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Expression in *Escherichia coli* of a Dominant Immunogen of *Trypanosoma cruzi* Recognized by Human Chagasic Sera

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A genomic clone expressing a *Trypanosoma cruzi* antigen in *Escherichia coli* was identified using human chagasic sera. Chagasic antibodies affinity purified on extracts of this clone recognized a high-molecular-weight protein expressed in all developmental stages of the parasite life cycle, as well as in various *T. cruzi* strains. The antigen is associated with the cytoskeleton of the parasite and localizes along the attachment region between the flagellum and the cell body. Antibodies to the recombinant antigen were detected in the sera of 115 chagasic patients from different endemic regions, but not in sera of patients with leishmaniasis, *T. rangeli* infection, or other parasitic diseases. Our data suggest that the presence of antibodies to this antigen may be specifically associated with Chagas' disease.

Trypanosoma cruzi, the etiological agent of Chagas' disease, displays a complex life cycle, including an invertebrate triatomine vector and mammalian hosts. During its morphogenesis, it displays both stage-specific and common antigens (1, 19), all part of a complex repertoire that may eventually be exposed to the host immune system. Recent studies indicate that this protozoan synthesizes several proteins and presents a large number of antigenic determinants to the host immune system (5, 11, 15, 23). The role of these antigens in the pathogenesis and immunopathology of Chagas' disease, however, is poorly understood. One possible approach to this question involves the identification and characterization of parasite antigens and analysis of the immune responses that they elicit in the mammalian host.

We have used DNA cloning techniques to identify and characterize *T. cruzi* antigens of clinical interest. We describe the isolation and characterization of a recombinant clone that expresses an immunodominant *T. cruzi* antigen which is specifically recognized by antibodies from human chagasic patients. The recombinant antigen shares common antigenic determinants with a high-molecular-weight cytoskeletal protein associated with the flagellum. Our results suggest that this antigen contains immunodominant epitopes present in all of the developmental stages of the parasite which could be used to develop diagnostic procedures.

MATERIALS AND METHODS

Parasites. *T. cruzi* was grown in liver infusion tryptose liquid medium supplemented with 10% heat-inactivated fetal calf serum at 28°C without shaking. Metacyclic trypomastigotes were isolated from cultures at the late stationary growth phase by chromatography on DEAE-cellulose columns. Cell culture trypomastigotes and amastigotes were obtained from infected monolayers of Vero and HeLa cells. Amastigotes were also obtained from spleens of infected mice.

Library construction and screening. Nuclear DNA from metacyclic trypomastigotes (G strain) was cloned in λgt11 as described by Young et al. (22). In brief, random genomic fragments were generated by partial digestion with DNase I and size fractionated on agarose gel (18). DNA fragments of 6.0 to 1.0 kilobases were eluted, ligated to *Eco*RI synthetic linkers, and cloned into *Eco*RI sites of λgt11. Part of the library was directly used for screening without further purification.

Immunoscreening of the recombinant DNA expression library was performed by the procedure of Ozaki et al. (13). Human chagasic sera were preabsorbed with induced λgt11 in *Escherichia coli* Y1090 bound to nitrocellulose filters for 12 h at 4°C. A library was plated out on Y1090 at a density of 2,000 plaques per 15-mm dish and grown at 42°C for 2 h. Nitrocellulose filters were soaked in 10 mM isopropyl β-D-thiogalactopyranoside in water, blotted dry, and placed on the plates, and incubation was continued for 2 h at 37°C.

The filters were removed, washed, and blocked in PBSM (10 mM sodium phosphate [pH 7.5], 150 mM NaCl, 5% defatted powdered milk) and probed with a 1:100 dilution of human chagasic sera overnight at 4°C. Filters were washed three times in an excess of PBSM and assayed for bound antibodies, using ^{125}I-labeled protein A and rabbit anti-human immunoglobulins coupled to peroxidase.

Phage dot blot immunoassay. Individual recombinant phages were subjected to a number of screenings with several serum samples, using the phage plaque immunoassay. Phages of positive clones as well as nonrecombinant λgt11 were individually dotted in 1 μl of phage buffer (100 mM NaCl, 50 mM Tris [pH 7.5], 5 mM $MgSO_4$, 0.01% gelatin) containing 100 PFU onto a lawn of *E. coli* Y1090 cells growing on LB (Luria-Bertani medium) agar plates. Cultures were grown and probed as described above for library screening.

