

THE INTEREST OF IMMUNOPRECIPITATION TESTS IN THE IMMUNOLOGICAL DIAGNOSIS OF CHAGAS' DISEASE

by

Y. CARLIER¹, F. S. BRENIERE², L. J. LEMESRE³, R. CARRASCO², P. DESJEUX², D. AFCHAIN³

¹Laboratoire de Parasitologie, Faculté de Médecine U. L. B.,
Bd. de Waterloo 115, B-1000 Bruxelles

²Instituto Boliviano de Biología de Altura (IBBA), La Paz, Bolivie

³Centre d'Immunologie et de Biologie Parasitaire (CIBP),
Institut Pasteur, Lille, France

Summary — Immuno-electrophoresis (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 : Immuno-electrophoresis; 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 & Brener, 1966; 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 Guerreiro & 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.*, 1956; 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 helminthiasis, 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, 1983). 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.* (1970), 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 micro-test (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* (Brenière *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) 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 muco-cutaneous leishmaniasis (35 with primary cutaneous lesions and 10 with mutilations of the face). They lived in the northern low lands (Beni & Alto Beni), areas known 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 Decker-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/1v), 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/1v) 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 subscapular 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 lyophilisation (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).

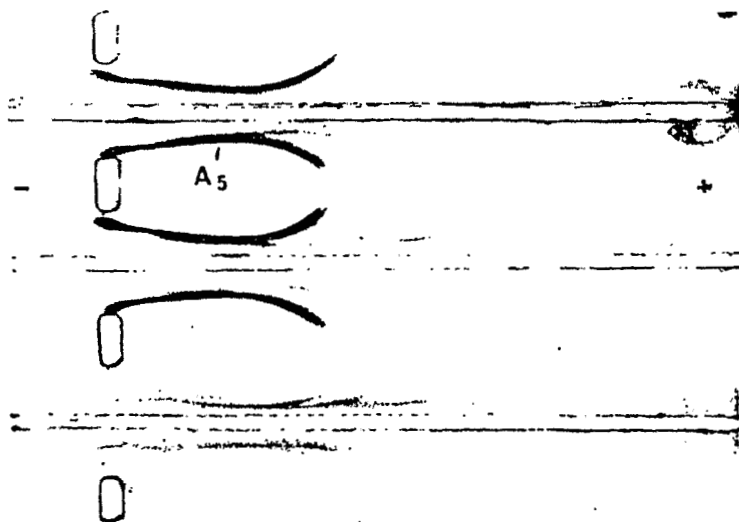


Figure 1.
Positive immunoelectrophoresis showing line 5 (A5) specific of *T. cruzi*.

MD was performed on microscope slides (25 × 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 μ l of human serum, concentrated to 12 μ l by lyophilization, were placed in four peripheral wells, whereas 12 μ l 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 μ l (24 μ g) of *T. cruzi* antigenic extract. The slides were incu-

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 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	39	72.2
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.

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 both in chagasic group 1 and in

TABLE 3
Relation between the *T. cruzi* and *L. braziliensis* IF serologies
and the frequency of the precipitation line 5

Serum groups	Total N	IF Results			MD Results	
		groups	n	%	MD+	%
1. Chagas' disease	88	Tc+, Lb+	64	72.7	57	89.0
		Tc > Lb	47	53.4	41	87.2
		Tc = Lb	12	13.6	12	100.0
		Tc < Lb	5	5.7	4	80.0
		Tc+, Lb-	24	27.3	17	70.8
2. Mucocutaneous Leishmaniasis (mixed infection)	41	Tc+, Lb+	15	36.6	12	80.0
		Tc > Lb	8	19.5	6	75.0
		Tc = Lb	4	9.7	3	75.0
		Tc < Lb	3	7.3	3	100.0
		Tc+, Lb-	1	2.4	1	100.0
3. Mucocutaneous Leishmaniasis	45	Tc-, Lb+	22	53.6	0	0.0
		Tc-, Lb-	3	7.3	0	0.0
		Tc+, Lb+	2	4.4	0	0.0
		Tc > Lb	1	2.2	0	0.0
		Tc < Lb	1	2.2	0	0.0
4. Controls	67	Tc-, Lb+	28	62.2	0	0.0
		Tc-, Lb-	15	33.3	0	0.0

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 as 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 antiserum.

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.

Acknowledgements — We are grateful for the diligent assistance of Hortensia Miguez, Clara Camacho & Martine Bailly.

