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ARTIGOS

IMMUNOCYTOCHEMICAL IDENTIFICATION OF *LEISHMANIA* AND *TRYPANOSOMA CRUZI* AMASTIGOTES *IN SITU* WITH HOMOLOGOUS AND HETEROLOGOUS POLYCLONAL ANTIBODIES

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The unlabelled antibody peroxidase-antiperoxidase method was used to study the immunocytochemical properties of Leishmania *and* Trypanosoma cruzi *amastigotes* in situ *after tissues had been submitted to different fixation procedures. Antisera were obtained from rabbits chronically infected with different strains of* T. cruzi *or immunized with* L. mexicana amazonensis *and* L. braziliensis guyanensis, *and were applied on 5 μm thick sections.* T. cruzi *antigens were well stained by the three anti-*T. cruzi *sera and the two anti-*Leishmania *sera at optimum dilution between 1:1,000 and 1:2,000, regardless the parasite strain. Differently, the leishmanial antigens were revealed by anti-*Leishmania *sera only at low dilutions (between 1:60 – 1:160), whereas the anti-*T. cruzi *sera, at these low dilutions, gave rather weak stainings. Although there is no clear explanation for this immunocytochemical "reverse-monodirectional" cross-reactivity between* Leishmania *and* T. cruzi, *the present results show that polyclonal antibodies agains* Leishmania *species, when used for immunocytochemical detection of these parasites* in situ, *react more strongly with* T. cruzi *amastigotes than with the homologous amastigotes.*

Key-words: Leishmania. Trypanosoma cruzi. *Antigenicity of amastigotes. Peroxidase-antiperoxidase method.*

Polyclonal antibodies have been used in immunocytochemical techniques for identification of both, *Leishmania* and *Trypanosoma cruzi* amastigotes in sections of tissue specimens. Two types of staining methods have been reported: (a) the peroxidase-labelled antibody for staining leishmanial antigens[16 24] and (b) the unlabelled antibody peroxidase-antiperoxidase (PAP) method for *T. cruzi* amastigotes[1 2]. The titres of the anti-*Leishmania* sera used in the former method ranged from 10 to 160 whereas the anti-*T. cruzi* sera had titres above 1,000 using the PAP method. The higher sensitivity of the unlabeled antibody PAP method in comparison to the labeled antibody immunoperoxidase technique is well known[3 18 21] and could be an explanation for the differences of the antibody titres used. Concerning this, Croker & Kuhn[6] found that an avidin-biotine-peroxidase complex technique was five to ten-fold more sensitive (best titres between 640 and 1,280) than peroxidase-labelled antibody method (best titres between 80 and 160) for revealing *T. cruzi* amastigotes in tissue sections. The different titres of the primary antibody could also be due to different affinities of the first antibody to the *Leishmania* and *T. cruzi* antigens. In addition, the tissue fixation could interfere in both parasite epitopes, changing differently their immunoreactivity.

Another question to be considered is how the antigenic cross-reactivity between *T. cruzi* and *Leishmania,* observed when serological methods are employed for diagnostic purpose[4 5 23 25] is expressed in the tissue sections. Comparative studies on the immunoperoxidase staining of these two microorganisms are not available in literature so far. The present work was carried out to study, by the PAP method, the *T. cruzi* and *Leishmania* immunoreactive properties in tissue sections using species-specific and heterologous antisera and different tissue fixation procedures.

MATERIAL AND METHODS

Antisera against *Leishmania*

Antisera against promastigotes of *L. braziliensis guyanensis* (code: MHOM/BR/70/M1176) were obtained from rabbits immunized by inoculation into the footpad of 1 ml of a promastigote suspension containing 2 mg of protein, emulsified with an equal volume of Freund's complete adjuvant and boosted 21 days later with another dose of antigen containing 200 μg of protein, without adjuvant, subcutaneously. The

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animals were bled 7 days afterwards. Antisera against *L. mexicana amazonensis* amastigotes (code: IFLA/BR/67/PH8) were obtained by immunization of rabbits with an amastigote suspension isolated from hamsters, purified as described by Shaw & Laison[25] and disrupted by ultrason. One ml of this suspension, containing 2 mg of protein, emulsified with an equal volume of Freund's complete adjuvant was used for the first inoculation of the animals and boosted as described above. The indirect immunofluorescence test (using promastigotes as antigen) was used to detect the presence of anti-*Leishmania* antibodies in both sera.

Antisera against T. cruzi

Antisera against *T. cruzi* were obtained from rabbits chronically infected with CL, Ernani and Y strains. The test of indirect immunofluorescence was used for monitoring the titres of circulating specific antibodies.