Antibody selection (plaque antibody selection). Recombinant phages were plated at a density of 10^5 PFU/150-mm LB agar plate and incubated at 42°C for 2 to 3 h. A filter of nitrocellulose soaked in 10 mM isopropyl β-D-thiogalactopyranoside was layered on a petri dish for 2 h at 37°C, flipped over on the same plate, and incubated for another 2 h. The filters were incubated with serum diluted 1:50. After washing, bound antibodies were eluted with 5 ml of 0.2 M glycine, pH 2.8, and neutralized with Tris base (13). These antibodies

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were dialyzed against 10 mM Tris hydrochloride, pH 7.5, and subsequently used to probe immunoblots of parasite protein extract or in electron microscopy immunolocalization studies.

SDS-PAGE and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with parasites and bacterial lysates in 7.5 or 5.0% gels as described previously (8). Immunoblotting was carried out by the method of Towbin et al. (20). Bound antibodies were revealed with rabbit anti-human immunoglobulins coupled to peroxidase. Protein molecular weight markers (molecular weight in parentheses) were from Sigma Chemical Co.: myosin heavy chain (200,000), β-galactosidase (116,000), phosphorylase *b* (92,500), bovine serum albumin (67,500), ovalbumin (43,000), carbonic anhydrase (30,000), and lysozyme (14,300). Mouse erythrocytes were used as a source of spectrins (molecular weights, 240,000 and 220,000).

Antisera. Human chronic chagasic sera were obtained from patients with chronic Chagas' disease diagnosed by serological and clinical symptoms. Serological analyses were performed by indirect immunofluorescence, indirect hemagglutination, and complement fixation tests. Pools of chronic chagasic sera were obtained from blood transfusion centers at São Paulo, Brazil. Antibodies against the recombinant antigen were raised in BALB/c mice by injecting the partially purified recombinant antigen. Sera also were obtained from patients with other parasitic diseases diagnosed by serological and clinical symptoms. Indirect immunofluorescence and hemagglutination tests were used to detect specific antibodies in the sera from these patients. Sera from humans infected with *T. rangeli* were also analyzed by enzyme-linked immunosorbent assay, using epimastigote antigens from this parasite. These sera were kindly provided by Felipe Guhl, Universidad de los Andes, Bogota, Colombia.

Immunofluorescence and immunoelectron microscopy. Immunofluorescence of extracellular forms was carried out with formaldehyde-fixed parasites. Isolation of parasite cytoskeletons and immunofluorescence labeling were done as described previously (12). For intracellular staining of parasites, HeLa cells grown on glass cover slips were infected with *T. cruzi* metacyclic tryptomastigotes. At 72 to 96 h postinfection, cover slips were washed five times with 10 mM sodium phosphate (pH 7.5)–150 mM NaCl (PBS) and fixed with 3.5% formaldehyde–PBS for 30 min; excess aldehyde was quenched with a 15-min incubation in 50 mM NH_4Cl–PBS, and samples were soaked in PBS containing 0.25% gelatin–0.01% saponin to prevent nonspecific binding and permeabilize the cells. Cover slips were then inverted onto 30-μl drops of antibody for 1 h, rinsed in PBS, and exposed to 1:40 dilutions of fluorescein isothiocyanate-anti-human immunoglobulin G for 30 min. After a rinse with PBS, cover slips were mounted in 0.1 M glycine (pH 9.6)–90% glycerol. Samples were examined on a Leitz Laborlux D epifluorescence microscope with ×40 and ×100 objectives. Photographs were taken on 400-ASA Ilford HP-5 films, subsequently developed for 18 min with Microdol-X (Eastman Kodak Co.).

To localize the parasite antigen in the electron microscope, cytoskeletons fixed with 4% *para*-formaldehyde–0.1% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, were adsorbed onto poly-L-lysine-coated Parlodion grids; after 15 min, the grids were soaked in electron microscopy buffer (0.1 M Tris hydrochloride [pH 7.4], 0.1% gelatin, 1% ovalbumin, 0.5% Tween 20 [2]) for 1 h. Samples were then incubated with affinity-selected human anti-H49 antibodies diluted 1:1 in electron microscopy buffer for another hour. Unbound antibodies were removed by rinsing with running distilled water for 1 min. Grids were then soaked in electron microscopy buffer in a 1:40 dilution of protein A coupled to 10-nm colloidal gold particles (a kind gift from Paul Webster, ILRAD, Nairobi, Kenya) and incubated for 1 h. After being rinsed as above, samples were stained with 1% aqueous uranyl acetate, air dried, and examined on a JEOL 100 CXII electron microscope operated at 80 kV.

