Clin Exp Immunol 1994; 97:417–423

Cross-reactivity studies and differential serodiagnosis of human infections caused by *Trypanosoma cruzi* and *Leishmania* spp; use of immunoblotting and ELISA with a purified antigen (Ag163B6)

E. L. MALCHIODI, M. G. CHIARAMONTE, N. J. TARANTO*, N. W. ZWIRNER & R. A. MARGNI
*Instituto de Estudios de la Inmunidad Humoral (IDEHU-CONICET), Cátedra de Inmunología, FFyB-UBA, Buenos Aires, and *Hospital S. V. de Paul, Orán, Salta, Argentina*

(*Accepted for publication 6 May 1994*)

SUMMARY

Results of our studies on the reactivity of chagasic and leishmaniasic sera with the purified *T. cruzi*-specific antigen 163B6, as assessed by ELISA, and with complex antigenic mixtures from *T. cruzi* and *Leishmania mexicana*, by immunoblotting, are presented here. Our objective was to identify the antigens responsible for the exhibited cross-reactivity between trypanosomiasis and leishmaniasis, and to find a specific reactivity pattern corresponding to each parasitosis. In spite of the high cross-reactivity observed with the immunoblotting, the use of 7·5% A-B gels made it possible to identify a characteristic pattern for each parasitosis, that could be distinguished by the naked eye. The characteristic pattern corresponding to chagasic patients was ascribed to reactivity with *T. cruzi* bands of mol. wts 131, 125, 116, 111, 51–45 and 43 kD, that were not recognized by leishmaniasic sera. *Trypanosoma cruzi* antigens of mol. wts 85, 81, 70, 65–60, 37 and 32 kD were considered as crossing antigens, since they were recognized by leishmaniasis sera. With *L. mexicana*, most of the chagasic patients presented reaction with antigen of mol. wts 124, 107, 92, 59 and 32 kD, while bands of mol. wts 155, 140, 73, 56 and 48 kD were recognized only by leishmaniasic sera. In this study we found 12 out of 45 sera of patients with leishmaniasis, from a region endemic for both parasitoses, which exhibited a pattern of bands very similar to those corresponding to chagasic individuals, strongly suggesting a mixed infection. This hypothesis was verified by using a purified specific antigen of *T. cruzi*, Ag163B6, which would be the major cysteine proteinase of this specie (cruzipain). By ELISA, these 12 sera showed a positive reaction with this purified antigen, as those of chagasic patients, thus leading to the confirmation of the presence of a mixed infection.

Keywords trypanosomiasis leishmaniasis cross-reactivity differential serodiagnosis

INTRODUCTION

Trypanosoma cruzi is the causative agent of Chagas' disease, a parasitosis which affects 16–20 million people in the American Continent [1]. Furthermore, many species of Leishmania cause cutaneous, mucocutaneous and visceral leishmaniasis in this area, thus posing a serious public health problem [2]. The geographical distribution of both parasitoses overlaps in many areas [3,4].

Conventional serological reactions for the diagnosis of trypanosomiasis and leishmaniasis, such as the indirect immunoflorescence assay (IFA), ELISA, indirect haemagglutination (IHA), and direct agglutination, use as antigens either whole parasites or subcellular fractions of *T. cruzi* and *Leishmania* spp. As already discussed [5–9], when these complex antigen preparations are used, a marked cross-reactivity among sera from patients suffering from these parasitoses is found. This situation is worsened by the overlapping of the endemic regions of both zoonoses in Latin America. This has a great socioeconomical impact, since most employers reject workers having positive serology for Chagas' disease, even in those cases showing no pathology of heart or viscera.

The use of purified antigen for the conclusive and reliable diagnosis of Chagas' disease has been emphasized as being of paramount importance among the different issues currently under study concerning this parasitosis [10,11].

Correspondence: E. L. Malchiodi, Instituto de Estudios de La Inmunidad Humoral (IDEHU-CONICET), Cátedra de Immunologia, FFyB-UBA, Junín 956, 1113 Buenos Aires, Argentina.

We have recently purified by immunoaffinity, using an anti-*T. cruzi* MoAb, the antigen termed Ag163B6, which is not present in *L. mexicana*. This antigen reacted by ELISA with all sera of chronic chagasic patients, whereas it showed no reactivity with sera of the leishmaniasis patients tested [12]. Antigen Ag163B6 is a glycoprotein which exhibits two bands of mol. wt 45–55 kD by SDS–PAGE. Studies on the behaviour in acrylamide gels, immunological identity and enzymatic activity, suggest that Ag163B6 should be identical to the major cysteine proteinase (cruzipain) of *T. cruzi* [13] isolated by Cazzulo [14], which would be involved in the defence mechanisms of the parasite against the immunoresponse of the host [15,16].

