

## Design, Construction, and Evaluation of a Specific Chimeric Antigen To Diagnose Chagasic Infection

Sebastián Aguirre,<sup>1</sup> Ariel M. Silber,<sup>2</sup> Maria Edileuza F. Brito,<sup>3</sup> María E. Ribone,<sup>4</sup>  
Claudia M. Lagier,<sup>4</sup> and Iván S. Marcipar<sup>1\*</sup>

*Instituto de Tecnología Biológica, INTEBIO, Universidad Nacional del Litoral, Santa Fe, Argentina*<sup>1</sup>; *Departamento de Parasitologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, Brasil*<sup>2</sup>; *Centro de Pesquisas Aggeu Magalhães, Fundação Oswaldo Cruz, Recife, Brasil*<sup>3</sup>; and *Departamento de Química Analítica, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina*<sup>4</sup>

Received 18 May 2006/Returned for modification 4 July 2006/Accepted 31 July 2006

**Chagas' disease is routinely diagnosed by detecting specific antibodies (Abs) using serological methods. The methodology has the drawback of potential cross-reactions with Abs raised during other infectious and autoimmune diseases (AID). Fusion of DNA sequences encoding antigenic proteins is a versatile tool to engineer proteins to be used as sensitizing elements in serological tests. A synthetic gene encoding a chimeric protein containing the C-terminal region of C29 and the N-terminal region of TcP2 $\beta$  was constructed. A 236-serum panel, composed of 104 reactive and 132 nonreactive sera to Chagas' disease, was used to evaluate the performance of the chimera. Among the nonreactive sera, 65 were from patients with AID (systemic lupus erythematosus and rheumatoid arthritis) or patients infected with *Leishmania brasiliensis*, *Brucella abortus*, *Streptococcus pyogenes*, or *Toxoplasma gondii*. The diagnostic performances of the complete TcP2 $\beta$  (TcP2 $\beta_{FL}$ ) and its N-terminal region (TcP2 $\beta_N$ ) were evaluated. TcP2 $\beta_{FL}$  showed unspecific recognition toward leishmaniasis (40%) and AID Abs (58%), while TcP2 $\beta_N$  showed no unspecific recognition. The diagnostic utility of the chimera was evaluated by analyzing reactivity and comparing the results with those obtained with TcP2 $\beta_N$ . The chimera reactivity was higher than that of the peptide fractions (0.874 versus 0.564 optical density,  $P = 0.0017$ ). The detectability and specificity were both 100% for the whole serum panel tested. We conclude that the obtained chimera shows an improved selectivity and sensitivity compared with other ones previously reported, therefore displaying an optimized performance for *Trypanosoma cruzi* infection diagnosis.**

Chagas' disease is a parasitic illness affecting 16 to 18 million people, mainly in Latin America. Its etiological agent is the protozoan parasite *Trypanosoma cruzi* (<http://www.who.int/ctd/chagas/disease.htm>). Although 80% of the disease is vectorially transmitted, interhuman transmission is also significant. This is explained by the prevalence of Chagas' infected reservoirs in blood banks, which ranges from 1.7 to 53%, depending on the geographical location (<http://www.who.int/ctd/chagas/burdens.htm>). Considering there are chagasic blood donors who are nonsymptomatic, an accurate screening for chagasic infection is the main means to prevent interhuman transfusional transmission.

Most of the conventional serological reactions use whole extracts of the noninfective insect-stage epimastigote, since this is the easiest, cheapest, and safest parasite stage to be cultured, which allow for detection of antibodies (Abs) against the mammalian infective stages. Indeed, epimastigote-derived antigens (Ags) are broadly accepted for serological methods, and they have shown to be sensitive enough to be used as a screening primary tool at blood banks (15). However, the methodology is very difficult to standardize, since these kinds of Ags are constituted by largely undefined complex mixtures, and frequently render false-positive or undetermined results that lead to an unnecessary disposal of whole-blood reservoirs

(33, 34). In addition, the cross-reactivity of several components of these Ag mixtures with sera from patients infected with phylogenetically related organisms, such as *Leishmania* spp. or *Trypanosoma rangeli*, leads frequently to wrong diagnosis (3, 8, 32). To overcome this problem, many authors have proposed to use purified Ags (1, 2, 9, 10, 18, 29, 30, 36, 39, 45) or recombinant Ags to enhance specificity and sensitivity (some of them reviewed in reference 12).

Several recombinant proteins have shown to be suitable tools for the specific diagnosis of chagasic infection. However, none of them were antigenic enough to be recognized by all of the chagasic human sera. To address this drawback, combinations of more than one recombinant protein were used to enhance their overall performance, thus improving assay sensitivity to values close to that of conventional serology (44).

Recombinant Ags may also fail in specificity when some fragments of their amino acid (aa) sequence (not necessarily the whole sequence) are shared with their orthologues in other organisms. In these cases, the DNA fragment encoding for the unspecific region may be removed, yielding an optimized recombinant Ag in terms of specificity. This is the case for the *T. cruzi* calflagin, in which a fragment responsible for cross-reactivity with sera from *Leishmania* spp. has been identified, and the Ag has been successfully optimized for diagnostic purposes by the excision of this low-specificity fragment (28).

Some anti-*T. cruzi* Abs show cross-reactivity with epitopes of other orthologous proteins of phylogenetically related microorganisms and certain host proteins. The latter have been involved in the autoimmune pathological process occurring in

\* Corresponding author. Mailing address: Ciudad Universitaria, Paraje "El pozo," INTEBIO, CC 242, 3000 Santa Fe, Argentina. Phone: 54 342 4575206, ext. 125. Fax: 54 342 4575206, ext. 118. E-mail: [imarcipr@fcb.unl.edu.ar](mailto:imarcipr@fcb.unl.edu.ar).

the chronic form of Chagas' disease. This is the case for the TcP2 $\beta$  protein, which has a 13-aa C-terminal fragment, R13, sharing 12 aa with a homologue human protein (22). The diagnostic performance of TcP2 $\beta$  peptide was promising when evaluated by a multicenter study (21). However, the high cross-reactivity of TcP2 $\beta$  with sera of patients suffering autoimmune or related parasitic diseases remained a problem to be solved (25). Interestingly, the orthologous protein of *Leishmania* spp., whose C-terminal fragment is recognized by sera from chagasic patients, has been proposed to be used to detect anti-*Leishmania* spp. Abs, provided the cross-reactive fragment is eliminated from the protein sequence (41).

Fusion of DNA sequences encoding selected specific regions of antigenic proteins is a powerful tool to engineer synthetic genes encoding for chimeric proteins to be used in diagnostic tests. In the present work, we optimized the above-described Ag, TcP2 $\beta$ , for the detection of *T. cruzi* infection by excision of a fragment which diminished its specificity, and we fused its DNA coding sequence to that of a previously reported calflagin-derived protein. Finally, we evaluated the performance of this two-component chimeric protein for *T. cruzi* infection diagnostic purposes.

