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IMMUNOPEROXIDASE TECHNIQUE IN EXPERIMENTAL CHRONIC CHAGASIC MYOCARDITIS

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SUMMARY

Chagas'disease has been described as the commonest form of chronic myocarditis. An immunologic pathogenesis has been discribed for this form of the disease. So far, no immunoperoxidase technique has been used for the detection of immunological deposits in chromic experimental Chagas'myocardiopathy. Forty-one Swiss mice, three months old were inoculated intraperitoneally with doses between 10 and 10^5 Tulahuen trypomastigotes. Mice were reinoculated one month after with doses between 10^2 and 10^5 and sacrificed at 6 (n=21) and 9 months (n=9) after the first inoculation. ECGs were recorded before sacrifice. Immunoperoxidase technique (peroxidase-antiperoxidase method), immunofluorescence (direct and indirect) as well as histological studies were performed in myocardiums and skeletal muscles of the surviving animals. The most sensitive methods for detecting chronic chagasic infection were the routine histologic studies (73%) and the ECGs 83% and 89% on 6 and 9 mo. post-infected mice, respectively. Myocardial involvement varied from interstitial mild focal lymphocyte infiltrates up to replacement of myocytes by loose connective tissue. Atrial myocardiums (21/23, 91%) were more affected than ventricles (9/23, 39%). Typical chagasic nests were rarely found. Skeletal muscle involvement (11/18 and 7/9) varied from mild to extensive lymphocyte and plasmacell infiltrates, and necrotic fibers. The involved antigen were shown in skeletal muscles by the immunoperoxidase technique as diffusely arranged granular intracytoplasmatic deposit for both IgC and total immunoglobulins. The coincidence between this technique and histologic muscle lesions was 11/18 (61%) in 6 mo. and 6/8 (75%) at 9 mo. post-infection. In heart, delicate granular deposits of total immunoglobulins were seen diffusely arranged within the ventricular myocytes; coincidence between immunoperoxidase technique anl histologic involvement increased from 36 to 66% in animals sacrifeced 6 and 9 mo. post-infection. This strongly stressed the increase of immunologic phenomena with the chronification of infection. Concerning sensitivity, immunoperoxidase and direct immunofluorescence were highly sensitive in skeletal muscle (100%, $p < 0.01$). Conversely, direct immunofluorescence technique showed poor results in heart while immunoperoxidase increased its sensitivity from 21.4% (at 6 mo.) to 66.6% (at 9 mo.) post-infection ($p < 0.001$). Considering the necessity of obtaining an adequate vaccine in order to prevent this disease an experimental model like this, rendering immunological reactions as revealed by the immunoperoxidase technique, would be useful.

KEY WORDS: Chagas'disease; Immunoperoxidase technique; Chronic myocarditis

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INTRODUCTION

Among the many known causes of myocarditis, Chagas'disease has been described as the commonest form of chronic myocarditis in the world [11]. Reports of lines of research seem to demonstrate an immunological pathogenesis for this form of the disease [6,7,24,26].

Our team recently found deposits of immunoglobulins and C_3 detected "in vivo" by immunoperoxidase in myocardial biopsies of patients suffering from chronic Chagas'disease [20].

Despite these immunopathological progresses no immunoperoxidase technique has been used for the detection of immunological deposits in chronic experimental Chagas'myocardiopathy. We report here the method used in this department and the results obtained in the detection by immunoperoxidase technique of heart antibodies in experimental Chagas'disease, in comparison with electrocardiographic, histological and immunofluorescense patterns of alterations detected.

MATERIAL AND METHODS

1) **Animals.** Forty-one Swiss mice three-months old were used. The experimental scheme is detailed on Table I. Fourteen normal mice of the same age and weight were used as controls. ECGs were recorded before sacrifice with a Sanborn Twin Viso electrocardiograph at a speed of 100 mm/sec [18].

TABLE I
Experimental chagas'disease in the mice

Number of animals	First inoculation (*)	Second inoculation (*)	Dead animals	Sacrificed animals (**)
6	20 T.c. (+)	10^2 T.c.	1	6th month (n=5)
12	20 T.c.	10^3 T.c.	3	6th month (n=9)
6	20 T.c.	10^4 T.c.	2	6th month (n=4)
6	20 T.c.	10^5 T.c.	3	6th month (n=3)
5	20 T.c.	10^4 T.c.	—	9th month (n=5)
6	10 T.c.	—	2	9th month (n=4)

(*) Number of intraperitoneally inoculated parasites
(**) After either 6 or 9 months first inoculation
(+) Tulahuen trypomastigotes of forms of **Trypanosoma cruzi**

2) **Immunoperoxidase technique.** Specimens: The hearts and the sections of skeletal moscle were fixed in neutral formaldehyde (pH 7.0), embedded in paraffin and cut in 6 micra serial sections.

