Parasitol Res (1997) 83: 226–232 

ORIGINAL PAPER

F. Martín · C. Puertas · M.C. Thomas · C. Marañón
M.E. Patarroyo · J. Martín · C. Alonso · M.C. López

# Identification of a *Trypanosoma cruzi* antigenic epitope implicated in the infectivity of fibroblast LLC-MK2 cells

Received: 9 July 1996 / Accepted: 15 September 1996

**Abstract** In the present paper we describe the identification of an antigenic epitope that appears to be associated with the surface membrane of the *Trypanosoma cruzi* parasite, probably implicated in infectivity. Anti-TcMe antibodies inhibited the infectivity of fibroblast LLC-MK2 cells by 34% relative to a preimmune serum. The epitope was specifically recognized by 55% of the sera from 80 chagasic patients. The anti-TcMe antibody immunoprecipitated proteins of about 60 and 40 kDa from epimastigote and cell-culture trypomastigote forms, respectively. It is likely that the 60- and 40-kDa proteins are processed from higher-molecular-weight precursors, since the antibody immunoprecipitated protein fractions in the range of 115–150 kDa from in vitro translation products of poly $A^+$ RNA.

## Introduction

The protozoan parasite *Trypanosoma cruzi* is the etiological agent responsible for Chagas' disease. According to recent data, Chagas' disease presently affects 15 million people, and at least 70 million live in endemic areas. The annual death rate associated with this infection is 50,000 (Marsden 1995). The health problem posed by Chagas' disease has yet to be resolved since in many cases there is neither a defined clinical frame in which the disease can be included nor a specific, highly sensitive technique for its diagnosis (Luqueti 1990). Moreover, chemotherapy is not very effective, particularly during the chronic phase of the disease. The infection of the host cell, essentially nonphagocytes such as fibroblasts, by *T. cruzi* is accomplished in two phases. In the first phase the parasite adheres to the membrane of the target cell, and in the second one it actively penetrates into the host (Zingales and Colli 1985; Boschetti et al. 1987; Abuin et al. 1989; Van Voorhis et al. 1989). Since the adhesion and penetration processes are likely to be carried out by specific receptors located in the membrane of the target cell (Araujo-Jorge 1989; Davis and Kuhn 1990; Schenkman et al. 1991; De Cassia et al. 1993; Franco et al. 1993; Ramírez et al. 1993), it becomes obvious that elucidation of the mechanisms implicated in infection and the development of protective tools will require identification of the molecules involved in these processes. In the present paper we report the identification of a membrane-associated epitope of *T. cruzi* that appears to be implicated in the infectivity in vitro of host cells, since antibodies against the epitope inhibited the infection process by 33% and 34% relative to an unrelated serum and to preimmune serum, respectively. It was observed, moreover, that 55% of the sera from chagasic patients recognized the membrane-associated epitope.

F. Martín · C. Puertas[1] · M.C. Thomas · C. Marañón · J. Martín
M.C. López (✉)
Departamento de Biología Molecular,
Instituto de Parasitología y Biomedicina, López Neyra,
CSIC, Calle Ventanilla,
n° 11, E-18001 Granada, SPAIN,
Fax: (58) 203323;
e-mail: MLOPEZ@samba.cnb.uam.es

M.E. Patarroyo
Instituto de Immunología, Bogotá, Colombia

C. Alonso
Centro de Biología Molecular, CSIC-UAM, Madrid, Spain

*Present address:*
[1] Departamento de Parasitología, Universidad Javeriana,
Bogotá, Colombia

## Materials and methods

### Parasites

Epimastigotes of the Tulahuen strain were cultivated at 28 °C in RPMI 1640 medium containing glutamine and supplemented with 10% inactivated fetal bovine serum. Trypomastigote forms were obtained from in vitro culture according to the method described by Andrews et al. (1987).