#### MATERIALS AND METHODS

**Reagents.** All standard reagents were purchased from Sigma (St. Louis, Mo.), unless otherwise indicated.

**Parasite cultures and homogenates.** Epimastigotes of *T. cruzi* (Tulahuen strain) were grown in infusion tryptose medium supplemented with 10% fetal calf serum (Cultilab, São Paulo, Brazil) (7). Total homogenates of epimastigotes were obtained by resuspension of the washed cells in five volumes of 1 mM *N-p*-tosyl-L-lysine chloromethyl ketone and 1 mM phenylmethylsulfonyl fluoride in distilled water, freeze and thaw (four cycles), and sonication (20 kHz, 30 W, 2 min).

**Patients' serum samples.** Serum samples from *T. cruzi*-infected patients ( $n = 104$ ) were obtained from a region of endemicity located in northeast Argentina. The *T. cruzi* infection status of the patients was established by using two different conventional tests, namely, commercial enzyme-linked immunosorbent assay (ELISA) (Chagatest ELISA) and indirect hemagglutination (Chagatest IHA) from Wiener Lab (Argentina), both of them based on epimastigote total homogenate Ags. The serological status was established by the WHO recommended criterion, i.e., any sample is considered to be positive or negative to *T. cruzi* infection when concordant results are obtained by using both conventional tests (11). All individuals were serologically negative for syphilis, human immunodeficiency virus, and hepatitis B or C virus. Negative sera were obtained from healthy blood donors ( $n = 117$ ) from the same Argentinean region. Fifteen sera from individuals infected with *Leishmania (Viannia) braziliensis* were obtained from patients recruited at the Centro de Pesquisas Aggeu Magalhães, Fundação Oswaldo Cruz, Recife PE, Brazil, with clinical manifestations of cutaneous leishmaniasis. All of these individuals live in Pernambuco State, Brazil, a region where the infection by *L. (V.) braziliensis* is endemic (6). The patients were defined as epidemiologically negative for *T. cruzi* infection, since there are no reports of the presence of the insect vector or cases of infection in this region. These individuals reported not having traveled to areas where *T. cruzi* is endemic or having received blood transfusion or organ grafting.

Serum samples were gathered into different groups as follows. Group Ch+, containing 32 Chagas-positive serum samples, without other reactivity, was used as a positive control in all trials. Group Ch-, containing 32 Chagas-negative serum samples, without other reactivity, was used as a negative control in all trials. Control groups Ch+ and Ch- were enlarged with an additional 72 Chagas' disease positive and 35 Chagas' disease negative serum samples, respectively, to evaluate only the chimeric protein. Group AID consisted of 17 and 7 serum samples from patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), respectively, all of them being negative for Chagas' disease; group AID was used as an autoimmune disease serum control. Group ID contained 10, 9, and 7 serum samples from patients infected with *Toxoplasma gondii*, *Brucella abortus*, and *Streptococcus pyogenes*, respectively, all of them being negative for chagasic infection; group ID was used as an infectious disease serum control.

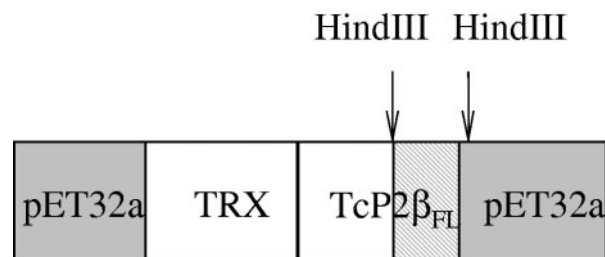


FIG. 1. Schematic representation of the method used to obtain the plasmid encoding TcP2 $\beta$ <sub>N</sub>.

Group L consisted of 15 serum samples from patients infected with *L. (V.) braziliensis*, all of them being negative for Chagas' disease; group L was used as a leishmaniasis disease serum control.

**Polyclonal serum.** The recombinant C-terminal region of the 29-kDa calflagin protein (C29<sub>C</sub>) was expressed and purified as previously described (28). Serum against C29<sub>C</sub> was obtained from rabbits inoculated twice subsequently, as previously described (37). Polyclonal sera were kept at  $-20^{\circ}\text{C}$ .

**Expression vector engineering.** Molecular biology reagents were purchased from Promega (Madison, WI). The *T. cruzi* cDNA encoding the full-length TcP2 $\beta$  protein (TcP2 $\beta$ <sub>FL</sub>) was obtained by screening a *T. cruzi* tripomastigote cDNA library, gently gifted by Mariano Levin (INGEBI—University of Buenos Aires, Buenos Aires, Argentina), as previously described (26). The cDNA encoding TcP2 $\beta$ <sub>FL</sub> was subcloned in the EcoRI site of the vector pET-32a (named pET-32a/TcP2 $\beta$ <sub>FL</sub>), digested with HindIII enzyme, and ligated without the excised fragment (pET-32a/TcP2 $\beta$ <sub>N</sub>) (Fig. 1 A). The *T. cruzi* cDNA encoding the full-length calflagin (C29<sub>FL</sub>) subcloned in the EcoRI site of the vector pMALC2 (pMALC2/C29<sub>FL</sub>) was obtained as previously described (28). The construction was digested with HindIII, and the 3' fragment of 400 bp was purified by GFX PCR (Amersham), ligated in the HindIII site of the pET-32a/TcP2 $\beta$ <sub>N</sub> vector, and used to transform BL21(DE3) competent cells. The clones expressing the chimera (named pET-32a/TcP2 $\beta$ <sub>N</sub>-C29<sub>C</sub>) were selected by immunoscreening, using the immune serum against C29<sub>C</sub>. The obtained plasmids were tested for the presence of the expected DNA fragment by restriction endonuclease analysis and automatic sequencing by the dideoxy chain termination method (35).

**Expression and purification of recombinant proteins.** *Escherichia coli* cells bearing the plasmids pET-32a, pET-32a/TcP2 $\beta$ <sub>FL</sub>, pET-32a/TcP2 $\beta$ <sub>N</sub>, and pET-32a/TcP2 $\beta$ <sub>N</sub>-C29<sub>C</sub> were grown overnight in 10 ml of LB medium, supplemented with 0.1 mg/ml ampicillin at 37°C, with agitation. The respective TcP2 $\beta$ <sub>FL</sub>, TcP2 $\beta$ <sub>N</sub>, TcP2 $\beta$ <sub>N</sub>-C29<sub>C</sub>, and thioredoxin reductase (TRX) peptides were purified according to the manufacturer's specifications. Briefly, cells were induced for 3 h with isopropyl- $\beta$ -D-thiogalactopyranoside, washed with phosphate-buffered saline (PBS), centrifuged, and resuspended in 50 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 8), 300 mM NaCl, 10 mM imidazole buffer. Once the supernatants were applied to the columns, they were washed with the same buffer and then eluted into different fractions using the mentioned buffer plus 50, 100, and 250 mM imidazole, respectively. Protein quantification was performed by using the Bradford assay (5). The purity of the recombinant protein was analyzed by 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), followed by staining with Coomassie blue.