Peroxidase-antiperoxidase method was performed as described by STERNBERGER[23], using different dilutions of primary antiserum, ranging from 1/250 to 1/500. Primary antisera: 1) antibodies against mouse immunoglobulins (Dako Lab); 2) antibodies against mouse IgG (Sigma Chemical); 3) Linking antisera and 4) PAP complex were obtained from Dako Lab. Peroxidase activity was developed with 30 mg % 3.3'-diaminobenzidine tetrahydrochloride in 0.01% aqueous hydrogen peroxide.

Specific anti-G immunoglobulins and anti-total immunoglobulins antibodies titer of antisera were compared with antikeration antisera (Dako Lab).

Other negative controls were performed by incubating control sections with 0.05 M tris/HCl buffer (pH 7.6) replacing the primary antisera.

Spleens of normal mice were used as positive controls for immunoglobulins. Several tests, as detailed in figure 1, were made in order to assure the reliability of the technique.

3) **Immunofluorescence technique.** Frozen sections were used and indirect immunofluorescence technique was employed on heart slices, skeletal muscle of murine and bovine origin,

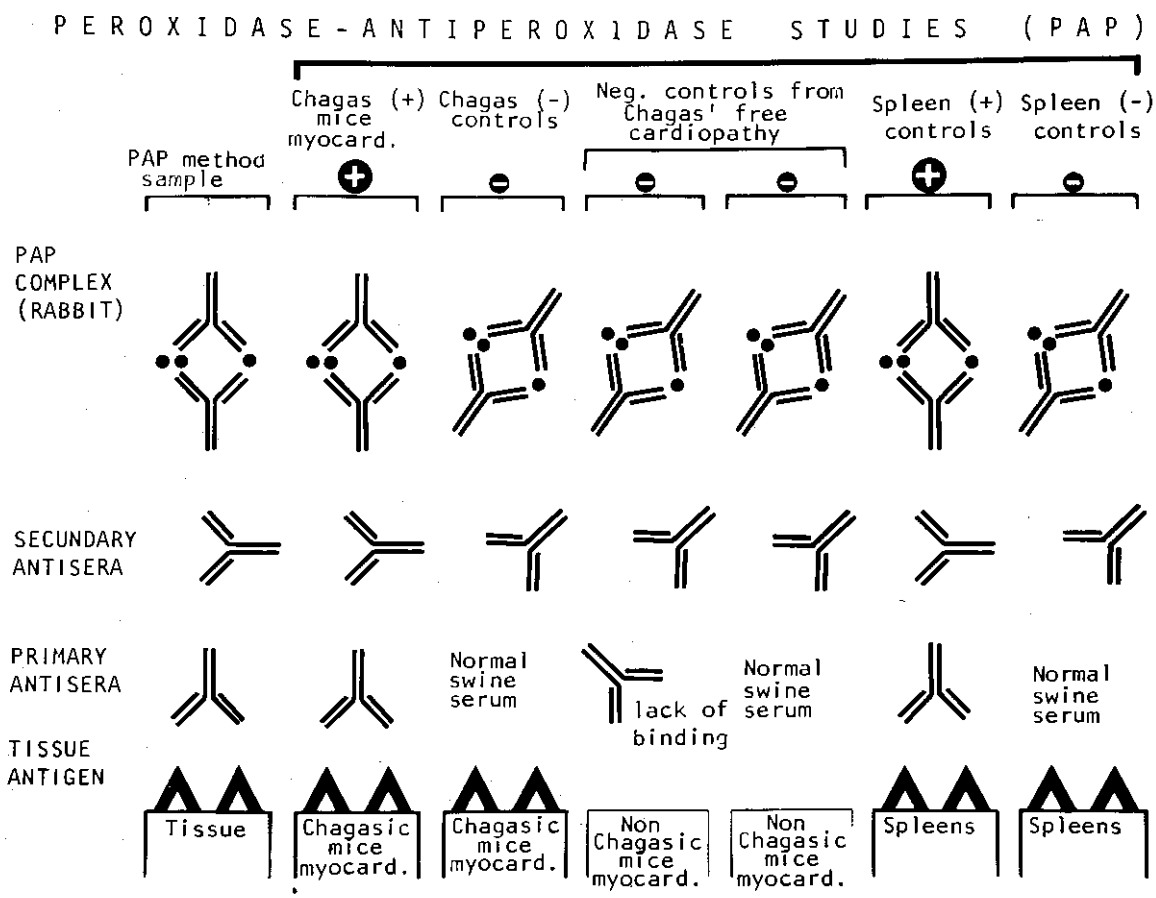

Fig. 1 — Immunohistochemical (PAP) tests performed on mice myocardium and spleens.

and stomach and liver of mice. The conjugate used was a goat anti-mouse immunoglobulin coupled to fluorescein (Cappel Lab. USA) (FITC) at dilutions of 1/32 and 1/64. Some positive sera from anti-striated muscle antibodies were adsorbed with skeletal muscle or bovine heart homogenates, and then again tested by means of indirect immunofluorescence technique [10]. Direct immunofluorescence technique was performed according to already published methods (see references 6,7).