## Isolation of proteins

An enriched extract of soluble membrane proteins from *Trypanosoma cruzi* was obtained from an epimastigote culture 24 h after the stationary phase had been reached. The percentage of trypomastigote forms present in the culture was about 8%. The proteins were prepared in lysis buffer [50 m*M* TRIS-HCl (pH 7.8), 1% NP 40, 2 m*M* $SO_3HNa$, 5 m*M* ethylenediaminetetraacetic acid (EDTA), 1% sodium dodecyl sulfate (SDS)] in the presence of protease inhibitors [2 m*M* phenylmethyl-sulfonyl fluoride (PMSF), 10 μg leupeptin/ml, 0.05 mg tosyllysine-chloromethylketone (TLCK)/ml, 1 mg Pefabloc/ml (Boehringer Mannheim)] and incubated at 4 °C for 20 min. After centrifugation at 7,000 *g* for 5 min at 4 °C the pellet was resuspended in SB buffer (Martín et al. 1993) and placed on ice for 5 min at 20 °C. The lysates were sonicated three times for 10 s and heated for 3 min at 95 °C. After being centrifuged at 12,000 *g* for 15 min, an aliquot of the supernatant was electrophoresed in 12% SDS-polyacrylamide gel electrophoresis (PAGE) gel. The Western-blot analysis was carried out by standard methods (Towbin et al. 1979). The sera were used at a dilution of 1/100. The blots were revealed with an anti-rabbit IgG alkaline phosphatase conjugate F(ab')2 fragment at a dilution of 1/1,000. NTB → Nitroblue tetrazolium chloride BCIP → 5-bromo-4-chloro-3-indolyl phosphate (NTB/BCIP) was used as a substrate. The protein fraction of approximately 60 kDa, corresponding to one of the most intensely marked bands, was cut out from the gel, electroeluted, and dialyzed against water. The dialysis membrane used had a cutoff point of 10,000 Da. The protein concentration was determined by Lowry's method (Lowry et al. 1951). The protein fractions were detected as a single band in SDS-PAGE electrophoresis after conventional silver staining (Guillemette and Lewis 1983).

## Sequencing and synthesis of peptides

The sequence of the amino-terminal end of the electroeluted protein was determined in a Beckman Sequencer 890M using the method described by Edman and Begg (1967); 350 pmol of the protein was used for sequencing. The residual phenytiohidantoin derivative (PTH-aa) was identified by high-performance liquid chromatography (HPLC) according to the method described by Hunkapiller and Hood (1978). A peptide corresponding to the sequence obtained was synthesized by the method described by Houghten (1985).

## Antigenicity and immunogenicity of the synthetic peptide

The reactivity of the peptide against the sera was determined using the Falcon assay screening test–enzyme-linked immunoabsorbant assay (FAST-ELISA; Becton Dickinson Labware, Linco Park, N.J.) technique as described by López et al. (1994). The concentration of the antigen was 1 μg/well. The cutoff point for positivity was set at an absorbance value of 0.050. This value represents the mean value for the absorbance of 40 normal sera plus 5 SD. The sera were used at a dilution of 1/64. In all, 80 serum samples from chagasic patients, 80 normal sera (provided by the INS and CINTROP-UIS, Colombia), and 100 sera from patients with different infectious diseases (leishmaniasis, tuberculosis, leprosy, and malaria; provided by the Bogotá Institute of Immunology, Colombia) were used. Specific anti-peptide antibodies were obtained in New Zealand rabbits immunized with 1 mg of the synthetic peptide coupled to KLH by glutaraldehyde (Reichilin 1990). A control rabbit was inoculated with KLH. The inoculation schedule was set at 0, 15, 30, 60, 80, and 100 days. The first dose, emulsified in complete Freund's adjuvant (1/1, v/v), was injected subcutaneously into the axillary region at ten different points. The rest of the injections, emulsified in incomplete Freund's adjuvant (1/1, v/v), were given intramuscularly as two equal aliquots in each leg. Prior to inoculation of the peptide, preimmune serum samples were obtained for control purposes. The reactivity against the peptide was determined on days 50, 70, 90, and 110 by the FAST-ELISA technique. The titer of the sera after the 5th dose of immunization was 1/25,600.

## Radioiodination and immunoprecipitation

The parasites were labeled using the lactoperoxidase glucose oxidase method (Hubbard and Cohn 1976). In all, $10^7$ epimastigote and $10^7$ trypomastigote forms of *T. cruzi* from a cell culture were resuspended in 100 μl of PBS buffer. Then, 50 μl of lactoperoxidase glucose oxidase, 400 μCi of iodine 125, and 10 μl of 0.1 *M* glucose in PBS were added to the cell suspension and incubated for 10 min at 20 °C. The free $^{125}I$ was removed by successive washes with PBS. The parasites were resuspended in 50 μl of PBS, to which 1 ml of lysis buffer [10 m*M* TRIS-HCl (pH 7.4), 1% NP 40, 0.15 *M* NaCl, 1 m*M* EDTA, 1 mg bovine serum albumin (BSA)/ml, 1 m*M* PMSF] was added. The samples were incubated for 30 min on ice and centrifuged for 5 min at 400 *g*. The supernatant containing the radioactively labeled proteins was incubated with preimmune serum and protein-A agarose (Boheringer Mannheim) for 1 h at 4 °C and then centrifuged at 12,000 rpm for 15 s. The resulting supernatant was incubated with the anti-peptide antibody at a dilution of 1/100 for 1 h at 4 °C and then with protein-A agarose for 1 h at 4 °C. After centrifugation at 12,000 rpm for 15 s the precipitate was washed three times with different buffers [buffer 1: lysis buffer + 0.5 *M* NaCl, buffer 2: lysis buffer + 0.1% SDS, buffer 3: 10 m*M* TRIS-HCl (pH 7.4), 0.1% NP 40] to increase the stringency of washing. Immunoprecipitated proteins were solubilized in Laemmli (1970) buffer, heated at 95 °C for 4 min, and analyzed in 10% polyacrylamide gels containing 0.1% SDS.

