A Multi-Epitope Synthetic Peptide and Recombinant Protein for the Detection of Antibodies to *Trypanosoma cruzi* in Radioimmunoprecipitation-Confirmed and Consensus-Positive Sera

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Peptide epitopes of *Trypanosoma cruzi* have been identified through expression cloning. A tripeptide (2/D/E) containing three epitopes (TcD, TcE, PEP-2) was used in ELISA to detect antibodies to *T. cruzi* in 239 of 240 consensus-positive sera and 41 of 42 sera confirmed positive by radioimmunoprecipitation assay. The 1 discrepant consensus-positive serum was used to expression-clone a novel gene that contained a repeat sequence. A peptide corresponding to this sequence, TcLo1.2, was specific for *T. cruzi.* This antigen detected the discrepant consensus-positive serum and enhanced reactivity of low-positive sera in the tripeptide assay. A branched synthetic peptide, 2/D/E/Lo1.2, or a linear recombinant, r2/D/E/Lo1.2, realized all of the diagnostic features of the four epitopes, including the ability to boost reactivity of low-reactive sera. These studies show that peptides and recombinants containing multiple repeat epitopes are powerful tools for developing assays for *T. cruzi* antibody detection and have direct application in blood screening.

Chagas' disease is caused by the parasite *Trypanosoma cruzi* and affects a large number of persons in Central and Latin America [1–4]. Transmission is by human contact with the reduviid bug, which transmits the parasite, or by blood transfusion, which has overtaken the natural route of transmission in many endemic areas. This suggests that the blood supplies in many countries of Central and South America [5–8] as well as the United States [9–22] should be tested for the presence of antibodies to *T. cruzi.* Direct detection of the organism in blood samples by direct examination, hemoculture, or xenodiagnosis is difficult and has low sensitivity [23]. For these reasons, serologic methods for the detection of antibodies to *T. cruzi* antigens have been the most effective methods for demonstrating exposure to the parasite, and consensus-positive sera are determined by use of multiple assays. Most of these methods, such as hemagglutination, complement fixation, or immunofluorescence titration (IFAT), as well as some ELISAs employ fixed whole organisms or crude antigens, resulting in specificity problems due to cross-reactivity with other organisms (e.g., leishmania, syphilis) [24–28]. In terms of blood screening assays, the ELISA is the most amenable to automation and is the most frequently used assay format in blood banks. More recently, radioimmunoprecipitation (RIPA) with specific *T. cruzi* antigens has been used as a confirmatory assay in the United States [29–31].

Gene cloning has enabled the identification of several important *T. cruzi* antigens that can be used in the development of assays for detection of *T. cruzi* infection [32–37]. The recombinant protein TcD, a major antigenic epitope of a 260-kDa *T. cruzi* trypomastigote antigen, is highly sensitive and specific for *T. cruzi* [38]. It contains a 10–amino acid repeat epitope that was synthesized as one and one-half repeat units and was comparable in sensitivity to the parent recombinant [39]. In addition, this peptide was shown to complement another peptide with repeating epitopes (PEP-2), thus leading to increased sensitivity in serologic assays for *T. cruzi* [39]. Because this two-peptide combination did not achieve 100% sensitivity for detecting *T. cruzi* infection, another immunodominant antigen (rTcE) was identified by expression cloning. rTcE is a 35-kDa ribosomal protein identified by screening a cDNA expression library from *T. cruzi,* using known Chagas' disease sera that were negative for antibodies against TcD [40–41]. In the case of rTcE, 16 copies of a 7–amino acid conserved repeat sequence were identified, with some diversity in specific amino acids within the repeat. A polymorph of this repeat sequence (rTcHi29) has also been identified and is discussed below. Peptides TcE and TcHi29, containing three repeats, were synthesized and had the full reactivity of the full-length recombinants.

To develop a useful diagnostic and blood-screen reagent, the three sequences (TcD, TcE, PEP-2) were combined into a single multi-epitope synthetic peptide called 2/D/E [40–41]. This peptide was tested by ELISA with *T. cruzi*–infected sera and with

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sera from blood donors: The peptide was highly sensitive, detecting 239 of 240 consensus-positive sera. The 1 discrepant serum from this study was then used in the expression-cloning of another antigen containing a 15–amino acid repeat motif described below. A peptide constructed with two repeats (TcLo1.2) was strongly immunoreactive.

Herein, we describe the utility of 2/D/E and TcLo1.2 as tools in the sensitive detection of antibodies to *T. cruzi.* We also describe their combination in a branched tetrapeptide synthetic antigen or recombinant (TcF) containing all four epitopes for further development of reproducible ELISAs that would be adaptable for use in blood banks.

