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Use of a 24-Kilodalton *Trypanosoma cruzi* Recombinant Protein to Monitor Cure of Human Chagas' Disease

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A 24-kDa recombinant protein from *Trypanosoma cruzi* (rTc24) was evaluated by enzyme-linked immunosorbent assay (ELISA) and Western blot (immunoblot) tests to identify treated chagasic patients considered parasitologically cured on the basis of persistently negative tests of hemocultures and lytic antibodies. Some of these patients were termed dissociated because their sera, although negative by the complement-mediated lysis test, were positive by conventional serology. The negative lysis test indicates the absence of active infection after specific treatment, but this assay requires live and infectious parasites and cannot be used easily in a laboratory routine. Here we tested rTc24 by ELISA and Western blotting as an alternative for the complement-mediated lysis test. For the group of patients with active infection despite the treatment (uncured patients), all the sera tested recognized rTc24 in both tests. For the dissociated patients, approximately 80% of the sera did not react with rTc24 in the ELISA or in Western blots, in agreement with the negative complement-mediated lysis tests. Thus, the 24-kDa *T. cruzi* recombinant antigen, when used for initial trials to evaluate cure of chagasic patients submitted to specific treatment, will allow the identification of most, but not all, cases.

Chagas' disease is one of the main problems of public health in Latin America, where approximately 16 to 18 million people are infected with *Trypanosoma cruzi*. Characterized by an acute phase that lasts approximately 2 months, this infection progresses to a lifelong chronic phase. Most patients are diagnosed during the chronic phase by conventional serology tests (CS), i.e., indirect immunofluorescence (IIF), the complement fixation reaction, and indirect hemagglutination. During the chronic phase, the circulating trypomastigotes are scarce and are detected only by indirect methods such as xenodiagnosis and hemoculture. By repeated testing and the use of modifications of the established hemoculture technique, the sensitivity has recently been increased, allowing the detection of the parasite in 95% of chronic patients (19).

The treatment of Chagas' disease with nitroimidazole derivatives in both acute and recent chronic infections may prevent pathologic effects in the later stages of disease (6). The indication of such treatment in the chronic phase is still controversial because most treated patients continue to have positive CS, even though their hemocultures become less frequently positive than those of the untreated, chronically infected patients (7). The serological test of complement-mediated lysis (CoML) has been used as an alternative method to determine cure, and it detects the presence of protective antibodies that circulate during chronic infections but not in the uninfected immunized host (13). The protective antibodies, so-called lytic antibodies, recognize antigens on the surface of living trypomastigotes and represent a class of antibodies distinct from those detected by CS. The latter, found in nonprotected immunized hosts, do not recognize living trypomastigotes in CoML tests (13) or their antigens of high molecular masses (150 to 160 kDa) in immunoprecipitation assays (20).

Patients in the chronic phase, consistently positive by the CoML assay, may become negative 6 to 8 months after specific treatment. Among treated patients, about 20% presented repeatedly negative CoML tests for years, although their CS remained positive. These patients, termed "dissociated," have been considered cured (14). After a 10-year follow-up, the negative hemoculture confirmed that they had in fact resolved the infection (7). Thus, the CS is not a reliable method to assess cure after treatment.

A negative CoML test predicts elimination of *T. cruzi* in treated patients, but this test has practical limitations since it requires live and infectious parasites (12). To identify an antigen(s) specifically recognized by the lytic antibodies, and thus to allow detection of cure through more practical tests such as enzyme-linked immunosorbent assay (ELISA) and Western blotting (immunoblotting), has been our goal. Purified *T. cruzi* antigens were recently tested by ELISA, which included the GP57/51 glycoprotein characterized as a cysteine proteinase (21) and trypomastigote-excreted and -secreted antigens. High correlations (70 and 90%, respectively) between negative ELISA and CoML results were observed in two studies (8, 11). Additionally, the glycoconjugate, F2, extracted from trypomastigotes (74 and 96 kDa) was recognized by the sera of chagasic patients with positive CoML results but not by sera from dissociated patients (1).