## Immunoprecipitation of in vitro translation products

The in vitro synthesis of proteins for immunoprecipitation of in vitro translation products was performed in a rabbit reticulocyte system (Pelham and Jackson 1976). First, 2 μg of poly $A^+$ RNA from epimastigotes were incubated with 10 μCi of L-[$^{35}S$]-Met and R Nasin for 90 min at 30 °C. The transcripts were then immunoprecipitated as described above.

## Immunoelectron microscopy

Epimastigote forms of the parasites obtained from the exponential phase of growth were washed three times in PBS buffer, centrifuged at 400 *g* for 20 min, and fixed in a solution of 1.5% formaldehyde and 1% glutaraldehyde. The sample was dehydrated by increasing ethanol concentrations at −20 °C and was included in Lowicryl K4M. The polymerization was done at −20 °C under UV light. The samples were immediately sectioned and mounted on grids. The grids were incubated in blocking solution (TBS, 1% BSA, 0.1% Tween-20) for 1 h at room temperature, incubated for 1 h with the anti-peptide antibody at a dilution of 1/100, washed several times with buffer (0.1% TBS, 0.1% BSA, 0.1% Tween-20), and incubated with anti-rabbit IgG (whole-molecule) gold conjugate for 1 h at room temperature. Finally, the samples were washed three times with 0.1% TBS/0.1% Tween-20. The last wash was done with sterile water and the samples were left to dry.

## Fluorescence-activated cell-sorting analysis

Flow-cytometry analysis was performed on a FACScan device (Becton Dickinson). The parasites were incubated with the anti-peptide antibody at dilutions of 1/10 and 1/50 for 1 h at 4 °C. The control sample was incubated with preimmune serum at a dilution of 1/10. Afterward the parasites were washed and incubated for 1 h at 4 °C with a rabbit anti-IgG conjugated with fluorescein isothiocyanate (FITC). The samples were then fixed with paraformaldehyde in a way similar to that described by Loken et al. (1990).

## Infectivity of T. cruzi

Trypomastigote forms of *T. cruzi* were used to infect LLC-MK2 fibroblast cells grown on 24-well microplates at a parasite:cell ratio

of 5:1 for 3 h at 37 °C in an atmosphere containing 5% $CO_2$. Before infection the trypomastigote forms were incubated for 2 h at 37 °C with PBS, anti-TcMe, anti-KLH, and control preimmune sera at a 1/200 dilution. After incubation the parasites were washed in PBS. The parasites were then used to infect LLC-MK2 fibroblast cells. The extracellular parasites were removed by successive washes in PBS. The infected LLC-MK2 cells were incubated at 37 °C in an atmosphere containing 5% $CO_2$. After 72 h of incubation the trypomastigote forms present in the supernatant of the infected cultures were counted and removed every 12 h by washing in PBS.

## Results

Using a highly reactive pool of sera obtained from chagasic patients, several protein fractions of *Trypanosoma cruzi* were identified by Western blotting (Fig. 1, lane 1). One of the most intensely marked bands, corresponding to a molecular weight of 60 kDa, was cutout from the gel and electroeluted. The sequence of the amino-terminal region, designated TcMe, was TAEDVLTFEGEGTGE. Antibodies raised against the peptide recognized the TcMe peptide and a protein band of a molecular weight similar to that recognized by the pool of sera (Fig. 1, lane 3). For determination of the spectrum of the antigenicity of the TcMe epitope, sera from 80 chagasic patients and sera from patients with other infectious diseases were made to react with the TcMe peptide. We observed that 44 of the sera from the chagasic patients specifically recognized the TcMe peptide (Table 1). Thus, the sensitivity of the diagnostic value of TcMe, defined as the ability to distinguish the sera of truely chagasic patients, was observed to be 55%. However, the specificity, defined as the ability to distinguish negative sera from both normal individuals and patients with other infectious diseases, was 95% for tuberculosis patients' sera and 100% for the remaining sera analyzed.

