The interest of immunoprecipitation tests in the immunological diagnosis of Chagas' disease

by

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Summary — Immunoelectrophoresis (IEP) and a double diffusion microtest (MD) were evaluated for immunological diagnosis of Chagas’ disease, using 527 sera from Bolivian patients. The specificity of the tests was given by the identification of precipitating antibodies anti-component 5, previously demonstrated as specific of Trypanosoma cruzi. IEP showed 1 to 14 precipitation lines in 96 per cent of the sera, both with parasitological (positive xenodiagnosis) or serological confirmation of T. cruzi infection, whereas the control sera were all negative. Precipitation line 5, identified by its particular pattern, was present in 70 per cent of the same sera. In MD, precipitation line 5, identified by identity reaction with a rabbit anti-component 5 specific serum, was found in 80.2 per cent of all the serological positive sera: 84.1 per cent of the sera with positive and 72.2 per cent of the sera with negative xenodiagnosis (P 0.05). Line 5 was never found, neither in the leishmaniasis group (coming from an area without Chagas' disease) nor in the control group. In another particular leishmaniasis group, coming from an area endemic for both infections, 31.7 per cent of the sera were line 5 positive, indicating associated Chagas' disease. Consequently, the immunoprecipitation test, allowing the detection of T. cruzi specific line 5, is cheap and simple to perform. It can be recommended in association with the other serological tests which are more sensitive, when highly specific immunodiagnosis of Chagas' disease is required.

Keywords : Immunoelectrophoresis; Immunodiffusion; Immunodiagnosis; T. cruzi, Chagas’ Disease; Specificity; Antigen 5.

Introduction

In the acute phase of Chagas' disease, blood trypomastigotes of Trypanosoma cruzi are easy to detect by direct microscopy. By contrast, in the chronic stage of the infection, parasitological investigations, such as xenodiagnosis or blood culture, only result in 31 to 50 per cent case detection (Chiari & Bruner, 1968; Pifano et al., 1973; Cerisola et al., 1974; Neal & Miles, 1977). In chronic cases without patent blood forms, the diagnosis of T. cruzi infection is based only on the presence of anti-T. cruzi circulating antibodies, emphasizing the importance of the immunological diagnosis of Chagas' disease.

Various sensitive serological techniques have been applied: the complement fixation test (CFT), pioneered by Guerriero & Machado (1913), direct agglutination (Vattuone & Yanovsky, 1971), hemagglutination (IHA) (Romana, 1961; Cerisola et al., 1962; Neal & Miles, 1970; Camargo et al., 1973), immunofluorescence (IF) (Fife & Muschel, 1959; Sadun et al., 1963; Camargo, 1966; Alvarez et al., 1968; Petana, 1975) and more recently
enzyme-linked-immunosorbent assay (ELISA) (Voller et al., 1975; Anthony et al., 1979; Tandon et al., 1979; Spencer et al., 1980; Guimaraes et al., 1981; Schechter et al., 1983) and thin layer immunoassay (Nilsson & Voller, 1982).

However, many human sera with positive serological reactions for *T. cruzi* also have positive reactions with other flagellate antigens such as *T. rangeli* (Anthony et al., 1979); *Leishmania donovani* (Camargo et al., 1966; Nery Guimaraes et al., 1969; Salfelder & Mannweiler, 1981; Guimaraes et al., 1981) or *L. braziliensis* (Chaffee et al., 1958; Duxbury & Sadun, 1964; Camargo & Rebonato, 1969; Nery Guimaraes et al., 1969; Gam & Neva, 1977; Guimaraes et al., 1981). Such observations can mainly be explained by the cross-reactions demonstrated between flagellate antigens (Afchain et al., 1979; Bronzina et al., 1980; Anthony et al., 1981) and the likely existence of mixed infections in patients from areas endemic for various flagellate infections (Souza & Johnson, 1971; Anthony et al., 1979), emphasizing the need for specific immunological diagnosis of *T. cruzi* infection.

The immunoprecipitation tests are widely used and recommended for the diagnosis of parasitosis like helminthiases, taking advantage of the analysis of the different precipitation lines to identify some genus- or species-specific line for a highly specific immunological diagnosis (Biguet et al., 1965; Carlier & Wery, 1963). Curiously few works have evaluated the immunoprecipitation tests for the diagnosis of Chagas' disease. Muniz (1974) and Pellegrino et al. (1959) used liquid phase precipitation in tube, whereas Aguilar-Torres et al. (1976) and Knight et al. (1976) tested counter-immunoelectrophoresis. Only Afchain et al. (1979), in a preliminary work with few sera, studied specific precipitation lines in immunoelectrophoresis (IEP).

