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ANTIGENS OF *TRYPANOSOMA CRUZI* WITH CLINICAL INTEREST CLONED AND EXPRESSED IN *ESCHERICHIA COLI*

JOSÉ FRANCO DA SILVEIRA; GLÁUCIA S. PARANHOS*; PAULO C. COTRIM; RENATO A. MORTARA; MÁRIO E. CAMARGO**; ANIS RASSI***; JAMIRO WANDERLEY****; RICARDO CORRAL*****; HECTOR L. FREILIJ*****; SAUL GRINSTEIN***** & WIN DEGRAVE******

Disciplina de Parasitologia, Escola Paulista de Medicina, Caixa Postal 20207, 04023 São Paulo, SP, Brasil *Laboratoire de Biologie Cellulaire, Bio-Merieux, Lyon, France **Instituto de Medicina Tropical da USP, SP, Brasil ***Faculdade de Medicina de Goiânia, UFGo, Goiânia, GO, Brasil ****Hospital das Clínicas, UNICAMP, Campinas, SP, Brasil *****Hospital de Ninos Ricardo Gutierrez, Buenos Aires, Argentina ******Departamento de Biologia Molecular, FIOCRUZ, Rio de Janeiro, RJ, Brasil

INTRODUCTION

Trypanosoma cruzi has a complex antigenic structure. Recent studies indicate that is synthesizes many proteins and presents a large number of antigenic determinants to the host's immune system (Zingales et al., 1984; Dragon et al., 1985; Martins et al., 1985; Rangel-Aldao et al., 1986; Yoshida, 1986; Israelski et al., 1988; Nogueira, 1988). Although there are several antigens identified it is not yet known which antigens are important in the pathogenesis and immunopathology of Chagas' disease (Zingales et al., 1984; Yoshida, 1986; Nogueira, 1988). The development of DNA cloning and bacterial expression techniques has opened up a new approach to studying the antigenicity of *T. cruzi* components. It is hoped that the use of recombinant DNA techniques will provide new insights into the biology of the parasite and new approaches to diagnosis and, perhaps, towards the development of a vaccine.

We have used DNA cloning techniques to identify and characterize *T. cruzi* antigens of clinical interest. In this report we describe the characterization of two recombinant clones that express immunodominant antigens of *T. cruzi* recognized by human chagasic sera. One of these clones, named A13, encodes a polypeptide recognized by IgM and IgG antibodies from sera of acute and congenital chagasic patients (Paranhos, 1989; Paranhos et al., 1990). Another recombinant phage, clone H49, encodes a polypeptide containing tandemly repeat amino acid sequence motifs (Cotrim et al., 1990; Paranhos et al., 1990). This repetitive antigen is a good marker of the chronic stage of Chagas' disease.

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RESULTS

Isolation of T. cruzi *antigen genes* – In an attempt to define antigens involved in the development of acute and chronic phases of Chagas' disease, we decided to isolate a large number of antigenic determinants from a genomic expression library using human chagasic sera. A genomic library was constructed because, unlike a cDNA library, all unique genes are equally well represented regardless of the level of transcription or the developmental stage in which they are expressed.

An expression genomic library was made directly from randomly generated fragments of *T. cruzi* nuclear DNA. Genomic DNA isolated from metacyclic trypomastigote forms was digested with DNase I, size fractionated on agarose gels, ligated to EcoRI synthetic linkers and cloned into the EcoRI site of vector lambda gt11 (Cotrim et al., 1990).

Approximately 50,000 recombinant phages were screened with a pool of chronic chagasic sera and 30 recombinant phages expressing *T. cruzi* antigens were detected. Out of 30 positive clones, twelve were purified to homogeneity and then subjected to screening with acute and chronic human chagasic sera. The differential immunoscreening allowed us to identify a recombinant phage (clone A13) encoding a *T. cruzi* antigen which is recognized by anti-

bodies from acute and congenital chagasic patients. Other recombinant phages encode antigens that are preferentially recognized by antibodies from sera of chronic chagasic patients. One of these recombinant phages (clone H49) encodes a polypeptide containing tandemly arranged repeats which are 68 amino acids in lenght.

Characterization of a T. cruzi *antigen recognized by acute and chronic human chagasic sera* (Paranhos, 1989; Paranhos et al., 1990) – Clone A13 encodes a beta-galactosidase fusion protein of approximately 145 kDa which strongly reacts with IgM and IgG antibodies present in the sera from acute and congenital chagasic patients. IgG antibodies to A13 antigen are also detected in the sera of chronic patients with different clinical forms of Chagas' disease.