The reactivity of chagasic and leishmaniasic sera with the purified *T. cruzi* Ag163B6, assessed by ELISA, and with complex antigenic mixtures, by immunoblotting, was tested to identify the antigens responsible for the cross-reactivity between these parasitoses, and to find specific reactivity patterns corresponding to each parasitosis, in order to evaluate the possibility of developing a specific diagnostic procedure.

MATERIALS AND METHODS

Parasites

Trypanosoma cruzi epimastigotes, Tulahuen strain, were grown in biphasic medium, as previously described [17]. *Leishmania mexicana* promastigotes were grown in a liquid medium [18].

Both parasites were harvested during the exponential growth phase by centrifugation at 5000 *g* for 15 min and washed three times with 0·15 M NaCl, 0·01 M phosphates (PBS). Each pellet was treated as described later to be used in different reactions.

Soluble fractions from T. cruzi *and* L. mexicana

One gram (wet weight) of washed epimastigotes or promastigotes was broken by three cycles of freezing and thawing. They were then resuspended in 2·65 ml of 0·25 M sucrose and 5 mM KCl containing protease inhibitors (2 μM PMSF, 5 μM leupeptin, 5 μM pepstatin and 5 μM E-64; Sigma, St Louis, MO). After centrifugation at 6000 *g* for 10 min at 4°C, the supernatant was separated (S1), the pellet was resuspended again in the same solution and centrifuged at 17 000 *g* for 10 min at 4°C. The supernatant thus obtained was pooled with S1 and centrifuged at 45 000 *g* for 30 min at 4°C. The resulting supernatants were called F45 from *T. cruzi* and F45 from *L. mexicana*.

Protein concentration was determined according to Bradford [19] using bovine serum albumin (BSA) as standard.

Purification of Ag163B6 of T. cruzi

Purified MoAb 163B6 [12] was fixed to Sepharose 4B-BrCN [20] and used as immunosorbent for F45 from *T. cruzi*. The retained antigen was eluted with 0·1 M glycine-HCl buffer pH 3·0, dialysed against PBS and stored at −70°C until use.

Indirect immunoflorescence assay

Formalin-treated epimastigotes or promastigotes were used (10 000 parasites/area) for indirect immunoflorescence assay as described by Alvarez *et al.* [21].

Sera were assayed in serial two-fold dilutions from 1 : 30. Fluorescein-conjugated $F(ab')_2$ fragment goat immunoglobulins to human total globulins (Kallestad) in 1 : 50 dilution in Evans blue 0·005% in PBS were used as second antibody.

ELISA

An indirect method for antibody detection was used as described by Voller *et al.* [22]. Plates (Nunc, Roskilde, Denmark) were sensitized with F45 from *L. mexicana* (4 μg/well) and sera were assayed in serial three-fold dilutions. Titres were determined as the reciprocal of the dilution producing 50% of the maximal absorbance determined under saturation conditions.

Sera were also analysed against F45 from *T. cruzi* and Ag163B6 (4 μg/well and 0·1 μg/well, respectively) at single 1 : 1000 dilution. Sera having an absorbance higher than the mean absorbance of 25 control negative sera +3 s.d. were considered positive.

As second antibody, peroxidase-conjugated goat immunoglobulins to human total immunoglobulins (Dako, Hamburg, Germany) were used at 1 : 4000 dilution.

SDS–PAGE and immunoblotting

Whole cell lysates of *T. cruzi* epimastigotes and *L. mexicana* promastigotes containing protease inhibitors (2 μM PMSF, 5 μM leupeptin, 5 μM pepstatin and 5 μM E-64; Sigma) were electrophoretically separated in SDS–PAGE according to Laemmli [23], with a 3% stacking gel and 7·5%, 10% or 12·5% resolving gels. Samples were electrophoresed at 200 V during 1 h using a Mini-Protean II apparatus (Biorad, Richmond, CA) and blotted onto nitrocellulose at 300 mA for 1·5 h in a Trans Blot apparatus (Biorad) [24].

After blocking in Tris buffer solution (50 mM Tris-HCl, 150 mM NaCl pH 7·4) with 0·5% skim milk, the nitrocellulose was cut into 4-mm strips and incubated for 1 h at room temperature with 1 : 500 dilution of human sera. After three washes with TBS, strips were incubated with 1 : 4000 dilution of peroxidase-conjugated goat immunoglobulins to human total immunoglobulins and developed with H_2O_2/4-Cl-l-naphthol.

The molecular weight was calculated by a calibration curve obtained with molecular weight standards (Sigma).

Sera

Forty-five sera from leishmaniasic patients with cutaneous and mucocutaneous lesions, parasitologically confirmed by skin ulcer smear or by *in vitro* culture, were analysed. The patients were from different Latin America regions (five from Brazil, five from Venezuela, six from Bolivia and 31 from Argentina; 27 out of the last 31 were from the Northern province of Salta).