The aim of this work was to evaluate IEP and a double diffusion microtest (MD) for the immunological diagnosis of Chagas' disease. The required specificity of the test was given by the identification of precipitating antibodies to component 5, previously demonstrated as specific of *T. cruzi* and without cross-reactions with other flagellates, particularly *T. rangeli*, *L. donovani*, *L. mexicana* (Afchain et al., 1978, 1979) and *L. braziliensis* (Brenibre et al., in press). Precipitation line 5 was identified either by its particular pattern in IEP or by identity reaction with a rabbit anti-component 5 specific serum in MD.

**Material and Methods**

**Human sera**

Sera were obtained from 527 Bolivian patients divided in four groups according to their geographical origin:

1. The first group (1) included 374 patients, all of them with a positive serology for *T. cruzi* (see below). They came from southern low lands (Camiri), areas known to be highly endemic for Chagas' disease, but where leishmaniasis had never been found. They were asymptomatic or with cardiac or digestive pathologies compatible with the chronic phase of Chagas' disease. The performance of xenodiagnosis was possible for 142 of them of whom 88 (62.0 per cent) were positive.

2. The second group (2) comprised 54 sera and completed with CFT titers of *T. cruzi* epimastigotes (see below) and pretested according to previous studies to 1:40 detection titre for both leishmaniasis and Chagas' disease. The extinction value of ELISA was taken into account. The *L. braziliensis* serology xenodiagnosis group 1 and group 2 were performed by counter-immunoelectrophoresis according to the procedures described above.

3. The third group (3) comprised 10 sera from patients with positive serological reactions for *T. cruzi* and *L. braziliensis* infection, the association between *L. braziliensis* and *T. cruzi* infection was studied by a double diffusion microtest (MD)

4. The fourth group (4) was a control group with sera obtained from patients of highland areas (Altiplano) who had travelled to the endemic areas.

The *T. cruzi* serology was performed with CFT titers of *T. cruzi* epimastigotes (see below) and pretested according to previous studies to 1:40 detection titre and the extinction value of ELISA was determined. The *L. braziliensis* serology xenodiagnosis group 1 and group 2 were performed by counter-immunoelectrophoresis according to the procedures described above. The *L. braziliensis* promastigotes (Tehuantepec) were used as a control group. The supernatant was dialyzed against 1:40 titre saline and centrifuged at 18,000 rpm.
2. The second group (2) concerned 41 patients with clinical evidence of mucocutaneous leishmaniasis. 21 of them presented typical primary cutaneous ulcerations with surrounding indurations and the other 20 had typical mutilations of the face. They lived in the Yungas valleys, where both leishmaniasis and Chagas' disease are known to be endemic, allowing the association between *L. braziliensis* and *T. cruzi* infections.

3. The third group (3) concerned 45 patients also with clinical evidence of mucocutaneous leishmaniasis (35 with primary cutaneous lesions and 10 with mutilations of the face). They lived in the northern low lands (Beni & Alto Beni), areas know to be endemic for leishmaniasis but free of Chagas' disease.

4. The fourth group (4) was a control group of 67 asymptomatic patients, from highland areas (Altiplano) exempt of both infections, and who never had travelled to the endemic areas.

**Serological tests**

The *T. cruzi* serology was performed using IF and ELISA for all the sera and completed with CFT for group 1 sera, with the same batch of *T. cruzi* epimastigotes (see below). The tests were performed and interpreted according to previous studies (Brenière *et al.*, in press). The positive detection titres were 1:40 and 1:2 for IF and CFT respectively, and the extinction value of ELISA was 10.17. Only the sera positive for all the serological tests performed were considered as positive.

The *L. braziliensis* serology was carried out for the sera of the positive xenodiagnosis group 1 and groups 2, 3, 4 using IF and *L. braziliensis* promastigotes (see below), according to Guimaraes *et al.* (1974). Previous studies showed a 1:40 titre as the positive detection limit.

**T. cruzi and L. braziliensis antigenic extracts**

*T. cruzi* epimastigotes (Tehuantepec strain) were obtained from culture in cell-free GLSH monophasic medium at 28 °C (Le Ray *et al.*, 1975). The parasites were collected by centrifugation at 2,000 g, washed three times and divided into two samples. The first one was 1 per cent glutaraldehyde fixed and used directly in smears for IF. The second one was frozen in 1 per thousand NaCl, disintegrated five times in a hydraulic press (LKB X Press) at 18,000 psi and centrifuged at 26,000 g for 1 hr at 4 °C. The supernatant was dialyzed and lyophilized for use in immunodiffusion, IEP, CFT, and ELISA.