Southern and Northern hybridizations. Southern blots were performed as described earlier (10). DNA samples were electrophoresed in 0.7% (wt/vol) agarose gels and transferred to nitrocellulose. Prehybridization was performed at 45°C for 4 h in hybridization buffer (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.5], 5× Denhardt solution, 50% formamide, 0.1% SDS, 100 μg of sonicated herring sperm DNA per ml, 50 μg of yeast tRNA per ml). The hybridization was carried out at 45°C for 24 h with 5 to 10 ml of fresh hybridization buffer per filter supplemented with 2×10^6 cpm of the probe per ml. Posthybridizations washes were carried out at 60°C two times for 30 min each in 2× SSC, twice for 30 min each in 1× SSC, and for 60 min in 0.1× SSC. For Northern (RNA) blot analysis, RNA was electrophoresed on formaldehyde-agarose gels and transferred to nitrocellulose. Hybridizations and washings were carried out as described for Southern blotting.

RESULTS

Clone H49 encodes a *T. cruzi* dominant immunogen in humans. A *T. cruzi* genomic library was constructed in the expression vector λgt11, using nuclear DNA from metacyclic trypomastigotes. About 50,000 recombinant phages were screened with a pool of human chronic chagasic sera. Based on the signal intensity, one clone (designated clone H49), of 30 recombinant phages identified, was chosen for further characterization.

Clone H49 encodes a β-galactosidase fusion protein of approximately 150 kilodaltons (kDa) (Fig. 1A, lanes 2 and 4). Human chagasic sera reacted with the recombinant fusion protein but not with lysates of nonrecombinant λgt11-infected bacteria (Fig. 1A, lanes 1 and 2). Antisera to β-galactosidase reacted with β-galactosidase (116 kDa) in λgt11 lysates as well as with the fusion protein in H49 lysates (150 kDa), indicating that the insert is located in the β-galactosidase gene (Fig. 1A, lanes 3 and 4).

The reactivity of the recombinant antigen with human chagasic antibodies was analyzed by immunoblot and phage plaque immunoassay. A positive reaction was obtained with up to 1:10,000 dilutions in immunoblot (Fig. 1B) and 1:5,000 dilutions in phage plaque immunoassay (Fig. 1C). This shows that the reactivity of the recombinant antigen is not significantly affected under the reducing conditions used in the immunoblot.

The specificity of H49 recombinant antigen was tested with sera from patients with the various clinical forms of Chagas' disease and other parasitic diseases. The recombinant antigen reacted with all 115 chronic chagasic sera tested but not with sera from patients having other parasitic diseases (Table 1). Most significantly, there was no reaction with antibodies from patients with visceral or mucocutaneous leishmaniasis and *T. rangeli* infection (Table 1; Fig. 1C). These results suggest that the presence of serum antibodies to H49 antigen may be specifically associated with Chagas' disease.

