USE OF AN IRON SUPEROXIDE DISMUTASE EXCRETED BY *TRYPANOSOMA CRUZI* IN THE DIAGNOSIS OF CHAGAS DISEASE: SEROPREVALENCE IN RURAL ZONES OF THE STATE OF QUERETARO, MEXICO

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Abstract. Four iron superoxide dismutase (SODI, SODII, SODIII, and SODIV) activities with pI values of 6.9, 6.8, 5.25, and 3.8, respectively, were isolated from epimastigote forms of the Maracay strain of *Trypanosoma cruzi* cultivated at 28°C in Grace’s medium supplemented with 10% heat-inactivated fetal bovine serum. The activity of SODe (pI 3.8), which coincides with that of SODIV, was detected in Grace’s medium without serum in which *T. cruzi* epimastigotes were cultivated for 24 hours at 28°C. SODe, which was excreted into the medium by the parasite, was immunogenic and antibodies to SODe were detected in serum to a dilution of 1:2,500 by Western blot. The role of SODe is related to the establishment of the parasite within the host, and its high immunogenicity and specificity make it a useful molecular marker in diagnosing infection with this parasite. To validate a Western blot result using SODe as a antigen fraction, 1,029 sera of individuals from 11 municipalities in the state of Queretaro, Mexico were analyzed. Sampling was done randomly and results were compared with those for the same sera with three conventional serologic methods: an enzyme-linked immunosorbent assay (ELISA), indirect hemagglutination (IHA), and an indirect immunofluorescence assay (IFA) to detect antibodies to *T. cruzi* SODe. Samples that were positive by these three techniques were also positive by the Western blot method. The seroprevalence values for SODe (8.16% by ELISA and Western blot) in Queretaro were considerably higher than those reported in regions of Mexico considered to be endemic for Chagas disease. These results support the use of SODe in the serodiagnosis of Chagas disease.

INTRODUCTION

Chagas disease, which is caused by the protozoan parasite *Trypanosoma cruzi*, is now ranked as the most serious parasitic disease in Latin America, and has a heavy economic impact. The disease is transmitted in two ways: by vectors (triatomes) and by transfusions with infected blood. In recent years, because of migration from rural to urban areas, Chagas disease has become a serious health problem in cities in Latin America, where it can be transmitted vertically as well as by blood transfusions or organ transplants.

Infection with this parasite is characterized by an acute parasitemic phase of 1–2 months, followed by a life-long chronic phase in which bloodstream parasites are detectable only by indirect methods such as xenodiagnosis or hemoculture. Most patients are diagnosed during the chronic phase by conventional serologic tests, such as an indirect immunofluorescence assay (IFA), an enzyme-linked immunosorbent assay (ELISA), a complement fixation test, or indirect hemagglutination (IHA).

Although these assays when used with crude parasite extracts are very sensitive in detecting *T. cruzi* infections, a problem with these assays is that they yield a small but significant number of false-positive results, especially when sera from patients with leishmaniasis are tested. This can make a correct diagnosis difficult, especially in areas in which both diseases are endemic, resulting in the rejection of a large number of blood donations. Another problem of the serologic methods concerns their sensitivity, which is not always adequate, leading to increased transmission of the disease.

This situation has resulted in the search for antigens from *T. cruzi* that could be used for the detection of this parasite. Two molecules have been detected: 1) a cysteine proteinase (GP57/51) that is released during metacyclogenesis and a 70-kD heat-shock protein that may be useful in diagnosing infection with this parasite.

For many intracellular parasites and bacterial pathogens, superoxide dismutase (SOD), a metalloenzyme present in all oxygen-breathing organisms, constitutes the first line of defense against oxidative damage caused by the formation of superoxide radicals. This enzyme catalyzes the reduction of superoxide radicals to molecular oxygen and hydrogen peroxide. Three classes of SODs, each with different metal cofactors (iron, manganese, and copper-zinc), have been identified. They also differ in their location in the cell (cytosol, other cell organelles, and cell secretions).

