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Isolation of a *Trypanosoma cruzi* antigen by affinity chromatography with a monoclonal antibody. Preliminary evaluation of its possible applications in serological tests

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SUMMARY

By affinity chromatography with a monoclonal antibody (163B6), obtained in our laboratory, we have isolated a *T. cruzi* antigen which could be useful for differential diagnosis of Chagas' disease from leishmaniasis. This antigen, a 52-kD protein, reacted with all sera from Chagas' disease patients tested but not with sera from patients with leishmania, in ELISA. The 52-kD antigen is widely distributed in the *Trypanosoma* genus since the 163B6 monoclonal antibody reacts with *T. rangeli* and 8 strains and a clone of *T. cruzi* epimastigotes.

Keywords *Trypanosoma cruzi* antigen Chagas' disease serological tests monoclonal antibodies

INTRODUCTION

Chagas' disease (trypanosomiasis americana) is a parasitosis produced by the protozoan *T. cruzi*, which affects millions of people in Central and South America. The disease can be diagnosed by looking for the parasites in blood, or by other methods (reviewed by Camargo & Takeda, 1979) used to detect antibodies in patients' sera. Since in vast regions of Latin America different types of leishmaniasis are also found, and taking into account the high degree of cross-reactivity of *T. cruzi* and *Leishmania* (Walton, Chinel & Eguia, 1973; Desjeux, Quilici & Lapierre, 1974; Desjeux *et al.*, 1983; Araujo, 1986), the need for safe and specific diagnostic tests is evident. Serological techniques at present use either subcellular fractions or whole parasites; thus, it is not possible to rule out false-positive reactions due to cross-reaction with sera from individuals with other parasitic diseases. Diagnosis of Chagas' disease using purified antigens would lead to more specific tests.

Hybridoma technology has largely contributed to a better knowledge of the antigenic structure of *T. cruzi* (Snary *et al.*, 1981; Araujo *et al.*, 1982; Orozco *et al.*, 1984; Tachibana, Nagakura & Kaneda, 1986) and to the development of new methods of diagnosis (Lemesre *et al.*, 1986).

The present study was carried out with the purpose of improving the diagnosis of Chagas' disease. The isolation of anti-*T. cruzi* monoclonal antibodies, the purification by affinity chromatography of an antigenic component recognized by one of these antibodies, and the possible use of that antigen in serological diagnosis are described.

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MATERIALS AND METHODS

Parasites

Epimastigotes of *T. cruzi*, strains Tulahuen, LP, RA, AMP, AF, Y, Mesa, CA1 and its CA1 K98 clone (Muller, Añasco & Gonzalez Cappa, 1986; Muller *et al.*, 1987) and *T. rangeli* were cultured in a cell-free biphasic medium. Promastigotes of *L. mexicana* were grown in the same medium containing 20% (v/v) fetal calf serum (FCS) (GIBCO). Parasites were collected during exponential growth, washed four times with phosphate-buffered saline (PBS), resuspended at 10% (v/v) and treated with 3% formalin (v/v) in PBS for 5 days. After centrifugation at 3500 ***g*** for 10 min, the original volume was restored with PBS.

Subcellular fraction of T. cruzi

The supernatant from 30 000 *g* of epimastigotes (Tulahuen strain) lysed by pressure–descompression (F30) in the presence of PMSF (2 mM), iodoacetamide (10 mM) and EDTA (10 mM) was used (Segura *et al.*, 1974).

Human sera

Seventy-five serum samples were selected for comparative purposes as follows: 25 sera that were negative for *T. cruzi* by

IIF, ELISA and haemaglutination tests; 31 sera from chronic Chagas' disease patients; 10 samples from acute Chagas' disease patients with Romaña sign; and nine sera from leishmanial patients.

*Monoclonal anti-*T. cruzi *antibodies*

*Immunization.*BALB/c mice were immunized by i.p. injection of 0·2 ml of F30 (0·1 mg) in Freund's complete adjuvant. Inoculations were repeated on days 35 and 50. After assaying sera by ELISA, animals with appropriate titres received a further i.p. injection with no adjuvant, four days before fusion (day 60).

Fusions. These were performed according to Koehler & Milstein (1975) with minor modifications (Fossati, 1985) using splenic cells from hyperimmunized mice and NSO myeloma cells.

Screening. Antibody detection was done by ELISA, as described below. Antibody isotypes were determined by bidimensional double diffusion (Ouchterlony, 1958) with commercial monospecific antisera (Sigma).

Immunoenzymatic assay (ELISA)

An indirect method for antibody detection was used as described by Nieto & Carbonetto (1989). The following antigens were used: F30 (4 μg/well), formalin-treated parasites (1×10^5/well) (Carbonetto *et al.*, 1989) or an affinity-purified antigen (0·1 μg/well).