Materials and Methods

Study population. Serum samples were obtained from patients with different diseases in order to evaluate the sensitivity and specificity of peptides in serologic assays for the diagnosis of *T. cruzi* infection. Sera from patients with confirmed Chagas' disease (as determined by clinical diagnosis or consensus testing with IFAT and crude lysate ELISA) and sera positive for leishmania, tuberculosis, or malaria were obtained from Roberto Badaro (University of Bahia, Salvador, Brazil). Serum samples were also obtained from patients in Ribeirào Prêto, Brazil, with cardiac abnormalities or Chagas' disease; the samples were positive for *T. cruzi* by crude lysate and commercial ELISAs. Serum samples positive for the 72- and 90-kDa *T. cruzi*–specific proteins were obtained from David A. Leiby (American Red Cross, Rockville, MD). These samples, which originated from US blood banks, were *T. cruzi*–positive, as determined by ELISA (Abbott, Abbott Park, IL) and RIPA. Sera positive for *T. cruzi* crude lysate in ELISA were also obtained from the El Oro region of Ecuador and were supplied by Ron Guderian (Laboratory of Clinical Investigations, Hospital Vozandes, Quito, Ecuador). Other samples, which were consensus positive for *T. cruzi* infection by a variety of tests, were obtained from Frank Steurer (Centers for Disease Control and Prevention [CDC], Atlanta).

Recombinant proteins and synthetic peptides. The recombinant protein rTcD is a major antigenic epitope of a 260-kDa *T. cruzi* antigen that is prevalent in trypomastigotes. It was cloned from *T. cruzi* and expressed in *Escherichia coli* as previously described [38] and contains a 10–amino acid repeat sequence. The reactive peptide TcD is a 15–amino acid peptide (AEPKSAEPKPAEPKS) containing this repeat. The rTcE recombinant is the 35-kDa L19E ribosomal sequence and was also cloned from *T. cruzi* by use of TcD-negative sera and expressed in *E. coli.* It contains a 7–amino acid repeat sequence, and three repeats (TcE) were necessary for full activity (KAAIAPAKAAAAPAKAATAPA). A sequence related to TcE, namely TcHi 29 (KTAAPPAKTAAPPAKTAAPPA), was obtained by screening an amastigote cDNA library with high-titered *T. cruzi* antibody–positive sera. A third repeating epitope, PEP-2 (GDKPSPFGQAAAGDKPSPFGQA), was also shown, as previously described [27, 28, 39], to be serologically active.

Initially the three sequences (TcD, TcE, PEP-2) were combined into a single multi-epitope peptide, 2/D/E (GDKPSPFGQAA-AGDKPSPFGQAGCGAEPKSAEPKPAEPKSGCGKAAIAPA-KAAAAPAKAATAPA), and tested with consensus-positive sera to determine its ability to detect antibodies to *T. cruzi.* Subsequently, a fourth repeating epitope, TcLo1.2 (GTSEEGSRGG-SSMPSGTSEEGSRGGSSMPA), was obtained by expression cloning, using a serum sample that was negative for reactivity with 2/D/E. This epitope was further combined into a branched tetrapeptide with 2/D/E on one arm and TcLo1.2 on the other, and it was later expressed as a linear recombinant, r2/D/E/Lo1.2.

The individual linear peptides TcLo1.2 and 2/D/E were synthesized using Fmoc chemistry employing HATU (O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) or HBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) activation on a peptide synthesizer (9050; Millipore, Bedford, MA). They were purified by reverse-phase high-performance liquid chromatography on a Delta Pak C18 column (Waters Associates, Milford, MA), using a gradient of 0%–60% acetonitrile in water containing 0.05% trifluoroacetic. Fractions were lyophilized and characterized by use of electrospray mass spectrometry and amino acid analysis.

The branched tetrapeptide 2/D/E/Lo1.2 was synthesized from a lysine core residue using orthogonal protection, whereby Fmoc is used to protect the α amino group, and Dde (1-(4,4-Dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl) is used to protect the ϵ amino group. Selective deprotection allows the synthesis of the first peptide chain from either the α or ϵ group on the lysine. This first peptide chain is terminated with a protecting group that is not removed during the course of the synthesis of the second peptide chain. A tert-butoxy carbonyl (Boc) amino acid was used for this purpose with the Dde and Fmoc combination. The remaining lysine amino protecting group, Dde, was removed with 20% hydrazine before a second amino acid chain was synthesized. Cleavage of the branched peptide from the resin and removal of the N-α-Boc moiety was carried out using trifluoroacetic acid as described above. This approach was used to synthesize a peptide with 2/D/E (or TcHi29) on the α amino group and TcLo1.2 on the ϵ amino group. Purification of the peptides was done by methods described for the linear peptides above.

The recombinant r2/D/E/Lo1.2 was created by synthesizing overlapping phosphorylated oligonucleotides, annealing the matched oligonucleotides pairs to create double-stranded DNA. The annealed pairs were then ligated into a pT7 construct. The expression construct was transformed into BLR(DE3) pLys S *E. coli* (Novagen, Madison, WI) and grown overnight in LB broth with kanamycin (30 μg/mL; Sigma, St Louis) and chloramphenicol (34 mg/mL; Sigma). Overnight culture was then used to inoculate 500 mL of 2× yeast extract and tryptone with the same antibiotics and the culture with 1 m*M* isopropyl-β-D-thiogalactopyranoside. Four hours after induction, cells were harvested; sonicated in 20 m*M* tris, 100 m*M* NaCl, 0.1% deoxycholate (pH 8) containing 20 m*M* phenylmethylsulfonyl fluoride and 20 μg/mL leupeptin; and centrifuged at 26,000 *g.* The recombinant TcF is purified from the resulting supernatant by metal ion affinity chromatography, using nickel resin (Pro-bond; Invitrogen, Carlsbad, CA) and a stepwise gradient elution with 50, 100, and 500 m*M* imidazole in the wash buffer. TcF fractions were pooled, concentrated by ultrafiltration, and dialyzed against 10 m*M* tris, pH 8. Further purification to remove endotoxin was achieved on an anion exchange (High Q; Bio-Rad, Hercules, CA) eluted with a gradient of 0–1 *M* NaCl in 10 m*M* tris, pH 8.