All parasitic protozoa studied to date have SODs with only iron cofactors. Ever since it was discovered that SODs from parasitic protozoa had iron cofactors and that they differed from those of the vertebrate host not only structurally but also terms of specific inhibitors, they have been proposed as potential chemotherapeutic targets. In some parasitic protozoa, it has been demonstrated that some SODs involved in oxidative attack are highly immunogenic, raising the possibility of using them in the diagnosis of Chagas disease.

We recently characterized two iron superoxide dismutase activities (SODI and SODII) from a plant trypanosomatid isolated from *Euphorbia characias*. The isoenzyme SODII is immunogenic and is excreted into the culture medium by this plant trypanosomatid (Villagrán ME and others, unpublished data). In *T. cruzi*, SODs have been detected in the epimastigote stage; however, their presence and role in other developmental stages of the parasite is unknown.

The purpose of this study was to determine whether *T. cruzi* excretes an SOD (SODe) similar to that excreted by plant trypanosomatids and to analyze its potential immuno-
logic properties and usefulness in diagnosing infection with this parasite. To demonstrate the diagnostic usefulness of SODe, we tested 1,029 sera from individuals in the state of Queretaro, Mexico, by an IFA, IHA, and an ELISA, as well as by a Western blot, against the antigenic SODe fraction obtained from cultures of the Maracay strain of *T. cruzi*. This enables us to obtain new data on the prevalence of Chagas disease in this area. In addition, assays were conducted with sera from patients with Chagas disease, as well as with sera from patients in Peru with leishmaniasis and from patients in Spain infected with *Toxoplasma gondii* and *Mycobacterium leprae*.

**MATERIALS AND METHODS**

**Parasites and culture.** The Maracay strain of *T. cruzi* was isolated from a clinical specimen at the Institute of Malariology and Environmental Health in Maracay, Venezuela. Epimastigotes were grown in axenic Grace’s medium (Gibco-BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum at 28°C. Epimastigote cultures (in the exponential growth phase) were concentrated by centrifugation at 600 × g for 10 minutes, and cells were washed twice and resuspended in ice-cold STE buffer (0.25 M sucrose, 25 mM Tris-HCl, 1 mM EDTA, pH 7.8) (buffer 1).

**Extraction and purification of SOD isoenzymes.** Cells were suspended (0.5–0.6 grams wet weight/mL) in 3 mL of buffer 1 and disrupted by three cycles of sonic disintegration (each for 30 seconds at 60 volts). The sonicated homogenate was centrifuged at 1,500 × g for 10 minutes at 4°C, and the pellet was washed three times with buffer 1 and resuspended in this buffer to obtain a total supernatant fraction of 9 mL. This fraction was centrifuged (2,500 × g for 10 min at 4°C), the supernatant was collected, and solid ammonium sulfate was added. The protein fraction, which precipitated at salt concentrations of 30–85%, was centrifuged (9,000 × g for 20 minutes at 4°C), redissolved in 2.5 mL of 20 mM potassium phosphate buffer, pH 7.8, containing 1 mM EDTA (buffer 2), and purified on a Sephadex G-25 column (PD10; Pharmacia, Piscataway, NJ) that was equilibrated with buffer 2. This resulted in a final volume of 3.5 mL (fraction P5)

**Enzyme assay.** The SOD activity was determined by NAD(P)H oxidation according to Paoletti and Mocali. A unit was the amount of enzyme required to inhibit the rate of NAD(P)H reduction by 50%. The protein content was determined in all fractions by the Bio-Rad (Hercules, CA) test, which was based on the Bradford method (Sigma, St. Louis, MO), with bovine serum albumin as a standard.

**Gel electrophoresis.** Isoelectric focusing in a polyacrylamide Phast (Pharmacia) gel pH 3–9 was performed as previously described. The gels were stained according to a protocol reported elsewhere and stained for protein with silver nitrate as described by the manufacturer.