This study has been supported by French Ministry of Foreign Affairs by Ministry of Research and Industry (Grant n° PVD/81-L11423); Belgian FNRS (Grant n° 1.5.603.83F); EEC Grant n° TS-M 024B (RS); WHO (Special program for research and training in tropical diseases).

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

Résumé — Les tests d'immunoélectrophorèse 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 96 p. cent des sérums, confirmés parasitologiquement (xénodiagnostic 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 : 84,1 p. cent des sérums avec un xénodiagnostic 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 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.

REFERENCES

- Afchain, D., Fruit, J., Yarzabal, L. & Capron, A. (1978) : Purification of a specific antigen of *Trypanosoma cruzi* from culture forms. *Am. J. Trop. Med. Hyg.*, 27 : 478-482.
- Afchain, D., Le Ray, D., Fruit, J. & Capron, A. (1979) : Antigenic make-up of *Trypanosoma cruzi* culture forms : identification of a specific component. *J. Parasitol.*, 65 : 507-514.
- Afchain, D., Capron, A. & Prata, A. (1970) : Les anticorps précipitants dans la trypanosomiase américaine humaine. *Gaz. Med. Bahia*, 3 : 141-147.
- Aguilar-Torres, F. G., Rytel, M. W. & Kagan, I. G. (1976) : Comparison of counter immunoelectrophoresis (CIE) with other serologic tests in the detection of antibodies to *Trypanosoma cruzi*. *Am. J. Trop. Med. Hyg.*, 25 : 667-670.
- Alvarez, M., Cerisola, J. A. & Rohweder, R. W. (1968) : Test de immunofluorecencia para diagnostico de la enfermedad de Chagas. *Bol. Chil. Parasitol.*, 23 : 4-9.
- Anthony, R. L., Cody, T. S. & Constantine, N. T. (1981) : Antigenic differentiation of *Trypanosoma cruzi* and *Trypanosoma rangeli* by means of monoclonal-hybridoma antibodies. *Am. J. Trop. Med. Hyg.*, 30 : 1192-1197.
- Anthony, R. L., Johnson, C. M. & Sousa, O. E. (1979) : Use of micro-ELISA for quantitating antibody to *Trypanosoma cruzi* and *Trypanosoma rangeli*. *Am. J. Trop. Med. Hyg.*, 28 : 969-973.