**SDS-PAGE.** The samples were resuspended in loading buffer, subjected to 12% SDS-PAGE, and stained with Coomassie brilliant blue, according to the method of Laemmli (20).

**Western blotting.** Protein extracts or the purified fraction (50  $\mu\text{g}$  per lane) were subjected to 12% SDS-PAGE and electroblotted to a nitrocellulose membrane. Membranes were incubated with sera diluted 1:1,000 in PBS with 1% bovine serum albumin and, subsequently, with anti-rabbit immunoglobulin G peroxidase conjugate, diluted 1:1,000 in PBS with 1% bovine serum albumin. The reaction was developed with 0.05% diaminobenzidine and 0.04 volumes of H<sub>2</sub>O<sub>2</sub> in PBS.

**ELISA using recombinant proteins.** The optimal concentrations of serum, Ag, and conjugate were determined by a chessboard titration. The optimal Ag concentration (500 ng/well) was determined by using 50, 100, 500 and 1,000 ng per well of each protein and analyzing the best discrimination between two positive and two negative sera. Polystyrene microplates (Costar) were then sensitized with 500 ng/well of each protein. The plates were incubated overnight at 4°C, washed three times with 0.01% Tween in PBS, and blocked for 1 h at 37°C with 1% bovine serum albumin in PBS. Microplates were incubated with a 1:100

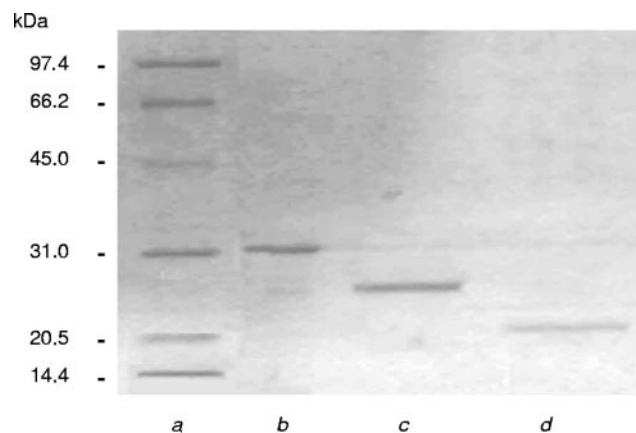


FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins, followed by staining with Coomassie blue. Lanes: *a*, molecular marker; *b*, TcP2 $\beta$ <sub>FL</sub>; *c*, TcP2 $\beta$ <sub>N</sub>; *d*, TRX.

dilution of human serum in 1% low-fat milk in PBS. After washing, microplates were incubated with anti-rabbit immunoglobulin G-peroxidase conjugate (Sigma, St. Louis, MO), diluted 1:1,000 in 1% milk in PBS. The reaction was developed using tetramethyl benzidine in H<sub>2</sub>O<sub>2</sub>. All incubations were performed at 37°C for 60 min. Absorbances were read at 450 nm.

**Data analysis.** The results, recorded as optical density at 450 nm (OD<sub>450</sub>), were distributed by using a scatter computer graphic software (GraphPad Prism, version 2.00). The cutoff values of ELISA were calculated as the mean OD<sub>450</sub> of the true negative sera plus 3 standard deviations. Results of ELISAs were compared with the serologic status obtained by the respective reference technique, as described above. The GraphPad Prism software was used to perform Student's *t* test to compare population distributions. Sensitivity was expressed as 100 × detectability index of positives, i.e., the number of positive samples detected by using our Ags over the number of true-positive samples evaluated, confirmed by two alternative commercial kit assays (ELISA and indirect hemagglutination [IHA]) (43). Specificity was expressed as 100 × detectability index of negatives, defined as the number of negative samples detected by using our Ags over the number of true-negative samples evaluated, confirmed by two alternative commercial kit assays (ELISA and IHA) (43).

**Nucleotide sequence accession numbers.** Calflagin, ribosomal TcP2 $\beta$ , and TcP2 $\beta$ <sub>N</sub>-C29<sub>C</sub> were deposited in GenBank under the accession numbers X75030, AF192980, and DQ648783, respectively.

## RESULTS

**Evaluation of TcP2 $\beta$  lacking the C-terminal fraction.** Since the ribosomal 13-aa TcP2 $\beta$  C-terminal fragment shows cross-reactivity toward SLE (25, 38), we excised this C-terminal coding sequence from the full-length gene and expressed both TcP2 $\beta$ <sub>FL</sub> and TcP2 $\beta$ <sub>N</sub>. The protein TRX, which is fused to proteins expressed in pET-32a, was also expressed alone as control. The expressed proteins were purified and run in a 12% SDS-PAGE (Fig. 2). The obtained products were successfully purified to apparent homogeneity; the molecular weights were those expected in each case.

The three obtained proteins were then evaluated as capture Ags in ELISAs, using three different serum groups from the panel previously described. When using Ch+ and Ch- groups, the sensitivities obtained for TcP2 $\beta$ <sub>FL</sub> and TcP2 $\beta$ <sub>N</sub> were 100% and 94%, respectively (Fig. 3A). When testing TcP2 $\beta$ <sub>FL</sub> with sera from the AID group (autoimmune patients), 58% of them showed cross-reactivity (Fig. 3B). The same holds true for samples from group L (cutaneous leishmaniasis patients), with the reactivity being 40% (Fig. 3C). However, cross-reactions