Collecting and handling of tissue specimens

Leishmania infected tissues were obtained from nasal ulcers of hamsters infected several weeks before with promastigotes of the following species: *L. mexicana mexicana,* code MHOM/BR/60/BH6; *L. mexicana amazonensis,* code IFLA/BR/67/PH8 and *L. braziliensis guyanensis,* code MHOM/BR/70/M1176. Different samples from each animal were fixed in neutral, buffered 4% formaldehyde and in Bouin's fluid for 18-24 hours and then processed routinely for paraffin inclusion. Other samples from animals infected with *L. braziliensis guyanensis* and *L. mexicana amazonensis,* were snap frozen in melting Freon immediately after the biopsy procedure; freeze-dried (Freeze Dryer "Edwards do Brasil") for 6 – 8 hours at -40°C and then divided into three sets: one set was embedded directly in paraffin wax under vacuum, at 60°C for 40 minutes (unfixed tissue); the others were left at 60°C under vapour of either benzoquinone crystals or paraformaldehyde, for 3 hours. After that, they were embedded in paraffin wax under vacuum, at 60°C for 40 minutes.

Tissues infected with *T. cruzi* were obtained as follows: (a) heart fragments from two chagasic patients who died with congestive heart failure. Both presented chronic myocarditis with numerous amastigote nests (relapsing myocarditis)); (b) fragments of placenta, from a case of spontaneous abortion from a mother with chronic Chagas' disease. On routine histology the placental fragments presented chorioamnionites and frequent nests of amastigotes; (c) myocardial and liver tissues from mice inoculated with the Y strain of *T. cruzi* and from dogs infected with *Berenice* and *Colombiana* strains. Tissue specimens infected with *T. cruzi* were fixed in either, 4% formaldehyde or Bouin's fluid, then embedded in paraffin wax.

Immunocytochemical staining method

The PAP method[28] was used for identification of *Leishmania* and *T. cruzi.* Phosphate buffered saline (PBS) 0.01M, pH 7.2, was used throughout as the washing buffer after each step and as a diluent. The sections were treated with 0.3% hydrogen peroxyde to block endogenous peroxidase activity, then incubated with 1:30 diluted normal swine serum to reduce non-specific binding of the link antibody. The sections were incubated with the primary antibody (sera anti-*Leishmania* or anti-*T. cruzi*) at 4°C overnight afterwards. A range of dilutions from 20 to 4,000 times was used. Swine anti-rabbit immunoglobulins (Dako Laboratories, Copenhagen) at 1:80 dilution was used as the second (link) antibody layer and rabbit peroxidase-antiperoxidase complex (Miles-Yeda Ltd, Israel), at 1:200 dilution as the third layer. Visualization of the peroxidase-antiperoxidase complex was achieved by the diaminobenzidine method. Finally, the sections were counterstained with hematoxylin and mounted for examination.

For negative control of the *T. cruzi* staining, the anti-*T. cruzi* and anti-*Leishmania* sera were adsorbed in trypomastigote-sepharose affinity column as previously reported[7]. The anti-*Leishmania* sera were adsorbed by incubation overnight with promastigotes of *L. braziliensis guyanensis.*

Aliquotes of anti-*Leishmania* and anti-*T. cruzi* sera were also pre-adsorbed with dried red blood cells of cattle, goat and rabbit and then used as primary antibodies to stain tissue samples known to be positive for *T. cruzi* in order to verify the possibility of a false-positive reaction due to attachment of heterophyle antibodies to the surface membrane of *T. cruzi*[15]. Sera obtained from rabbits after immunization against *Leishmania/T. cruzi*-unrelated antigens, using the same immunization schedule as described above for *Leishmania* antibodies production, were used for control of cross-reactivity between *T. cruzi* and constituents of the Freund's adjuvant.

RESULTS

Leishmania *staining with anti-*Leishmania *sera.*

The best results of immunocytochemical stainings of tissue sections from animals with leishmaniasis, incubated with anti-*L. braziliensis guyanensis* and anti-*L. mexicana amazonensis* sera were achieved with titres about 80 (range: 60 – 160) using liquid fixed tissues (Table 1), and about 160 (range: 80 – 320) using freeze-dried tissues. In spite of the presence of non-specific background staining which was stronger

in freeze-dried tissues, the parasites were identified within hypodermal macrophages (Figure 1).