- Biguet, J., Rose, F., Capron, A. & Tran Van Ky (1965) : Contribution de l'analyse immuno-électrophorétique à la connaissance des antigènes vermineux. Incidences pratiques sur leur standardisation, leur purification et le diagnostic des helminthiases par immuno-électrophorèse. *Rev. Immunol. (Paris)*, **29** : 5-15.
- Bout, D., Carlier, Y. & Capron, A. (1979) : Immunodiagnosis of hydatidosis using a mono-specific immune serum Anti-Ag. 5. *Biomedicine*, **31** : 214-215.
- Brenière, S. F., Carrasco, R., Miguez, H., Lemesre, J. L. & Carlier, Y. (1985) : Comparisons of immunological tests for serodiagnosis of Chagas disease in Bolivian patients. *Trop. & Geogr. Med.*, a, in press.
- Brenière, F. S., Carrasco, R., Molinado, S., Lemesre, J. L., Desjeux, P., Afchain, D. & Carlier, Y. : Specific immunodiagnosis of chagas disease : immunodiffusion test using a specific serum anti *Trypanosoma cruzi* component 5. *Z. Parasitenk.*, b, in press.
- Bronzina, A. A., D'Alessandro, A. & Segura, E. (1980) : Diferencias y similitudes antigenicas entre *T. rangeli* y *T. cruzi*. *Medicina (Buenos Aires)*, **40** : 45-49.
- Camargo, M. E. (1966) : Fluorescent antibody test for the serodiagnosis of American trypanosomiasis. Technical modification employing preserved culture forms of *Trypanosoma cruzi*. *Rev. Inst. Med. Trop. São Paulo*, **8** : 227-234.
- Camargo, M. E., Hoshino-Shimizu, S., Macedo, F., Peres, B. V. & Castro, C. (1977) : Diagnostico serologico da infecção humana pelo *Trypanosoma cruzi*. Estudo comparativo de testes de fixação do complemento, imunofluorescência, hemaglutinação e floculação em 3624 soros. *Rev. Inst. Med. Trop. São Paulo*, **19** : 254-260.
- Camargo, M. E., Hoshino-Shimizu, S. & Siqueira, G. R. V. (1973) : Hemagglutination with preserved, sensitized cells, a practical test for routine serologic diagnosis of American trypanosomiasis. *Rev. Inst. Med. Trop. São Paulo*, **15** : 81-85.
- Camargo, M. E. & Rebonato, C. (1969) : Cross-reactivity in fluorescence test for *Trypanosoma* and *Leishmania* antibodies. A simple inhibition procedure to ensure specific results. *Am. J. Trop. Med. Hyg.*, **18** : 500-505.
- Carlier, Y. & Wéry, M. (1983) : Méthodes actuelles de diagnostic immunologique en parasitologie. *Ann. Biol. Clin.*, **41** : 435-444.
- Cerisola, J. A., Chaben, M. F. & Lazzari, J. O. (1962) : Test de hemaglutinacion para el diagnostico de la enfermedad de chagas. *Prensa Med. Argentina*, **49** : 1761-1767.
- Cerisola, J. A., Rohweder, R., Segura, E. L., Del Prado, C. E., Alvarez, M. & De Martini, G. J. (1974) : in « El xenodiagnostico », Instituto Nacional de diagnostico e investigacion de la enfermedad de Chagas « Dr. Mario Fatala Chaben », Buenos-Aires.
- Chaffe, E. F., Fife, E. H. & Kent, J. F. (1956) : Diagnosis of *Trypanosoma cruzi* infection by complement fixation test. *Am. J. Trop. Med. Hyg.*, **5** : 763-771.
- Chiari, E. & Brener, Z. (1966) : Contribuição no diagnóstico parasitológico de doença de Chagas na sua fase crônica. *Rev. Inst. Med. Trop. São Paulo.*, **8** : 134-138.
- Deker-Jackson, J. F. & Honigberg, I. B. M. (1978) : Glycoproteins released by *Leishmania donovani* : immunological relationship with host and bacteriae antigen and preliminary biochemical analysis. *J. Protozool.*, **25** : 514-525.
- Duxbury, R. E. & Sadun, E. H. (1964) : Fluorescent antibody test for the serodiagnosis of visceral leishmaniasis. *Am. J. Trop. Med. Hyg.*, **13** : 525-529.
- Fife, E. H. & Muschel, L. H. : Fluorescent antibody technique for serodiagnosis of *Trypanosoma cruzi* infection. *Proc. Soc. Exp. Biol. Med.*, **101** : 540-543.
- Fuchs, A. P., Fioratti, V. L., Mello, V. A. & Boainain, E. (1980) : Diagnostico serologico na doença de Chagas. Estudos comparativos de diferentes tecnicas. *Rev. Inst. Med. Trop. São Paulo*, **22** : 242-245.
- Gam, A. A. & Neva, F. A. (1977) : Comparison of cell culture with epimastigote antigens of *Trypanosoma cruzi*. *Am. J. Trop. Med. Hyg.*, **26** : 47-57.
- Guerreiro, C. & Machado, A. (1913) : Da reação de Bordet e Gengou na molestia de Carlos Chagas como elemento diagnostico. *Brasil Médico*, **27** : 225-226.
- Guimarães, M. C., Celeste, B. J., Ayres, E. C., Mineo, J. R. & Diniz, J. M. P. (1981) : Immunoenzymatic assay (ELISA) in mucocutaneous leishmaniasis, Kala-Azar and Chagas' disease : an epimastigote *Trypanosoma cruzi* antigen able to distinguish anti-*Trypanosoma* and anti-*Leishmania* antibodies. *Am. J. Trop. Med. Hyg.*, **30** : 942-947.
- Guimarães, M. C., Giovannini, V. L. & Camargo, M. E. (1974) : Antigenic standardization for mucocutaneous leishmaniasis immunofluorescent test. *Rev. Inst. Med. Trop. São Paulo*, **16** : 145-148.
- Knight, R. A., Rocha, H. & Kaye, D. (1976) : Comparison of the complement fixation test and counter-electrophoresis test for the detection of antibodies in Chagas' disease. *J. Clin. Microbiol.*, **3** : 67-69.
- Le Ray, D. (1975) : Structure antigénique de *Trypanosoma brucei* (Protozoa, Kinetoplastida). Analyse immunoélectrophorétique et études comparatives. *Ann. Soc. Belge de Méd. Trop.*, **55** : 129-311.