Chronic chagasic patients from the province of Santa Fe (Argentina), a non-endemic area for leishmaniasis, where cases had never been reported, were studied as well. These patients presented positive reactions in conventional serology.

Twenty-five sera from healthy donors were used as negative controls.

RESULTS

Reactivity of leishmaniasic sera by IFA and ELISA with conventional antigens

Forty-five sera from leishmaniasis patients were analysed

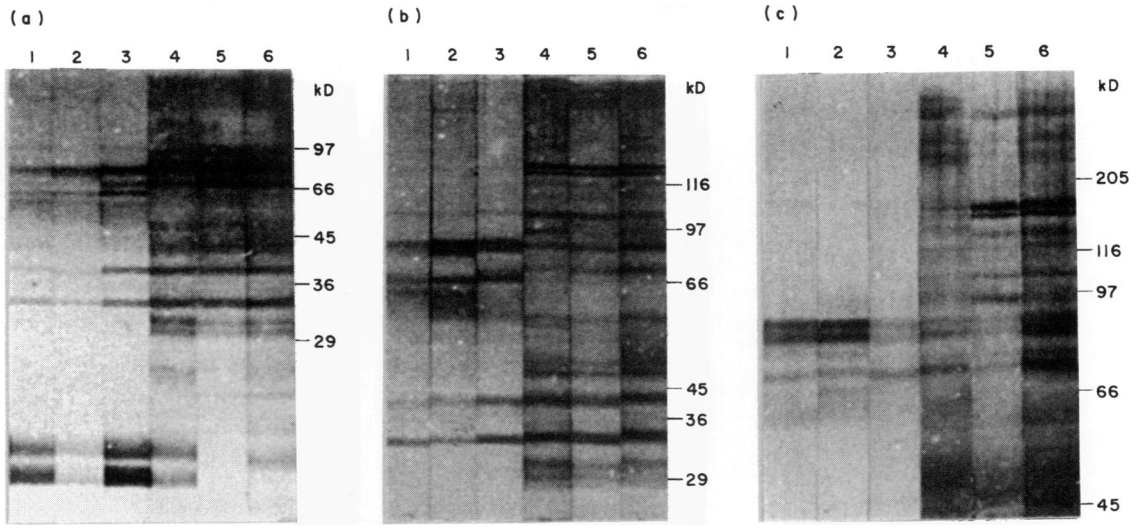

Fig. 1. Immunoblotting of *T. cruzi* epimastigotes with leishmaniasic and chagasic sera. Epimastigotes (Tulahuen strain) were treated with SDS–PAGE sample buffer and running in 12·5% (a), 10% (b) and 7·5% (c) acrylamide-bisacrylamide (A-B) gels. The nitrocellulose was cut in strips and incubated with leishmaniasic (lanes 1–3) and chagasic (lanes 4-6) sera. The molecular weight markers are indicated.

by IFA with formalin-treated parasites. All sera reacted with *L. mexicana* promastigotes, with titres ranging from 30 to 960. On the other hand, a high percentage of the leishmaniasis patients (87·5%) reacted against *T. cruzi* epimastigotes, with titres ranging from 30 to 960. The same sera were analysed by ELISA to determine antibodies against complex and soluble antigenic fractions of *L. mexicana* and *T. cruzi*. Antibodies against fraction F45 of *L. mexicana* were observed in 100% of sera, whereas 75·5% showed antibodies against F45 of *T. cruzi* with varying titres.

Reactivity pattern by immunoblotting

All sera of chronic chagasic patients tested by immunoblotting exhibited similar reactivity patterns with *T. cruzi* epimastigotes. These results were independent of the acrylamide-bisacrylamide (A-B) concentration used in the electrophoretic separation of the antigens (Fig. 1a–c, lanes 4–6). Sera from leishmaniasis patients (Fig. 1a–c, lanes 1–3) also recognized numerous antigens (mainly bands of mol. wt 85, 81, 70, 65–60, 37 and 32 kD) when the same parasite was used. It was not possible to differentiate clearly between the