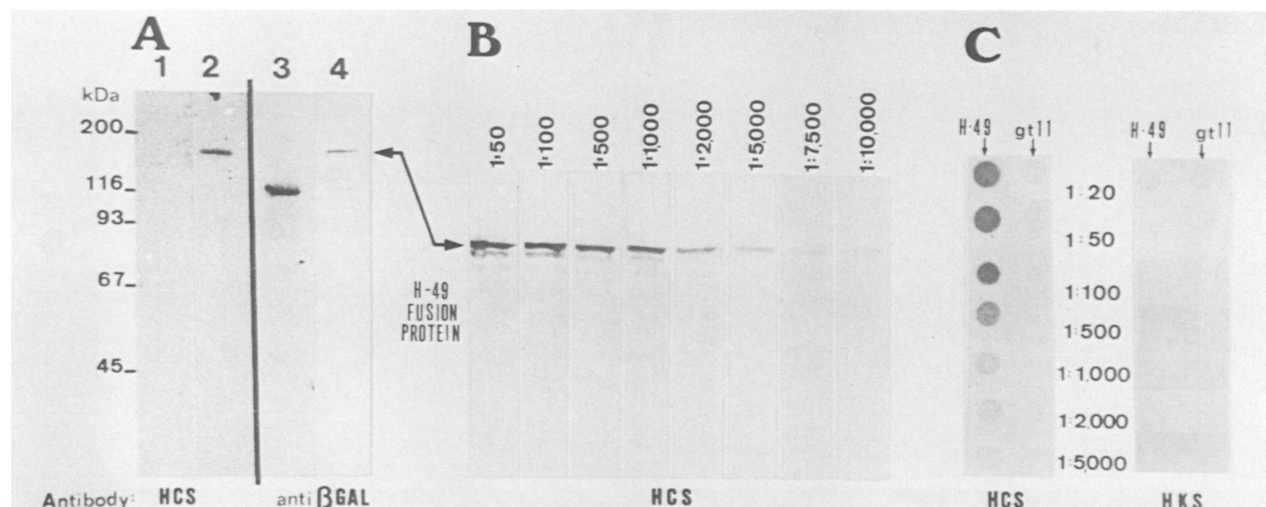

FIG. 1. Reactivity of human chagasic serum to H49 recombinant antigen. (A) Lysates of induced nonrecombinant λgt11 (lanes 1 and 3) and clone H49 (lanes 2 and 4) were separated by SDS-PAGE, Western blotted, and probed with either human chronic chagasic sera (HCS) (lanes 1 and 2) or mouse anti-β-galactosidase (anti-βGAL) (lanes 3 and 4). (B) Lysates of clone H49 were separated by SDS-PAGE (7.5% polyacrylamide gel), Western blotted, and probed with a serial dilutions of human chagasic serum (HCS). (C) Phage plaque immunoassay. Drops of clone H49 and nonrecombinant λgt11 were arrayed on plates and probed with serial dilutions of human chronic chagasic sera (HCS) or human kala-azar sera (HKS). Immunocomplexes were revealed with either rabbit anti-human total immunoglobulins or anti-mouse total immunoglobulins coupled to peroxidase.

Comparison of cloned gene product with *T. cruzi* native polypeptides. Identification of *T. cruzi* native polypeptides that share antigenic determinants with the product of clone H49 was carried out with antibodies purified by the plaque antibody selection technique (13). Human chagasic serum-selected antibodies were used to identify the corresponding parasite polypeptides on immunoblots of protein extracts of metacyclic trypomastigotes, amastigotes, and epimastigotes (Fig. 2). The selected antibodies strongly reacted with a *T. cruzi* polypeptide of very large apparent size, 300 kDa (Fig. 2, lanes 1 to 3). In spite of the lack of adequate size markers at this extreme molecular weight, the mobility of this antigen was considerably greater than that of the 220- to 240-kDa mouse spectrins. Antibodies purified from the same chagasic serum on nonrecombinant λgt11 did not react with *T. cruzi*

TABLE 1. Reactivity of H49 recombinant antigen with antibodies in serum samples from chagasic and nonchagasic patients[a]

Diagnosis	No. of serum samples	No. of positive results with H49 antigen
Chronic Chagas' disease		
Chronic cardiopathy	47	47
Digestive form	7	7
Cardiac and digestive forms	29	29
Indeterminate form	32	32
Other diseases		
Malaria	10	0
Schistosomiasis	8	0
Toxoplasmosis	11	0
Tegumental leishmaniasis	8	0
Visceral leishmaniasis	29	0
T. rangeli infection	4	0

[a] Reactivity of the recombinant antigen was assayed by phage dot blot immunoassay, using sera from chagasic patients (1:250 dilution) and from those with other parasitic diseases (1:40 dilution). Chagasic sera represented populations from the Brazilian states of São Paulo, Minas Gerais, Goiás, Bahia, and Paraíba.