ELISA. ELISAs for *T. cruzi* lysate and specific peptides were

performed as follows. Corning Easiwash 96-well microtiter plates (Corning Costar, Cambridge, MA) were coated overnight at 4°C with *T. cruzi* lysate (100 ng/well), PEP-2 (10 ng/well), TcE (25 ng/well), TcD (500 ng/well), tripeptide 2/D/E (40 ng/well), branched tetrapeptide (200 ng/well), TcLo1.2 (1 μg/well), or recombinant TcF (200 ng/well). Plates were then aspirated and blocked with PBS containing 1% (wt/vol) bovine serum albumin for 2 h at room temperature and then washed in PBS containing 0.1% Tween 20 (PBST). A 1/50 dilution of serum in PBS containing 0.1% bovine serum albumin was added to wells and incubated for 30 min at room temperature. This was followed by six washes with PBST and incubation with protein A–horseradish peroxidase conjugate (1/20,000 dilution; Sigma) for a further 30 min. Plates were then washed six times in PBST and incubated with tetramethylbenzidine substrate (Kirkegaard & Perry, Gaithersburg, MD) for 15 min. The reaction was stopped by the addition of 1 *N* sulfuric acid, and the plates were read at 450 nm with an ELISA plate reader (EL311; Biotek Instruments, Hyland Park VA).

The cutoff for assays was determined from the mean of the negative population +3 SD of the mean. Samples falling within 10% of the cutoff were considered equivocal. The lysate-based Chagas' ELISA (Gull Laboratories, Salt Lake City) was performed according to the manufacturer's specifications. Cutoff and determination of positive, equivocal, and negative samples was achieved by reference to the standards and controls supplied with the test.

T. cruzi lysate for use in ELISAs was prepared as described [39]. IFAT titers were determined according to published methods [25].

Results

Although TcD is superior to other *T. cruzi* antigens thus far identified for its ability to detect specific anti–*T. cruzi* antibodies in sera from infected persons, the sensitivity of TcD alone was only ~93% [39]. Therefore, the use of TcD alone will not constitute an ideal serodiagnostic test for blood-screening purposes.

Most serologic *T. cruzi* antigen genes reported thus far were identified through expression screening of genomic DNA libraries. The screening of such libraries may allow antigens that are encoded by genes that are present in low copy numbers and are expressed only in mammalian stages to escape detection. Therefore, screening strategies that would select for antigen genes that are abundant in the mammalian stages (e.g., amastigote) of the parasite would be ideal.

An amastigote cDNA expression library was constructed and screened with a pool of sera from *T. cruzi*–infected persons. The criteria for antigen selection were based on strong reactivity with *T. cruzi* patient sera, positive serology on TcD-negative sera, antigen conservation in a variety of *T. cruzi* isolates, and lack of reactivity on normal and heterologous patient sera. Forty-two recombinant clones that have immunodominant B cell epitopes abundant in the amastigote stage of the parasite were initially isolated. Within this group of clones, 6 had sequence similarity with other members of the eukaryotic ribosomal proteins (L19E, S8, and S-phase specific) and were novel *T. cruzi* genes, while 12 represented novel sequences not present in GenBank.

The ribosomal proteins L19E and S8 and the S-phase–specific antigen were selected for further analysis on the basis of their relatively strong reactivities with TcD-negative sera. Unlike S8 and the S-phase–specific proteins that showed sequence homology throughout their entire lengths with their eukaryotic homologue, L19E was unique. Following the region of homology, the *T. cruzi* L19E homologue (rTcE) contained 16 copies of a tandem-arrayed 7–amino acid repeat at the carboxy end. This repeat sequence is absent in other sequenced L19E proteins. The clone encoded a protein of 35 kDa. Thus, the repetitive domain appears to be present in the *T. cruzi* L19E homologue as part of the tail region.

Because rTcE was exceptionally reactive with the test serum and repeat sequences often represent immunodominant B cell epitopes, we pursued the repeat region of rTcE as a candidate to complement TcD. The repeat sequence for TcE was identified as KAAXAPA, in which X can be A, T, or I. A synthetic peptide (TcE) was constructed to contain a single repeat of each motif (i.e., three repeats total, see Materials and Methods) and was comparable in reactivity to the parent recombinant (data not shown). The enhancement of TcD-negative or TcD–low-positive sera with TcE is shown in figure 1. The reactivity of 10 sera that were low positive or negative with TcD were enhanced by inclusion of TcE in the solid phase. In addition, this formulation of TcE was also complementary with PEP-2, and when used in combination with PEP-2 and TcD, it provided a highly sensitive mix of peptides for use in detecting Chagas'-positive sera [40–41]. Similar effects were seen with TcHi29, which had the repetitive sequence KTAAPPA, which was similar to the sequence for TcE. A tripeptide containing all three epitopes (TcD, TcE, PEP-2) was synthesized and tested for its ability to detect antibodies to *T. cruzi* in consensus-positive sera. The structure of this peptide, referred to as 2/D/E, is described in Materials and Methods, and its activity in serologic detection of *T. cruzi* antibodies is described below. Its overall reactivity was greater than that seen for the individual mix of peptides since it minimized any problems related to competition for solid phase; such problems are expected when individual peptides are mixed [40–41].