4) Histological studies. Sections were stained with Mayer's hematoxilyn-eosin, Barbeito-Lopez Trichome stain [17] and Nissl technique.

Grading of lesions were assessed according to the following categorization: —: no lesions; +: mild lymphocyte infiltrates; ++: diffusely distributed moderate lymphocyte infiltrates; +++: abundant lymphocyte infiltrates with involvement of myocytes or neurons or skeletal fibers and even replacement of these cells by loose connective tissue.

5) Statistical studies. Fisher's exact probability test was used to compare sensitivity between techniques after 6 and 9 months post infection.

RESULTS

The most sensitive methods were the routine histologic studies and the electrocardiographic recordings. As already reported [18], ECGs showed pathological changes in 15/18 (83%) and 8/9 (89%) on 6 and 9 month post-

infected mice, respectively (Figure 2). When both methods were considered together, sensitivity came up to 100%. The routine histologic sections showed a high percentage of myocardial lesions (14/19, 73%). In brief, myocardial involvement varied from interstitial mild focal lymphocyte infiltrates up to replacement of myocytes by loose connective tissue (Figure 3) Atrial specimens were more affected (21/23, 91.3%) than ventricle ones (9/23, 39.1%).

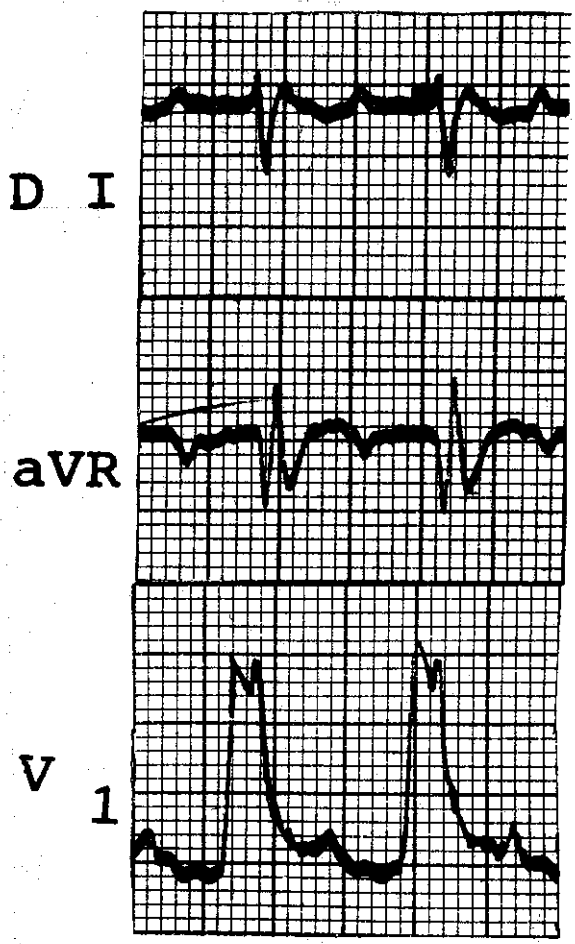

Fig. 2 — Mouse ECG. Six months post chagasic infection. A wide S wave in D_1, a r' wave in Avr and a wide and high notched QRS complex in V_1 precordial lead is shown, suggesting a right bundle branch block. Paper speed at 100 mm/sec.

Regarding severity of damage, mild (+) and moderate lesions (++) were most frequent (25/35 and 8/35, 94.3%), while the most severe lesions (+++) were rare (2/35, 5.7%). Typical chagasic cysts were rarely found. Skeletal muscle involvement had a frequency of 11/18 and 7/9 in 6 and 9 months post-infections, respectively.