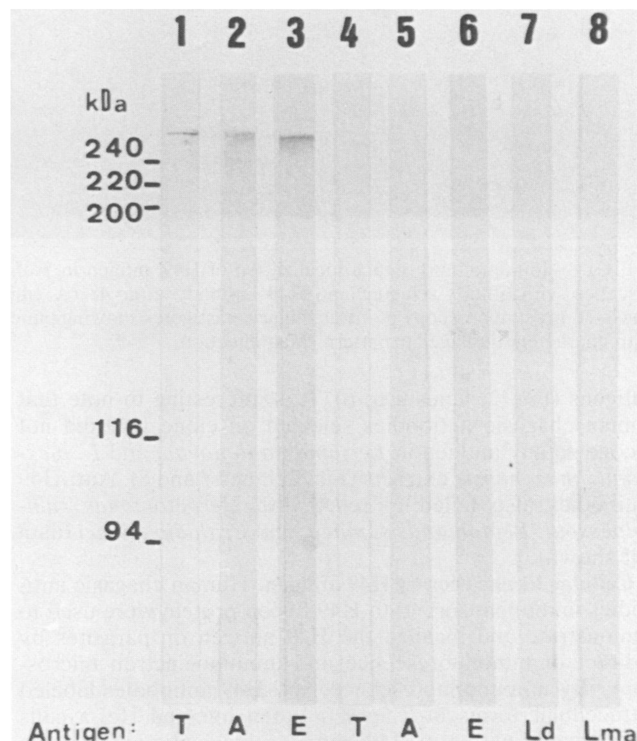

FIG. 2. Identification of native *T. cruzi* protein with selected antibodies. Chagasic antibodies were purified on phages expressing H49 antigen or nonrecombinant λgt11 and used as probes in Western blot (5.0% polyacrylamide gel) containing *T. cruzi* or *Leishmania* extracts. Human chagasic antibodies immunopurified on clone H49 were reacted with metacyclic trypomastigotes (T), amastigotes (A), and epimastigotes (E) from *T. cruzi* (lanes 1 to 3) and *L. donovani* (Ld) and *L. mexicana amazonensis* (Lma) (lanes 7 and 8). Antibodies immunopurified on nonrecombinant λgt11 were reacted with metacyclic trypomastigotes, amastigotes, and epimastigotes (lanes 4 to 6). Immunocomplexes were revealed with rabbit anti-human total immunoglobulins coupled to peroxidase.