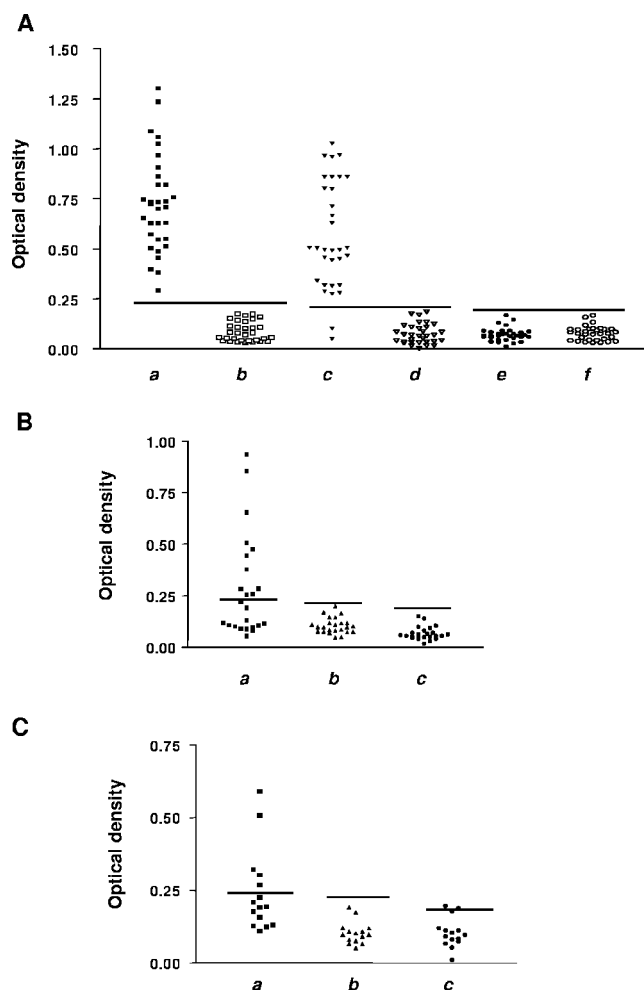


FIG. 3. Optical density values obtained by performing ELISA with several serum groups. Horizontal lines indicate the cutoff values calculated for each peptide. (A) Results obtained for each fragment when assaying Ch+ and Ch- control serum groups. Points in columns *a* and *b* show results for TcP2 $\beta$ <sub>FL</sub>, points in columns *c* and *d* show results for TcP2 $\beta$ <sub>N</sub>, and points in columns *e* and *f* show results for TRX. Points in columns *a*, *c*, and *e* correspond to results for Chagas' disease-positive sera; points in columns *b*, *d*, and *f* correspond to results for Chagas' disease-negative sera. (B) Results obtained for each fragment when assaying the AID serum group. Points in column *a* show results for TcP2 $\beta$ <sub>FL</sub>, points in column *b* show results for TcP2 $\beta$ <sub>N</sub>, and points in column *c* show results for TRX. (C) Results obtained for each fragment when assaying the L serum group. Points in column *a* show results for TcP2 $\beta$ <sub>FL</sub>, points in column *b* show results for TcP2 $\beta$ <sub>N</sub>, and points in column *c* show results for TRX.

were avoided when using TcP2 $\beta$ <sub>N</sub>. TRX protein was also evaluated in each assay for all of the serum samples, showing no unspecific reactivity. All of these results are summarized in Table 1. The obtained results prompted us to explore the possibility of expressing this protein fused to C29<sub>C</sub>, which presents lower sensitivity than C29<sub>FL</sub> but higher specificity toward chagasic Abs (28).

The pET-32a vector containing the TcP2 $\beta$ <sub>N</sub>-encoding DNA was cut with the HindIII enzyme and then purified. The C29<sub>C</sub>-encoding fragment, previously subcloned into a pMAL vector (28), was excised by digestion with the HindIII enzyme. The

TABLE 1. Reactivity obtained for TRX, TcP2 $\beta$ <sub>FL</sub>, TcP2 $\beta$ <sub>N</sub>, and TcP2 $\beta$ <sub>N</sub>-C29<sub>C</sub> proteins when assaying the Ch+, Ch-, AID, L, and ID serum groups

Serum group	% Reactivity with:			
	TRX	TcP2 $\beta$ <sub>FL</sub>	TcP2 $\beta$ <sub>N</sub>	TcP2 $\beta$ <sub>N</sub> -C29 <sub>C</sub>
Ch+	0 <sup>a</sup>	100 <sup>a</sup>	94 <sup>a</sup>	100 <sup>b</sup>
Ch-	0 <sup>a</sup>	0 <sup>a</sup>	0 <sup>a</sup>	0 <sup>c</sup>
AID	0	58.3	0	0
L	0	40	0	0
ID	0	— <sup>d</sup>	—	0

<sup>a</sup> 32 serum samples tested.

<sup>b</sup> 104 serum samples tested.

<sup>c</sup> 67 serum samples tested.

<sup>d</sup> —, assay not performed.

obtained fragment corresponded to a sequence encoding a 6-kDa peptide (Fig. 4B). The fragment was ligated to the HindIII site, which was present in the vector containing the TcP2 $\beta$ <sub>N</sub>-encoding sequence (Fig. 4A). Then, a synthetic gene encoding a chimera of 113-aa was obtained (Fig. 4B). The expression of this synthetic protein was induced and evaluated by 12% SDS-PAGE (Fig. 5A), followed by Western blotting, using a specific polyclonal anti-C29<sub>C</sub> serum (Fig. 5B). It should be noted that, even when the hyperimmune serum unspecifically recognized some protein bands of the bacteria extract, it did not recognize TcP2 $\beta$ <sub>N</sub>. Contrarily, the serum showed reactivity towards C29<sub>FL</sub> and the fusion peptide TcP2 $\beta$ <sub>N</sub>-C29<sub>C</sub>.

**Evaluation of the TcP2 $\beta$ <sub>N</sub>-C29<sub>C</sub> chimera.** To assess the Chagas' disease diagnostic utility of the protein obtained, we assayed the reactivity toward different sets of sera from the panel previously typified; the results were compared with those ob-

tained when using TcP2 $\beta$ <sub>N</sub> as Ag. When assaying the Ch+ and Ch- control serum groups, the samples rendered 100% specificity and sensitivity with the TcP2 $\beta$ <sub>N</sub>-C29<sub>C</sub> chimera (Fig. 6 and Table 1), even when they had previously failed to recognize the TcP2 $\beta$ <sub>N</sub> Ag (Fig. 3 and Table 1). The mean OD value for positive sera was increased when using the new Ag, whereas the mean OD value for negative samples showed no significant variation (Table 2). The serum samples from patients with leishmaniasis or autoimmune disease were not recognized by the new protein, thus rendering 100% specificity (Fig. 6A and Table 1).

An additional evaluation of the new chimera was performed by testing extra control samples, containing 72 Chagas' disease-positive and 35 Chagas' disease-negative serum samples, previously verified to be so by two other conventional serological tests, without other serological reactivity. We also studied cross-reactivity with the ID serum group, which includes sera from patients with the infectious diseases that have been reported in the literature as causative of cross-reactions when performing tests for diagnosis of *T. cruzi* infection. The ID serum group, consisted of Chagas-negative serum samples showing reactivity toward *Toxoplasma gondii*, *Streptococcus pyogenes*, or *Brucella abortus* (Fig. 6B). The sensitivity and specificity obtained with these additional serum panels was 100%, indicating that this synthetic protein behaves as an optimized Ag for the diagnosis of the chagasic infection.