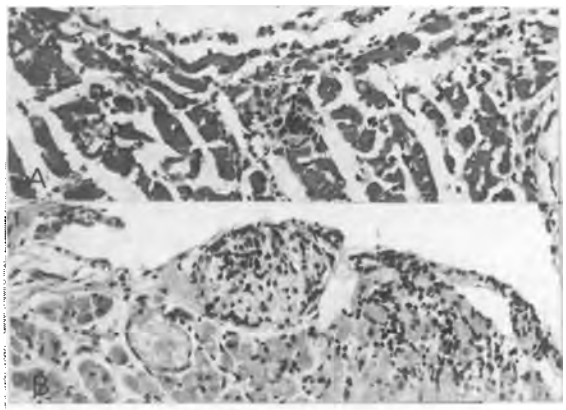

Fig. 3 — Mouse myocardium. A — Mild lymphocyte infiltrate in atrial myocardium. B — Moderate lymphocyte infiltrate surrounding ventricular myocardium fibers H-E (x 500).

Lesions is skeletal muscle fibers varied from mild lymphocyte infiltrates either surrounding nerve branches or in the interstitium up to extensive lymphocyte and plasma cell infiltrates.

The latter cells were seen surrounding vessels and nerve branches, as well as within and among the muscle fascicles (Figure 4). Necrotic fibers were commonly observed. Only in one case amastigotes nests were found. Severity of fiber damage were categorized as being, 8/30, + (26%); 5/30, ++ (13%) and 5/30, +++ (16%).

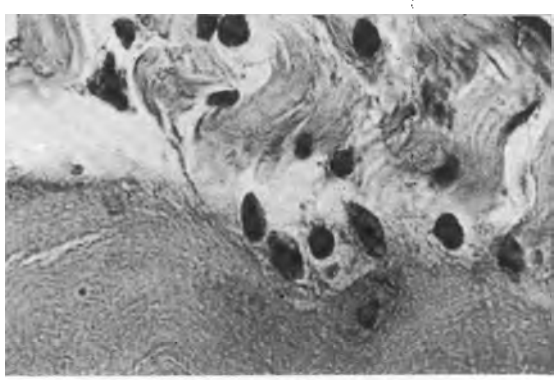

Fig. 4 — Mouse skeletal muscle. Mild lymphocyte infiltrates. Focal damage of sarcolemma (x 500).

Immunopathological studies

Agreement between immunofluorescence and immunoperoxidase technique was present

in 68 and 50% of hearts studied 6 and 9 months post-infection and in 61 and 85% of skeletal muscles at the same time.

Separate analysis of results according to the the patterns of immunoperoxidase and immunofluorescence showed no consistent differences between groups. The following patterns were observed: Skeletal muscle: The antigens had an intracellular localization (Figure 5), for both 6 and 9 months post-infected mice. It was shown by the immunoperoxidase technique as diffusely arranged granular intracytoplasmic deposits. In 6 months post-infected mice, 13/18 showed positive immunoperoxidase deposits for both, IgG and total immunoglobulins. Seven of these mice showed consistent histological lesions related to chronic chagasic infection. It must be pointed out that 4/18 negative cases were also negative from the anatomopathological point of view. Then, the coincidence between the immunoperoxidase technique and histologic muscle lesions was 61% (11/18). In 9 month post-infected mice the positive immunoperoxidase deposits were found in all cases (8/8). The percentage of coincidence between immunoperoxidase and histologic muscle lesions was 75% (6/8).

It was possible to stablish a comparison between immunoperoxidase and direct immunofluorescence technique as referred only to sarcolematic deposits, because the latter did not show intracellular deposits. This percentage of coincidence was 61 and 85% for 6 and 9 months post-infected mice.

In control animals neither intracytoplasmic nor sarcolemmatic deposits were observed.

Heart: In 6 month post-infected mice, delicate granular deposits of total immunoglobulins were seen diffusely within the ventricular myocytes, in 4/9 cases (1/250 and 1/500 dilutions) (Figure 6).

Conversely, IgG deposits were less frequent (2/19) and gave a weak reaction. In no cases was immunoperoxidase activity detected in endothelial cells. As we obtained serial sections for both immunoperoxidase and routine histological technique, a close comparison was made; coincidence between both techniques was observed in 7/19 cases (36%).