The potential location of TcMe on the parasite was studied by immunoprecipitation of labeled epimastigote and trypomastigote surface proteins from intact parasites. We observed that the anti-TcMe antibody immunoprecipitated an epimastigote protein fraction of about 60 kDa in addition to a set of proteins of higher molecular weight (range 115–150 kDa; Fig. 2A). Interestingly, when trypomastigote membrane proteins were incubated with the anti-TcMe antibody a protein of about 40 kDa was specifically immunoprecipitated (Fig. 2B). After a long period of autoradiographic exposure, high-molecular-weight bands similar to those immunoprecipitated with epimastigote forms could be

**Table 1** Reactivity as determined by FAST-ELISA of the TcMe epitope to sera from chagasic patients, sera from healthy individuals, and sera from patients with other infectious diseases

| Group and form of disease | Number of sera | Number of positive sera |
|---|---|---|
| Chagas' | 80 | 44 |
| Malaria | 30 | 0 |
| Leprosy | 20 | 0 |
| Tuberculosis | 20 | 1 |
| Leishmaniasis | 30 | 0 |
| Healthy | 80 | 0 |

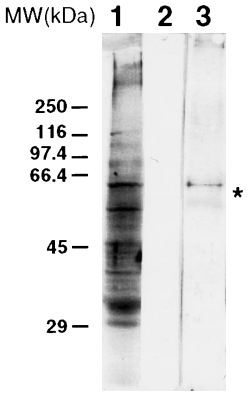



**Fig. 1** Western blot of membrane proteins from epimastigotes of *Trypanosama cruzi* (Tulahuen strain). (*Lane 1* Pool of six sera from Chagas patients – the sera were provided by the Instituto Nacional de Salud de Bogota, Colombia; l*ane 2* preimmune rabbit serum; l*ane 3* hyperimmune anti-TcMe serum) Molecular-weight markers (*MW*) are indicated at the *left*

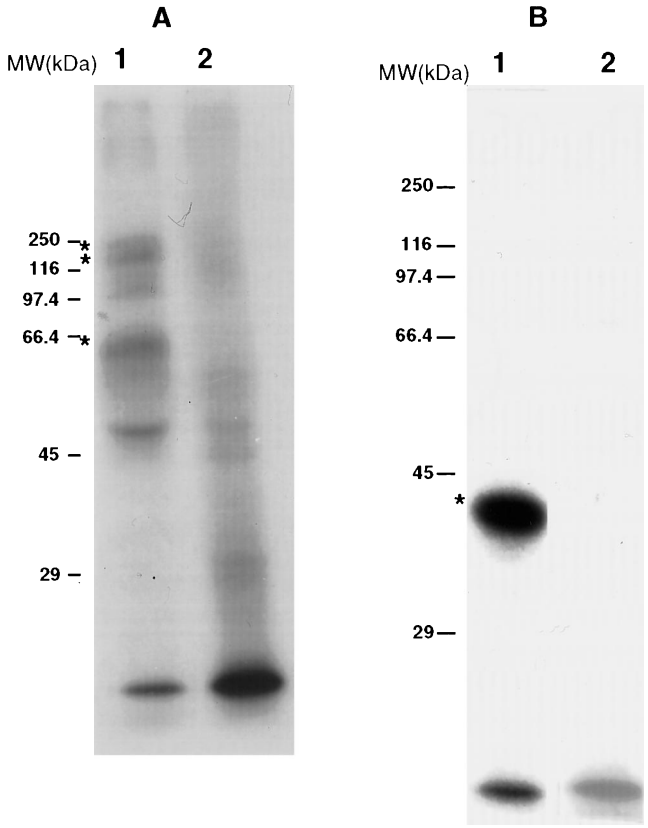



**Fig. 2A, B** Immunoprecipitation of surface-radioiodinated *T. cruzi*. **A** Epimastigote forms. **B** Trypomastigote forms from cell cultures (*Lane 1* Rabbit hyperimmune anti-TcMe serum, *lane 2* preimmune serum from the same immunized rabbit). The bands specifically immunoprecipitated by the anti-TcMe antibodies are indicated by *asterisks*. Molecular-weight markers are indicated at the *left*