*L. braziliensis* promastigotes were obtained from cell-free culture on NNN medium modified according to Deker-Jackson and Honigberg (1978), centrifuged, washed and used in smears for IF as the *T. cruzi* epimastigotes.

**T. cruzi component 5 and specific anti-component 5 serum**

The previous crude antigenic solution of *T. cruzi* was extracted three times with chloroform/methanol (2v/v), stirring it 30 min at room temperature and centrifuging it 30 min at 11,400 g. The last aqueous phase was
incubated overnight at 37°C before ethanol precipitation (3v/lv) at −20°C during 4 h. After centrifugation (11,400 g), the sediment was solubilized in distilled water and used as a component 5-rich fraction.

The rabbits were immunized by simultaneous multiple intradermic injections according to Vaitukaitus et al. (1971) with 2 mg of fraction 5 and boosted by weekly subcutaneous injections of 1 mg over 6 weeks. The presence of precipitating anti-component 5 antibodies in the rabbit sera was controlled in IEP (see below) and in immunodiffusion by identity reaction with a reference monospecific anti-component 5 serum prepared according to Afchain et al. (1979).

**Immunoprecipitation tests**

IEP was carried out according to Biguet et al. (1965) in 1 per cent agarose, using sera concentrated threefold by lyophilization (Fig. 1). It was considered positive when showing at least one sharp precipitation line. Precipitation line 5 was identified by its particular intensity and position in IEP (Afchain et al., 1979).

![Figure 1. Positive immunoelectrophoresis showing line 5 (A5) specific of T. cruzi.](image)

MD was performed on microscope slides (25 x 80 mm) covered with 4 ml of 1 per cent agarose (IBF-France) in 0.1 M veronal buffer pH 8.2. Three sets of wells for sera and antigenic extract were punched on one slide, allowing simultaneous study of 12 different human sera. Sixty μl of human serum, concentrated to 12 μl by lyophilization, were placed in four peripheral wells, whereas 12 μl of 1:4 diluted rabbit anti-5 immune serum were put in the central well. Furthermore (Fig. 2) two micro-wells were filled with 2 μl (24 μg) of T. cruzi antigenic extract. The slides were incubated in a moist chamber by immersion in water for dried and stained by Coomassie blue. Human serum produced a line 5 obtained with the A5.

![Double diffusion microtest: wells for precipitation line 5 (A5), microwell T = T. cruzi antigenic extract.](image)

The frequencies of the lines obtained for sera of the group IEP (Fig. 1), with 1 to 14 percent, and line 5 in 70 per cent (positive xenodiagnosis) or sera whereas the control sera showed no line 5 in 70 per cent.

<table>
<thead>
<tr>
<th>Frequencies of total count</th>
<th>Positive xenodiagnosis group I</th>
<th>total sera (76)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of lines</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>0</td>
<td>3</td>
<td>4.0</td>
</tr>
<tr>
<td>1-2</td>
<td>2</td>
<td>2.6</td>
</tr>
<tr>
<td>3-4</td>
<td>22</td>
<td>29.0</td>
</tr>
<tr>
<td>5-6</td>
<td>22</td>
<td>29.0</td>
</tr>
<tr>
<td>&gt; 6</td>
<td>21</td>
<td>27.6</td>
</tr>
<tr>
<td>Total positive</td>
<td>73</td>
<td>96.0</td>
</tr>
</tbody>
</table>

n = number of sera.

As shown in table 2, 30% of the sera of group 1 presented a line obtained with the specific T. cruzi antigenic extract.
Chagas' disease and 39 out of 54 (72.2 per cent) sera with a negative one showed precipitation line 5, but these results were not statistically different (P > 0.05).

No sera of leishmaniasis group 3 and of control group 4 presented any precipitation line.

In leishmaniasis group 2, with a possible associated T. cruzi infection, 13 out of the 41 (31.7 per cent) sera showed the presence of precipitation line 5.

Since the IF detection limit was 1:4 for L. braziliensis and T. cruzi serologies, it was easy to compare titres of both serologies. As shown in table 3, an IF titre higher for L. braziliensis than for T. cruzi did not imply the absence of Chagas' disease, or inversely a higher one for T. cruzi than for L. braziliensis did not eliminate leishmaniasis, since equal titres or inversed serologies could be observed in chagasic group 1 and in

### TABLE 2

Frequencies of precipitation line 5 in MD in the different groups of patients.

| Patients groups          | n    | MD+ | %
|-------------------------|------|-----|---
| 1. Chagas' disease      | 374  | 300 | 80.2
| Xeno +                  | 88   | 74  | 84.1
| Xeno -                  | 54   | 22  | 41.3
| 2. Possible mixed infection | 41  | 13  | 31.7
| 3. Mucocutaneous leishmaniasis | 45  | 0   | 0.0
| 4. Control              | 67   | 0   | 0.0

n = number of sera; MD+ = number of sera with line 5 in MD; Xeno +/- = positive or negative xenodiagnosis.