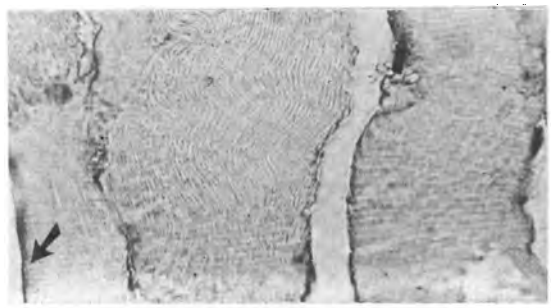

Fig. 5-A

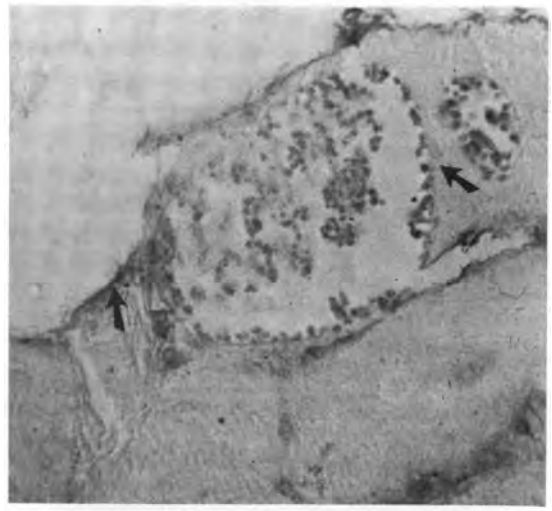

Fig. 5-B

Fig. 5 — Skeletal muscle immunoperoxidase. A — Difusely distributed intracellular and sarcolemmal positive granular staining of total immunoglobulins. B — Amastigote nest; focal sarcolemmal positive staining with total immunoglobulins. Immunoperoxidase technique (PAP) (x 500).

Fig. 6 — Mouse myocardium. Diffusely distributed intracellular (granular) and sarcolemmal (pseudolinear appearance) positive staining of total immunoglobulins (x 190).

Correlation between direct immunofluorescence and pathologic involvement was found in 2/19 cases, while the indirect immunofluorescence coincidence was 8/19.

In 9 month post-infection mice, notorious high coincidence was found between immunoperoxidase and routine histological studies, 6/9 (66%).

In control animals no deposits were observed.

Correlation of results among the different techniques

An important correlation was found between microscopic myocardial involvement and electrocardiographic changes. Correlation between immunopathologic methods (direct immunofluorescence and immunoperoxidase) in heart was adequate (68 and 50%).

Coincidence between immunoperoxidase technique and histologic involvement increased from 36 to 66% in animals sacrificed 6 and 9 months post-infection. This finding strongly stressed the increase of immunologic phenomena with the chronification of infection.

As regards skeletal muscle, a good correlation was found between immunofluorescence and immunoperoxidase technique (61 and 85%), and among histological involvement, direct immunofluorescence and immunoperoxidase technique (38 and 71%). These last figuures stress again the increase in correlations as chronic infection progressed.

Concerning sensitivity, both methods, immunoperoxidase and direct immunofluorescence were highly sensitive in skeletal muscle. (100%, $p < 0.01$) (see Table II).

Conversely, direct immunofluorescence technique showed poor results in heart (7.1 and 37.5%, N.S.), while immunoperoxidase increased its sensitivity from 21.4% (at 6 months post-infection) to 66.6% (at 9 months post-infection) ($p < 0.01$) (Table II).

TABLE II

Sensitivity of the immunopathologic methods (+)

No of mice	6 months post infection	Techniques (•)	9 months post infection	No of mice
A) **Myocardium**				
6/14	42.9%	IIMF*	87.5%	7/8
1/14	7.1%	DIMF	37.5%	3/8
3/14	21.4%	IMPX**	66.6%	6/9
B) **Muscle**				
7/11	63.6%	IIMF**	100%	6/6
7/11	63.6%	DIMF**	100%	6/6
6/11	54.5%	IMPX**	100%	6/6

(+): The histopathology was used as reference
(•): Sensitivity given in percentages
*: $p < 0.10$ (Fischer's Exact Probability Test)
**: $P < 0.01$
IIMF: Indirect immunofluorescence technique
DIMF: Direct immunofluorescence technique
IMPX: Immunoperoxidase technique

DISCUSSION

In this paper, it was shown that both immunoperoxidase and direct immunofluorescence techniques highly correlate with microscopical skeletal muscle involvement (100% of sensitivity at 9 mo. post-infection) ($p < 0.01$), in chronic experimental Chagas infection in mice. Conversely, in myocardium, immunoperoxidase technique gave better results than direct immunofluoorescence technique (the so-called EVI pattern) [6,7,10] as compared histological involvement (66.6 vs 37.5% of sensitivity, at 9 mo. post infection). The localization of the immunoglobulin deposits along the fibers further suggests that they represent an autoimmune reaction to antigens on the fibers and not a trapping of circulating antigen-antibody complexes. In general, there was correlation with the serum EVI antibody detected by indirect immunofluorescence technique.

However, it should be pointed out that both techniques use different substrates (rat myocardium in the immunofluorescence technique and mice infected myocardiums in the immunoperoxidase). Furthermore, immunofluorescence is used as an indirect method for the detection of circulating antibodies, while the immunoperoxidase technique as used by us detects "in vivo" deposited serum factors in a direct way.