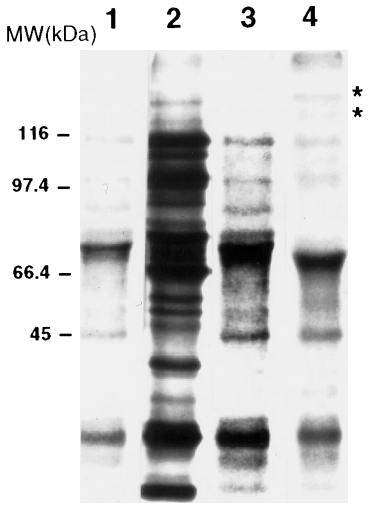
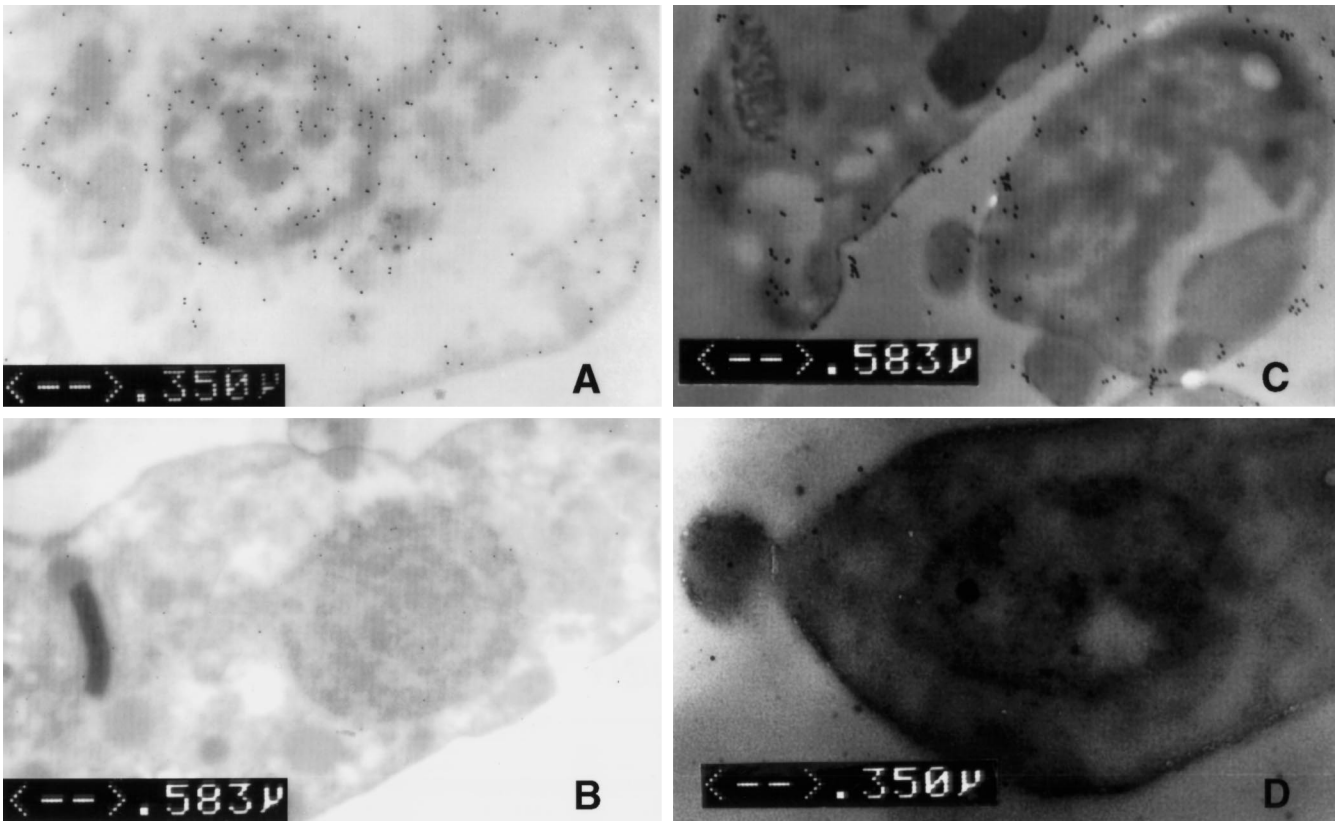



**Fig. 3** Immunoprecipitation of the in vitro translation products of poly $A^+$ RNA of epimastigotes of *T. cruzi*. (*Lane 1* Immunoprecipitation of the translation products using preimmune serum from a rabbit inoculated with epimastigote forms of *T. cruzi, lane 2* immunoprecipitation of the translation products by the hyperimmune serum made against epimastigote forms of *T. cruzi, lane 3* immunoprecipitation of the translation products by the preimmune serum made against the rabbit inoculated with the TcMe epitope, *lane 4* immunoprecipitation of the products by anti-TcMe, *asterisks* protein fractions specifically immunoprecipitated by the anti-TcMe antibody). Molecular-weight markers are shown at the *left*

detected, although the 60-kDa band was never present (data not shown). That the 60- and 40-kDa protein fractions could have resulted from processing of high-molecular-weight fractions was suggested by immunoprecipitation of in vitro translation products of poly $A^+$ RNA from epimastigotes using anti-TcMe. Figure 3 (lane 4) shows that the antibody immunoprecipitated two bands of a molecular size of 115–150 kDA. Bands of similar molecular size were also immunoprecipitated with anti-epimastigote antibodies (Fig. 3, lane 2).