The ideal antigen to be used in tests to replace the CoML test is the 160-kDa glycoprotein, considered one of the target epitopes of the lytic antibodies on the surface of the parasite, since only sera from patients that had the lytic antibodies recognized GP160 (20). GP160 purified from trypomastigote surfaces (24) has been characterized as a regulatory protein for C3 convertase activity in the complement system (22), being capable of binding the C3b component. Indeed, C3b had been

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found on the surfaces of circulating blood trypomastigotes from mice (15), and it binds to acellular cultured parasites (9). Antibodies against GP160 induce trypomastigote lysis, probably because they block the regulatory activity of GP160, allowing the complete activation of the complement system (22, 24). Favoring this hypothesis is the previous finding that the immunoglobulin (Ig)-Fc portion of the lytic antibody from chronic chagasic patients is not necessary for the lysis (10), making more likely the process of activation through the alternative pathway (16).

GP160, isolated in a C3b affinity column, has recently been tested by using sera from chagasic patients in Western blots. All sera from nontreated chagasic patients were positive, while sera from dissociated patients were negative, correlating with the negative results of CoML and confirming its applicability as a specific indicator of parasitological cure after treatment (23). Purification of GP160 and the ability to obtain it in large amounts are limiting factors, since 10^{10} parasites yield only 5 μg of protein; moreover, the protein is rapidly degraded, even when stored at −70°C (24). Because of these technical limitations, we have not used GP160 in the present studies; however, when it is available as a recombinant protein, its use will be more feasible.

Since purified antigens in ELISAs discriminate most patients with negative CoML tests (1, 8, 11) and considering the importance of appropriate monitoring of treated chagasic patients, we proceeded in a search for an ideal antigen for identifying all cured patients. In this work we describe, for the first time, the use of a recombinant *T. cruzi* protein, rTc24, in ELISAs and Western blots in an attempt to identify all the dissociated individuals. Our interest in testing this protein resulted from the recent demonstration of its ability to induce lytic antibodies and to induce protection against *T. cruzi* infections in 30 to 40% of all immunized mice (28).

MATERIALS AND METHODS

Patients. Sera from chronic chagasic patients with cardiac and indeterminate forms of the disease, all treated and clinically evaluated by one of us, were studied. The group was composed of 72 chagasic patients treated with either 3-methyl-4(5′-nitrofurfurylideneamino)-tetrahydro-4H-1,4-thiazine-1-dioxide (nifurtimox) or *N*-benzil-1-nitro-1-imidazoleacetamide (benznidazole). On the basis of positive CoML and IIF tests, 40 of these 72 treated patients were considered noncured. This group included 14 patients with positive parasitologic tests (hemoculture) and 26 patients with negative hemoculture results. Another group of 28 similarly treated patients had repeatedly negative results by the hemocultures and the CoML test and positive CS for Chagas' disease, i.e., they were dissociated and were considered cured. A third group of four cured patients was consistently negative by serology (CoML and IIF) and parasitologic tests (hemoculture and xenodiagnosis). The control group, consisting of 35 nonchagasic subjects, included 11 individuals from areas of endemicity and 24 individuals from areas of nonendemicity who had at least three negative serologic results for *T. cruzi*. Informed consent was obtained from the patients or their guardians, and the human experimentation guidelines of the Federal University of Minas Gerais (Belo Horizonte, Brazil) were followed in conducting the clinical research.

Subcloning and purification of Tc24. A *T. cruzi* trypomastigote cDNA clone encoding a 24-kDa protein (Tc24) (28) was purified and subcloned in a pGEX plasmid known to be a high-expression vector. The pGEX-2T vector directs the synthesis of foreign polypeptides in *Escherichia coli* as a fusion polypeptide with the 26-kDa *Schistosoma japonicum* gluthatione *S*-transferase (GST). The DNA insert encoding the Tc24 protein was ligated in the corresponding cloning site of the plasmid, and the products were expressed in *E. coli*. Positive clones were selected, and the purification of the fusion protein was carried out as described previously (27). The purity of the recombinant protein was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (17).