- Muniz, J. (1947) : Do valor da reação de precipitina no diagnostico das formas agudas e subagudas da doença de Chagas (*Trypanosomiasis americana*). Mem. Inst. Oswaldo Cruz, 45 : 537-549.
- Neal, R. A. & Miles, R. A. (1977) : The sensitivity of culture methods to detect experimental infections of *Trypanosoma cruzi* and comparison with xenodiagnosis. Rev. Inst. Med. Trop. São Paulo, 19 : 170-176.
- Neal, R. A. & Miles, R. A. (1970) : Indirect hemagglutination test for Chagas' disease with a simple method for survey work. Rev. Inst. Med. Trop. São Paulo, 12 : 325-332.
- Nery-Guimarães, F., Lage, H. A., Venancio, I. A. & Grynberg, N. F. (1969) : Estudo comparativo da reação indirecta de anticorpos fluorescentes em doença de Chagas, leishmanioses tegumentares e calasar com varios antigens de *Leishmania* e *Trypanosoma*. O Hospital (Rio de Janeiro), 75 : 1811-1825.
- Nilsson, L. A. & Voller, A. (1982) : A comparison of thin layer immunoassay (TIA) and enzyme linked immunosorbent assay (ELISA) for the detection of antibodies to *Trypanosoma cruzi*. Trans. Roy. Soc. Trop. Med. Hyg., 76 : 95-97.
- Pellegrino, J., Brener, Z. & Jacomo, R. (1956) : A reação de precipitina na fase aguda da doença de Chagas. Rev. Brasil. Malariol., 8 : 247-2525.
- Petana, W. B. (1975) : Sensitivity of the indirect fluorescence test for Chagas' disease in large scale serology surveys. Pan. Am. Hlth. Org., 318 : 289-291.
- Pifano, F. C., Morrel, J. R. & Ortiz, M. D. (1973) : Estudio comparativo entre el *Rhodnius prolixus* (Stal 1849) y el *Triatoma pallidipennis* (Stal 1872, Pinto 1927) en la prueba xenodiagnostico realizada en casos cronicos de enfermedad de Chagas. Arch. Venez. Med. Trop., 5 : 85-94.
- Romaña, C. (1961) : Aplicacion del metodo de hemaglutinacion al diagnostico de la enfermedad de chagas. Rev. Soc. Arg. Biol., 37 : 73-76.
- Sadun, E. H., Duxbury, R. E., Williams, J. S. & Anderson, R. I. : Fluorescent antibody test for the serodiagnosis of African and American trypanosomiasis in man. J. Parasitol., 49 : 385-388.
- Salfelder, A. & Mannweiler, E. (1981) : Immunodiagnostische Befunde an Seren von Leishmaniose, Chagas, Malaria und Amöbiasis Patienten in Endemiegebieten Venezuelas. Tropenmed. Parasitol., 32 : 194-196.
- Schechter, M., Voller, A., Marinkelle, C. S., Flint, J. E., Guhl, F. & Miles, M. A. (1983) : Purified *Trypanosoma cruzi* specific glycoprotein for discriminative serological diagnosis of South American trypanosomiasis (Chagas' disease). Lancet, 2 : 934-941.
- Sousa, O. E. & Johnson, C. M. (1971) : Frequency and distribution of *Trypanosoma cruzi* & *Trypanosoma rangeli* in the Republic of Panama. Am. J. Trop. Med. Hyg., 20 : 405-410.
- Spencer, H. C., Allain, D. S., Sulzer, A. J. & Collins, W. E. (1980) : Evaluation of the micro enzyme-linked immunosorbent assay for antibodies to *Trypanosoma cruzi*. Am. J. Trop. Med. Hyg., 29 : 179-182.
- Tandon, A., Zahner, H. & Lämmler, G. (1979) : CELISA (complement enzyme-linked immunosorbent assay) : a new method for the estimation of complement fixing antibodies; its use for Chagas' disease. Tropenmed. Parasitol., 30 : 189-193.
- Vaitukaitis, J., Robbins, J. B., Nieschlag, E. & Ross, G. T. (1971) : A method for producing specific antigen with small doses of immunogen. J. Clin. Endocr., 33 : 988-991.
- Vattuone, N. H. & Yanovsky, J. F. (1975) : *Trypanosoma cruzi* : agglutination activity of enzyme treated epimastigote. Exp. Parasitol., 30 : 349-355.
- Voller, A., Draper, C., Bidwell, D. E. & Bartlett (1975) : Microplate enzyme linked immunosorbent assay for Chagas' disease. Lancet, 1 : 426-429.
- Yarzabal, L. A., De Albornoz, M. B., De Cabral, M. A. & Santiago, A. R. (1978) : Specific double diffusion microtechnique for the diagnosis of aspergillosis and paracoccidioidomycosis using monospecific antisera. Sabouraudia, 16 : 55.