Identification of *T. cruzi* native polypeptides that share antigenic determinants with the product of clone A13 was carried out with antibodies purified by the plaque antibody selection technique (Paranhos et al., 1990; Ozaki et al., 1986). Human chagasic serum-selected antibodies were used to identify the corresponding parasite polypeptides on immunoblots of protein extracts of different developmental forms of *T. cruzi.* The selected antibodies strongly reacted with a polypeptide of 230 kDa in metacyclic trypomastigotes and amastigotes, but not in the non-infective epimastigotes. By indirect immunofluorescence, we have also demonstrated the presence of A13 antigen in amastigotes, metacyclic and tissue culture-derived trypomastigotes. Metacyclic trypomastigotes from different *T. cruzi* strains (G, DM30, Y, CL, Tulahuen) also reacted positively with anti-A13 antibodies.

The cellular location of A13 antigen is not yet known. Immunofluorescence studies using formalidehyde-fixed parasites showed that anti-A13 antibodies stained the whole cellular body of amastigotes and trypomastigotes. In contrast, no staining was obtained using living parasites. Several attempts to immunoprecipitate surface-labeled polypeptides using anti-A13 antibodies gave negative results (Paranhos, 1989). Taken together, these results suggest that A13 antigen has an intracellular location in the parasite.

The DNA sequence of clone A13 reveals one open reading frame encoding 251 amino acids without tandemly repeated sequences. Comparison of the sequence with nucleotide and amino acid sequences available at GeneBank and with *T. cruzi* published sequences did not reveal any significant similarities (Paranhos et al., 1990).

Since clone A13 encodes a polypeptide which is a target of the host immunologic response, we have examined this polypeptide for putative antigenic epitopes using a computer program that predicts the secondary structure of proteins superimposed with values for hydrophilicity (Chow & Fasman, 1974; Hopp & Woods, 1981). This analysis indicated that the polypeptide contains a number of sites that meet the criteria for likely antigenic epitopes (Paranhos, 1989). Peptides corresponding to the antigenic regions predicted from this analysis will be synthesized and used to detect antibodies in sera of chagasic patients.

Studies on the genomic organization of A13 gene suggest that this gene exists as a single copy in *T. cruzi* haploid genome. The A13 gene locus maps to a single chromosome of about 3.2 megabases (Paranhos, 1989).

The specificity and sensitivity of A13 recombinant antigen were analysed with a panel of patient sera using a phage dot-blot immunoassay (Paranhos et al., 1990; Ozaki et al., 1986). The recombinant antigen reacted with 90 and 73% of the acute and congenital chagasic sera tested, respectively. It was also detected by 90% of the chronic chagasic sera tested (Table I). Most significantly, there was no reaction with antibodies from patients with visceral or mucocutaneous leishmaniasis and *T. rangeli* infection. Our data suggested that A13 antigen may be useful for the development of serodiagnostic procedures of acute and chronic phases of Chagas' disease.

Characterization of a dominant immunogen of T. cruzi *recognized by human chagasic sera* (Cotrim et al., 1990; Paranhos et al., 1990) – Clone H49 produces a fusion protein of approximately 150 kDa which strongly reacted with IgGs antibodies in the sera from chronic chagasic patients. A positive reaction was obtained with up to 1:10,000 dilutions of chagasic sera in immunoblots containing the recombinant antigen.

Chagasic antibodies affinity purified on the recombinant antigen recognized a high-molecular

TABLE I

Reactivity of A13 recombinant antigen with antisera from patients with Chagas' disease and other parasitic diseases

Origin of human serum	No. of serum samples	No. of positive results with A13 antigen	Percentage of individuals anti-A13 positive
Patients with:			
Congenital Chagas' disease	11	8	73
Acute Chagas' disease	32	29	91
Chronic Chagas' disease	145	132	91
Malaria	10	0	0
Schistosomiasis	8	0	0
Toxoplasmosis	11	0	0
Tegumentar leishmaniasis	11	0	0
Visceral leishmaniasis	35	0	0
T. rangeli infection	4	0	0
Normal	12	0	0

Drops containing 100 pfu of recombinant phage were arrayed on plates and probed with sera from patients with Chagas' disease (1:200 dilution), and sera from patients with other parasitic diseases (1:40 dilution). Sera from Chagas' disease and other parasitic diseases were obtained from patients diagnosed by serological and clinical manifestations. Chagasic serum represents populations of Brazil and Argentina (Paranhos et al., 1990).

weight protein (~ 300 kDa) expressed in all developmental stages of 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 zone between the flagelum and the cell body.