Tripeptide (2/D/E) ELISA with consensus-positive sera. Table 1 summarizes the reactivity of the tripeptide 2/D/E with consensus-positive sera from different geographic sources. These samples were positive for *T.cruzi* antibodies by IFAT, crude lysate ELISA, or alternate commercial EIA. Of the 240 sera tested, 239 were above the cutoff (mean of the negatives +3 SD), indicating a sensitivity of 99.6%. A population histogram showing the distribution of these consensus-positive sera tested in relation to the cutoff is shown in figure 2. In contrast, of the 149 test serum samples from healthy, random doners, 148 were below the designated cutoff, indicating a specificity of 99.33%.

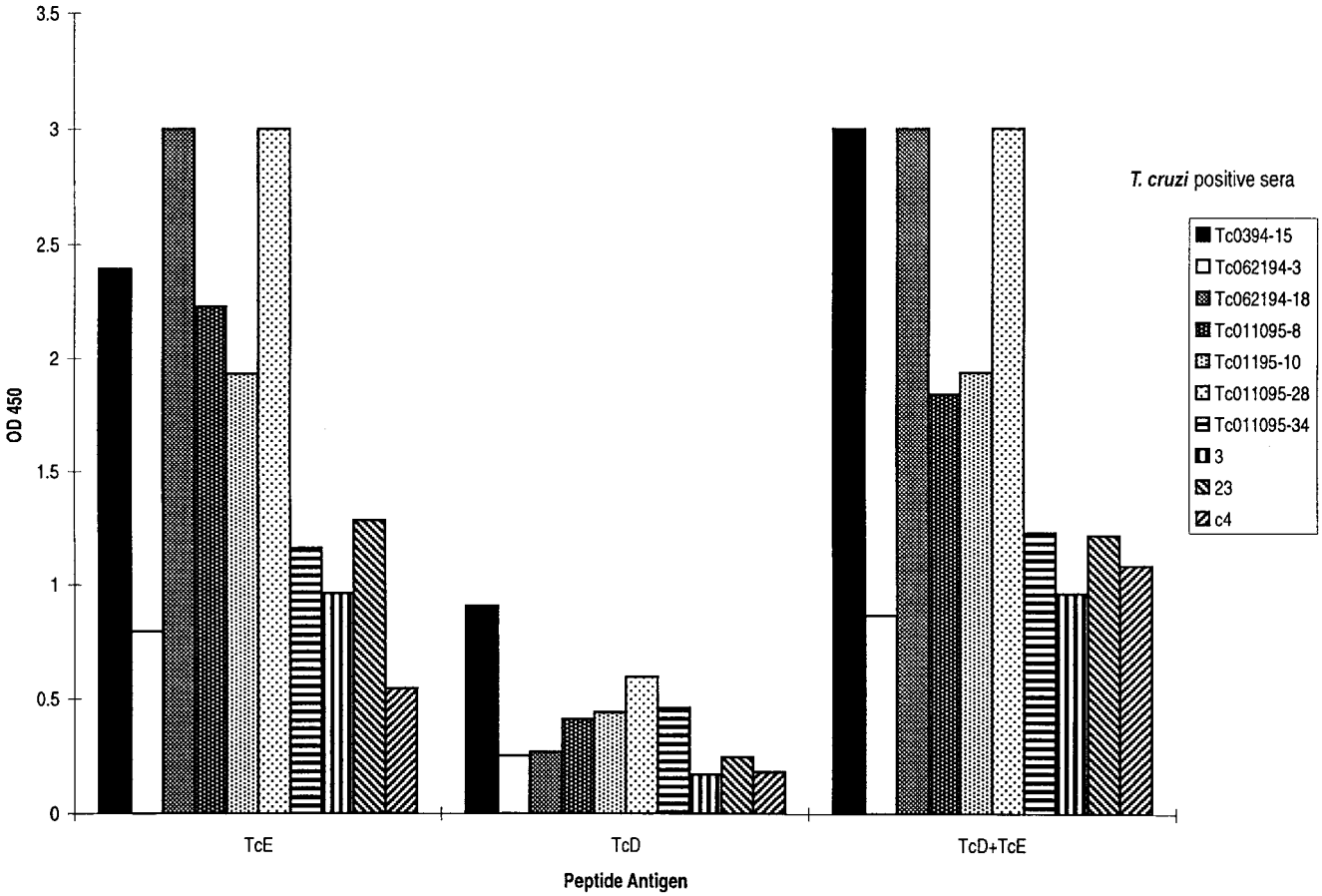

Figure 1. TcE enhancement of reactivity of *T. cruzi* antibody–positive, TcD-negative, and TcD–low-positive sera. Ten sera that were low positive or negative for TcD peptide were tested with TcE peptide alone or in combination with TcD. Peptides were coated on ELISA plates as described in Materials and Methods. OD 450 = optical density at 450 nm.