## DISCUSSION

Chagasic infection is currently diagnosed serologically by detecting anti-*T. cruzi* immunoglobulin G Abs in the patient's

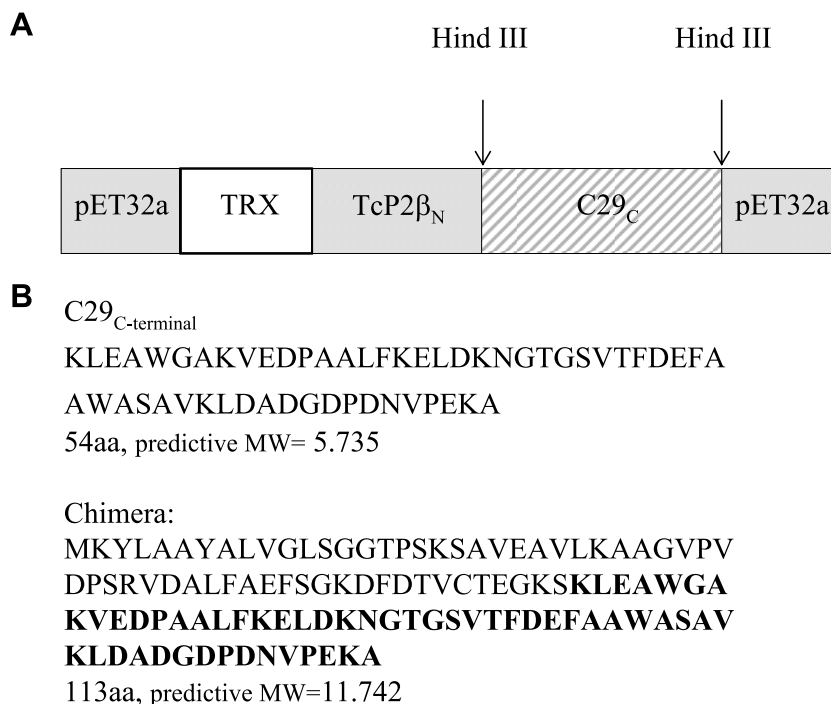


FIG. 4. (A) Schematic representation of the method used to obtain the plasmid encoding the chimera protein. (B) C29<sub>C</sub> and chimera amino acid sequences. Letters in bold correspond to the C29<sub>C</sub> fraction fused to TcP2 $\beta$ <sub>N</sub>.



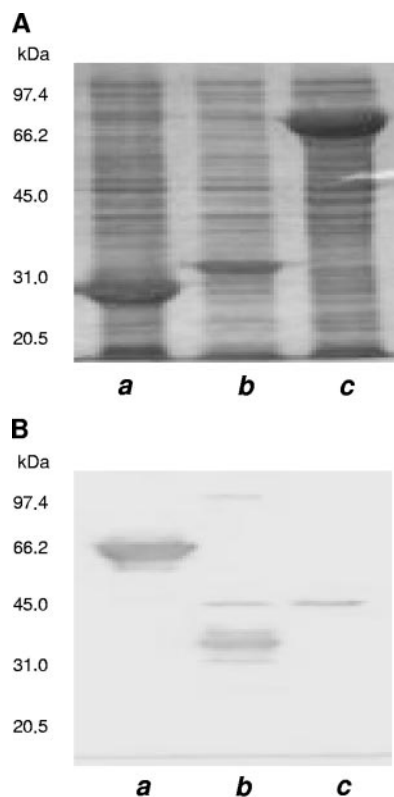


FIG. 5. (A) Coomassie blue staining of protein extracts after electrophoresis in a 12% sodium dodecyl sulfate–polyacrylamide gel. Lane *a*, *E. coli* BL21(DE3)-(TcP2 $\beta$ <sub>N</sub>-TRX) cell extract, used as a negative control; lane *b*, *E. coli* BL21(DE3) cell extract of the isolated clone; lane *c*, *E. coli* DH5 $\alpha$  (C29-MBP) cell extract. (B) Western blot assay for TcP2 $\beta$ <sub>N</sub>, the C29<sub>C</sub> fraction fused to TcP2 $\beta$ <sub>N</sub>, and C29<sub>C</sub> in different *E. coli* extracts, using a polyclonal anti-C29<sub>C</sub> serum. Lane *a*, *E. coli* DH5 $\alpha$  (C29-MBP) cell extract, used as a positive control; lane *b*, *E. coli* BL21(DE3) cell extract of the chimera clone; lane *c*, *E. coli* BL21(DE3)-(TcP2 $\beta$ <sub>N</sub>-TRX) cell extract, used as a negative control.

serum. However, conventional serological methods often show cross-reactivity with related protozoan infections, particularly leishmaniasis. Recombinant Ags are specific, but a panel of them must be used to obtain appropriate sensitivity (12). Moreover, the utilization of several antigenic determinants gathered in the same molecule, which allows for an equilibrated adsorption of the Ag to the ELISA plaque, thus facilitating standardization during reagent production has also been reported (12, 16).

Late in the 1980s, Levin et al. obtained a 34-aa TcP2 $\beta$  protein decoding a DNA sequence by using a *T. cruzi* cDNA library (22). As mentioned above, this protein has a 13-aa, C-terminal fragment, R13, which shares 12 aa with a homologue human protein. Other works have described the presence of Abs against the complete TcP2 $\beta$  peptide, or against a fraction of it, in sera from patients suffering from several infections and autoimmune diseases (13, 38, 40, 42). These facts led researchers to propose that this peptide might be involved in the development of an autoimmune response in patients with Chagas' disease (24, 31). A multicenter study evaluated the diagnostic performances of some chagasic recombinant Ags, among which TcP2 $\beta$  peptide had been included (21). The