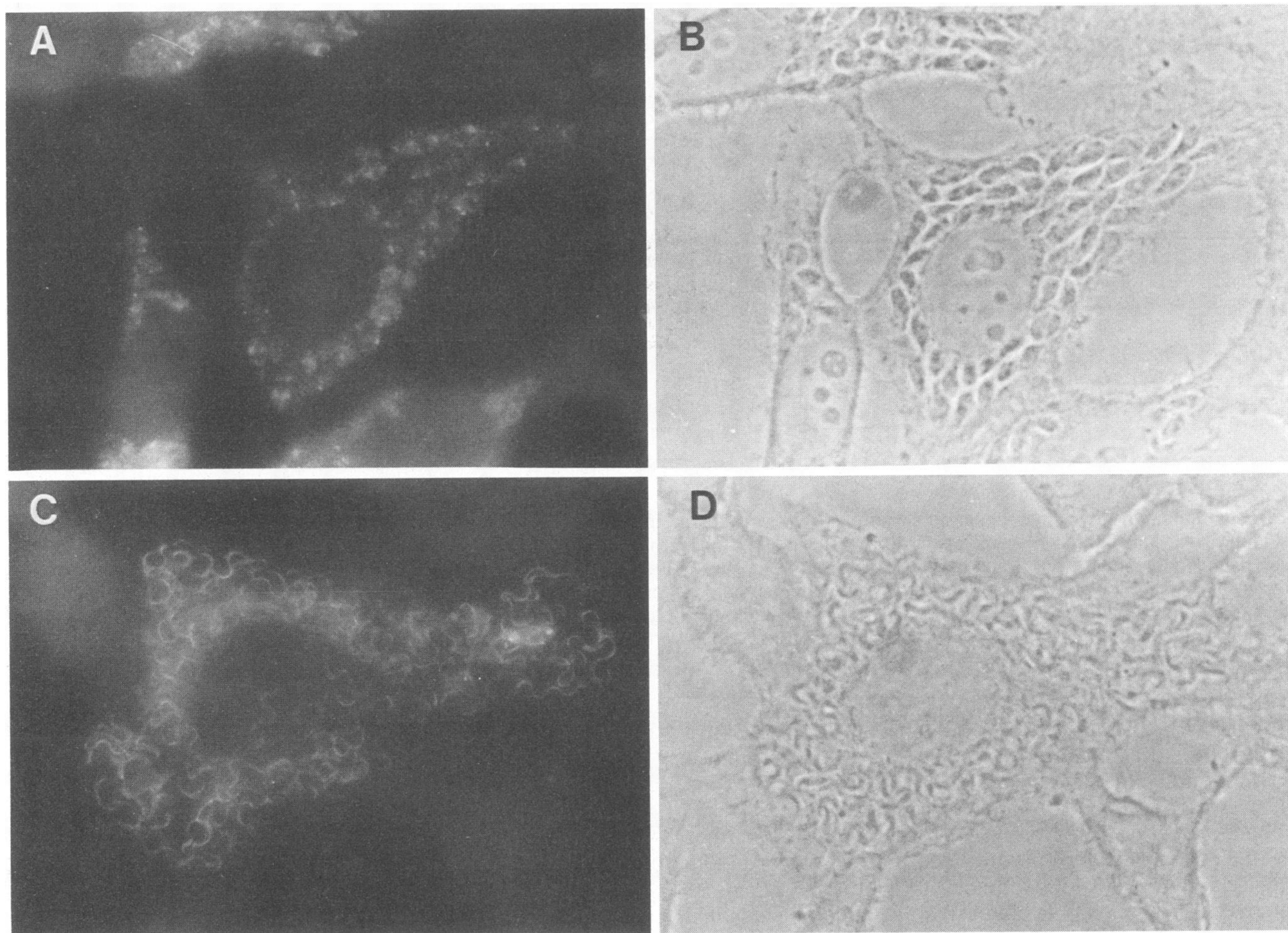

FIG. 3. Immunofluorescence localization of H49 antigen in *T. cruzi*. HeLa cells infected with *T. cruzi* were processed for immunofluorescence with affinity-selected anti-H49 antibody after 48 (A and B) or 72 (C and D) h. (A and B) Fluorescence and corresponding phase-contrast microscopy of intracellular amastigotes showing punctate pattern. (C and D) Anti-H49 labeling of intracellular trypomastigotes with characteristic flagellar pattern. Magnification, ×960.

antigens (Fig. 2, lanes 4 to 6). It is interesting to note that human chagasic antibodies selected on clone H49 did not recognize any antigen in *Leishmania donovani* and *L. mexicana amazonensis* extracts (Fig. 2, lanes 7 and 8). Anti-H49 antibodies also failed to react with *Herpetomonas samuelpessoai*, *Leptomonas samueli*, and *Crithidia deanei* (data not shown).

Cellular localization of H49 antigen. Human chagasic antibodies immunoadsorbed to H49 fusion protein were used to demonstrate and localize the H49 antigen on parasites by indirect immunofluorescence and immunoelectron microscopy. By immunofluorescence, anti-H49 antibodies labeled intracellular forms of *T. cruzi*. When infected HeLa cells were treated with anti-H49, intracytoplasmatic amastigotes reacted, yielding a punctate staining (Fig. 3A and B), whereas trypomastigotes displayed a more organized flagellar pattern (Fig. 3C and D). Anti-H49 antibodies also stained isolated metacyclic trypomastigotes and cytoskeletons of both metacyclics and epimastigotes along the length of the flagellum (not shown). A similar flagellar pattern was also observed with metacyclic trypomastigotes from other *T. cruzi* strains (Y, CL, Tulahuen, DM 28, and DM 30). There was no reaction with *L. mexicana amazonensis*, *T. brucei brucei*, or *L. donovani* (data not shown).

The immunofluorescence pattern obtained with formaldehyde-fixed parasites suggests that H49 antigen could be associated with the *T. cruzi* cytoskeleton. To extend these observations to the ultrastructural level, *T. cruzi* cytoskeletons were obtained by extracting cells in a microtubule-stabilizing buffer containing Nonidet P-40 (10) and incubated with anti-H49 antibodies followed by protein A coupled to 10-nm colloidal gold particles. In epimastigotes (Fig. 4A) and trypomastigotes (metacyclic or culture derived; Fig. 4B and C), anti-H49 antibodies gave a linear labeling along the contact region between the flagellum and the cell body. Furthermore, it is apparent that H49 antigen is a component of neither the axoneme nor the flagellar paraxial structure (see arrowheads in Fig. 4B and C). These localization studies confirmed that H49 antigen is expressed in all developmental stages of *T. cruzi* and is associated with the parasite's cytoskeleton.