ELISA technique. A standard ELISA (5) was used to detect antibodies against the recombinant Tc24 protein in sera from treated chagasic patients. The optimal concentrations of serum, antigen, and conjugate were determined by checkerboard titration. Briefly, Immulon I plates (Dynatech Laboratories, Alexandria, Va.) were coated with 100 μl of 0.5-μg/ml Tc24 recombinant antigen in 0.05 M carbonate bicarbonate buffer (pH 9.6), and the plates were incubated overnight at 4°C. The unbound material was discarded, and all plates were blocked with 200 μl of 0.15 M phosphate-buffered saline (PBS) (pH 7.2) containing 0.05% Tween 20 (Sigma Chemical Co., St. Louis, Mo.) and 1% bovine serum albumin (BSA; Sigma Chemical Co.) for 2 h at 37°C. After five washes with PBS-Tween 20, 100 μl of human serum diluted 1:100 in PBS-Tween 20 and 0.3% BSA were added and the plates were incubated for 2 h at 37°C. The plates were washed 10 times with PBS-Tween 20, 100 μl of a 1:1,000 dilution of peroxidase-conjugated anti-human IgG (γ chain specific) (Sigma Chemical Co.) was added to the wells, and the plates were incubated for 1 h at 37°C. After 10 washes with PBS-Tween 20, 100 μl of a 1:1 mixture of 2,2-azino-di(3-benzthiazondine sulfonate) and hydrogen peroxide (Sigma Chemical Co.) was added to each well and the plates were incubated for 15 min at 37°C. The reaction was stopped by the addition of 50 μl of 10% SDS solution to each well. Optical density was measured at 405 nm by using an automated ELISA reader (model 2550 EIA Reader; Bio-Rad Laboratories). All the assays were carried out in duplicate. Since the Tc24 recombinant protein was expressed as a fusion protein with GST, parallel ELISAs using the GST recombinant protein alone as the antigen were carried out under the same conditions as were used for the Tc24 fusion protein.

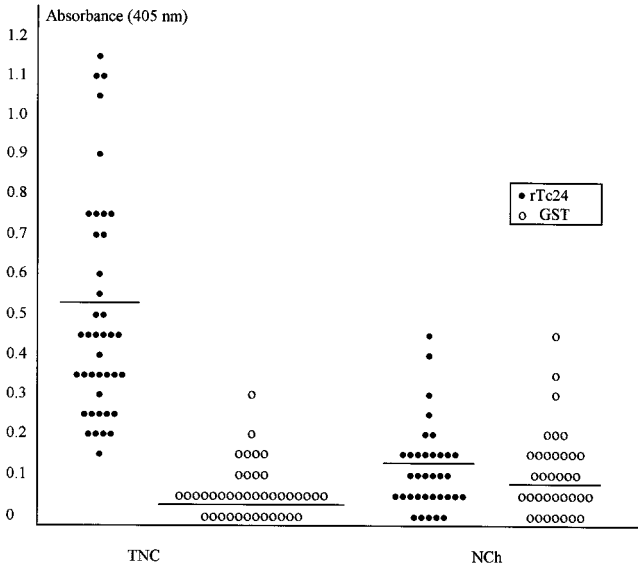

FIG. 1. Individual and average reactivities of sera from 40 chagasic patients who were treated but not cured (TNC) and from 35 nonchagasic controls (NCh) with the 24-kDa recombinant protein of *T. cruzi* (rTc24) and the GST of *S. japonicum* as determined by ELISA.

Western blot analyses. Seventy-five micrograms of recombinant Tc24 was electrophoresed in an SDS–12% polyacrylamide gel, and the proteins were transferred from the gels to nitrocellulose paper (Bio-Rad) (29). After transfer, the nitrocellulose paper was blocked by using a solution consisting of PBS (pH 7.2), 0.05% Tween 20, and 5% delipidized milk and incubated overnight at 4°C. Strips of paper were then cut and incubated separately with a 1:100 dilution of human chagasic serum for 2 h at room temperature. Control sera from both nonchagasic individuals and chagasic treated and cured individuals were also analyzed. Strips were then washed three times in PBS-Tween 20 and incubated for 2 h at room temperature with alkaline phosphatase-conjugated anti-human IgG (heavy and light chain specific) (Promega, Madison, Wis.) diluted 15,000 times in PBS-Tween 20. The strips were washed as before, and a 1:1 mixture of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate toluidinium (Promega) was added as a substrate. After color development, the reaction was stopped by immersion of the strips in distilled water.