Dot-immunoenzymatic assay (dot-IEA)

The assay was performed as described by Carbonetto *et al.*, (1989). Formalin-treated epimastigotes were fixed to nitrocellulose (1×10^5 parasites/dot), and ascitic fluids were assayed at a 1/500 dilution. Peroxidase-conjugated rabbit anti-mouse immunoglobulin was used as a second antibody (Dako).

Indirect immunofluorescence (IIF)

The technique of Alvarez, Cerisola & Rohwedder (1968), which employs formalin-treated parasites, was used. Ascitic fluids were assayed at a 1/30 dilution in PBS.

Purification of antigens by affinity chromatography

The gammaglobulin fraction was purified from 163B6 ascites by DEAE-cellulose chromatography (Margni, 1982). Elution was done with a phosphate-buffered gradient (7·5–50 mM, pH 8·0). The gammaglobulin obtained was fixed to Sepharose 4B-BrCN (Cuatrecasas & Anfinsen, 1971) and used as immunosorbent for F30. The retained antigen was eluted with 0·1 M glycine-HCl buffer, pH 3·0, dialysed against PBS and stored at −20°C until used.

SDS-PAGE

The discontinuous method in the presence of SDS described by Hames (1981) was used. The proteins were stained by the silver technique (Merril *et al.*, 1981) and the relative masses (M_r) were determined by using reference standards (Sigma).

RESULTS

Fusion of NSO myeloma cells with splenocytes from mice immunized with F30 resulted in 12 cellular clones producing antibodies able to react with the immunogen in ELISA. Eight of these clones were expanded as ascitic fluids.

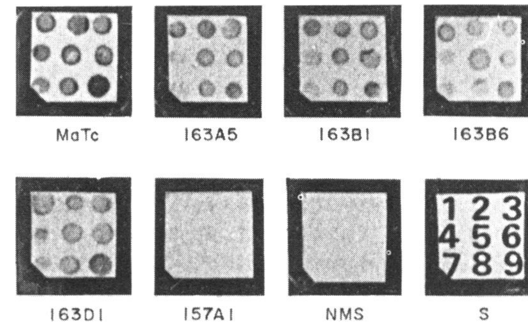

Fig. 1. Reactivity of monoclonal antibodies against different *T. cruzi* strains. MaTc, mouse anti-*T. cruzi* antiserum; NMS, normal mouse serum; S, distribution scheme of *T. cruzi* strains, as follows: 1, Tulahuen; 2, LP; 3, RA; 4, AWP; 5, AF; 6, Y; 7, Mesa; 8, CA1; and 9, CA1 K98.

Table 1. Isotype and reactivity of anti *T. cruzi* monoclonal antibodies

Antibody (ascites)	Isotype	ELISA* F30	ELISA* Epi.	IIF *T.c.*	IIF *T.r.*	IIF *L.m.*
157A1		< 100	< 100	−	−	−
155a	IgM	300	2000	+	+	+
163A1	IgM	1500	500	+	+	−
163A5	IgM	2700	1000	+	+	+
163B1	IgM	8900	1500	+	+	+
163B6	IgG1	60000	< 100	+	+	−
163C3	IgG1	32000	300	+	+	−
163C6	IgM	4000	1000	+	+	+
163D1	IgM	2300	700	+	+	+

* Titres are expressed as the reciprocal of the dilution producing 50% of the maximal absorbance determined under saturation conditions.

Epi., epimastigotes; *T.c.*, *T. cruzi*; *T.r.*, *T. rangeli*; *L.m.*, *L. mexicana*.

Reactivity of the monoclonal antibodies with different strains of *T. cruzi* was analysed by the dot-IEA technique. All anti-*T. cruzi* monoclonal antibodies reacted with all strains tested (Fig. 1); 157A1 ascites was used as a negative control. Both a negative and a positive control serum were included in the same figure. These sera were obtained either from normal mice or from mice infected with *T. cruzi*, respectively.

Isotypes of each antibody and their reactivities with F30, formalin-treated epimastigotes of *T. cruzi* and *T. rangeli* and formalized *L. mexicana* promastigotes are shown in Table 1.

Monoclonal antibodies tested by IIF reacted with both *T. cruzi* and *T. rangeli* epimastigotes, whereas 163A1, 163B6, and 163C3 ascites did not react with *L. mexicana* promastigotes. Fluorescent images obtained with epimastigotes of the Tulahuen strain were characteristic for each monoclonal antibody. Some exhibited a strong fluorescence throughout the parasite (163A5, 163B1, 163C6 and 163D1); others showed scarce, fluorescent large granules without membrane fluorescence (163B6 and 163C3), whereas antibody 155a showed a typical

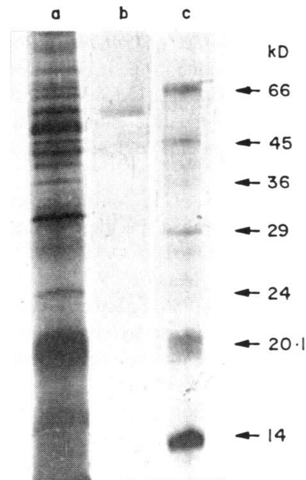

Fig. 2. SDS–PAGE of (a) F30; (b) 163B6 affinity chromatography purified antigen; (c) mol. wt markers.