**Determination of the isoelectric point of purified enzymes.** Isoelectric points were determined in the Phast System using trypsinogen, lentil-lectin acidic band, horse heart myoglobin, carbonic anhydrase II, β-lactoglobulin A, soybean trypsin inhibitor, and amyloglucosidase from *Aspergillus niger* (pI values of 9.3, 8.15, 6.8, 5.9, 5.1, 4.6, and 3.6, respectively) as standards. The gels were stained for protein with silver nitrate and for SOD activity as described elsewhere.

**Antigen preparations.** Epimastigote forms in the exponen-
Serologic assays. Epimastigotes of the Maracay strain of *T. cruzi* were used as the antigenic fraction in these assays. For the ELISA, optimal concentrations of antigen, serum, and conjugate were determined by checkerboard titration, and epimastigote homogenates were used at a concentration of 1.25 µg/mL, following the procedure of Krautz and others. Samples showing reactivity at dilutions > 1:32 were considered positive. The IFA was carried out following the method of Camargo, and samples with titers > 1:16 were considered positive. Indirect hemagglutination was performed according to the method of Camargo and others. All samples were analyzed in triplicate in polystyrene microtiter plates, and samples with titers > 1:32 were considered positive. In all cases, positive and negative controls were also analyzed simultaneously.

RESULTS

When a partially purified fraction of the epimastigote forms of the Maracay strain of *T. cruzi* was used for isoelectric focusing polyacrylamide gel electrophoresis (pI 3–9), followed by staining for SOD, four different SOD bands (SODI, SODII, SODIII, and SODIV) were detected (Figure 1, lane A). The SODI and SODII bands had similar isoelectric points (6.8–6.9) and were considered a double-banded isoenzyme. The other two bands (SODIII and SODIV) were single bands with isoelectric points of 5.25 and 3.8, respectively.

To identify the metal cofactors of the SODs, we tested inhibitors that have known effects on various cofactors (Cu/Zn, Mn, or Fe) of these enzymes. The four SODs were inhibited by H$_2$O$_2$, but not by cyanide, indicating that all four enzymes had an iron cofactor.

When epimastigote forms of *T. cruzi* (5 × 10$^{10}$ parasites) were cultured for 24 hours in Grace’s medium without serum and the cell-free supernatant was collected, concentrated, and subjected to isoelectric focusing (pI 3–9), followed by staining for SOD activity, only a single SOD band was detected (Figure 1, lane B). The isoelectric point of this SOD was consistent with the isoelectric point of the SODIV band detected in the partially purified fraction of *T. cruzi* epimastigotes cultured under normal conditions.

We identified specific antibodies against SOD of *Trypanosoma cruzi* obtained from immunized BALB/c mice and identified by Western blot using SOD as the antigen fraction (Figure 2). With the control serum (Figure 2, lane 4), the reaction was negative at a dilution of 1:100. Sera containing antibodies to SOD (Figure 2, lanes 1–3) showed reactivity at dilutions of 1:100, 1:500, and 1:2,500.

To confirm the usefulness of SOD in diagnosing Chagas disease, we tested 1,029 blood samples from 11 municipalities in Queretaro, Mexico by Western blot, using the purified SOD extract as the antigen fraction (Table 1). We used the results of three conventional serologic methods (ELISA, IHA, and IFA) as standards. Eighty-five samples at a dilution of 1:100 were positive by Western blot against the SOD antigen fraction (Figure 3, lanes 1–6), giving a seroprevalence of 8.26% (Table 1). The seroprevalences of the different

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**Figure 1.** Determination of the isoelectric points by isoelectric focusing (pI 3–9) of isoenzymes of superoxide dismutase (SOD) of the Maracay strain of *Trypanosoma cruzi*. Lane M, marker proteins with various pI values stained with Coomassie Blue; lane A, purified fraction of *T. cruzi*; lane B, SOD fraction excreted by *T. cruzi*. The SOD activity was determined according to the technique of Beyer and Fridovic.