RESULTS

The ability of the fusion protein, rTc24, to detect chagasic infections was evaluated by ELISA and by comparing its average reactivity with that obtained with GST isolated from the nonrecombinant plasmid. Forty sera from treated patients considered noncured on the basis of the consistently positive results of CS and CoML and 35 sera from nonchagasic individuals were tested (Fig. 1). Sera from chagasic patients recognized specifically the *T. cruzi* epitope expressed by rTc24 (average absorbance = 0.501), with little reactivity to GST (average absorbance = 0.066) (Fig. 1). Sera from healthy in-

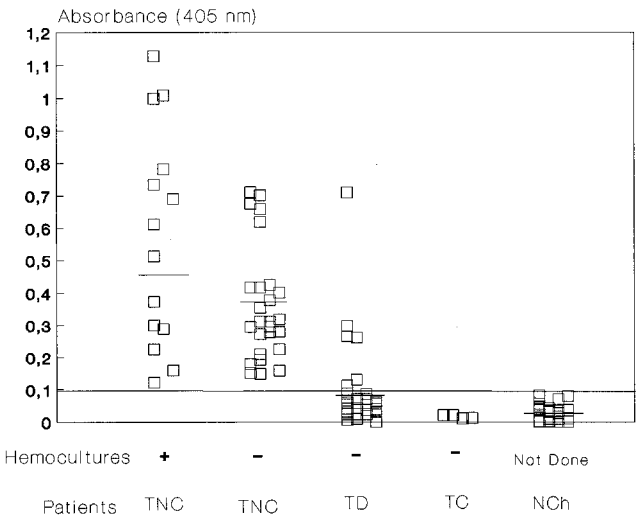

FIG. 2. IgG antibodies against the recombinant protein of *T. cruzi*, rTc24, detected by ELISA, in sera of chagasic patients treated but not cured (TNC), with positive (+) or negative (−) hemocultures, treated dissociated patients (TD), treated cured patients (TC), and nonchagasic individuals (NCh).

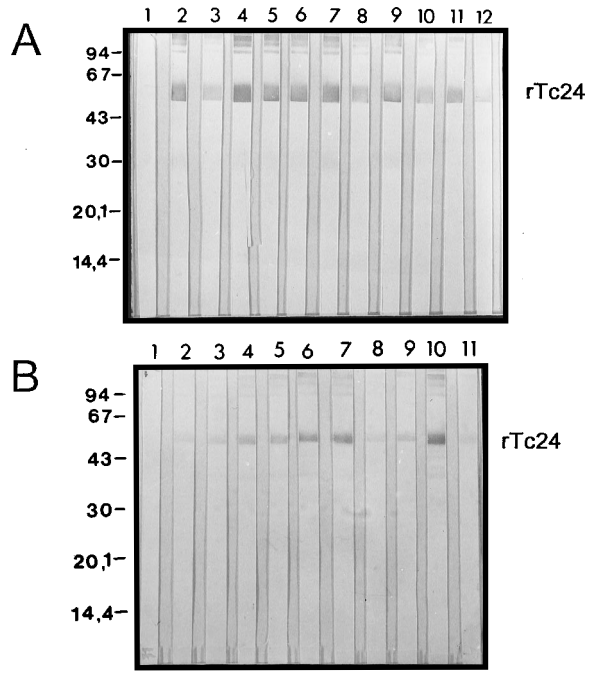

FIG. 3. Western blot of the fusion protein rTc24 with sera from nonchagasic individuals (lanes 1) and sera from treated but uncured chagasic patients with positive (panel A, lanes 2 to 12) or negative (panel B, lanes 2 to 11) hemocultures.