At present, the significance of these immunological components remain unknown and interpretation of the role of humoral antibodies in the pathogenesis of Chronic Chagas'myocardiopathy (ChCHM) is very controversial.

The presence of "in vivo" bound immunoglobulins, detected by the direct immunofluorescence technique in myocardial biopsies of 4

EVI (+) chagasic individuals was reported by Cossio et al [9]. Since immunoglobulins were bound to the sarcolemma of myocytes and to the plasma membrane of endothelial cells, authors theorized that they interfere with some of the transmembrane difusion and transport processes [9].

Ribeiro Dos Santos and Hudson [21] suggested that during the acute fase of the disease, parasite antigens are released from the disruption of infected host cells or due to immune lysis of **T. cruzi** and that these parasite antigens could bind to these cells and thus render them susceptible to damage by the host's own immune response.

At present, the originally described "EVI antibody" may be considered as a complex system represented by several antibodies. In this sense, it is likely that the pure endocardial-vascular-interstitial staining is represented by laminin, the major, or even only tissue antigen in this system [26], because passage of sera from Chagas'patients or infected monkeys over laminin-sepharose beads eliminates the tissue reactions.

On the contrary, simultaneous studies from Cossio's laboratory showed that no EVI pattern can be observed on human tissue employing indirect immunofluorescence and absortion test [15-16]. This fact suggested a heterophil nature of the EVI antibody, which renders it incapable of direct pathogenic role. Accordingly, in humans the specificity of EVI antibody for **T. cruzi** infection was only 38% [2].

In a more recent paper, those authors [4] have investigated by the indirect immunofluorescent technique the presence of antibodies in chagasic patients and controls. The patterns observed were intracellular, following striations. Absorption test demonstrated that the cellular reaction is independent of the EVI antibody, and is removed by myosin; IgG was always involved and he antibody did not fix C. These findings in humans closely resemble the patterns of immunoperoxidase found by us in the chronically infected chagasic mice. Also according to Cossio and co-workers' last report the anti-laminin antibody does not represent the EVI antibody, and it only should react with a heterophilic contaminant of the murine laminin, used in Szarfman's experiment [5].

It is conceivable that an autoimmune response, whether primary or secondary could be activated in other clinical conditions in which inflamatory cells and myocardium bound immunoglobulins coexist: idiopathic chronic congestive myocarditis [13], rheumatic fever [14] or heart transplant rejection [22].

As a probable autoimmune reaction is present in ChCHM [23], a reliable and simple technique like immunoperoxidase would be welcome.

Furthermore, files may be reviewed and formaldehyde fixed material both human and experimental [3-8-19], used for different purposes in the study of ChCHM and other heart diseases (see above) could be studied by pathologists with advantage over immunofluorescence. Accordingly, anti myosin [1] and anti-laminin deposits [26] are now being investigated in chronically infected mice in our laboratory.

RESUMEN

Tecnica de inmunoperoxidasa en miocardiopatia chagásica crónica experimental

La enfermedad de Chagas ha sido considerada como una de las causas más frecuentes de miocarditis crónica. Siendo descriptas las alteraciones inmunológicas, como patogenia para este tipo de enfermedad.

Por tal motivo, se empleó la técnica de inmunoperoxidasa para la detección de depósitos de inmunoglobulinas en la miocardiopatía chagásica crónica experimental.

Se utilizaron 41 ratones Swiss de 3 meses de vida, los mismos fueron inoculados intraperitonealmente con dosis entre 10 y 10^5; tripomastigotas de la cepa Tulahuen. La reinoculación se realizó 1 mes después con dosis entre 10^2 y 10^5; siendo sacrificados a los 6 (n=21) y 9 meses (n=9) de la primera inoculación, previos estudios electrocardiográficos.

Posteriormente se estudiaron los miocardios y músculos esqueléticos con técnicas histológicas de rutina, inmunoperoxidasa (método peroxidasa anti-peroxidasa) e inmunofluorescencia (directa e indirecta).

Los metodos más sensibles para la detección de la enfermedad de Chagas crónica resultaron ser los estudios histológicos (73%) y la electrocar-

diografía (83%) a los 6 meses p.i.y (89%) a los 9 meses p.i. (pos-infección).

Se observaron diferentes alteraciones miocárdicas, desde infiltrados linfocitarios leves y focales en intersticio hasta reemplazo de miocitos por tejido conectivo.