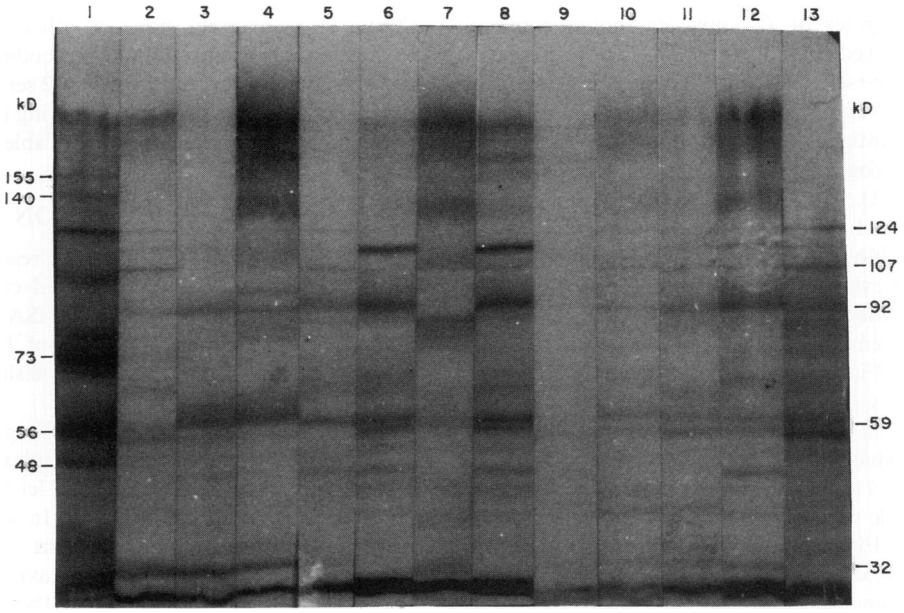

Fig. 2. Reactivity by immunoblotting of chagasic sera against *L. mexicana* promastigotes; 7·5% acrylamide-bisacrylamide (A-B) gels were used. Lane 1, serum from leishmaniasic patient; lanes 2–13, sera from chagasic patients from an area free of leishmaniasis. On the left, antigens not recognized by chagasic sera are indicated. On the right, antigens recognized by 64–91% of the chagasic sera tested are indicated.

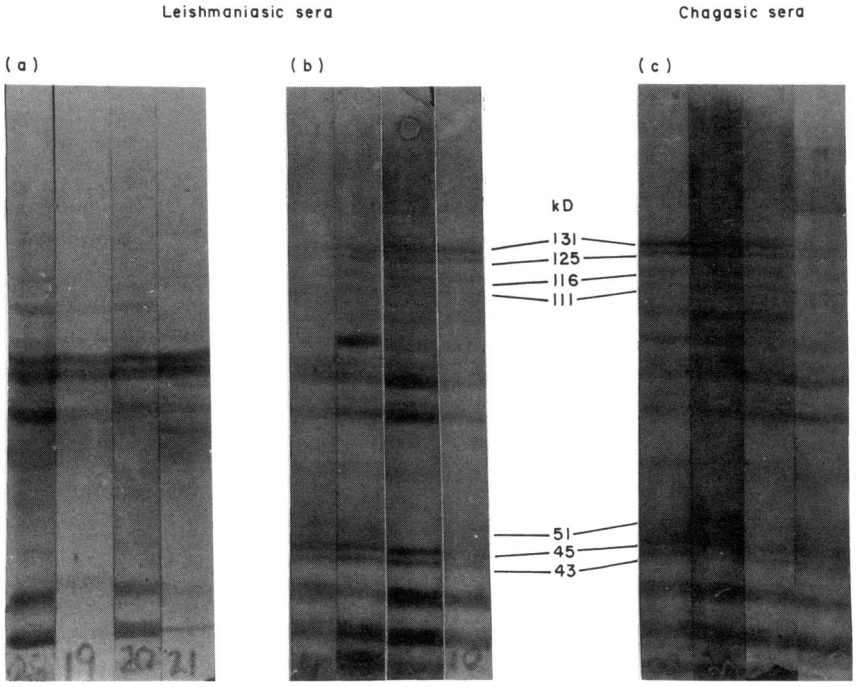

Fig. 3. Reactivity by immunoblotting of sera from chagasic and leishmaniasic patients with *T. cruzi* epimastigotes; 7·5% acrylamide-bisacrylamide (A-B) gels were used. (a) Sera from leishmaniasic patients. (c) Sera from chagasic patients with a characteristic pattern. (b) Sera from leishmaniasic patients with a pattern of reactivity similar to the chagasic one. Characteristic antigens recognized by chagasic sera are indicated.

pattern exhibited by these sera and that corresponding to chagasic patients by means of 12·5% or 10% A–B gels (Fig. 1a,b). Instead, the difference became evident with 7·5% A-B gels, in which the antigens between 40 and 200 kD are more efficiently separated (Fig. 1c).

On the other hand, sera of chronic chagasic patients from non-endemic leishmaniasis areas recognized a great number of antigens in promastigotes of *L. mexicana*, but a common pattern was not observed (Fig. 2, lanes 2–13). Nevertheless, a range of 64–91% of the sera tested did recognize those antigens of mol. wt 124, 107, 92, 59, 32 kD. Instead, none of the sera tested reacted with antigens of mol. wt 155, 140, 73, 56 and 48 kD, which were recognized in tests by sera of leishmaniasic patients (Fig. 2, lane 1).