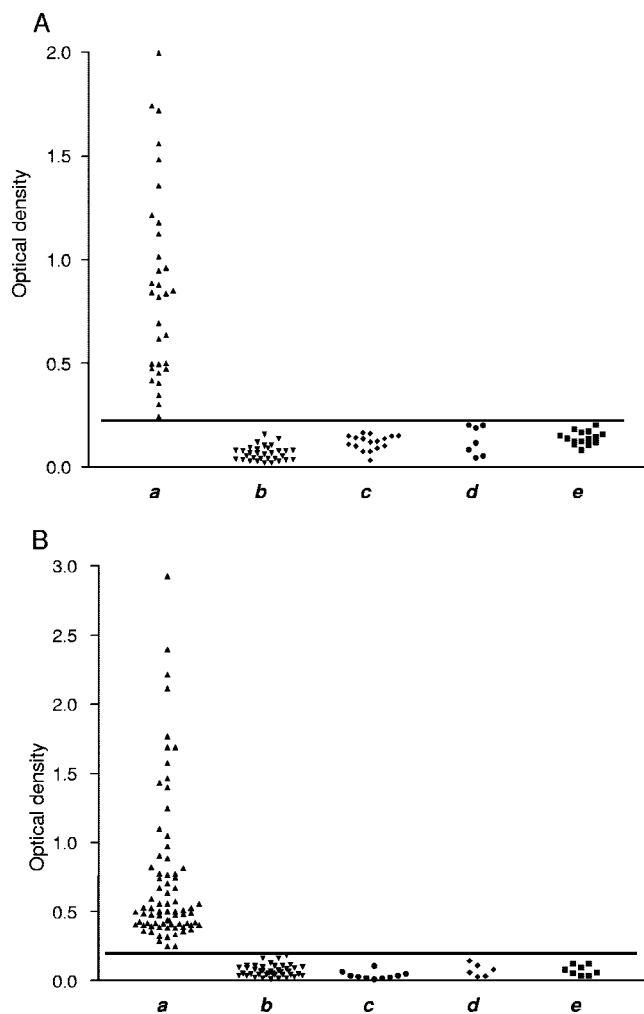


FIG. 6. Optical density values obtained by performing ELISA with several serum groups. Horizontal lines correspond to calculated cutoff values. (A) Results obtained for the chimera by assaying the Ch<sup>+</sup>, Ch<sup>-</sup>, AID, and L serum groups. Columns *a* and *b* show results for Chagas' disease-positive and Chagas' disease-negative sera, respectively, columns *c* and *d* show results for the AID serum group for SLE and RA sera, respectively, and column *e* shows results for the L serum group. (B) Results obtained for the chimera when assaying additional control serum samples and the ID serum group. Columns *a* and *b* show results for 72 Chagas' disease-positive and 35 Chagas' disease-negative control serum samples, respectively, columns *c*, *d*, and *e* show ID group results for sera of patients infected with *Toxoplasma gondii*, *Brucella abortus*, and *Streptococcus pyogenes*, respectively.

results obtained for TcP2 $\beta$  (76% sensitivity) showed that the peptide did not enhance immunoassay sensitivity, compared with other Ags, and displayed high cross-reactivity with sera of patients with autoimmune or related parasitic diseases. This seems to be the reason why this peptide was not used in tests for diagnosis of chagasic infection. However, since anti-R13 peptide Abs were found in serum of chagasic patients with cardiopathies, quantification of these Abs was proposed to be useful to evaluate the patient's status in terms of cardiopathy development (4). Additionally, the orthologous protein of *Leishmania* spp., whose C-terminal fraction showed cross-reactivity when assaying sera from chagasic patients, has been

TABLE 2. Comparison of the mean OD obtained by ELISA for Ch+ and Ch- control panels, when using TcP2 $\beta$ <sub>N</sub> peptide and the new chimera as sensitizing agent

Control panel (n)	Mean OD with sensitizing agent:		P value
	TcP2 $\beta$ <sub>N</sub>	Chimera	
Ch+ (32)	0.564	0.874	0.0017
Ch- (32)	0.073	0.065	0.45

proposed to be used as a diagnostic tool to detect anti-*Leishmania* spp. Abs, provided the controversial region is eliminated from the protein sequence (41). Indeed, this *Leishmania*-truncated protein exhibits no cross-reactivity toward chagasic sera when used as the sensitizing element.

We have previously described that the use of TcP2 $\beta$ <sub>FL</sub> protein as a sensitizing Ag detects all of the positive sera assayed (26). Considering that it has been described that the chagasic antigenic determinants are among the first 15 aa, whereas the final 20 aa are not immunogenic (23), we used this information as a criterion to eliminate more than 13 terminal amino acids, generating the Ag TcP2 $\beta$ <sub>N</sub>. The peptide obtained showed a higher specificity but lower sensitivity than TcP2 $\beta$ <sub>FL</sub>. The enhanced specificity was displayed by the absence of reactivity when assaying sera from SLE and RA patients, together with the lack of cross-reactivity when assaying sera from *L. (V.) brasiliensis*-infected patients.

To increase the Ag sensitivity, we fused TcP2 $\beta$ <sub>N</sub> to another studied protein region. Taking into account that previous works have shown that *T. cruzi* calflagins are useful as sensitizing agents for diagnosis of chagasic infection (14, 19, 22) and considering that it has also been demonstrated that the C-terminal fraction of this protein shows high specificity when assaying leishmanial sera (28), C29<sub>C</sub> was chosen to assemble the chimera. We therefore built up an artificial protein composed of C29<sub>C</sub> and TcP2 $\beta$ <sub>N</sub>. The usage of this fused peptide as a sensitizing Ag rendered a sensitivity of 100% (Fig. 6 and Table 1). The results obtained for the serum samples of the Ch+ and Ch- control groups (Table 2) additionally showed an increased reactivity for the positive sera with respect to that obtained when testing the same samples against TcP2 $\beta$ <sub>N</sub> ( $P = 0.0017$ ). In contrast, the negative serum samples showed no reactivity enhancement ( $P = 0.45$ ). These results show that this new Ag displays better discrimination efficiency between positive and negative samples than that of TcP2 $\beta$ <sub>N</sub> alone. The evaluation of the fusion protein obtained also included testing those Chagas' disease-positive sera that had rendered positive results when using the complete TcP2 $\beta$  peptide but had rendered negative results when using only TcP2 $\beta$ <sub>N</sub>. Again, 100% consistency with the sera typification was obtained, indicating that the sensitivity drop occurring when using TcP2 $\beta$ <sub>N</sub> alone was fully prevented.

To complete the assessment of the new Ag, we analyzed Chagas' disease-negative sera of patients with SLE, AR, and leishmaniasis. Once more, the recognizing effectiveness of TcP2 $\beta$ <sub>N</sub>-C29<sub>C</sub> was 100%, since all of the samples studied rendered negative results. However, it should be noted that 4 of the 132 negative and 3 of the 108 positive serum samples evaluated presented OD values close to the cutoff which be-

long to the undetermined zone, as demarked by the cutoff value,  $\pm 10\%$ . These results show that we achieved the work objective, i.e., obtaining an Ag which displays improved features to diagnose chagasic infection compared to others currently used. Incidentally, taking into account that the concentration of anticallagins Abs has been correlated with parasitological cure (19) and that the level of anti-TcP2 $\beta$  Abs has been proposed as indicative of the patient's cardiopathy development (4), it is interesting to evaluate this new chimera's clinical predictive value, for which further studies are programmed.

Very few reports of these chimeric Ags used for the detection of anti-*T. cruzi* Abs have been published, in spite of being this a promising strategy. Houghton et al. published in 1999 the development of a multicomponent protein for the diagnosis of chagasic infection (17), which was further improved later (16). This protein allowed a very important discrimination among positive and negative sera. However, the validation did not include sera from patients with leishmaniasis.

The optimization of the diagnosis of infection with microorganisms is a multivariable task involving, among others, the search for suitable Ags, the analysis of their avidity (27), and in the case of recombinant Ags, the possibility of optimizing their antigenic profile by selecting an adequate expression system (26). Regions that are responsible for undesired cross-reactivity can be removed (28), and regions showing high specificity can be grafted one to another to raise sensitivity (16, 17). In line with this, the construction of the chimeric protein herein reported constitutes a promising land to obtain Ags that allows for standardization of diagnosis techniques and provides a novel platform to obtain new immunogens.

#### ACKNOWLEDGMENTS

This work was funded by ANPCyT, PICTR2002-00057 (C.M.L., A. Marcipar, and I. Malan Borel).

We are grateful to Alberto Marcipar for essential preliminary suggestions and discussion. We also dedicate this work to his lovely memory.

