

TRYPANOSOMA CRUZI: IDENTIFICATION OF SPECIFIC EPIMASTIGOTE ANTIGENS BY HUMAN IMMUNE SERA

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Soluble antigens from epimastigotes of Trypanosoma cruzi were analyzed by western blot in terms of their reactivity with sera from patients with Chagas' disease. In addition, sera from patients with visceral (AVL) and tegumentar leishmaniasis (ATL) were also tested in order to identify cross-reactivities with Trypanosoma cruzi antigens. Twenty eight polypeptides with molecular weights ranging from 14 kDa to 113 kDa were identified with sera from Chagas' disease patients. An extensive cross-reactivity was observed when sera from human visceral leishmaniasis were used, while only a slight cross-reaction was observed with sera from tegumentar leishmaniasis. On the other hand, 10 polypeptides specifically reacting with sera from Chagas' disease patients were identified. Among them, the antigens with molecular weights of 46 kDa and 25 kDa reacted with all sera tested and may be good candidates for specific immunodiagnosis of Chagas' disease.

Key words: western blot – *Trypanosoma cruzi* – antigens – specificity – cross-reactivity

Several serological methods such as complement fixation, haemagglutination, direct agglutination and indirect immunofluorescence have been described for the serological diagnosis of Chagas' disease (Camargo & Takeda, 1979). However, cross-reactivity is frequently observed among visceral and tegumentar leishmaniasis and Chagas' disease sera. As there is a frequent overlap on the distribution of Chagas' disease and leishmaniasis distribution the identification of specific antigens is important for the establishment of discriminative serological methods.

Some specific antigens have already been identified and their potential usefulness for immunodiagnosis was analyzed (Snary, 1980; Scharfstein et al., 1983, 1985; Schechter et al., 1983; Morgado et al., 1985; Lemesre et al., 1986). However, preliminary results of our laboratory on the reactivity of human sera to

T. cruzi indicated specific reactions to some polypeptides rather different from those observed by these authors (Morgado et al., 1984). Indeed, we observed that the polypeptide of 46 kDa seems to be specific for patients with Chagas' disease.

In this paper we confirmed two specific polypeptides (25 and 46 kDa) from epimastigotes of *T. cruzi* that were recognized by all the Chagas' disease sera tested, but not by sera from individuals with leishmaniasis.

MATERIAL AND METHODS

Human sera – Fifty-six sera were obtained from patients with both indeterminate and chronic clinical forms of Chagas' disease. The former patients were identified either by xenodiagnosis or by the positivity in indirect immunofluorescence (IIF). The latter ones were classified based on cardiac, digestive and cardiac and digestive associated manifestations according to several criteria as described elsewhere (Macedo, 1976; Rezende et al., 1960). Thirteen sera were obtained from patients with American visceral leishmaniasis (AVL) and ten from patients with tegumentar leishmaniasis (TL). These patients were diagnosed by the

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visualization of Giemsa-stained parasites in bone marrow aspirates or by detection of parasites in biopsies, as well as by the detection of *Leishmania* antibodies in IIF.

Normal controls consisted of 10 sera from individuals living in endemic areas for Chagas' disease but with no evidence of *T. cruzi* infection, and 11 sera from healthy individuals living in non-endemic areas.

Parasite and *T. cruzi* antigen – The Y strain of *T. cruzi* (Silva & Nussenzweig, 1953) was used. Culture forms were obtained by haemocultures in liver infusion tryptose (LIT) from acutely infected mice. The parasites were grown at 28 °C by serial passages every 2 days. They were harvested on the 2nd day of culture (10^{11} cells), washed 6 times (2000 g, 15 min) with 150 mM phosphate buffered saline (PBS) pH 7.2 containing 1 mM N- α -tosyl-L-lysyl-chloromethyl ketone (TLCK) and 1 mM phenyl-methyl-sulphonyl fluoride (PMSF), protease inhibitors. The pellet was resuspended in 10 ml of deionized water containing the same concentration of protease inhibitors, sonicated 5 times (30 sec pulses) on ice at 18 A (Sony-prep 150 Sonicator, MSE Ltd, Sussex, England) and centrifuged at 35,000 g for 30 min at 4 °C. The protein concentration of the supernatant was determined according to Lowry et al. (1951) and stored at -70 °C until use.