Forty-five sera of patients with leishmaniasis were studied by immunoblotting against *T. cruzi* epimastigotes, which had been previously separated in 7·5% A-B gels. Thirty-three sera exhibited a band pattern (Fig. 3a) which could be easily differentiated from the pattern corresponding to chagasic patients (Fig. 3c). Conversely, the other 12 sera of leishmaniasic patients had a band pattern showing no differences (Fig. 3b) from the pattern corresponding to chagasic patients (Fig. 3c), which were able to recognize antigens of mol. wt 131, 125, 116, 111, 51–45 and 43 kD, in the same way as the sera from chagasic patients.

Results shown in Fig. 4 were obtained when the study was performed with *L. mexicana* promastigotes. Figure 4a depicts the reactivity of leishmaniasic sera. As can be seen in Fig. 4c, chagasic sera exhibited a lower number of bands, although some of them were quite strong and definite, corresponding to antigens of mol. wt 124, 107, 92, 59 and 32 kD. This reactivity is either absent or very weak in most of the leishmaniasic sera (Fig. 4a); but, as can be seen in Fig. 4b, the same sera of Fig. 3b react with those bands, similarly to the chagasic sera.

Reactivity with purified Ag163B6

When all sera were analysed by ELISA with a purified specific antigen of *T. cruzi*, i.e. Ag163B6, it was observed that all chronic chagasic patients showed a positive reaction (Table 1). Conversely, none of either healthy individuals or leishmaniasic patients showed antibodies specific against the antigens, with the exception of the 12 sera with which a pattern of bands similar to those corresponding to chagasic individuals was obtained by immunoblotting (Table 1).

DISCUSSION

The aim of this study was to test the reactivity of chagasic and leishmaniasic sera with purified and complex antigens. Our findings indicate that the use of ELISA with the Ag163B6 or immunoblotting with epimastigotes of *T. cruzi*, would make it possible to distinguish chagasic, leishmaniasic and mixed infection patients.

In the serological diagnosis of Chagas' disease, the use of conventional reactions with complex antigenic mixtures presents high cross-reactivity with leishmaniasis patients, as reported by several authors [5–9]. In some laboratories, it is usual to work with high dilutions of both chagasic and leishmaniasic sera in an attempt to avoid cross-reactivity, and to obtain a reliable diagnosis of these parasitoses [25]. As reported here, we found that sera from the leishmaniasic patients tested exhibited reactivity at both high and low dilutions, either with homologous or with heterologous antigens, either by using IFA with formalin-treated parasites or

Table 1. Reactivity of sera from chronic chagasic and leishmaniasic patients with Ag163B6 by ELISA and *T. cruzi* epimastigotes by immunoblotting (IB)

Sera	Immunoblotting pattern: Similar to chagasic	Immunoblotting pattern: Different from chagasic	Reactivity with Ag163B6 by ELISA*
Chronic chagasic ($n = 35$)	35/35	0/35	35/35
Leishmaniasic ($n = 45$)	12/45†	33/45	12/45†

* Sera having an absorbance higher than the mean absorbance of 25 negative control sera +3 s.d. were considered positive.

† Twelve leishmaniasic sera with IB patterns similar to the chagasic one are the same leishmaniasic sera as those exhibiting positive reactivity against Ag163B6.

ELISA with soluble extracts. These results indicate that the use of high dilutions to avoid cross-reactions is inappropriate.

Study of chagasic sera by immunoblotting with *T. cruzi* epimastigotes made it possible to find a pattern characteristic of this parasitosis (Fig. 1). By analysing leishmaniasic sera with epimastigotes, 7·5% A-B was found to be the percentage of choice to obtain a pattern of reactivity different from that of the chagasic sera (Fig. 1c). In this way, observation of the bands presented by the leishmaniasic sera with the naked eye made it possible to differentiate them from the control chagasic sera. This represents an important advantage, since it would be unnecessary to determine exactly the mol. wt of the antigens recognized by each serum to obtain the diagnosis.