To define the subcellular localization of the TcMe epitope, we carried out immunoelectron microscopy studies and flow-cytometry analysis of intact parasites using anti-TcMe antibodies and preimmune sera. Figure 4C shows that the colloidal gold deposits are predominantly associated with the surface of the parasite. As a control of unspecific membrane binding an antibody against heat-shock protein 70 (anti-hsp70 antibody) was used. As expected (Martín et al. 1993), the labeling was associated with the nuclear and cytoplasmic fractions (Fig. 4A). The surface localization of the TcMe epitope was also corroborated by flow-cytometry analysis. Figure 5 shows the presence of high fluorescence intensity in parasites labeled with the anti-TcMe antibody relative to control sera.

**Fig. 4A–D** Immunoelectron microscopy studies. **A, C** Hyperimmune sera against hsp70 protein and TcMe epitope, respectively. **B, D** Preimmune sera from the animal inoculated with hsp70 and TcMe, respectively



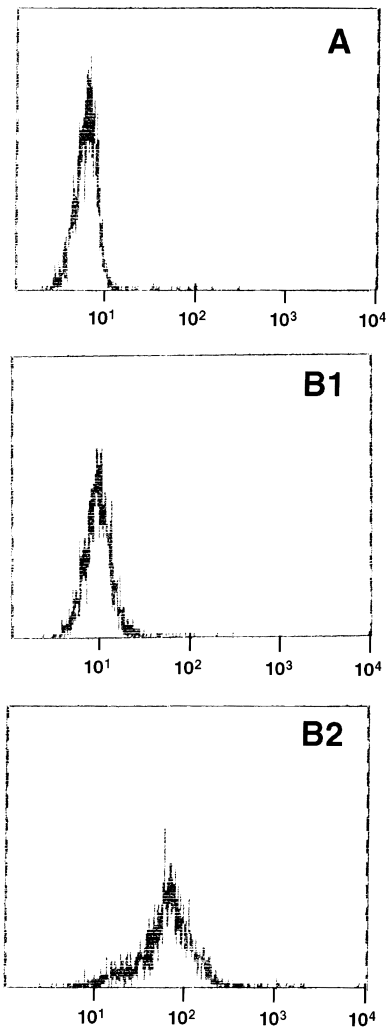



**Fig. 5A, B** Immunofluorescent flow-cytometry analysis of *T. cruzi*. Number of cells is indicated in the *ordinate* and fluorescence intensity is shown in the *abscissa*. **A** cells stained with preimmune serum at 1/10 dilution. **B** cells stained with anti-TcMe serum at 1/50 ($\mathbf{B}_1$) and 1/10 ($\mathbf{B}_2$) dilution, respectively

To evaluate the potential functional role of the TcMe epitope, we evaluated the ability of anti-TcMe antibodies to inhibit the invasion of LLC-MK2 monolayer cells by trypomastigotes derived from cell cultures (Table 2). We observed that the anti-TcMe antibody inhibited the capacity of the parasites to infect host cells by 50% relative to PBS, by 34% relative to the preimmune serum, and by 33% relative to anti-KLH. The adhesion of the parasites to the cells did not seem to be affected by anti-TcMe antibodies.

**Table 2** Inhibition of infectivity

| Time postinfection (h) | % of inhibition[a,b] | | |
|---|---|---|---|
| | Anti-TcMe | Preimmune | Anti-KLH |
| 72 | 0 | 0 | 0 |
| 84 | 59 ± 4 | 18 ± 4 | 17 ± 3 |
| 96 | 55 ± 5 | 20 ± 5 | 20 ± 4 |
| 108 | 44 ± 5 | 14 ± 4 | 16 ± 3 |
| 120 | 45 ± 3 | 18 ± 3 | 16 ± 4 |
| 132 | 46 ± 4 | 15 ± 3 | 18 ± 3 |

[a]Mean values ± SD for 5 experiments
[b]Calculated relative to the infection observed in cultured LLC-MK2 cells incubated with PBS

## Discussion

In the present paper we show the identification of an antigenic epitope (TcMe) from *Trypanosoma cruzi* that is associated with the membrane of the parasite. The TcMe epitope is immunogenic in vivo in *T. cruzi*-infected people, since the peptide was recognized by 55% of the sera obtained from 80 chagasic patients. The recognition of TcMe by the chagasic sera is highly specific (95%), since there was no reactivity with sera from healthy individuals or with sera from patients with other infectious diseases. Although the recognition sensitivity of TcMe is lower than that reported for other proteins and/or epitopes (Ibáñez et al. 1988; Almeida et al. 1990; Gruber and Zingales 1993; Solana et al. 1995), we understand that it can increase the diagnostic value of existing proteins by participating in a pool of antigens for use in the serodiagnosis of Chagas' disease. The application of a pool of antigens is particularly useful in the diagnosis of a disease such as trypanosomiasis, in which there is a high variability of antigen recognition. Almeida et al. (1990) report that the use of combined antigens gives better results than that of individual antigens since some sera can display a low titer response to individual antigens.