#### REFERENCES

- Aguillon, J. C., R. Harris, M. C. Molina, A. Colombo, C. Cortes, T. Hermosilla, P. Carreno, A. Orn, and A. Ferreira. 1997. Recognition of an immunogenetically selected *Trypanosoma cruzi* antigen by seropositive chagasic human sera. *Acta Trop.* **63**:159-166.
- Almeida, I. C., D. T. Covas, L. M. Soussumi, and L. R. Travassos. 1997. A highly sensitive and specific chemiluminescent enzyme-linked immunosorbent assay for diagnosis of active *Trypanosoma cruzi* infection. *Transfusion* **37**:850-857.
- Araujo, F. G. 1986. Analysis of *Trypanosoma cruzi* antigens bound by specific antibodies and by antibodies to related trypanosomatids. *Infect. Immun.* **53**:179-185.
- Aznar, C., P. Lopez-Bergami, S. Brandariz, C. Mariette, P. Liegeard, M. D. Alves, E. L. Barreiro, R. Carrasco, S. Lafon, D. Kaplan, et al. 1995. Prevalence of anti-R-13 antibodies in human *Trypanosoma cruzi* infection. *FEMS Immunol. Med. Microbiol.* **12**:231-238.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248-254.
- Brito, M. E., M. G. Mendonca, Y. M. Gomes, M. L. Jardim, and F. G. Aboth. 2000. Identification of potentially diagnostic *Leishmania braziliensis* antigens in human cutaneous leishmaniasis by immunoblot analysis. *Clin. Diagn. Lab. Immunol.* **7**:318-321.
- Camargo, E. P. 1964. Growth and differentiation in *Trypanosoma cruzi*. I. Origin of metacyclic trypanosomes in liquid media. *Rev. Inst. Med. Trop. Sao Paulo* **12**:93-100.
- Camargo, M. E., E. L. Segura, I. G. Kagan, J. M. Souza, R. Carvalheiro Jda, J. F. Yanovsky, and M. C. Guimaraes. 1986. Three years of collaboration on the standardization of Chagas' disease serodiagnosis in the Americas: an appraisal. *Bull. Pan. Am. Health Organ.* **20**:233-244.