Western blot analysis – Gradient gels of 7-15% polyacrylamide were used with the discontinuous buffer system of Laemmli (1970). Electrophoretic transfer of protein from the gel to the nitrocellulose paper (NCP) was performed at 240-400 mA, 12 V, 4 °C, for 16 hours in 25 mM Tris/192 mM glycine/20% methanol, according to Batteiger et al. (1982). The efficiency of transfer was assessed by staining the NCP with 2% Ponceau-red in 3% acetic acid for 3 min and destaining with distilled water. The nitrocellulose papers were blocked with PBS containing 0.5% of Tween 20 (PBST) for 90 min at room temperature and washed with PBST. The NCP was cut into 5 mm strips and incubated with 1 ml of human sera diluted 1/100 in PBST for 16 hours at 4 °C. The strips were then washed 6 times, 20 min each, with PBST, and incubated with peroxidase-conjugated goat anti-human IgG diluted 1/1000 in PBST for 90 min at room temperature. The strips were washed again as described before and soaked in a solution of

0.003% H₂O₂ with 0.25 mg/ml of diaminobenzidine in 20 mM citrate phosphate buffer pH 5.0. The colour development was stopped by washing in distilled water.

RESULTS

Representative patterns of reaction of Chagas' disease patient sera with *T. cruzi* epimastigotes polypeptides are shown in Fig. 1. At least 28 polypeptides with molecular weights of 14 kDa to 113 kDa could be identified. The intensity of the reaction to different polypeptides was variable but similar patterns of reactivity were observed with almost all sera analyzed, mainly with polypeptides of molecular weights above 24 kDa.