The TcMe epitope was identified in epimastigotes and in trypomastigotes as forming a part of protein fractions of about 60 and 40 kDa respectively in addition to a set of proteins of higher molecular weight (range 115–150 kDa). The immunoelectron microscopy and flow-cytometry data suggest that the TcMe epitope is located on the surface of the parasite. The nonuniform distribution of the label is probably indicative of a nonuniform arrangement of the protein over the surface of the parasite as has been indicated for other *T. cruzi* membrane proteins (Prioli et al. 1991; Frevert et al. 1992). The data cannot rule out the possibility that TcMe may be associated functionally rather than structurally with the surface of the parasite. The immunoprecipitation of a set of high-molecular-weight proteins in both epimastigote and trypomastigote forms, together with the existence of anti-TcMe-labeled stage-specific proteins (60 kDa from epimastigotes and 40 kDa from trypomastigotes), suggests that both of these proteins may come from a common precursor. This hypothesis is supported by the immunoprecipitation of high-molecular-weight protein fractions in the range between 115 and 150 kDa from in vitro translation of poly $A^+$ RNA and by the absence of the protein fractions of 60 and 40 kDa. Although there was no sequence homology of the TcMe epitope with any of the protein sequences described, the immunoprecipitation profiles generated by the anti-TcMe

antibodies are quite similar to those reported for the proteins encoded by the TCR39 (Hoft et al. 1989) and B13 (Gruber and Zingales 1993) clones. Thus, it may well be that the TcMe epitope is partially shared by different proteins. Albuin et al. (1989) suggest that proteins from *T. cruzi* strains or clones containing epitopes involved in cell invasion and recognized by the same antibodies vary in amino acid composition and/or in the structure of chemical residues added during post-translational events.

The functional potential of TcMe was shown by the observation that anti-TcMe antibodies inhibited the infection of LLC-MK2 by 34% relative to unrelated antibodies. The infectivity inhibition percentage observed is similar in magnitude to the 46–50% value obtained by Abuin et al. (1989) using the McAbH1A10 monoclonal antibody against the 85-kDa surface glycoprotein. Also De Cassia et al. (1993) report that monoclonal antibodies against the 35/50-kDa surface glycoconjugate inhibit 60% of metacyclical-form infection of Vero cells as an indication that the glycoproteins may participate in the host-cell invasion process. The question as to whether the percentage of inhibition of infectivity induced by anti-TcMe antibodies increases when metacyclical forms derived from axenic cultures are used as indicated by Abuin et al. (1989) is under investigation.

**Acknowledgements** We thank Ms. F. Guzmán (Instituto de Inmunologia, Bogotá, Colombia) for the synthesis of peptide and Ms A. López-Barajas for excellent technical assistance. We are also grateful to Drs. A. Corredor (INS, Bogotá, Colombia), V.M. Angulo, and C.I. González Rugeles (CINTROP, UIS, Bucaramanga, Colombia) for providing the human sera. This work was supported by grant BIO93-0043 from Plan Nacional I+D (CICYT), Spain.

## References

Abuin G, Colli W, Souza W, Alves MJ (1989) A surface antigen of *Trypanosoma cruzi* involved in cell invasion (Tc-85) is heterogeneous in expression and molecular constitution. Mol Biochem Parasitol 35: 229–238

Almeida E, Krieger MA, Carvalho MR, Oelemann W, Goldenberg S (1990) Use of recombinant antigens for the diagnosis of Chagas' disease and blood bank screening. Mem Inst Oswaldo Cruz 85: 513–517

Andrews NW, Abrams CK, Slatin SL, Griffiths G (1987) Stage-specific surface antigens expressed during the morphogenesis of vertebrate forms of *Trypanosoma cruzi*. Exp Parasitol 64: 474–484

Araujo-Jorge TC (1989) The biology of *Trypanosoma cruzi* macrophage interaction. Mem Inst Oswaldo Cruz 84: 441–462

Boschetti MA, Piras MM, Henríquez D, Piras R (1987) The interaction of a *Trypanosoma cruzi* surface protein with Vero cells and its relationship with parasite adhesion. Mol Biochem Parasitol 24: 175–184

Davis CD, Kuhn R (1990) Detection of antigens with affinity for host cell membrane polypeptides in culture supernatants of *Trypanosoma cruzi*. Infect Immun 58: 1–26

De Cassia R, Rigoni VL, González J, Yoshida N (1993) The 35/50 kDa surface antigen of *Trypanosoma cruzi* metacyclic trypomastigotes, an adhesion molecule involved in host cell invasion. Parasite Immunol 15: 121–125