Table 2 shows the reactivity of a group of consensus-positive sera received from the CDC, where other tests had been used to confirm the presence of *T. cruzi* antibodies or to isolate parasites. These tests included IFAT, complement fixation, culture, and alternative *T. cruzi* antibody ELISA. In all of the 7 sera described in table 2, the tripeptide assay was positive and the samples were from several distinct geographic areas, including Central and North America—areas with low endemicity for *T. cruzi.*

Tripeptide (2/D/E) ELISA with sera confirmed positive for T. cruzi by RIPA. RIPA to detect the 72- and 90-kDA *T. cruzi*–specific proteins are frequently used in the United States as the confirmatory assay for *T. cruzi* antibody–positive sera as determined, for example, by ELISA [29–31]. The tripeptide ELISA was used to test two panels of sera containing samples confirmed positive by RIPA. In the first panel, serum samples found positive and samples found negative for *T. cruzi* by RIPA were tested, including samples from patients with *Leishmania*

Table 1. Sensitivity of tripeptide 2/D/E ELISA for detection of *T. cruzi* antibodies in consensus-positive sera.

Serum identification (geographic source)	ELISA reactivity (no. positive/no. total)
Consensus positive samples (Bahia, Brazil)	158/159
Consensus positive samples (El Oro, Ecuador)	55/55
Samples from patients with cardiac abnormalities (Ribeirão Prêto, Brazil)	12/12
Samples from patients with Chagas' disease (Ribeirão Prêto, Brazil)	7/7
CDC consensus positive samples	7/7
Total	239/240

NOTE. CDC = Centers for Disease Control and Prevention.

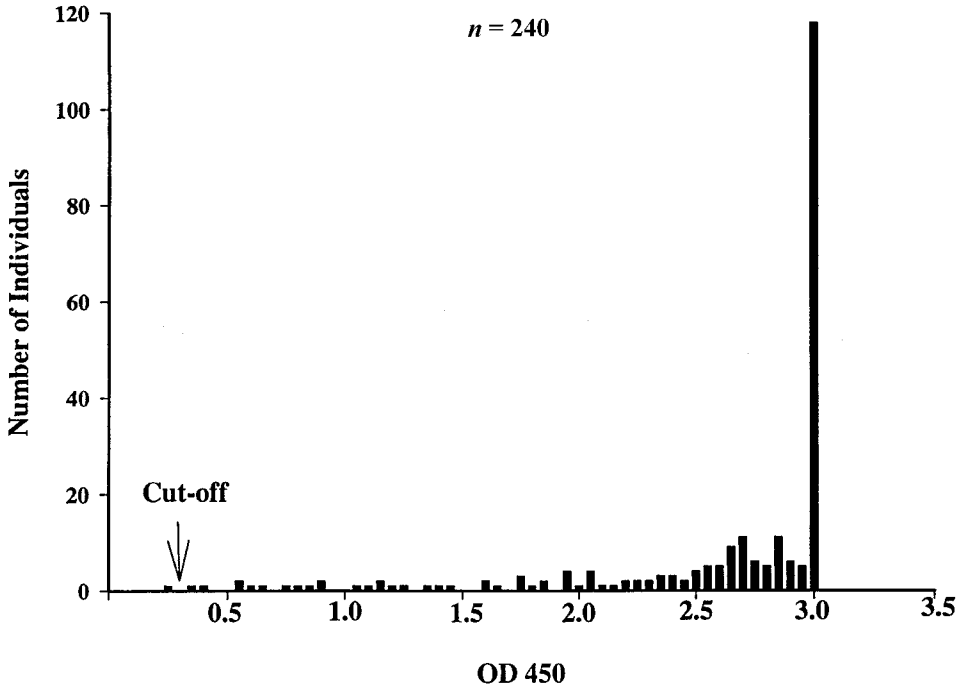

Figure 2. Histogram showing distribution of sera from consensus-positive *T. cruzi*–infected patients ($n = 240$) in 2/D/E peptide ELISA in relation to cutoff. OD 450 = optical density at 450 nm.

infection. The reactivity of this panel is shown in table 3 along with its reactivity with a commercially available Chagas' test from Gull Laboratories. This was not the same ELISA that was used by the Red Cross to identify positive samples that were later confirmed by RIPA [29–31].

The tripeptide EIA showed good correlation with RIPA, with 9 of 9 RIPA-confirmed positive samples being positive in the EIA. Of 11 samples that were negative by RIPA, 10 correlated with and 1 was equivocal with results of the 2/D/E ELISA. In particular, sera from leishmania patients were negative by ELISA. This is in contrast to the commercial ELISA, which showed several discrepancies: 4 of the 9 sera considered positive by RIPA were positive, 4 of the 11 sera considered negative by RIPA were positive, and 1 sample was equivocal. The second panel consisted of 33 sera confirmed positive by RIPA. Of these, the tripeptide assay reacted with 32, of which 3 were borderline positive and 1 was equivocal. The Gull Chagas' assay reacted with 22 of the 33 sera reconfirmed positive by RIPA, and 1 further sample was equivocal.