dividuals reacted weakly with both rTc24 and GST (average absorbances, 0.120 and 0.107, respectively). However, four sera in this group which had high absorbance values for GST (0.469, 0.310, 0.275, and 0.149) also had the highest values for rTc24 (0.485, 0.389, 0.276, and 0.242, respectively) (Fig. 1). This reinforces the idea that the reactivity of normal sera with rTc24 is not specific and that it is directed against the GST portion of the recombinant protein. There was no statistically significant difference in reactivity with the GST between sera from nonchagasic controls and sera from individuals who were treated but not cured. Although sera from nonchagasic individuals reacted more strongly with GST than did sera from chagasic patients, these values were not significantly different ($P > 0.05$). The values obtained by the rTc24-specific ELISA were analyzed after they were subtracted from values obtained by the GST-specific ELISA for each individual serum. Therefore, the cutoff value (0.1) for the rTc24 protein was defined as the average of the normal serum absorbances after subtraction of the GST reactivity (0.025) plus 2 standard deviations (Fig. 2).

The levels of antibodies against rTc24 measured by ELISA in individual serum samples from treated chagasic patients from the different groups as well as those measured in samples from nonchagasic controls are shown in Fig. 2. All sera from the treated but uncured patients (14 of them parasitologically positive and 26 of them negative) reacted with rTc24, showing absorbance values of greater than 0.1 (Fig. 2). There was no significant difference between the two groups of treated chagasic patients. Of 28 sera from treated dissociated patients (negative CoML result but positive IIF result), 22 (79%) showed absorbance values of less than 0.1, similar to the case with sera from nonchagasic individuals. Sera from six dissociated patients showed antibody levels similar to those observed in the sera from treated but uncured chagasic patients, whereas sera from four cured patients (negative CoML and IIF results) showed reactions with rTc24 similar to those of 35 normal sera from nonchagasic individuals of whom 11 were living in the area of endemicity.

When tested by rTc24-specific Western blotting (Fig. 3), all the sera from treated but uncured chagasic patients recognized the fusion protein, regardless of positive (Fig. 3A, lanes 2 to 12) or negative (Fig. 3B, lanes 2 to 11) hemocultures. Of the 15 sera from treated dissociated patients, 11 lost their ability to recognize rTc24 or had it decreased (Fig. 4A, lanes 14 to 23, and Fig. 4B, lane 24), the results thus correlating with the

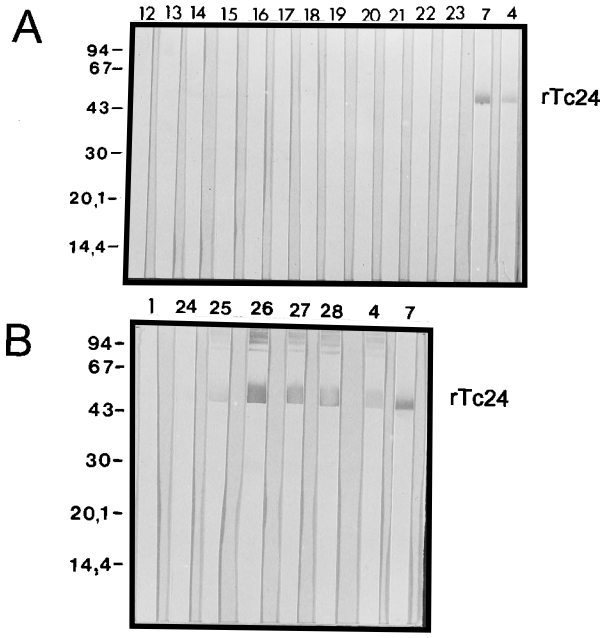

FIG. 4. Western blot of the fusion protein Tc24 with sera from two nonchagasic individuals (panel A, lane 12, and panel B, lane 1), 2 treated but uncured patients (lanes 4 and 7), 1 treated cured patient (panel A, lane 13), and 15 treated dissociated patients. Of the last group, 11 sera were negative by ELISA (panel A, lanes 14 to 23, and panel B, lane 24) and 4 were positive (panel B, lanes 25 to 28).