Table 2. Reactivity of 52-kD antigen with human sera, determined by ELISA

Patient sera	Ag163B6	F30
Negatives	0/25	0/25
Acute Chagasic	4/10	6/10
Chronic Chagasic	31/31	31/31
Leishmanial	0/9	8/9

Sera having an absorbance higher than the mean absorbance of negative sera + 3 s.d. were considered positive.

membrane fluorescence. Two of these antibodies (163C3 and 163B6) exhibited a high titre against F30; there was little or no reactivity with formalin-treated whole espimastigotes (Table 1).

Monoclonal antibody 163B6 was purified from ascitic fluid by DEAE–cellulose chromatography and fixed to Sepharose 4B–BrCN. An antigenic component was isolated by immuno-adsorption and further acid dissociation from fraction F30. This component, when analysed in SDS–PAGE under reducing and non-reducing conditions, exhibited a single band at 52 kD (Fig. 2).

This component was used as antigen in an indirect ELISA for the detection of antibodies in sera from patients with Chagas' disease. Results of assays of human sera from patients infected either with *T. cruzi* or leishmanias were compared with those obtained when fraction F30 was used as an antigen. As shown in Table 2, all sera from chronic Chagasic individuals yielded positive results with both antigens, whereas six of the sera from acute cases reacted with antigen F30 and only four reacted with the antigen purified with monoclonal antibody 163B6. None of the nine sera from the leishmaniasic patients reacted with the 52-kD antigen, and eight reacted with fraction F30.

DISCUSSION

Traditional diagnostic tests for Chagas' disease have a limited reliability due to the high proportion of false-positive reactions, difficulties in obtaining a clear cut-off value in rapid tests, and the high rate of cross-reactivity with other parasites (Walton *et al.*, 1973; Desjeux *et al.*, 1983; Araujo, 1986). These disadvantages arise largely from the heterogeneity of the antigens used, since they are usually derived from whole parasites, or complex subcellular fractions. These methodological deficiencies are largely overcome by using purified antigens (Scharfstein *et al.*, 1985; Dragon *et al.*, 1985).

Monoclonal antibodies are useful for identification and characterization of antigens that have possible diagnostic importance. Hudson *et al.*, (1987) reported the use of monoclonal antibodies for the differential identification of *T. cruzi* and *T. rangeli* epimastigotes. Lemesre *et al.*, (1986) also developed an immunoenzymatic competitive assay for detecting anti-*T. cruzi* antibodies by means of a species-specific monoclonal antibody and a C5-enriched antigenic preparation.

Both monoclonal antibody technology and the traditional methods used for separation of antigens were used in the present study. A series of monoclonal antibodies were prepared by fusion of somatic cells from mice hyperimmunized with fraction F30. These antibodies were reactive with eight *T. cruzi* strains and with a clone of one of them (Fig. 1). Since the strains analysed differ in origin and virulence (Muller *et al.*, 1986, 1987), it is assumed that epitopes recognized by the antibodies are widely distributed in the genus. Indeed all of them recognize antigenic determinants present in *T. cruzi* and *T. rangeli* (Table 1).

Most described here are IgM and only two of them are IgG. This high proportion of anti-*T. cruzi* IgM antibodies has been reported by other investigators (Orozco *et al.*, 1984; Chao & Dusanic, 1985; Giovanni-De-Simone *et al.*, 1987). IgG1 monoclonal antibodies (163B6 and 163C3) recognize antigens that would have an intracytoplasmic distribution, as suggested by the images obtained by IIF of formalin-treated epimastigotes fixed by heating. This was supported both by negative results obtained by ELISA when formalin-treated, non-dried epimastigotes were used, and by the high titres obtained with the F30 subcellular fraction.

The antigen isolated with 163B6 did not give false-positive results, and shows 100% specificity in differentiating between *T. cruzi* and leishmanial infections. It shows 100% sensitivity in the detection of chronic patients but, like the F30 fraction frequently used in diagnostic tests, is not effective in the detection of acute cases (Table 2).

It has not been possible to characterize the antigen recognized by the monoclonal antibody 163B6 by means of immunoblotting. Nevertheless, both the specificity and the affinity of this antibody were suitable for affinity chromatography. This is consistent with the hypothesis of Wong, Hudson & Hindmarsh (1986) and Stott (1989), that although it is likely that during immunoblotting the ionic detergent (SDS) could be removed by the electrophoretic transfer, the strong non-covalent forces which bind polypeptides to nitrocellulose would avoid the

complete renaturalization of the epitope, thus preventing recognition by the paratope.

Nevertheless, we have demonstrated that the antigen described could be employed in differential diagnosis of leishmaniasis and Chagas' disease.

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