The native H49 antigen is very sensitive to endogenous proteases, since the conditions of extraction strongly influence the resulting extent of proteolysis. When the extraction is carried out without protease inhibitors, more than 20 bands on Western blots can be detected by anti-H49 antibodies, with an apparent size difference between adjacent bands of about 10 kDa (Paranhos et al., 1990). This characteristic pattern of proteolysis is consistent with a higly repeated internal structure of H49 antigen.

This hypothesis wás confirmed by nucleotide sequence analysis of the 978 bp insert from clone H49. About 85% of the sequence consists of highly conserved, tandemly arranged 68 amino acid-long repeats. The basic repeat unit of H49 gene is homologous to those found in the clones 1 and FRA described by Ibanez et al. (1988) and Lafaille et al. (1989), respectively.

The number of repeat units of H49 gene was estimated by partial digestion of *T. cruzi* genomic DNA with enzymes that have only one restriction site in all repeat units (for example, Taq I and Hind III). We estimate that there are at least twelve repeat units. There are several tetranucleotide restriction enzymes that not cleave within the H49 repetitive sequence. When *T. cruzi* genomic DNA is digested with these enzymes and hybridized to labeled H49 insert, the probe hybridizes to high molecular fragments, suggesting that a very large proportion of the H49 gene consists of the conserved 204-bp repeat.

In order to study the antigenic relevance of the recombinant antigen, the fusion protein of clone H49 was subjected to a number of screenings with sera from patients with Chagas' disease and other parasitic diseases. The detection of anti-H49 antibodies in patients' sera was carried out by phage dot-blot immunoassay (Ozaki et al., 1986). The recombinant antigen reacted with 99% of the chronic chagasic sera tested (Table II). There was no reaction with sera from patients with malaria, schistosomiasis, toxoplasmosis, tegumentar leishmaniasis and *T. rangeli* infection. One serum, out of 35 sera from patients with visceral leishmaniasis, gave a weak positive reaction with H49 antigen. Based on these results, we conclude that H49 antigen is a good marker for the chronic phase of Chagas' disease and may be potentially important in the serodiagnosis of this disease.

TABLE II

Reactivity of H49 recombinant antigen with antisera from patients with Chagas' disease and other parasitic diseases

Origin of human serum	No. of serum samples	No. of positive results with H49 antigen	Percentage of individuals anti-H49 positive
Patients with:			
Chronic Chagas' disease	145	144	99
Malaria	10	0	0
Schistosomiasis	8	0	0
Toxoplasmosis	11	0	0
Tegumentar leishmaniasis	11	0	0
Visceral leishmaniasis	35	1	3
T. rangeli infection	4	0	0
Normal	12	0	0

Drops containing 100 pfu of recombinant phage were arrayed on plates and probed with sera from patients with Chagas' disease (1:200 dilution), and sera from patients with other parasitic diseases (1:40 dilution). Sera from Chagas' disease and other parasitic diseases were obtained from patients diagnosed by serological and clinical manifestations. Chagasic serum represents populations of Brazil and Argentina (Paranhos et al., 1990).

CONCLUSIONS AND PERSPECTIVES

Two *T. cruzi* antigens of clinical and diagnostic interest have been identified and characterized by gene cloning. Antibodies against A13 antigen are detected at the beggining of *T. cruzi* infection, persist in the chronic phase, and are present in patients with the different clinical forms of Chagas' disease. A13 antigen is a good candidate to be used in the serological diagnosis of acute and chronic phases of Chagas' disease.

H49 antigen is a strong immunogen of *T. cruzi*. This repetitive antigen is easily detectable by phage plaque immunoassay, as demonstrated by the positive reaction with 99% of chagasic sera from individuals living in endemic areas. This antigen is a good immunological marker for the chronic phase of Chagas' disease.

The serologic survey presented in this work was performed with a dot-blot immunoassay using *Escherichia coli* extracts containing recombinant fusion proteins. We believe that the detection of antibodies against A13 and H49 antigens will be improved using purified recombinant antigens and ELISA techniques. Subcloning and expression of H49 and A13 genes in vectors that produce large amounts of recombinant antigens are currently underway. To avoid the cross-reactivity of the recombinant antigen with anti-*E. coli* antibodies present in human sera, the genes are being subcloned in vectors that express recombinant proteins not fused with *E. coli* proteins.

We hope that improvements of serological tests with respect to specificity, sensitivity and standardization will be possible using recombinant antigens. In our view, the available data suggest that additional antigenic determinants will be required for the development of a reliable serological test based on recombinant antigens.

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