TcLo1.2-based immunoassays. The 1 consensus-positive sample that was missed by the 2/D/E ELISA was used to screen DNA libraries obtained from *T. cruzi.* This resulted in the identification of a recombinant protein (rTcLo1.2) containing an immunodominant repeat sequence, which was synthesized as a peptide, TcLo1.2 (see Materials and Methods), and evaluated in *T. cruzi* antibody assays [40–41]. Figure 3 shows the specificity of this peptide in ELISA compared with the specificity of *T. cruzi* lysate from epimastigotes. Compared with lysate,

Table 2. Reactivity of Centers for Disease Control and Prevention *T. cruzi* consensus-positive sera.

Patient identification	Location	IFAT	Complement fixation	ELISA (Abbott[a])	Culture	ELISA		
						2/D/E	2/D/E/Lo1.2	TcF
Tc103195-1	US laboratory accident	256	256	+	+	+	+	+
Tc103195-2	California	ND	512	+	+	+	+	+
Tc103195-3	Texas	256	32	+	+	+	+	+
Tc103195-4	Bolivia	512	16	+	+	+	+	+
Tc103195-5	Mexico	256	ND	+	+	+	+	+
Tc103195-6	Brazil	≥256	ND	+	ND	+	+	+
Tc103195-7	Brazil	≥256	ND	+	ND	+	+	+

NOTE. IFAT = immunofluorescence titration; ND = not done.
[a] Abbott Laboratories, Abbott Park, IL.

Table 3. ELISA reactivity of serum samples positive and negative for *T. cruzi* by radioimmunoprecipitation (RIPA).

Sample identification	RIPA	2/D/E	2/D/E/Lo1.2 EIA	TcF	Chagas' EIA[a]	Status
CPI-1	−	−	−	−	−	Negative
CPI-2	+	+	+	+	+	*T. cruzi* positive
CPI-3	−	−	−	−	+	Negative
CPI-4	+	+	+	+	−	*T. cruzi* positive
CPI-5	−	−	−	−	+	Negative
CPI-6	−	−	−	−	+	Negative
CPI-7	−	−	−	−	−	Negative
CPI-8	+	+	+	+	+	*T. cruzi* positive
CPI-9	+	+	+	+	+	*T. cruzi* positive
CPI-10	−	−	−	−	+	Negative
CPI-11	+	+	+	+	−	*T. cruzi* positive
CPI-12	+	+	+	+	−	*T. cruzi* positive
CPI-13	−	−	−	−	−	*Leishmania* species positive
CPI-14	+	+	+	+	−	*T. cruzi* and *Leishmania* species positive
CPI-15	−	−	−	−	−	*Leishmania* species positive
CPI-16	+	+	+	+	−	*T. cruzi* positive
CPI-17	−	−	−	−	E	Negative
CPI-18	+	+	+	+	+	*T. cruzi* positive
CPI-19	−	E	E	E	−	At cutoff in EIA; unknown bands on RIPA
CPI-20	−	−	−	−	−	*T. cruzi* positive

NOTE. E = equivocal as defined in Materials and Methods (within 10% cutoff).
[a] Gull Laboratories, Salt Lake City.