**Figure 2.** Detection of the excreted superoxide dismutase (SODe) isoenzyme of *Trypanosoma cruzi* by Western blot with various dilutions of polyclonal antibodies to SODe. Lane 1, 1:100; lane 2, 1:500; lane 3, 1:2,500; lane 4, control serum diluted 1:100.
Mexican municipalities ranged from 22.44% in San Juan del Río to 2.75% in Toliman (Figure 4). Serum at a dilution of 1:100 from a patient in Peru with Chagas disease was also positive (Figure 3, lane 10), while sera from a patient in Peru with leishmaniasis (Figure 3, lane 7) and from patients in Spain with leprosy (Figure 3, lane 8) and toxoplasmosis (Figure 3, lane 9) were negative.

Of the 1,029 samples assayed, 54 were positive by least two of the conventional serologic tests used, giving a seroprevalence of 5.25%. However, when the ELISA results were compared with those of the Western blot, the seroprevalence increased to 8.16% (Figure 5). The concordance between various combinations of the tests used is shown in Figure 6.

**DISCUSSION**

We recently observed that a trypanosomatid isolated from a plant (*Phytomonas* spp. isolated from *Euphorbia characias*) has two SODs isoenzymes with iron cofactors. On studying its subcellular location, we found that 20% of the activity was not solubilized even at high concentrations of digitonin, indicating that some of the SOD activity could be associated with membranes or even be excreted to the exterior by the parasite. This has been confirmed in species of the genus *Phytomonas* in our laboratory (Villagráín ME and others, unpublished data), and it has also been demonstrated that this excreted SOD is highly immunogenic. *Phytomonas* shows metabolic similarities to other trypanosomatids of animals and humans, such as *T. cruzi*. For these reasons, we investigated whether *T. cruzi*, in which two SOD activities had been demonstrated, excreted an SOD that serves the parasite as a defense mechanism against toxic radicals generated by the host.

In the present study, we have demonstrated that *T. cruzi* excreted an SOD with an iron cofactor and a pI of 3.8. SODe was obtained from epimastigote forms cultured for 24 hours in Grace’s medium without serum. Marker enzymes (pyruvate kinase and hexokinase) confirmed that there was no lysis of the parasite during this culture period, and that the presence of the SOD was due its excretion by the parasite. In addition to this SODe, three other isoenzymes with different pIs were also detected. The presence of several isoenzymes can be explained by the fact that each has a different location in the parasite and carries out different functions in various developmental stages.

It is known that in other parasitic protozoa, SOD is an immunogenic protein. One aim of the present study was to study the immunogenic properties of SODe. To achieve this, we isolated polyclonal antibodies against SODe. Control se-

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**TABLE 1**

Seroprevalence of antibodies against *Trypanosoma cruzi* in individuals in different municipalities of the State of Queretaro, Mexico by conventional serologic tests and Western blot against the SODe antigen fraction of *T. cruzi*.

<table>
<thead>
<tr>
<th>Municipalities</th>
<th>No. of persons sampled</th>
<th>Seroreactive in ≥ 2 tests</th>
</tr>
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<tr>
<td></td>
<td>ELISA</td>
<td>IHA</td>
</tr>
<tr>
<td>Arroyo Seco</td>
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<td>Toliman</td>
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<td>8</td>
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<td>Cadereyta</td>
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<td>16</td>
</tr>
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<td>7</td>
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</tr>
<tr>
<td>El Marqués</td>
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</tr>
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<td>3</td>
</tr>
<tr>
<td>Corregidora</td>
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</tr>
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</tr>
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<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>1,029</td>
<td>84</td>
</tr>
</tbody>
</table>

* SOD = superoxide dismutase; ELISA = enzyme-linked immunosorbent assay; IHA = indirect hemagglutination; IFA = indirect immunofluorescence assay.