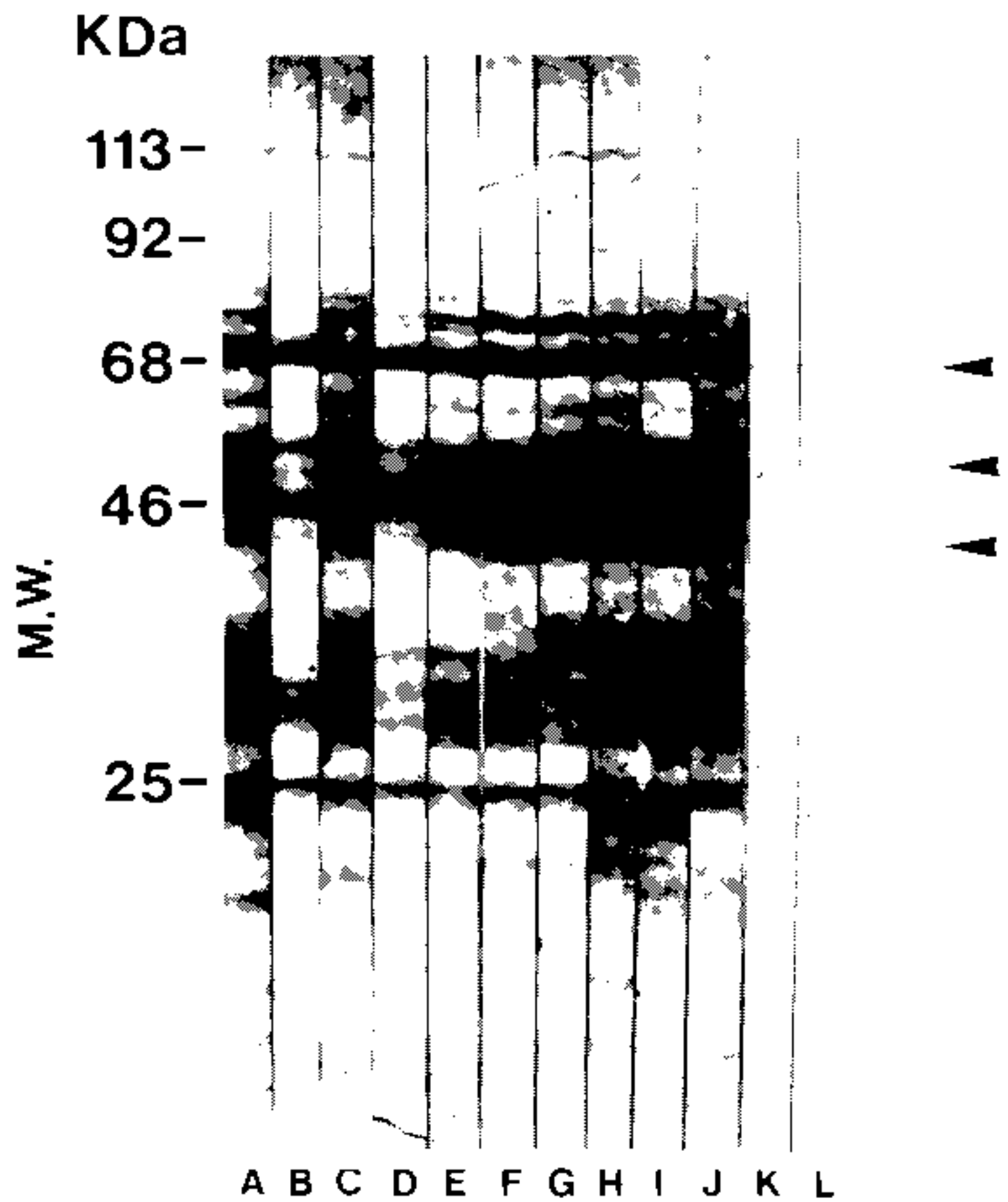


Fig. 1: reactivity of IgG antibodies to *Trypanosoma cruzi* epimastigotes soluble antigens after electrophoretic transfer of proteins from SDS-Page gels (7 to 15%) to nitrocellulose papers (6 μ g protein/strip). Antigens recognized by antibodies in sera of patients with Chagas' disease (A to J) and normal human serum (K and L).

When normal human control sera were incubated with blotted *T. cruzi* epimastigote polypeptides, some faint bands with molecular weights of 44 kDa, 55 kDa and 68 kDa were observed (Fig. 1) no reaction could be observed when culture medium was used as antigen in

the western blot. The reaction of sera from TL and AVL patients with *T. cruzi* soluble antigens were also analyzed. Representative reactions of such sera are shown in Fig. 2. Four of the 10 sera from TL patients which showed a positive reaction with 5 out 28 (18%) *T. cruzi* polypeptides. The other 6 sera were consistently negative in the western blot assay when *T. cruzi* was used as antigen, although they presented a positive IIF reaction when promastigotes of *Leishmania brasiliensis* were used as antigen (data not shown).