Edman P, Begg R (1967) A protein sequenator. Eur J Biochem 1: 80–91

Franco FRS, Paranhos-Bacalla GS, Yamauchi LM, Yoshida N, Da Silveira JF (1993) Characterization of a cDNA clone encoding the carboxy-terminal domain of a 90-kilodalton surface antigen of *Trypanosoma cruzi* metacyclic trypomastigotes. Infect Immun 61: 4196–4201

Frevert U, Schenkman S, Nussenzweig V (1992) Stage specific expression and intracellular shedding of the cell surface transsialidase of *Trypanosoma cruzi*. Infect Immun 60: 2349–2360

Gruber A, Zingales B (1993) *Trypanosoma cruzi:* characterization of two recombinant antigens with potential application in the diagnosis of Chagas' disease. Exp Parasitol 76: 1–12

Guillemette JG, Lewis PN (1983) Detection of subnanogram quantities of DNA and RNA on native and denaturing polyacrylamide and agarose gels by silver staining. Electrophoresis 4: 92–94

Hoft DF, Kim KS, Otsu K, Moser DR, Yost WJ, Blumin JH, Donelson JE, Kirchhoff LV (1989) *Trypanosoma cruzi* expresses diverse repetitive protein antigens. Infect Immun 57:1959–1967

Houghten RA (1985) General method for the rapid solid-phase synthesis of the large number of peptides: specificity of antigen-antibody interactions of the level of individual amino acids. Proc Natl Acad Sci USA 82: 5131–5135

Hubbard AL, Cohn ZA (1976) Biochemical analysis of membranes. In: Maddy AH (ed) Specific label for cell surface, cap. 12. Chapman and Hall, London, p 427

Hunkapiller MW, Hood LE (1978) Direct microsequence analysis of polypeptides using an improved sequenator, nonprotein carrier (Polybrene) and high pressure liquid chromatography. Biochemistry 17: 2124–2133

Ibáñez CF, Affranchino JL, Macina RA, Reyes MB, Leguizamon S, Camargo ME, Aslund L, Pettersson U, Frasch ACC (1988) Multiple *Trypanosoma cruzi* antigens containing tandemly repeated amino acid sequence motifs. Mol Biochem Parasitol 30: 27–34

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Loken MR, Brosnan JM, Bach BA, Ault KA (1990) Establishing optimal lymphocytes gates for immunophenotyping by flow cytometry. Cytometry 11: 453–459

López MC, Silva Y, Thomas MC, García A, Faus MJ, Alonso P, Martínez F, Del Real G, Alonso A (1994) Characterization of the chimeric SPf(66)n molecule used as a vaccine against malaria. Vaccine 12:585–591

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275

Luqueti AO (1990) Use of *Trypanosoma cruzi* defined proteins for diagnosis-multicentre trial-serological and technical aspects. Mem Inst Oswaldo Cruz 85: 497–505

Martin F, Requena JM, Martín J, Alonso C, López MC (1993) Cytoplasmic-nuclear translocation of the Hsp70 protein during environmental stress in *Trypanosoma cruzi*. Biochem Biophys Res Commun 196: 1155–1162

Marsden PD (1995) American trypanosomiasis. In: Cook GC (ed) Manson's tropical disease. Saunders, London, pp 1197–1212

Pelham M, Jackson RJ (1976) An efficient mRNA-dependent translation system from reticulocyte lysates. Eur J Biochem 76: 247–256

Prioli RP, Mejía JS, Aji T, Aikawa M, Pereira MEA (1991) *Trypanosoma cruzi*: localization of neuraminidase on the surface of trypomastigotes. Trop Med Parasitol 42: 146–150

Ramírez MI, Cassia Ruiz R de, Araya JE, Da Silveira JF, Yoshida N (1993) Involvement of the stage-specific 82-kilodalton adhesion molecule of *Trypanosoma cruzi* metacyclic trypomastigotes in host cell invasion. Infect Immun 61: 3636–3641

Reichilin M (1990) Use of glutaraldehyde as a coupling agent for proteins and peptides. Methods Enzymol 70: 159–165

Schenkman S, Diaz C, Nussenzweig V (1991) Attachment of *Trypanosoma cruzi* trypomastigotes to receptors at restricted cell surface domains. Exp Parasitol 72: 76–86

Solana ME, Katzin AM, Umezawa ES, Miatello CS (1995) High specificity of *Trypanosoma cruzi* epimastigote ribonucleoprotein

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

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76: 4350–4354

Van Voorhis W, Corhis WC, Eisen H (1989) A surface antigen of *Trypanosoma cruzi* that mimics mammalian nervous tissue. J Exp Med 169: 641–652

Zingales B, Colli W (1985) *Trypanosoma cruzi*: interaction with host cells. In: Hudson L (ed) Current topics in microbiology and immunology. Springer, Berlin Heidelberg New York, pp 129–153