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**Figure 3.** Immunoblot of sera from patients with various diseases against the superoxide dismutase excreted by the Maracay strain of *Trypanosoma cruzi*. Lanes 1–6, samples (diluted 1:100) from patients with Chagas disease in the state of Queretaro, Mexico; lane 7, sample from a patient with leishmaniasis in Peru; lane 8, sample from a patient with leprosy in Spain; lane 9, sample (diluted 1:50) from a patient with toxoplasmosis in Spain; lane 10, sample (diluted 1:100) from a patient with Chagas disease in Peru.
rum showed no reactivity against SODc by Western blot, while serum containing antibodies to SODc showed reactivity up a dilution of 1:2,500. This result indicates that SODc is a highly immunogenic protein, raising the possibility of its use in the diagnosis of Chagas disease.

To determine whether SODc could be used in the diagnosis of Chagas disease, we conducted serologic studies in Queretaro, Mexico. We investigated the use of SODc as the antigenic fraction in a diagnostic Western blot, and determined the seroprevalence of this disease in an area of Mexico in

\[\text{FIGURE 4. Distribution of antibodies to } \text{Trypanosoma cruzi} \text{ detected by an enzyme-linked immunosorbent assay and Western blot in individuals from different municipalities in the State of Queretaro, Mexico. Numbers indicate the percentages of seropositive individuals.}\]
which few data are available, but which is close to other states (Hidalgo, Guanajuato, and San Luis de Potosí) that are endemic for this disease. This is important because Chagas disease is a serious health problem in Mexico (e.g., in the Yucatan, its has a prevalence of 11.20%).

The Pan American Health Organization recommends that when performing an epidemiologic study of Chagas disease, more than one serologic procedure should be used to reduce diagnostic errors. Confirmation of undetermined cases of American trypanosomiasis by serologic diagnosis is made when there are positive results in at least two serologic tests. For this reason, in the present study samples were analyzed by three conventional serologic tests, in addition to the Western blot with SODe as antigen. In the ELISA, IHA, IFA test, and Western blot, the Maracay strain of T. cruzi was used as antigen because other investigators have reported that the strain of T. cruzi used does not appear to influence the results of serologic testing.

When we compared values obtained with two of the conventional serologic tests (ELISA and IFA), we found seroprevalence values of 5.64%. However, when we compared the results of the ELISA with those of the Western blot, the seroprevalence increased to 8.16% and the concordance between the two techniques was nearly 100%. Seroprevalence in a given municipality was random, fluctuating between 22.45% for San de los Ríos and 2.75% for Toliman. When we compared values obtained with two of the conventional serologic tests, in addition to the Western blot with SODe as antigen, we concluded that the Western blot with the SOD antigen excreted by the parasite was more specific and sensitive than the ELISA (seroprevalence of the Western blot = 8.26%).

It is known that at least 10% of patients with Chagas disease have serum antibodies that cross-react with some species of the genus Leishmania. Therefore, we also tested sera from patients with leishmaniasis, toxoplasmosis, and leprosy. None of these sera showed a positive reaction. However, the dilution titers used may have been too high to detect cross-reactivity.

The results of the present study confirm that T. cruzi excretes an SOD (with a pI of 3) that has a role in the establishment of the parasite in the host. This SOD is highly immunogenic and specific, and therefore should be a useful tool in the diagnosis of Chagas disease. The seroprevalence values found among the rural population of the state of Queretaro, Mexico are considerably higher than those published by the health authorities. It would be advisable to establish obligatory diagnosis of Chagas disease by means of serologic tests (including the Western blot with SODe) for all blood donors. This is necessary because as a consequence of demographic shifts, the parasite has migrated beyond the disease-endemic area due to transmission by blood transfusions. Received September 8, 2004. Accepted for publication December 2, 2004.

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