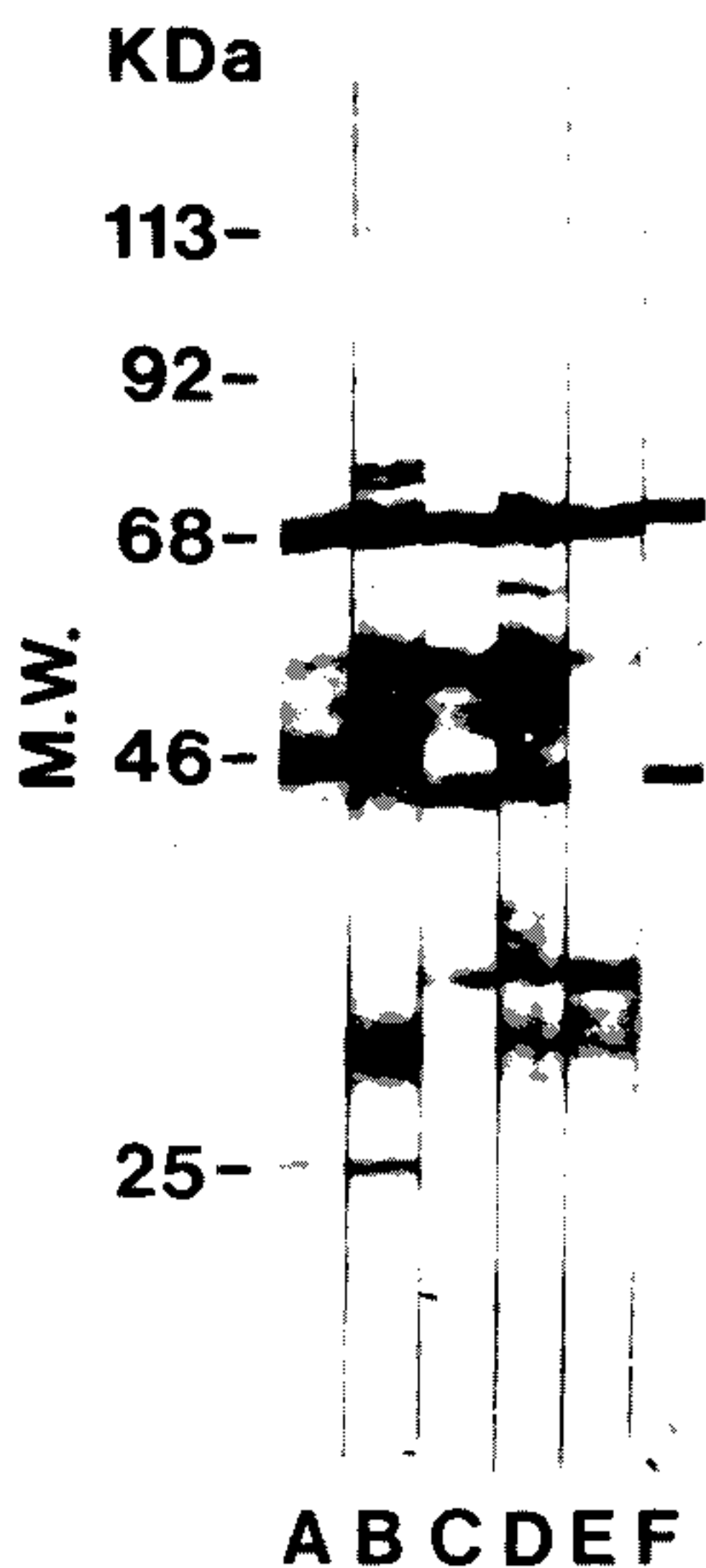


Fig. 2: reactivity of IgG antibodies to *Trypanosoma cruzi* soluble antigens after electrophoretic transfer of protein from SDS-Page gels (7 to 15%) to nitrocellulose papers (6 µg protein/strip). Antigens recognized by antibodies in sera from patients with Chagas' disease A and B; AVL (C and D) and ATL (E and F).

On the other hand, when sera from patients with AVL were analyzed at least 17 out 28 polypeptides (61%) of the *T. cruzi* polypeptides pattern were recognized. In 7 of the 13 sera analyzed the intensity of the reaction with each antigen was comparable to that observed with

sera from Chagas' disease patients. The other 6 sera showed a less intense pattern of reactivity, recognizing from 2 to 7 of the *T. cruzi* polypeptides.

The comparative analysis of patterns of reactivity of sera from Chagas' disease, TL and AVL indicated that at least 10 polypeptides specifically reacted with sera from Chagas' disease patients (Fig. 2). Among them, the polypeptides of molecular weights 46 kDa and 25 kDa reacted with all sera from Chagas' disease patients analyzed. The other 109 kDa, 42 kDa, 39 kDa, 26 kDa, 23 kDa, 17 kDa, 15 kDa and 14 kDa polypeptides were recognized by sera from Chagas' disease patients with variable frequency. The Table and the Fig. 3 present the data concerning the positivity with 109 kDa, 42 kDa, 39 kDa and 26 kDa polypeptides observed in sera from patients distributed according to the different clinical forms of Chagas' disease. The differences observed in the positivity with the first three polypeptides described above were not statistically significant. However, the antigen of 26 kDa seems to be preferentially recognized by patients with indeterminate form ($p < 0.05$), although some positive individuals could be observed in the other groups.