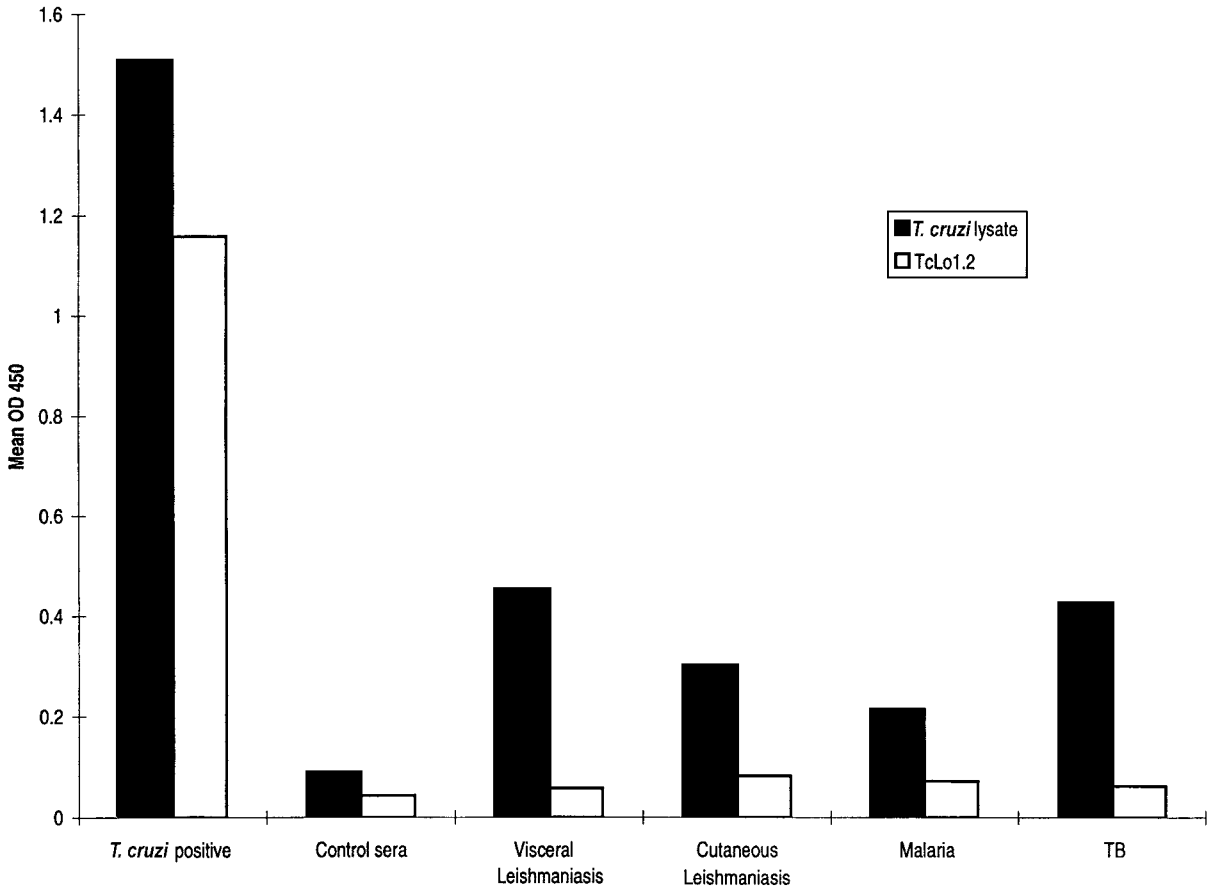

Figure 3. ELISA reactivities of sera from 8 patients each with *T. cruzi,* cutaneous leishmania, visceral leishmania, malaria, or tuberculosis (TB) and of 35 control sera with *T. cruzi* lysate and peptide TcLo1.2. OD 450 = optical density at 450 nm.

Table 4. Effect of TcLo1.2 alone or in combination with other peptides on signal cutoff of serum samples low positive for *T. cruzi* in 2/D/E peptide ELISA.

					Branched	
Sample identification	*T. cruzi* lysate	2/D/E	TcLo1.2	TcF	2/D/E/Lo1.2	2/D/Hi29/Lo1.2
Tc-011095-7	1.5	0.64	6.07	3.34	4.1	5.27
132003	4.02	1.01	5.57	6.70	4.13	7.26

NOTE. The cutoff for assays was determined from mean of negative population +3 SD of mean. Signal is optical density at 450 nm observed for sample in ELISA. Signal and cutoff of 1 means sample is at cutoff.

the TcLo1.2 peptide demonstrated a high degree of specificity when tested with sera from patients with visceral or cutaneous leishmaniasis, malaria, or tuberculosis.

The ability TcLo1.2 to improve reactivity of some low positive samples, as shown in table 4, and enable the detection of the 1 consensus-positive serum missed by tripeptide 2/D/E prompted the synthesis of a branched peptide (2/D/E/Lo1.2) and later a linear recombinant with all four epitopes (TcF). Both of these antigens detected all 240 consensus-positive samples.

Figure 4 compares the ELISA reactivity of the tripeptide, branched tetrapeptide 2/D/E/Lo1.2, and recombinant TcF with 2/D/E on a group of 38 sera that were determined to be *T. cruzi* positive by IFAT. These samples represent IFAT values of 1/40–1/640. This group of sera included the 1 sample that was missed by 2/D/E. The sample, which was from a patient with cardiac abnormalities, had an IFAT titer of 1/160 and was separately confirmed to be positive by RIPA. It is also possible to substitute the TcHi29 peptide in place of TcE in the branched tetrapeptide, as seen in figure 4; although, overall, the TcE construct performs better when tested on a broader panel of positive sera (data not shown). Both the tetrapeptide and the recombinant detected all 7 of the consensus-positive samples from the CDC. In addition, both detected all but 1 of the samples positive by RIPA. The sample they did not detect was clearly negative by the 2/D/E assay but equivocal in the tetrapeptide and recombinant ELISAs. Thus, of the 42 samples from a low-risk population that were positive by RIPA, both the