A thorough analysis of the reactive bands led to the determination of the cross-reactive antigens (mol. wts 85, 81, 70, 65–60, 37 and 32 kD) recognized by the leishmaniasic sera tested in *T. cruzi* epimastigotes. On the other hand, we found that while none of them reacted with antigens of mol. wt 131, 125, 116, 111, 51–45 and 43 kD, these bands were consistently recognized by all the chagasic sera tested. In a recent study, Chiller *et al.* [26] determined that in 10% A-B gels, bands of > 97, 38 and 25 kD were recognized by the chagasic sera, but not by the leishmaniasic sera. In our study, when the 10% gels were used, sera behaved in the same way with regard to the 25-kD antigen. Instead, the 38-kD antigen was recognized by the leishmaniasic sera in immunoblotting, as stated above. With regard to this point, it is worth mentioning that we had previously isolated a 37-kD antigen from *T. cruzi* (T37K) [27], which, when used in immunoprotection tests, had been able to lower the parasitaemia in *Cebus apella* monkeys challenged

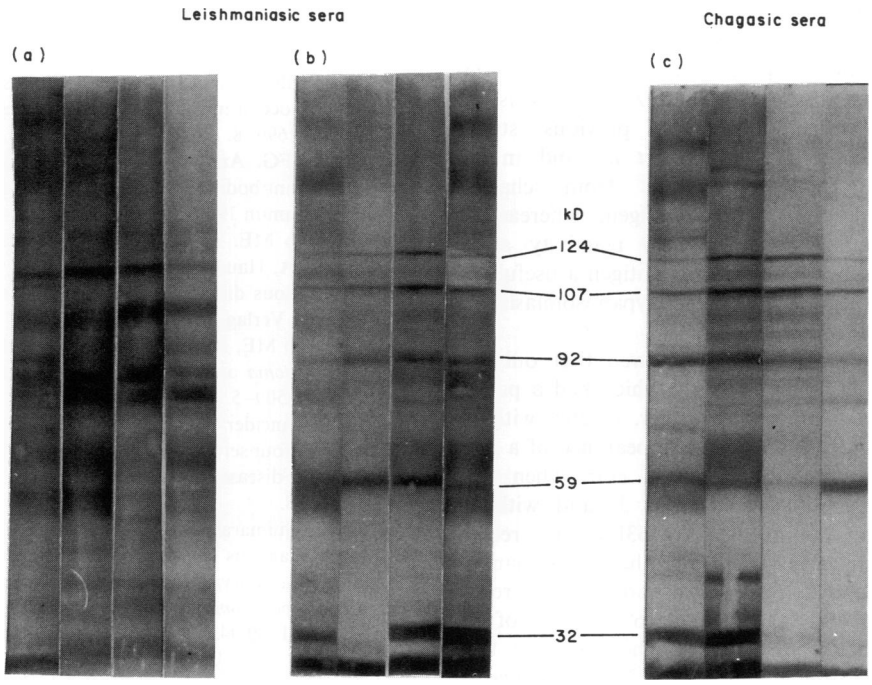

Fig. 4. Reactivity by immunoblotting of sera from chagasic and leishmaniasic patients with *L. mexicana* promastigotes; 7·5% acrylamide-bisacrylamide (A-B) gels were used. (a) Sera from leishmaniasic patients. (c) Sera from chagasic patients with a characteristic pattern. (b) Sera from leishmaniasic patients with a pattern of reactivity similar to the chagasic one. Characteristic antigens recognized by chagasic sera are indicated.

with trypomastigotes [28]. This antigen, however, would not be species-specific, since sera of the leishmaniasic patients tested reacted with it, as seen by ELISA (results not shown).

It should be mentioned that some of our results are not yet fully understood, i.e. that some leishmaniasic sera recognized a *T. cruzi* antigen of mol. wt 65–60 kD, with which chagasic sera do not react. A similar phenomenon, but within the same species, has been described by McDaniel *et al.* [29]; these authors, after infecting mice with different *T. cruzi* clones, detected specific antigens with sera obtained against the heterologous, but not against the homologous clone.

When sera of chronic chagasic patients from a leishmaniasis-free region were studied, a common reactivity pattern could not be found, but it was possible to identify the bands of mol. wt 124, 107, 92, 59 and 32 kD as the crossing antigens recognized by chagasic sera (Fig. 2). The antigens of mol. wt 155, 140, 73, 56 and 48 kD were recognized only by leishmaniasic sera (Fig. 2); therefore, they would be useful for a reliable diagnosis of leishmaniasis, eliminating the interference introduced by the cross-reactivity phenomenon [30–32].

As reported here, it was found that 12 out of 45 leishmaniasic patients tested exhibited a pattern of reactivity to *T. cruzi* similar to that of chagasic sera (Fig. 3), recognizing bands of mol. wt 131, 125, 116, 111, 51–45 and 43 kD. Moreover, these sera exhibited with *L. mexicana* a pattern similar to that of sera from chagasic patients (Fig. 4). These sera were from the Province of Salta (Argentina), where there is overlapping of endemic areas of leishmaniasis and trypanosomiasis. This would be compatible with the presence in these patients of a mixed infection.

Previous studies performed in our laboratory made it possible to obtain a glycoprotein from *T. cruzi*, the 163B6 antigen [12], which presents a double band at 55–45 kD.