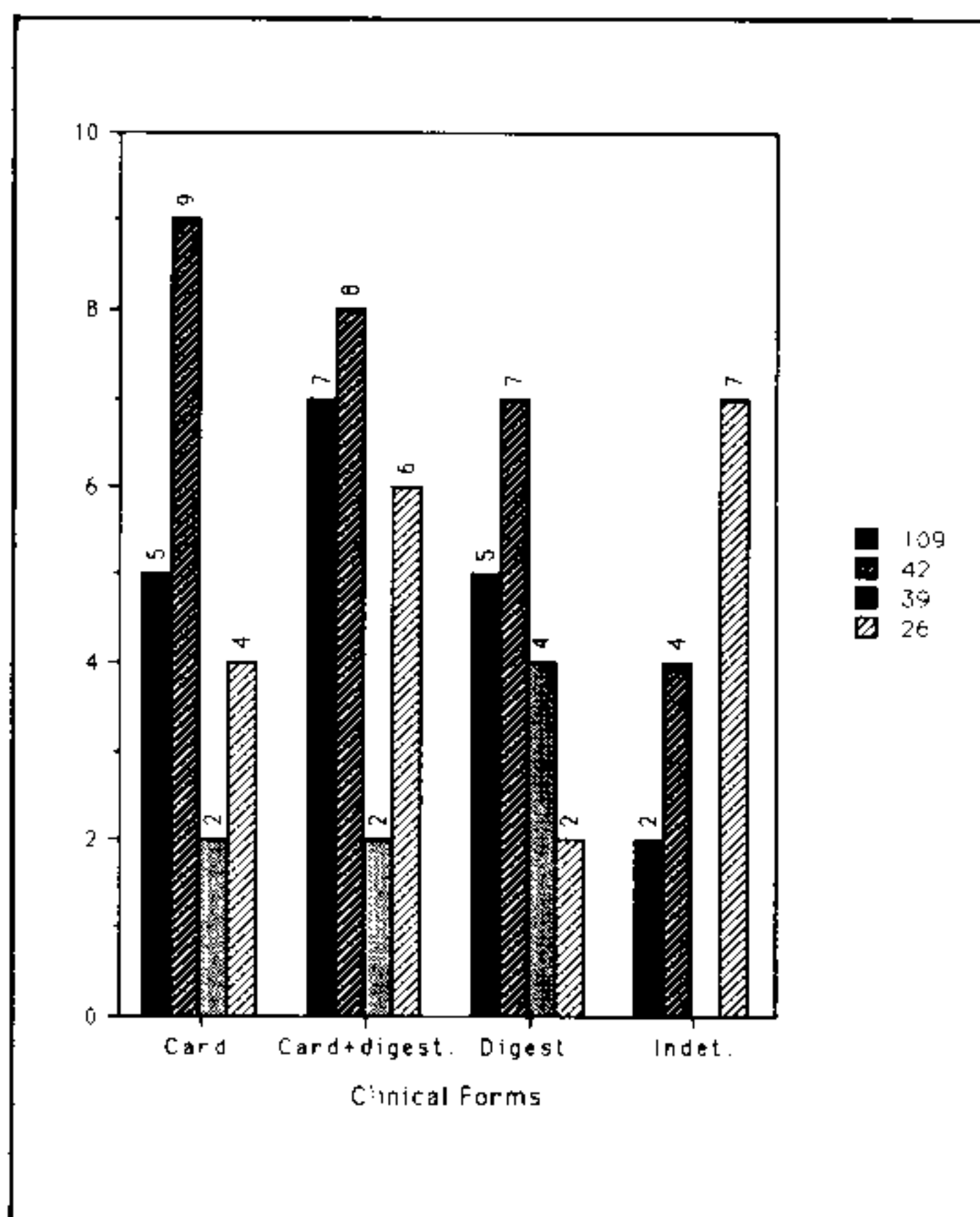


Fig. 3: distribution of the clinical forms of Chagas' disease patient according to the positivity with different antigens.

TABLE
Distribution of the patients according to the positivity with different antigens

Clinical form	Antigens (kDa)							
	109(a)		42(b)		39(c)		26(d)	
	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
Cardiac (N = 21)	5	16	9	12	2	19	4	17
Digestive (N = 11)	5	6	7	4	4	7	2	9
Card. + Dig. (N = 14)	7	7	8	6	2	12	6	8
Indeterm. (N = 10)	2	8	4	6	0	10	7	3
Total	19	37	28	27	8	48	19	37

(d) $\chi^2 = 8,08$ (with Yates correction) $p < 0,05$. All other are n. s.