negative results of CoML and hemoculture. Sera from dissociated patients with absorbance values higher than 0.2 (Fig. 2) recognized rTc24 in Western blots (Fig. 4B, lanes 25 to 28), whereas two that presented borderline absorbance values (0.1) (Fig. 2) did not react with rTc24 in a Western blot (Fig. 4A, lanes 16 and 23). Serum from one cured patient (negative CoML, IIF, and hemoculture results) was also negative for the reaction (Fig. 4A, lane 13).

DISCUSSION

CS, which are very important for the diagnosis of infection with *T. cruzi* in the chronic phase, have limitations when used as a criterion of therapeutic cure. In a follow-up study of 100 treated patients, the cure level was only 8% if the negative CS was used as a criterion. However, 60% of these patients had repeatedly negative results by xenodiagnosis (6). In another 10-year follow-up study of 82 treated patients, only 9% of the patients could have been considered cured on the basis of the negative results of CS, even though 26% of them, consistently positive by CS, had negative CoML results. Such patients, described as dissociated, have been considered cured, and their hemocultures, in an average of 3.3 tests for each patient, have been negative (7).

In the present study, we evaluated the use of ELISAs and Western blot tests using a recombinant 24-kDa protein of *T. cruzi* as an alternative to the CoML test. This protein is found in the membrane of all the evolutive stages of the parasite, particularly in the flagella of trypomastigote and epimastigote forms (4, 25, 28). Its gene sequence shows similarities to the sequences of several proteins that bind calcium (4, 18). Tc24 has high antigenicity, as determined by the high frequency with which it has been cloned by screening with sera from chagasic patients or animals in an experimental chronic phase of infection (4, 18, 25, 28). Tc24 purified from cultured *T. cruzi* has been used for the specific diagnosis of human Chagas' disease (26). Two synthetic peptides derived from the primary sequence of rTc24 were used to immunize BALB/c mice. One of them decreased by up to 50% the mortality in the acute phase, this protection being attributed to an epitope that stimulated the proliferation of T cells. The other peptide, despite inducing significant levels of IgG1 antibodies, did not produce protection (28).

Our results confirm the immunogenicity of Tc24, since all sera from treated but uncured patients who had active infections in spite of treatment recognized the fusion protein in ELISAs and Western blots. The reactivity was specific for the *T. cruzi* antigen, since those sera recognized weakly the GST from *S. japonicum*. In fact, sera from chagasic patients or healthy individuals react weakly with the portion of the recombinant protein that is not related to *T. cruzi*, a problem that can be solved by obtaining the recombinant antigens in expression vectors that allow the separation of the protein of interest.

Specific antibodies against rTc24 persist in approximately 20% of the sera from dissociated patients. It is possible that these patients produce high levels of antibody against Tc24 before treatment that persist after elimination of the parasite. Alternatively, residual antigens of *T. cruzi*, which have been demonstrated to occur in treated and cured mice (3), could induce specific anti-Tc24 antibodies without live parasites.

Despite the high positive correlation between the specific ELISA for rTc24 and CoML, this antigen is not the main target for lytic antibodies. Sera from mice immunized with rTc24 were able to induce lysis of only 15% of the trypomastigotes (28). Moreover, previous studies have indicated that important targets recognized by the lytic antibodies are glycoconjugates containing the galactosyl terminal residue (1, 2), as well as GP160 (24), a molecule that seems to block the activation of the C3 convertase present on the surface of the parasite (22).

In conclusion, we demonstrated that it is possible to distinguish between the majority of the dissociated treated patients and the treated but uncured patients by using the 24-kDa protein from *T. cruzi* in ELISAs and Western blot assays. This finding has practical implications, since it is possible to synthesize pure recombinant proteins in large amounts and since, additionally, these proteins are likely more stable than antigens purified from cultured parasites. Such factors make viable the use of recombinants for initial trials to evaluate cure of chagasic patients submitted to specific treatment.

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