Los miocardio auriculares (21/23,91%) fueron más afectados que los ventrículos (9/23, 39%); mientras que las típicos quistes chagásicos resultaron excepcionales

Los musculos esqueléticos (11/18 Y 7/9) presentaron distintos grados de lesión histológica, desdes leves a extensos infiltrados linfoplasmocitarios con presencia de fibras necróticas. Mientras que con la técnica de inmunoperoxidasa, los antígenos se revelaron como depósitos granulares intracitoplasmáticos difusamente distribuidos, tanto para IgG como para Ig totales. La coincidencia entre este método y las lesiones musculares histológicas fueron 11/18 (61%) a los 6 meses p.i.y 6/8 (75%) a los 9 meses p.i.

Por otra parte, los depósitos de lg totales en corazón se observaron dispuestos difusamente, en forma finamente granular dentro de los miocitos ventriculares La coincidencia entre ambas técnicas (inmunoperoxidasa e histología) resultó ser del 36% y 66% para los animales sacrificados a los 6 y 9 meses p.i. respectivamente.

Este fenómeno inmunológico se incrementó notablemente con el curso crónico de la enfermedad. Con respecto a la sensibilidad, tanto la inmunoperoxidasa como la inmunofluorescencia directa fueron altamente sensibles en músculo esquelético (100%, p 0,01).

Por otra parte, la técnica de inmunofluorescencia directa en corazón, evidenció pobres resultados, mientras que el método peroxidasa anti-peroxidasa incrementó su sensibilidad de 21,4% a los 6 meses p.i. al 66.6% a los 9 meses p.i. (p 0.001).

Este modelo experimental, en el cual se observan reacciones inmunológicas reveladas por la técnica de inmunoperoxidasa, podría resultar de utilidad, considerando la necesidad de obtener una vacuna adecuada para prevenir la miocardiopatía chagásica.

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REFERENCES

1. ARRAES-HERMANS, M. A. & TEXEIRA, M. L. — Anti-actin anti-myosin specificity of human antibodies in chronic and acute Chagas disease. In: CONGRESSO INTERNACIONAL SOBRE DOENÇA DE CHAGAS, Rio de Janeiro, 1979. **Anais.** Rio de Janeiro, Ind. Gráf. Cruzeiro do Sul, 1979. p. 195.

2. ATHANAZIO, P. R.; HOFF, R. & ANDRADE, Z. A. — EVI antibodies in individuals of the State of Bahia. In: CONGRESSO INTERNATIONAL SOBRE DOENÇA DE CHAGAS, Rio de Janeiro, 1979. **Anais.** Rio de Janeiro, Ind. Gráf. Cruzeiro do Sul, 1979. p. 200.

3. BOLOMO, N.; MILEI, J.; COSSIO, P. M.; SEGURA, E.; LAGUENS, R. P.; FERNANDEZ, L. & ARANA, R. M. — Experimental Chagas disease in a South American Primate (Cebus sp). **Medicina (B. Aires), 40:** 667, 1980.

4. COSSIO, P. M.; CASANOVA, M.; DIEZ, C.; BELLIDO, C.; ARCE, L.; STORINO, R. A.; DE LA VEGA, M. & ARANA, R. — Autoimmune response against myocardial tissue in Chagas'disease. **Medicine (B. Aires), 45:** 643, 1985.

5. COSSIO, P. M.; DENDUCHIS, B.; CASANOVA, M. B.; PODESTÁ, M. R.; STERIN-BORDA, L.; BORDA, E.; VILA, C.; ARANA, R. M. & LUSTIG, L. — Contrasting behaviour of EVI antigen in Chagas'disease with laminin: Studies on cryostat sections, cultured cells and isolated rat atrial preparation. **Clin. exp. Immunol.** (sent for publication, 1986).

6. COSSIO, P. M.; LAGUENS, R. P.; DIEZ, C.; SZARFMAN, A.; SEGAL, A. & ARANA, R. M. — Chagasic cardiopathy: antibodies reacting with plasma membrane of striated muscle and endothelial cells. **Circulation, 50:** 1252, 1974.

7. COSSIO, P. M.; DIEZ, C.; SZARFMAN, A.; KREUTZER, E.; CANDIOLO, B. & ARANA, R. M. — Chagasic cardiopathy. Demonstration of a serum gamma-globulin factor which reacts with endocardium and vascular structures. **Circulation, 49:** 13, 1974.

8. COSSIO, P. M.; DE LA VEGA, M. T.; BASOMBRÍO, M. A.; CASANOVA, M. B.; BOLOMO, N. J.; MILEI, J.; ARANA, R. M. & ALONSO, D. — Estudios inmunológicos y morfológicos en la enfermedad de Chagas experimental del ratón. **Medicina (B. Aires), 43:** 517, 1983.