The high frequency (96 per cent) with parasitological or serological usefulness among other symptoms of Chagas' disease.

The analytical properties of evaluating the frequency of T. cruzi patients with negative xenodiagnosis was observed in 70 per cent of the sera (p < 0.05) and in 80.2 per cent in the observed in 70 per cent of the sera. It is noteworthy: patients with negative xenodiagnosis can be observed in the control group, xenodiagnosis and 39 out of 54 (72.2 per cent) sera with a negative one showed precipitation line 5, but these results were not statistically different (P > 0.05).

### TABLE 3

Relation between the T. cruzi and L. braziliensis IF serologies and the frequency of the precipitation line 5.

<table>
<thead>
<tr>
<th>Serum groups</th>
<th>Total</th>
<th>IF Results</th>
<th>MD Results</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>groups</td>
<td>n</td>
<td>%</td>
<td>MD+</td>
</tr>
<tr>
<td>1. Chagas' disease</td>
<td>88</td>
<td>Tc+, Lb+</td>
<td>64</td>
<td>72.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tc &gt; Lb</td>
<td>47</td>
<td>53.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tc &lt; Lb</td>
<td>12</td>
<td>13.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tc&gt; Lb</td>
<td>5</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tc-, Lb-</td>
<td>24</td>
<td>27.3</td>
</tr>
<tr>
<td>2. Mucocutaneous Leishmaniasis (mixed infection)</td>
<td>41</td>
<td>Tc+, Lb+</td>
<td>15</td>
<td>36.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tc &gt; Lb</td>
<td>8</td>
<td>19.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tc = Lb</td>
<td>4</td>
<td>9.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tc &lt; Lb</td>
<td>3</td>
<td>7.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tc+, Lb-</td>
<td>1</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tc-, Lb+</td>
<td>22</td>
<td>53.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tc-, Lb-</td>
<td>3</td>
<td>7.3</td>
</tr>
<tr>
<td>3. Mucocutaneous Leishmaniasis</td>
<td>45</td>
<td>Tc+, Lb+</td>
<td>2</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tc &gt; Lb</td>
<td>1</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tc &lt; Lb</td>
<td>1</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tc-, Lb+</td>
<td>28</td>
<td>62.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tc-, Lb-</td>
<td>15</td>
<td>33.3</td>
</tr>
<tr>
<td>4. Controls</td>
<td>67</td>
<td>Tc-, Lb-</td>
<td>67</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Tc+/− = positive or negative IF for T. cruzi; Lb+/− = positive or negative IF for L. braziliensis; MD+ = number of sera with line 5 in MD.
leishmaniasis group 2. No relation could be noted between higher levels of titres of T. cruzi patients on L. braziliensis antigen and the frequency of precipitation line 5 in MD.

Discussion

The high frequency (96 per cent) of positive IEP in sera from patients with parasitological or serological confirmation of T. cruzi infection, suggests its usefulness among other serological tests for the immunological diagnosis of Chagas’ disease.

The analytical properties of the Immunoprecipitation tests were exploited evaluating the frequency of the T. cruzi specific anti-5-precipitating antibodies. By using a double diffusion microtest (MD) line 5 was found in 84.2 per cent of the sera of parasitologically confirmed T. cruzi infection and in 80.2 per cent in the serologically confirmed ones, and it was observed in 70 per cent of the sera from both groups by immunoelectrophoresis (IEP). It is noteworthy that MD was still positive in 72.2 per cent patients with negative xenodiagnosis. Moreover, no false positivity could be observed in the control group and in patients with mucocutaneous leishmaniasis and negative T. cruzi serology. Such results confirm the high specificity and immunogenicity of the component 5 previously demonstrated with rabbit hyperimmune sera (Afchain et al., 1978, 1979) and in a preliminary work in IEP with a few human sera (Afchain et al., 1970). Consequently in the group of patients with possible mixed infection the 31.7 per cent of the patients with a positive MD test had associated Chagas’ disease. The remaining 68.3 per cent with a negative MD test perhaps had an associated Chagas’ disease or, more likely, antibodies cross-reacting with Leishmania antigens.

