, complete concordance was observed with respect to the chagasic sera, but as many as 21 of 127 (17%) of the nonchagasic serum samples became positive. False positives were observed in patients with either cutaneous or visceral leishmaniasis. Higher ELISA values corresponded to the latter form, while cutaneous disease presented lower absorbance.

A test was considered positive when the optical density was above the median plus 5 standard deviations obtained from

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TABLE 1. Correlation between sensitivity and specificity of *T. cruzi* and *C. luciliae* extracts with serum from Chagas' disease patients and controls

Response to <i>C. luciliae</i> extract	No. of serum samples with response to <i>T. cruzi</i> extract ^a		Total no. of responses
	Positive	Negative	
Positive	91 (a)	21 (b)	112 (a + b)
Negative	0 (c)	106 (d)	
Total	91 (a + c)	127 (b + d)	218 (n)

^a Sensitivity, $(a/a + c)(100) = (91/91)(100) = 100\%$; specificity, $(d/b + d)(100) = (106/127) = 83.46\%$.

blood donor samples and the coefficient of variation was 9%. The sensitivity and specificity were 100 and 83.4%, respectively (Table 1).

On WB, the *C. luciliae* extract reacted with chagasic sera, and a 30-kDa band was dominant; 100% of the chagasic sera recognized it (Fig. 1). In the nonchagasic comparative group (WB profile not shown), a few of the serum samples from patients with visceral leishmaniasis showed a faint reaction. Specificity, with respect to the 30-kDa band, reached 96.8%.

Antibodies eluted by HCl-glycine buffer (pH 2.8) (20) were able to recognize a 30-kDa band in a second WB with either *T. cruzi* or *C. luciliae* antigens, although they recognized some other peptide bands as well. When *T. cruzi* extract was used in the second WB, bands with sizes of 30, 33, and 35 kDa were recognized. When *C. luciliae* was the protein source, two bands with sizes of 31 and 35 kDa reacted (Fig. 2 and 3). These results confirm that both extracts share epitopes recognized by IgG anti-*T. cruzi* antibodies.

To explore the role of carbohydrate compounds as antibody targets, we performed an oxidative reaction with sodium periodate, which attacks periodic structures with a repeating unit on extended linear molecules and no substituent at position 3' in the pyranoside form (2, 21). This experiment was performed according to the method of Woodward et al. (20). A sensitized plate was treated with sodium acetate (50 mM [pH 4.5] and

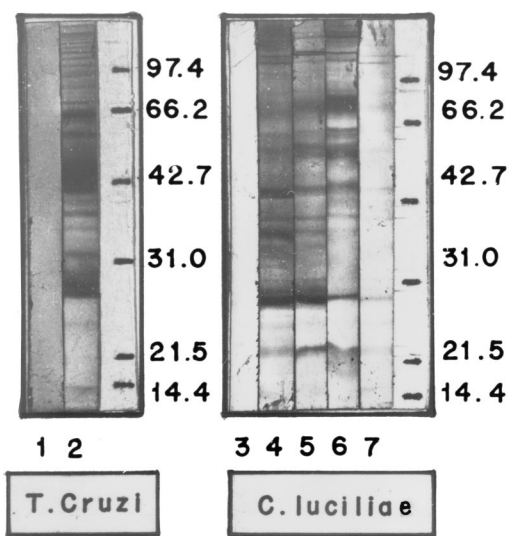


FIG. 1. Representative antigenic profile of *T. cruzi* (lanes 1 and 2) and *C. luciliae* (lanes 3 to 7) extracts reacted with chagasic sera. Lanes: 1 and 3, blanks; 2, 4, 5, 6, and 7, chagasic sera. Molecular masses are shown to the right of each panel in kilodaltons.

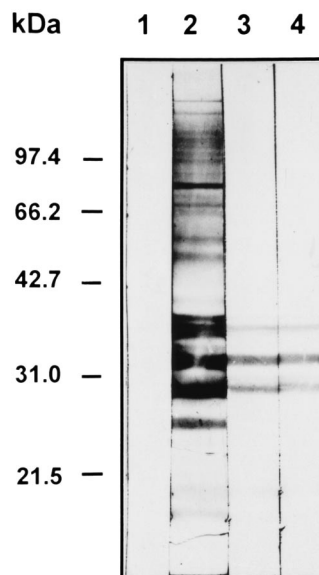


FIG. 2. WB assay of *T. cruzi* extract reacted with antibodies eluted from the 30-kDa region of *C. luciliae* with HCl-glycine (pH 2.8). Lanes: 1, blank; 2, positive; 3 and 4, eluted.