9. Carbonetto, C. H., E. L. Malchiodi, M. Chiaramonte, E. Durante de Isola, C. A. Fossati, and R. A. Margni. 1990. Isolation of a *Trypanosoma cruzi* antigen by affinity chromatography with a monoclonal antibody. Preliminary evaluation of its possible applications in serological tests. *Clin. Exp. Immunol.* **82**:93–96.
10. Cuna, W. R., C. Rodriguez, F. Torrico, D. Afchain, M. Loyens, and P. Desjeux. 1989. Evaluation of a competitive antibody enzyme immunoassay for specific diagnosis of Chagas' disease. *J. Parasitol.* **75**:357–359.
11. Cura, E., S. Wendel, F. P. Pinheiro, and M. Weinsbacher. 1996. Manual de procedimientos de control de calidad para laboratorios de serologias de los bancos de sangre. PAHO, Washington, D.C.
12. da Silveira, J. F., E. S. Umezawa, and A. O. Luquetti. 2001. Chagas disease: recombinant *Trypanosoma cruzi* antigens for serological diagnosis. *Trends Parasitol.* **17**:286–291.
13. Elkon, K., S. Skelly, A. Parnassa, W. Moller, W. Danho, H. Weissbach, and N. Brot. 1986. Identification and chemical synthesis of a ribosomal protein antigenic determinant in systemic lupus erythematosus. *Proc. Natl. Acad. Sci. USA* **83**:7419–7423.
14. Godsel, L. M., R. S. Tibbetts, C. L. Olson, B. M. Chaudoir, and D. M. Engman. 1995. Utility of recombinant flagellar calcium-binding protein for serodiagnosis of *Trypanosoma cruzi* infection. *J. Clin. Microbiol.* **33**:2082–2085.
15. Guhl, F., C. Jaramillo, J. C. Carranza, and G. A. Vallejo. 2002. Molecular characterization and diagnosis of *Trypanosoma cruzi* and *T. rangeli*. *Arch. Med. Res.* **33**:362–370.
16. Houghton, R. L., D. R. Benson, L. Reynolds, P. McNeill, P. Sleath, M. Lodes, Y. A. Skeiky, R. Badaro, A. U. Krettli, and S. G. Reed. 2000. Multi-epitope synthetic peptide and recombinant protein for the detection of antibodies to *Trypanosoma cruzi* in patients with treated or untreated Chagas' disease. *J. Infect. Dis.* **181**:325–330.
17. Houghton, R. L., D. R. Benson, L. D. Reynolds, P. D. McNeill, P. R. Sleath, M. J. Lodes, Y. A. Skeiky, D. A. Leiby, R. Badaro, and S. G. Reed. 1999. A multi-epitope synthetic peptide and recombinant protein for the detection of antibodies to *Trypanosoma cruzi* in radioimmunoprecipitation-confirmed and consensus-positive sera. *J. Infect. Dis.* **179**:1226–1234.
18. Kirchhoff, L. V., A. A. Gam, R. A. Gusmao, R. S. Goldsmith, J. M. Rezende, and A. Rassi. 1987. Increased specificity of serodiagnosis of Chagas' disease by detection of antibody to the 72- and 90-kilodalton glycoproteins of *Trypanosoma cruzi*. *J. Infect. Dis.* **155**:561–564.
19. Krautz, G. M., L. M. Galvao, J. R. Cancado, A. Guevara-Espinoza, A. Ouaissi, and A. U. Krettli. 1995. Use of a 24-kilodalton *Trypanosoma cruzi* recombinant protein to monitor cure of human Chagas' disease. *J. Clin. Microbiol.* **33**:2086–2090.
20. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680–685.
21. Levin, M. J., J. Franco da Silveira, A. C. Frascch, M. E. Camargo, S. Lafon, W. M. Degraive, and R. Rangel-Aldao. 1991. Recombinant *Trypanosoma cruzi* antigens and Chagas' disease diagnosis: analysis of a workshop. *FEMS Microbiol. Immunol.* **4**:11–19.
22. Levin, M. J., E. Mesri, R. Benarous, G. Levitus, A. Schijman, P. Levy-Yeyati, P. A. Chiale, A. M. Ruiz, A. Kahn, M. B. Rosenbaum, et al. 1989. Identification of major *Trypanosoma cruzi* antigenic determinants in chronic Chagas' heart disease. *Am. J. Trop. Med. Hyg.* **41**:530–538.
23. Levin, M. J., R. Rossi, G. Levitus, E. Mesri, S. Bonnefoy, N. Kerner, and M. Hontebeyrie-Joskowicz. 1990. The cloned C-terminal region of a *Trypanosoma cruzi* P ribosomal protein harbors two antigenic determinants. *Immunol. Lett.* **24**:69–73.
24. Levin, M. J., M. Vazquez, D. Kaplan, and A. G. Schijman. 1993. The *Trypanosoma cruzi* ribosomal P protein family: classification and antigenicity. *Parasitol. Today* **9**:381–384.
25. Levitus, G., M. Hontebeyrie-Joskowicz, M. H. Van Regenmortel, and M. J. Levin. 1991. Humoral autoimmune response to ribosomal P proteins in chronic Chagas heart disease. *Clin. Exp. Immunol.* **85**:413–417.
26. Marcipar, I. S., M. L. Olivares, L. Robles, A. Dekanty, A. Marcipar, and A. M. Silber. 2004. The diagnostic performance of recombinant *Trypanosoma cruzi* ribosomal P2 $\beta$  protein is influenced by its expression system. *Protein Expr. Purif.* **34**:1–7.
27. Marcipar, I. S., M. G. Risso, A. M. Silber, S. Revelli, and A. J. Marcipar. 2001. Antibody maturation in *Trypanosoma cruzi*-infected rats. *Clin. Diagn. Lab. Immunol.* **8**:802–805.
28. Marcipar, I. S., C. Roodveldt, G. Corradi, M. L. Cabeza, M. E. Brito, L. M. Winter, A. J. Marcipar, and A. M. Silber. 2005. Use of full-length recombinant calflagin and its c fragment for improvement of diagnosis of *Trypanosoma cruzi* infection. *J. Clin. Microbiol.* **43**:5498–5503.
29. Marcipar, I. S., E. Welchen, C. Roodveldt, A. J. Marcipar, and A. M. Silber. 2003. Purification of the 67-kDa lectin-like glycoprotein of *Trypanosoma cruzi*, LLGP-67, and its evaluation as a relevant antigen for the diagnosis of human infection. *FEMS Microbiol. Lett.* **220**:149–154.
30. Martinez, J., O. Campetella, A. C. Frascch, and J. J. Cazzulo. 1991. The major cysteine proteinase (cruzipain) from *Trypanosoma cruzi* is antigenic in human infections. *Infect. Immun.* **59**:4275–4277.
31. Masuda, M. O., M. Levin, S. F. De Oliveira, P. C. Dos Santos Costa, P. L. Bergami, N. A. Dos Santos Almeida, R. C. Pedrosa, I. Ferrari, J. Hoebeke, and A. C. Campos de Carvalho. 1998. Functionally active cardiac antibodies in chronic Chagas' disease are specifically blocked by *Trypanosoma cruzi* antigens. *FASEB J.* **12**:1551–1558.
32. Saez-Alquezar, A., A. O. Luquetti, J. Borges-Pereira, E. F. Moreira, M. Gadahela, M. T. Garcia Zapata, and A. H. Strugo Arruda. 1997. Estudo multicêntrico: validação do desempenho de conjuntos diagnósticos de hemaglutinação indirecta disponíveis no Brasil para o diagnóstico serológico da infecção pelo *Trypanosoma cruzi*. *Rev. Patol. Trop.* **26**:343–374.
33. Saez-Alquezar, A., E. C. Sabino, N. Salles, D. F. Chamone, F. Hulstaert, H. Pottel, E. Stoops, and M. Zrein. 2000. Serological confirmation of Chagas' disease by a recombinant and peptide antigen line immunoassay: INNO-LIA chagas. *J. Clin. Microbiol.* **38**:851–854.
34. Salles, N. A., E. C. Sabino, M. G. Cliquet, J. Eluf-Neto, A. Mayer, C. Almeida-Neto, M. C. Mendonca, P. Dorliach-Llacer, D. F. Chamone, and A. Saez-Alquezar. 1996. Risk of exposure to Chagas' disease among seroreactive Brazilian blood donors. *Transfusion* **36**:969–973.
35. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
36. Scharfstein, J., M. Schechter, M. Senna, J. M. Peralta, L. Mendonca-Previato, and M. A. Miles. 1986. *Trypanosoma cruzi*: characterization and isolation of a 57/51,000 m.w. surface glycoprotein (GP57/51) expressed by epimastigotes and bloodstream trypomastigotes. *J. Immunol.* **137**:1336–1341.
37. Silber, A., I. Marcipar, C. Roodveldt, P. Cabeza Meckert, R. Laguens, and A. Marcipar. 2002. *Trypanosoma cruzi*: identification of a galactose-binding protein that binds to cell surface of human erythrocytes and is involved in cell invasion by the parasite. *Exp. Parasitol.* **100**:217–225.
38. Skeiky, Y. A., D. R. Benson, J. A. Guderian, P. R. Sleath, M. Parsons, and S. G. Reed. 1993. *Trypanosoma cruzi* acidic ribosomal P protein gene family. Novel P proteins encoding unusual cross-reactive epitopes. *J. Immunol.* **151**:5504–5515.
39. Solana, M. E., A. M. Katzin, E. S. Umezawa, and C. S. Miatello. 1995. High specificity of *Trypanosoma cruzi* epimastigote ribonucleoprotein as antigen in serodiagnosis of Chagas' disease. *J. Clin. Microbiol.* **33**:1456–1460.
40. Soto, M., J. M. Requena, M. Garcia, L. C. Gomez, I. Navarrete, and C. Alonso. 1993. Genomic organization and expression of two independent gene arrays coding for two antigenic acidic ribosomal proteins of *Leishmania*. *J. Biol. Chem.* **268**:21835–21843.
41. Soto, M., J. M. Requena, L. Quijada, and C. Alonso. 1996. Specific serodiagnosis of human leishmaniasis with recombinant *Leishmania* P2 acidic ribosomal proteins. *Clin. Diagn. Lab. Immunol.* **3**:387–391.
42. Soto, M., J. M. Requena, L. Quijada, S. O. Angel, L. C. Gomez, F. Guzman, M. E. Patarroyo, and C. Alonso. 1995. During active viscerocutaneous leishmaniasis the anti-P2 humoral response is specifically triggered by the parasite P proteins. *Clin. Exp. Immunol.* **100**:246–252.
43. Tijssen, P. 1985. Practice and theory of enzyme immunoassays, vol. 15. Elsevier Science Publishers, Amsterdam, The Netherlands.
44. Umezawa, E. S., S. F. Bastos, M. E. Camargo, L. M. Yamauchi, M. R. Santos, A. Gonzalez, B. Zingales, M. J. Levin, O. Sousa, R. Rangel-Aldao, and J. F. da Silveira. 1999. Evaluation of recombinant antigens for serodiagnosis of Chagas' disease in South and Central America. *J. Clin. Microbiol.* **37**:1554–1560.
45. Umezawa, E. S., M. S. Nascimento, N. Kesper, Jr., J. R. Coura, J. Borges-Pereira, A. C. Junqueira, and M. E. Camargo. 1996. Immunoblot assay using excreted-secreted antigens of *Trypanosoma cruzi* in serodiagnosis of congenital, acute, and chronic Chagas' disease. *J. Clin. Microbiol.* **34**:2143–2147.