Tests of IFA with the MoAb against Ag163B6 [12] and of immunoblotting with a polyclonal serum (results not shown) indicated that the Ag163B6/cruzipain [13] is not present in *L. mexicana*. Moreover, previous studies performed independently by Cazzulo *et al.* and in our laboratory showed that most sera from chagasic patients reacted with the purified antigen, whereas the leishmaniasic sera analysed showed no reactivity at all [12,33]. These features would make this antigen a useful tool for the differential diagnosis between trypanosomiasis and leishmaniasis.

The use of Ag163B6 in ELISA showed that only the chagasic and the 12 leishmaniasic sera, which had a pattern by immunoblotting similar to the former, reacted with the antigen. This is in agreement with the appearance of a band at 51–45 kD in immunoblotting with *T. cruzi* when these 12 leishmaniasic sera were tested (Fig. 3) and with the immunoblotting using the purified Ag163B6, that reacted only with sera from chagasic patients and the 12 leishmaniasic patients mentioned above (results not shown). The results of the ELISA with the purified Ag163B6 and of the immunoblotting with *T. cruzi* support the idea that these 12 patients had a double infection. The existence of patients with mixed infection had been previously reported by Chiller *et al.* [26] and Levy-Yeyati *et al.* [34], based on methods differing from ours. According to our results, we believe it is not possible to diagnose a mixed infection by conventional serology, since there exists a high proportion of leishmaniasis patients, who, even though they are not infected with *T. cruzi*, give a positive reaction with this type of test.

It can therefore be concluded that it is possible to identify a reactivity pattern by immunoblotting of the chagasic sera with *T. cruzi* epimastigotes. Besides, in spite of cross-reactivity phenomena, this pattern can be differentiated from that exhibited by leishmaniasic sera with the same antigen. On the other hand, the fact that a leishmaniasic patient showed a pattern similar to that of chagasic patients strongly suggests the presence of a mixed infection, which could be alternatively confirmed and easily demonstrated by using Ag163B6 in a conventional ELISA.

ACKNOWLEDGMENTS

We thank Estela Lammel and Dr B. Franke de Cazzulo for the provision of parasites, and Dr Stella M. Gonzalez Cappa for critically reviewing the manuscript. This investigation received financial support from 'Consejo Nacional de Investigaciones Científicas y Técnicas' (CONICET) and Universidad de Buenos Aires. M.G.C. and N.W.Z. are fellows of CONICET. E.L.M. and R.A.M. are members of the Research Career of CONICET.

REFERENCES

1 Brener Z. Recent development in the field of Chagas' disease. Bull World Health Organ 1982; **60**:463–73.
2 World Health Organization. Public Health Aspects. In: WHO, Technical Report Series 701. The leishmaniasis. Geneva: WHO, 1984; 53–60.
3 Desjeux P, Aranda E, Aliaga O *et al.* Human visceral leishmaniases in Boliva: first proven case from "Los Jungas". Trans R Soc Trop Med Hyg 1983; **77**:851–5.
4 Walton BC, Chinel LV, Equia OE. Onset of espundia after many years of occult infection with L. braziliensis. Am J Trop Med Hyg 1973; **22**:696–8.
5 Araujo FG. Analysis of *Trypanosoma cruzi* antigens bound to specific antibodies and by antibodies to related trypanosomatids. Infect Immun 1986; **53**:179–85.
6 Camargo ME. American trypanosomiasis (Chagas' disease). In: Balows A, Hausler Jr WJ, Lennette E, eds. Laboratory diagnosis of infectious diseases, principles and practice, Vol 1, New York: Springer Verlag, 1988: 744–53.
7 Camargo ME, Rebonato C. Cross-reactivity fluorescence tests for *Trypanosoma* and leishmania antibodies. Am J Trop Med Hyg 1969; **18**:500–5.
8 Schattschneider W, Lopes ER, De Alencar JE *et al.* A comparative study of four serological methods for diagnosis of acute and chronic Chagas' disease in Brazilian patients. Trop Geogr Med 1992; **44**:210–8.
9 Soares Guimaraes MC, Celeste BJ, de Castilho EA *et al.* ELISA in mucocutaneous leishmaniasis, kala-azar and Chagas' disease: an epimastigote *trypanosoma cruzi* antigen able to distinguish between anti-*Trypanosoma* and anti-leishmania antibodies. Am J Trop Med Hyg 1981; **30**:942–7.
10 Luquetti AO. Use of *Trypanosoma cruzi* defined proteins for diagnosis-multicentre trial. Serological and technical aspects. Mem Inst Oswaldo Cruz 1990; **85**:497–505.
11 World Health Organization. Parasite antigens. Bull World Health Organ 1975; **52**:237–49.
12 Carbonetto CH, Malchiodi EL, Chiaramonte M *et al.* Isolation of

a *Trypanosoma cruzi* antigen by affinity chromatography with a monoclonal antibody. Preliminary evaluation of its possible applications in serological tests. Clin Exp Immunol 1990; **82**:93–96.