9. COSSIO, P. M.; LAGUENS, R. P.; KREUTZER, E.; DIEZ, C.; SEGAL, A. & ARANA, R. M. — Immunopathologic and morphologic studies in myocardial biopsies. **Amer. J. Path., 85:** 533, 1977.

10. COSSIO, P. M.; CORNEJO, L.; HERRERA, M.; VAZQUEZ, C. & ARANA, R. M. — Skeletal and heart muscle antibodies in myasthenia gravis. A new immunofluorescence pattern. **Biomedicine, 19:** 261, 1973.

11. Editorial. New light on Chagas'Disease. **Lancet: 1:** 1150, 1965.

12. GONÁLEZ CAPPA, S. M.; CHIALE, P. del PRADO, G. E.; KATZIN, A. M.; MARTINI, G. W. J.; ISOLA, E. L. D. de; ABRAMO ORREGO, L. & SEGURA, N. L. — Aislamiento de una cepa de **Trypanosoma cruzi** de un paciente con miocardiopatía chagásica crónica y su caracterización biológica. **Medicina (B. Aires), 40:** 63-68, 1980.

13. HATLE, L. & MELBYE, O. J. — Immunoglobulins and complement in Chronic myocardial disease. A myocardial biopsy study. **Acta med. scand., 200:** 385, 1976.

14. KAPLAN, M.; BOLANDE, R.; RAKITA, L. & BLAIR, J. — Presence of bound immunoglobulins and complement in the myocardium of acute rheumatic fever. **New Engl. J. Med., 271:** 637, 1964.

15. KHOURY, E. & FIELDS, K. L. — Chagas'disease and autoimmunity. (Letter to the Editor). **Lancet, 1:** 1088, 1980.

16. KHOURY, E.; DIEZ, C.; COSSIO, P. M. & ARANA, R. M. — Heterophil nature of EVI antibody in **Trypanosoma cruzi** infection. **Clin. Immunol. Immunopath., 27:** 283, 1983.

17. MILEI, J. & BOLOMO, N. J. — A routine method for diagnosis of early myocardial infarction. **Int. J. Cardiol., 4:** 319, 1983.

18. MILEI, J.; MORALES, M. C.; BOLOMO, N. J.; BASOMBRÍO, M. A. & COSSIO, P. M. — Correlación electrocardiográfica-anatomopatológica en la miocardiopatía chagásica crónica experimental en el ratón. **Medicina (B. Aires), 44:** 483, 1984.

19. MILEI, J.; BOLOMO, N. J.; VAZQUEZ, A. & NAGLE, C. — Normal and pathological electrocardiographic patterns in the Cebus Monkey. **J. med. Primat., 11:** 19. 1982.

20. MOLINA, H. A.; MILEI, J. & STORINO, R. A. — Chronic Chagas'myocardiopathy. Demonstration of "in vivo" bound immunoglobulins in heart structures by the immunoperoxidase technique. **Cardiology, 71:** 297, 1984.

21. RIBEIRO DOS SANTOS, R. & HUDSON, L. — **Trypanosoma cruzi:** immunological consequences of parasite modification of host cells. **Clin. exp. Immunol., 40:** 36, 1980.

22. ROSSEN, R. D.; BUTTER, W. T.; REISBERG, M. A.; BROODS, B. K.; LEACHMAN, R. D.; MILAM, J. D.; MITTAL, K.; MOTGOMERY, J. R.; NORA, J. & ROCHELLE, D. G. — Immunofluorescence localization of human immunoglobulins in tissues from cardiac allogeneic recipients. **J. Immunol., 106:** 17, 1971.

23. SANTOS-BUCH, C. A. & TEXEIRA, A. R. C. — The immunology of experimental Chagas'disease. III. Rejection of allogenic heart cells in vitro. **J. exp. Med., 140:** 38, 1974.

24. STERIN-BORDA, L.; COSSIO, P. M.; GIMENO, M. F.; GIMENO, A. L.; DIEZ, C.; LAGUENS, R. P.; CABEZA MECKERT, P.; ARANA, R. M. — Effect of chagasic sera on the rat isolated atrial preparation: Immunological, morphological and functional aspects. **Cardiovasc. Res., 10:** 613, 1976.

25. STERNBERGER, L. A. — Immunocytochemistry. 2nd. ed. New York, John Wiley, 1979.

26. SZARFMAN, A.; TERRANOVA, V. P.; RENNARD, S I.; FOIDART, J. M.; DE FATIMA LIMA, M.; SCHEINMAN, J. I. & MARTIN, G. R. — Antibodies to Laminin in Chagas'disease. **J. exper. Med., 155:** 1161, 1982.

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