Northern and Southern blot analyses. Northern blot analysis showed the hybridization of clone H49 to an 8-kilobase band present in trypomastigotes (Fig. 5A). A transcript of this length is sufficient to encode a protein of 300 kDa.

Clone H49 was hybridized to *T. cruzi* genomic DNA digested with *Eco*RI, *Bgl*II, *Alu*I, and *Kpn*I restriction endonucleases. Single bands were identified in each lane (Fig. 5B). We have also analyzed, by dot blot hybridization, several *T. cruzi* strains (Y, CL, DM 28, DM 30, and

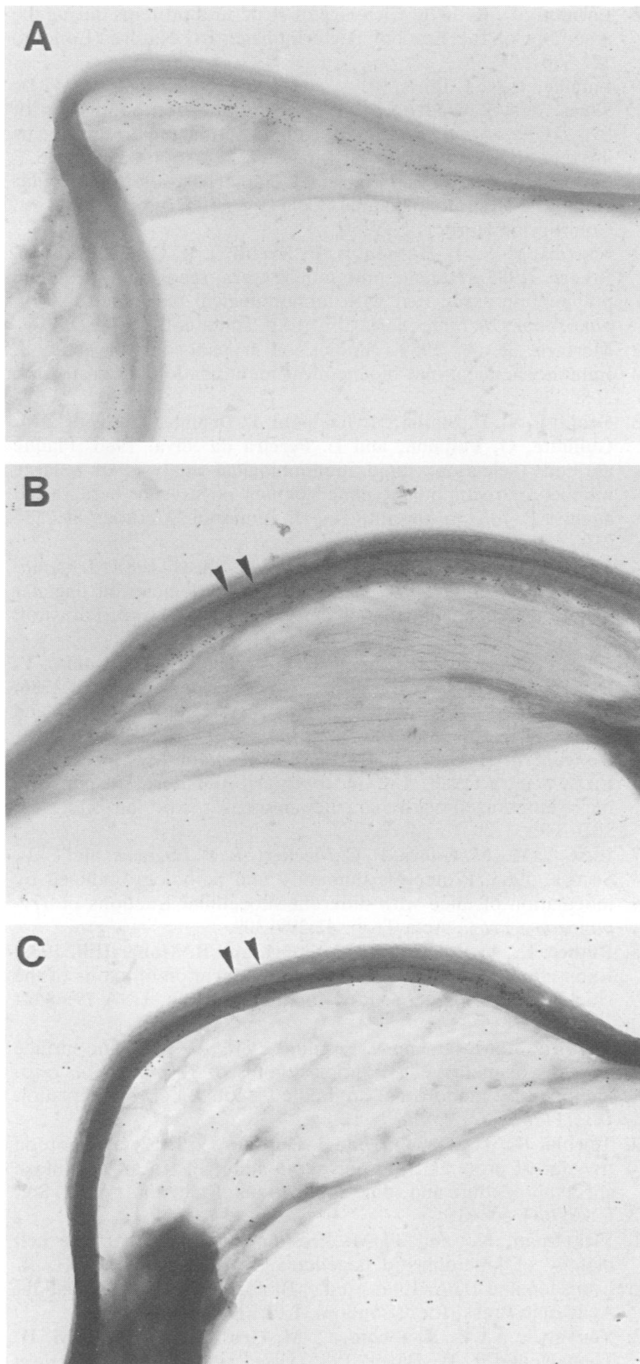

FIG. 4. Immunoelectron microscopic localization of H49 antigen on *T. cruzi* cytoskeletons. Cytoskeletons of *T. cruzi* epimastigotes (A), metacyclic trypomastigotes (B), and culture-derived trypomastogotes (C) were labeled with affinity-selected anti-H49 antibodies and bound immunoglobulins visualized with protein A coupled to 10-nm gold particles. Note the staining along the flagellum-body attachment in all forms. Arrowheads in panel B indicate the axoneme, and those in panel C show the paraxial structure. Magnification, ×27,000.