The higher sensitivity of line 5 detection in MD (84.2 per cent) than in IEP (70.0 per cent) can be explained by the absence, in MD, of the electrophoretic step, necessary to identify component 5 in IEP. Therefore, a higher antigen concentration is available in MD for diffusion, which, moreover, allows easier identification of line 5 by using the identity reaction with rabbit specific anti-component 5 immune serum. However, other serological tests, such as IF, CFT or ELISA, though demonstrated to be less specific, obtained a better relative sensitivity (Camargo et al., 1977; Fuchs et al., 1980; Brenière et al., in press). Consequently, the systematic use of MD for the Immunodiagnosis of Chagas’ disease, instead of more sensitive tests is unsuitable. On the contrary, MD would be useful for sera showing positive serological reactions with other flagellate antigens and/or sera from patients living in areas known to be endemic for flagellate infections (leishmaniasis, Chagas’ disease and T. rangeli infection). It is extremely important to obtain such confirmation of Chagas’ disease, since clinical and therapeutic management of leishmaniasis and Chagas’ disease are very different.

MD, with identity reaction (Fig. 2), is a technique simple to perform, as previously shown for other parasitic diseases (Yarzabal et al., 1978; Bout et al., 1979). It requires little human or rabbit serum or T. cruzi antigenic extract and allows simultaneous study of many sera on microscope
slide. The specific anti-component 5 serum is easy to produce. Forty ml of a high quality serum from one immunized rabbit allow 40,000 patients sera to be tested. Moreover, human sera with a major precipitation line to antigen 5 could be selected by IEP or immunodiffusion and used instead of the rabbit antisera.

In conclusion, immunoprecipitation allows the detection of T. cruzi specific antibodies by using a crude antigenic extract and it avoids the preparation of time-consuming and low yield, highly purified antigen. Moreover it does not require enzyme- or radioactive-labelled reagents and it is cheap and simple to perform. Thus, immunoprecipitation-in-gel tests can be recommended in association with the other serological tests, when highly specific immunodiagnosis of Chagas’ disease is required.

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L'intérêt des tests d’immunoprécipitation dans le diagnostic immunologique de la maladie de Chagas.

Résumé — Les tests d’immunélectrophorèse et de microimmunodiffusion ont été évalués pour le diagnostic de la maladie de Chagas, à l’aide de 527 sérums de patients boliviens. La spécificité des tests était liée à l’identification des anticorps précipitants anticomposants dont la spécificité pour Trypanosoma cruzi a déjà été démontrée. L’IEP a montré de 1 à 14 lignes de précipitation dans 95 p. cent des sérums, confirmés parasitologiquement (xénon-diagnostic positif) ou sérologiquement, alors que dans les sérums de contrôle, elle était négative. La ligne de précipitation 5, identifiable par son profil particulier, était présente dans 70 p. cent des mêmes sérums. En MD, la ligne de précipitation 5 identifiée par la réaction d’identité avec un sérum de lapin spécifique anti 5 était présente dans 80,2 p. cent de tous les sérums sérologiquement positifs : 82,1 p. cent des sérums avec un xénotest négatif (P 0,05).

La ligne 5 n’a été trouvée ni dans le groupe de leishmanioses (provenant de régions sans maladie de Chagas) ni dans le groupe de contrôle. Dans un autre groupe de leishmanioses, provenant d’une zone endémique pour les deux infections, 31,7 p. cent des sérums étaient positifs pour la ligne 5, indiquant une association avec la maladie de Chagas. Par conséquent, le test d’immunoprécipitation, identifiant la ligne 5 spécifique de T. cruzi, n’est pas cher et est facilement réalisable. Il peut donc être recommandé en association avec d’autres tests sérologiques plus sensibles quand un diagnostic immunologique très spécifique de la maladie de Chagas est requis.

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Los trabajos de Lumsden (1967), Sears et al. (1975) y la técnica de concentración rápida de Sangre (1978) han establecido la importancia de la concentración de sangre para el diagnóstico de Chagas. La técnica clásica utiliza tubos de microhematocrito, pero la versión rápida utiliza tubos de microhematocrito heparinizados. La concentración rápida es más rápida y puede ser realizada en caso de emergencia.

La técnica clásica se utiliza para el diagnóstico de enfermedad de Chagas en base a la concentración de sangre y la técnica rápida se utiliza para la concentración de sangre en caso de emergencia. Ambas técnicas son útiles para el diagnóstico de enfermedad de Chagas y malaria.

**Keywords:** Chagas' Disease; Malaria.