0.1, 5, and 20 mM sodium periodate in acetate buffer for 60 min at 4°C in complete darkness, and this was then reduced by 50 mM sodium borohydride in PBS for 20 min at 4°C in complete darkness. A conventional ELISA was run after treatment. Results showed a diminished reactivity, 21 and 29%, respectively, against both *T. cruzi* and *C. luciliae* antigenic extracts at a periodate concentration of 5 mM or higher. Periodate oxidation also produced a reduction of some polypeptide bands when WB was used instead of ELISA; however, the 30-kDa band was spared (Fig. 4 and 5). Enzymatic treatment of transferred total extracts, whether from *T. cruzi* or *C. luciliae*, with endoglycosidase F does not modify the antibody recognition pattern (data not shown). Therefore, there is evidence for

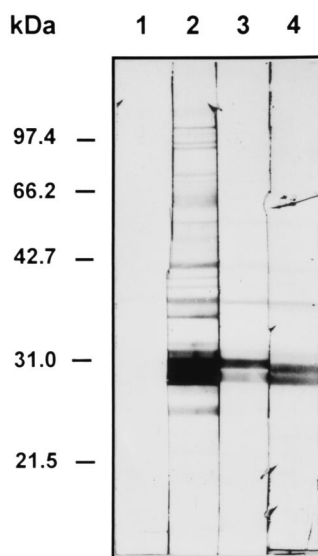


FIG. 3. WB assay of *C. luciliae* extract reacted with antibodies eluted from the 30-kDa region of *C. luciliae* by HCl-glycine (pH 2.8). Lanes: 1, blank; 2, positive; 3 and 4, eluted.

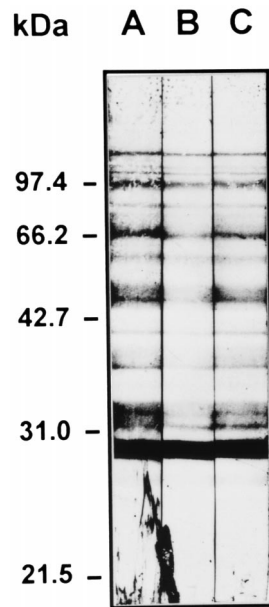


FIG. 4. WB assay of *C. luciliae* extract treated with 0.1, 5, and 20 mM periodate (lanes A, B, and C, respectively).

a major protein epitope on the dominant 30-kDa band, with a minor role for carbohydrate moieties in the reactivity disclosed by ELISA, in concordance with recent data by Umezawa et al. (19). Carbohydrate moieties are important for the antigenicity of *T. cruzi*, especially during acute infection, but not in the chronic phase.

The variability among *T. cruzi* strains isolated from reservoirs, triatomine vectors, or human patients is well known. In addition, there are clone-specific components (3) which contribute to the heterogeneity of the immune response. However, certain highly conserved epitopes have been recognized (13, 15) which are considered useful targets for improving diagnostic assays. Unfortunately, such moieties exist only in small amounts, making it difficult to use them for clinical testing (4).

Alternatively, recombinant proteins represent an interest-

ing possibility for the development of clinical diagnostic assays. Recently, 11 proteins, of both purified and recombinant moieties, were evaluated, with some of them reaching a high correlation (kappa index, ≥ 0.8) (10).

However, serodiagnosis of *T. cruzi* infections is still difficult, because no purified or recombinant proteins used as antigens in diagnostic tests are free of pitfalls and limitations, and they are expensive or not readily available in less-developed settings where the disease is an important public health issue, especially when urban Chagas' disease seems to menace blood supplies and as screening of blood donors becomes mandatory in most countries through the South American continent. In this sense, *Crithidia*-derived antigens, which are easily produced and contain significant moieties recognized by human antibodies, represent an interesting subject for studying the humoral immune response and, perhaps, an alternative diagnostic method with minimal expense and no risk.

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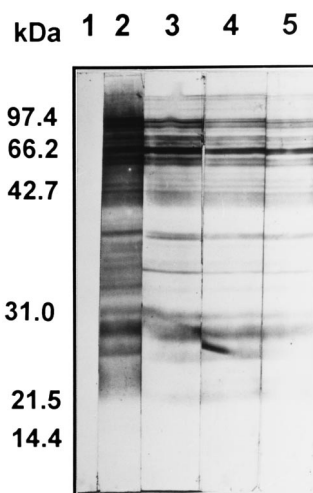


FIG. 5. WB assay of *T. cruzi* extract treated with 0.5, 5, and 20 mM periodate (lanes 3, 4, and 5, respectively). Lane 1, blank; lane 2, nontreated.

- antigen in serodiagnosis of Chagas' disease. *J. Clin. Microbiol.* **33**:1456–1460.
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