Tulahuen) and other trypanosomatids (*L. mexicana amazonensis*, *L. donovani*, *L. chagasi*, *H. samuelpessoai*, and *Crithidia luciliae termophila*) looking for sequences homologous to clone H49. Clone H49 hybridized exclusively with

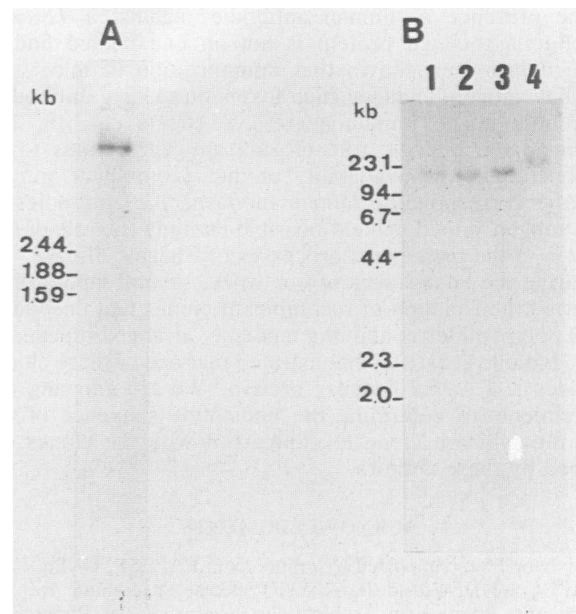

FIG. 5. Hybridization analyses of the H49 gene. (A) Northern blot hybridization of total RNA from trypomastigotes with the H49 insert. *T. cruzi* rRNAs were used as size markers. (B) Southern blot hybridization of *T. cruzi* genomic DNA with the H49 insert. The restriction digests were *Eco*RI (lane 1), *Bgl*II (lane 2), *Alu*I (lane 3), and *Kpn*I (lane 4). kb, Kilobases.

T. cruzi DNA, suggesting that it carries species-specific sequences (data not shown).

DISCUSSION

We have shown that a *T. cruzi* DNA clone (H49) encodes a recombinant protein that corresponds to a high-molecular-weight protein associated with the parasite cytoskeleton.

H49 antigen is apparently a dominant immunogen of *T. cruzi*. All chronic chagasic sera we tested for *T. cruzi* from 115 individuals living in endemic areas had sufficiently high antibody titers to register in a phage plaque immunoassay. In contrast to what has been found with other serologic methods (3, 4), we did not detect anti-H49 antibodies in samples from patients with either mucocutaneous or visceral leishmaniasis or *T. rangeli* infection. The presence of anti-H49 antibodies in chagasic patients from different regions of Brazil suggests that an antigenic region of H49 protein is conserved in many strains of *T. cruzi*. In fact, H49 antigen and its gene were detected in several *T. cruzi* strains from different geographical regions by indirect immunofluorescence and DNA hybridization. Taken together, these results suggest that the presence of antibodies to H49 antigen may be specifically associated with Chagas' disease; therefore, the H49 recombinant antigen could be useful in the diagnosis of this disease.

Immunoelectron microscopy showed that the H49 protein is a structural component of the *T. cruzi* cytoskeleton. The localization of H49 antigen in the contact region between the flagellum and cell body strongly suggests that it is a component of the desmosomelike flagellum-body attachment of trypanosomes (21). The absence of reaction of anti-H49 antibodies with other trypanosomatids suggests that this protein is a specific component of the *T. cruzi* flagellum-body attachment and could be used as a species-specific molecular marker for this structure.

The presence of human antibodies against a *T. cruzi* flagellum-associated protein is not an unexpected finding since it has been shown that immunization of mice with flagellar antigens induced high levels of specific antibodies with little or no immunoaggressive effects (14, 16, 17). Although the biologic role of H49 antigen remains to be demonstrated, the availability of the recombinant antigen and the corresponding human monospecific antibodies to H49 antigen should make it possible to study the role of this antigen in the pathogenic processes of Chagas' disease.

During the later stages of our work, several laboratories reported the isolation of recombinant clones that encode *T. cruzi* polypeptides containing tandemly arranged repeats (6, 7, 9). Lafaille et al. (9) demonstrated that one of these clones encodes a *T. cruzi* flagellar protein. We are carrying out experiments to determine the nucleotide sequence of the H49 recombinant clone to compare it with the clones described by these authors.

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