DISCUSSION

In this paper we identified some antigens which were specifically recognized by sera from Chagas' disease patients when compared with sera from AVL and TL patients, which partially confirms previous results (Araujo, 1986). Amongst them, those with molecular weights of 25 kDa and 46 kDa seemed to be good candidates for immunodiagnosis, since they were recognized by all the sera from Chagas' disease.

In addition, a preferential positivity with the 26 kDa polypeptide could be observed in the individuals presenting the indeterminate form of Chagas' disease. Studies with a larger sample should be performed in order to confirm this observation.

The polypeptide of 25 kDa, although present in a mixture of antigens obtained mainly from cytoplasm of the parasite was recognized by all the chagasic patients sera, and may be the same surface glycoprotein of epimastigotes described by Scharfstein et al. (1983) which reinforces the usefulness of this molecule as a good antigen for specific Chagas' disease immunodiagnosis. On the other hand, the specific component of 46 kDa has not yet been described by other authors and may be also an useful antigen for immunodiagnostic purposes. In addition, specific reactions to *T. cruzi* epimastigote antigens were not detected in the region of polypeptides around 90 kDa. In this region we could identify a polypeptide of apparent molecular weight of 92 kDa which reacted with sera from Chagas'

disease and AVL patients but not with those from TL patients. It is possible that this molecule is the same 90 kDa *T. cruzi* surface glycoprotein isolated by affinity chromatography using lectin of *Lens culinaris* by Snary (1980). This glycoprotein was described previously as being specific for Chagas' disease in a immunodiagnostic assay (Schechter et al., 1983), but, in a further report, Schechter et al. (1986) also verified positive reactions of this glycoprotein with sera from patients with visceral leishmaniasis of the Old World.

The reactivity of sera from AVL patients with *T. cruzi* polypeptides could be due to the polyclonal B cell activation observed in this disease (Ghose et al., 1980; Galvão-Castro et al., 1984). However, the intense reaction to *T. cruzi* polypeptides can be considered as a product of specific stimulation due to common epitopes between *T. cruzi* and *Leishmania* (Afchain et al., 1979). Indeed, high levels of antibodies in sera from Chagas' disease patients to the polypeptides present in the soluble extract of *Leishmania donovani chagasi* were also demonstrated when analyzed by western blot (Ivo-dos-Santos et al., 1987). The isolation and purification of these specific antigens could allow the establishment of a specific serological test for the diagnosis of Chagas' disease.

RESUMO

Trypanosoma cruzi: identificação de antígenos específicos de epimastigotas reconhecidos por soros humanos imunes — Antígenos solúveis de epimastigotas de *Trypanosoma cruzi* foram analisados por "imunoblot" a fim de ve-

rificar sua reatividade com soros de pacientes com doença de Chagas. Além disso, soro de pacientes com leishmaniose visceral (LVA) e tegumentar americana (LTA) foram também analisados com o objetivo de se identificar os antígenos de reação cruzada com o *Trypanosoma cruzi*. Pelo menos 28 polipeptídeos, com pesos moleculares variando de 14 a 113 kDa foram identificados com soros de pacientes com doença de Chagas. Uma intensa reatividade cruzada foi observada quando foram utilizados soros de pacientes com leishmaniose visceral, enquanto que uma fraca reação cruzada foi observada com soros de pacientes portadores de leishmaniose tegumentar. Por outro lado, pelo menos 10 polipeptídeos puderam ser identificados apresentando reação específica com soros de pacientes chagásicos. Entre estes, os polipeptídeos de pesos moleculares de 46 kDa e 25 kDa que reagiram com todos esses soros e são potencialmente bons candidatos a antígenos específicos no diagnóstico sorológico da doença de Chagas.

Palavras-chave: *Trypanosoma cruzi* – antígenos – especificidade – “imunoblot” – reação cruzada

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