13 Malchiodi EL, Chiaramonte MG, Martinez JA *et al.* Identity of the major cysteine proteinase (cruzipain) from *Trypanosoma cruzi* and an antigen (Ag163B6) isolated wtih a monoclonal antibody. Immunol Lett 1993; **35**:59–62.

14 Cazzulo JJ. Proteinases of *Trypanosoma cruzi.* In: Coombs GH, North MJ, eds. Biochemical protozoology. London: Taylor & Francis, 1991: 191–9.

15 Bontempi E, Cazzulo JJ. Digestion of human immunoglobulin G by the major cysteine proteinase (cruzipain) from *Trypanosoma cruzi.* FEMS Microbiol Lett 1990; **70**:337–42.

16 Souto-Padrón T, Campetella O, Cazzulo JJ *et al.* Cysteine proteinase in *Trypanosoma cruzi*: immunocytochemical localization and involvement in parasite–host cell interaction. J Cell Sci 1990; **96**:485–90.

17 Chiari E, Camargo EP. Culturing and cloning of *T. cruzi.* In: Morel CM, ed. Genes and antigens of parasites. Rio de Janeiro: Fundaçao Oswaldo Cruz, 1984: 23–26.

18 Jaffe CL, Grimaldi G, McMahon-Pratt D. The cultivation and cloning of leishmania. In: Morel CM, ed. Genes and antigens of parasites. Rio de Janeiro: Fundaçao Oswaldo Cruz, 1984: 43–91.

19 Bradford MN. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; **72**:248–54.

20 Fuchs S, Sela M. In: Weir ED, ed. Immunochemistry. Oxford: Blackwell Scientific Publications, 1986: 161.

21 Alvarez M, Cerisola JA, Rohwedder RW. Test de immuno-florescencia para el diagnóstico de la enfermedad de Chagas. Bol Chile Parasitol 1968; **23**:4–9.

22 Voller A, Bidwell D, Bartlett A. Enzyme-linked immunosorbent assay. In: Rose NR, Friedman H, eds. Manual of clinical immunology, 2nd edn. Washington, DC: American Society for Microbiology, 1988: 359–71.

23 Laemmli VK. Cleavage of strutural proteins during the assembly of the head of bacteriophage T4. Nature 1970; **227**:680–4.

24 Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrilamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 1979; **76**:4350–4.

25 World Health Organization. Surveillance methods. In: WHO, Technical Report Series 701. The leishmaniasis. Geneva: WHO, 1984: 85–89.

26 Chiller TM, Samudio MA, Zoulek G. IgG antibody reactivity with *Trypanosoma cruzi* and *Leishmania* antigens in sera of patients with Chagas' disease and leishmaniasis. Am J Trop Med Hyg 1990; **43**:650–6.

27 Malchiodi EL, Carbonetto CH, Margni RA. Isolation-characterization and preliminary immunological studies of a *Trypanosoma cruzi* antigenic component. Medicina (Buenos Aires) 1987; **47**:509–17.

28 Malchiodi EL, Carbonetto CH, Grana D *et al.* Immune response against *Trypanosoma cruzi* antigens in *Cebus apella* monkeys. Trop Med Parasitol 1993; **44**:86–90.

29 McDaniel JP, Howard RJ, Dvorak JA. Identification and analysis of epimastigote surface and metabolic proteins in *Trypanosoma cruzi.* Mol Biochem Parasitol 1986; **19**:183–94.

30 Jaffe CL, McMahon-Pratt D. Serodiagnostic assay for the visceral leishmaniasis employing monoclonal antibodies. Trans Roy Soc Trop Med Hyg 1987; **81**:587–94.

31 Jaffe CL, Zalis M. Purification of two *Leishmania donovani* membrane proteins recognized by sera from patients with visceral leishmaniasis. Mol Biochem Parasitol 1988; **27**:53–62.

32 Jaffe CL, Zalis M. Use of purified parasite proteins from *Leishmania donovani* for the rapid serodiagnosis of visceral leishmaniasis. J Infect Dis 1988; **157**:1212–20.

33 Martínez J, Campetella O, Frasch ACC *et al.* The major cysteine proteinase (cruzipain) from *Trypanosoma cruzi* is antigenic in human infections. Infect Immun 1991; **59**:4275–7.

34 Levy-Yeyati PS, Bonnefoy G, Mirkin G *et al.* The 70 kDa heat shock protein is a major antigenic determinant in human *Trypanosoma cruzi/Leishmania braziliensis braziliensis* mixed infection. Immunol Lett 1991; **31**:27–34.