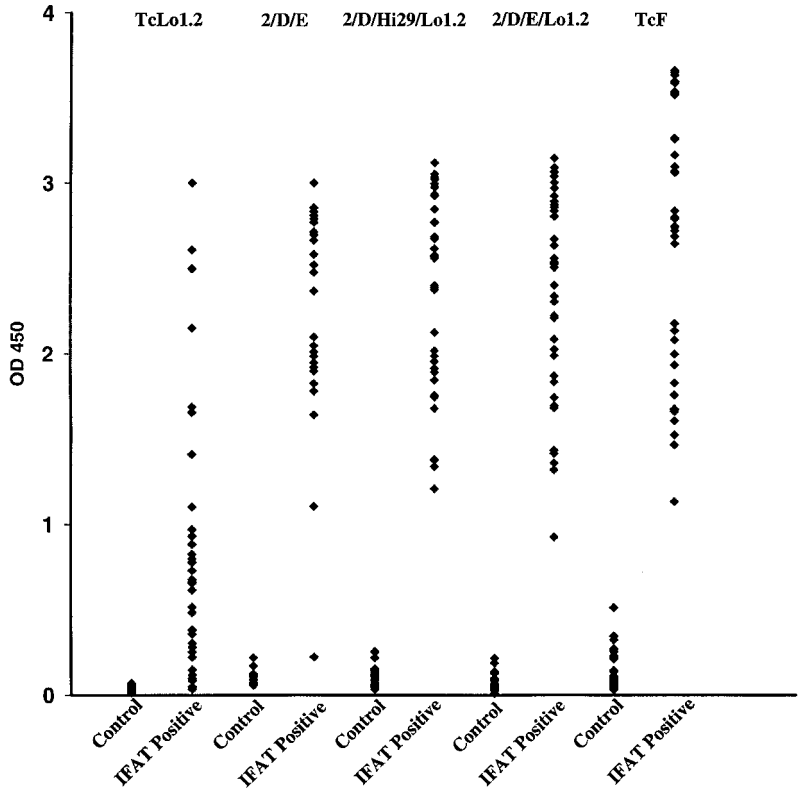

Figure 4. TcLo1.2, 2/D/E, branched tetrapeptides (2/D/Hi29/Lo1.2 and 2/D/E/Lo1.2), and TcF ELISA with 38 immunofluorescence titration (IFAT)–positive sera, including discrepant sample from 2/D/E peptide ELISA. OD 450 = optical density at 450 nm.

tetrapeptide and recombinant ELISAs detected 41 of 42, with the last sample being equivocal. This is in contrast to the Gull ELISA, which detected 26 of 42 samples positive by RIPA. The assay did however, detect all 38 of the consensus- and IFAT-positive samples that came from a high-risk population. This is consistent with the manufacturer's specifications for the Gull test with consensus-positive samples from endemic areas: It had a sensitivity of 96.5% compared with results for indirect hemagglutination and 99.1% compared with a combination of IFA and indirect hemagglutination.

Discussion

The data show that the use of repeating B cell epitopes in the form of synthetic peptides and recombinant proteins provides the means of developing very sensitive and specific assays for the detection of antibodies to *T. cruzi* in human sera. In this study, the consensus- and RIPA-positive sera were initially used to determine the utility of repeat epitopes combined in a tripeptide (2/D/E) in the detection of *T. cruzi* antibody. The tripeptide was highly sensitive, detecting 239 of 240 of the consensus-positive sera. The 1 consensus-positive serum not detected was used to identify a recombinant antigen, which contained a fourth repeating B cell epitope, TcLo1.2. This epitope, when tested alone as solid phase in ELISA was very specific for *T. cruzi,* detected the 1 discrepant sample with 2/D/E, and in some cases, boosted the reactivity of low-positive sera in the 2/D/E ELISA. Incorporation of this epitope into a branched tetrapeptide with an intact 2/D/E sequence attached to the α amino group of lysine and the TcLo1.2 epitope on the ϵ amino group produced a peptide with the reactivity of all four B cell epitopes. Mixing of the two dipeptides, TcD/TcE and PEP-2/TcLo1.2, also was effective in exhibiting all four epitope reactivities but with lower optical density values than for the tetrapeptide (data not shown) and slightly less sensitivity.

Substitution of the TcE in the branched peptide with a polymorph of TcHi29 gave rise to a peptide with activity comparable to that with the TcE version but with some subtle differences indicating higher sensitivity for the TcE epitope. For this reason, the TcE version was considered the most appropriate for further assay development. Incorporation of TcLo1.2 into a linear recombinant with 2/D/E (TcF) also resulted in detection of all 38 IFAT consensus-positive sera and may provide for an easier solid phase for assay development due to the ability to produce high levels of protein in *E. coli.*

The data indicate that the tetrapeptide- and recombinant-based assays provide a sensitive and specific alternative to lysate-derived assays and show a strong correlation to results with the RIPA test, which, especially in the United States, is currently used for confirmatory testing of blood donor samples considered positive for *T. cruzi* by ELISA. The reproducible synthesis of such a peptide or recombinant would prove advantageous in the development and subsequent manufacture of a standardized assay for eventual application to blood bank testing. In particular, the recombinant containing four distinct epitopes provides the most powerful diagnostic reagent to date for detection of *T. cruzi* antibodies in human serum and can be prepared reproducibly in large quantities. Lysate-derived assays detect considerably more positive US blood donors than can be confirmed by the RIPA confirmatory assay [30–31]. This is in contrast to the assays described here, which correlate well with RIPA, and would indicate that the tetrapeptide or recombinant or their individual epitopes could be used as an effective basis for developing a confirmatory assay. The data presented also indicate that both the tetrapeptide and recombinant TcF described have the potential for the development of sensitive and specific assays for detection of *T. cruzi* antibodies in both high- and low-risk populations, as would be encountered in Brazil (consensus positives) and the United States (RIPA confirmed).

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