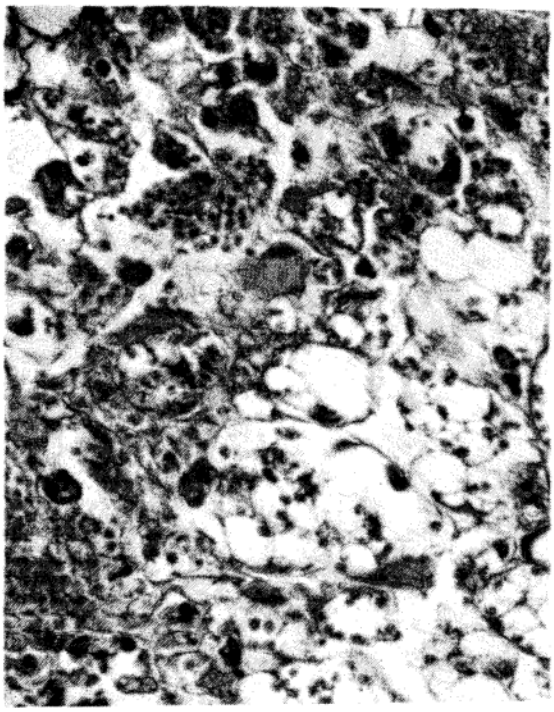

Figure 1 – Histological sections stained by the PAP method for demonstrating L. mexicana amazonensis amastigogotes using homologous antibodies. *Numerous parasites present in hamster's hypodermal nasal tissue as small, conspicuous dark-bodies. Antisera titre: 60, hematoxylin counterstain, original magnification: x 500.*

Leishmania *staining with anti-*T. cruzi *sera*

No positive specific staining of Leishmania amastigotes could be seen when serum anti-Y strain of *T. cruzi* was used. The sera anti-*CL* and anti-*Ernane* strains gave a weak positive staining of *L. braziliensis guyanensis* and *L. mexicana amazonensis* at titres up to 80 (Table 1). Nevertheless, these reactions were frequently dubious, due to nonspecific background staining observed mainly in freeze-dried tissues.

T. cruzi *staining with anti-*T. cruzi *sera*

T. cruzi amastigotes in tissue specimens of dogs and mice with experimental trypanosomiasis, and man naturally infected, were specifically stained with the three different anti-*T. cruzi* sera. The best stainings were obtained with titres between 1,000 and 2,000 (Table 1). The tissue sections displayed amastigotes as strong, dark-brown bodies within the cells (Figure 2A). The nest of parasites could be seen easily at x100 magnification of the light microscope. The nonspecific background staining was minimal or absent with titres above 1,000.

T. cruzi *staining with anti-*Leishmania *sera*

T. cruzi amastigotes in all tissue specimens were positively stained with the two anti-*Leishmania* sera. The best titres were 1,000 (anti-*L. mexicana amazonensis*) and 2,000 (anti-*L. braziliensis guyanensis*) (Table 1). The amastigotes were stained strongly as conspicuous, dark-brown bodies, against the hematoxylin contrasted host cells (Figure 2B, C, D). On examination, these tissue sections were not distinguishable from those stained with the anti-*T. cruzi* sera. The amastigotes were immunoreactive to antisera

*Table 1 – Titres of the anti-*Leishmania *and anti-*T. cruzi *sera for demonstrating* Leishmania *and* T. cruzi *antigens in formaldheyde and Bouin's fluid fixed tissue sections. PAP method.*

Antisera to	*Leishmania* antigens*			*T. cruzi* antigens**		
	80	160	320	1000	2000	4000
T. cruzi						
CL	+/–	–	–	+	+/–	–
Ernani	+/–	–	–	+	+	+/–
Y	–	–	–	+	+	+/–
Leishmania						
Lbg	+	+/–	–	+	+	+/–
Lma	+	+/–	–	+	+/–	–

* *L. braziliensis guyanensis* (Lbg); *L. mexicana amazonensis* (Lma) and *L. mexicana mexicana.*
** *Y, Colombiana and Berenice* strains (animal infections) and human tissue (natural infection).
\+ = positive staining; +/– = weak staining;
– = negative/very weak staining.

with titres up to 2,000 (anti-*L. mexicana amazonensis*) and 4,000 (anti-*L. braziliensis guyanensis*).

Controls

The pre-adsorptions of heterophyle antibodies resulted in a slightly weaker staining of *T. cruzi* amastigotes in most sections, however it did not change significatively the titres. The pre-adsorption of both anti-*T. cruzi* and anti-*Leishmania* sera in trypomastigote-sepharose column and the anti-*Leishmania* sera with *L. braziliensis guyanensis* promastigotes, abolished almost completely the stainings of *T. cruzi* amastigotes and of *T. cruzi* and *Leishmania* amastigotes, respectively. Antisera from rabbits that were immunized against *Leishmania/T. cruzi*-unrelated antigens, using Freund's adjuvant, were negative for staining of *T. cruzi* amastigotes.

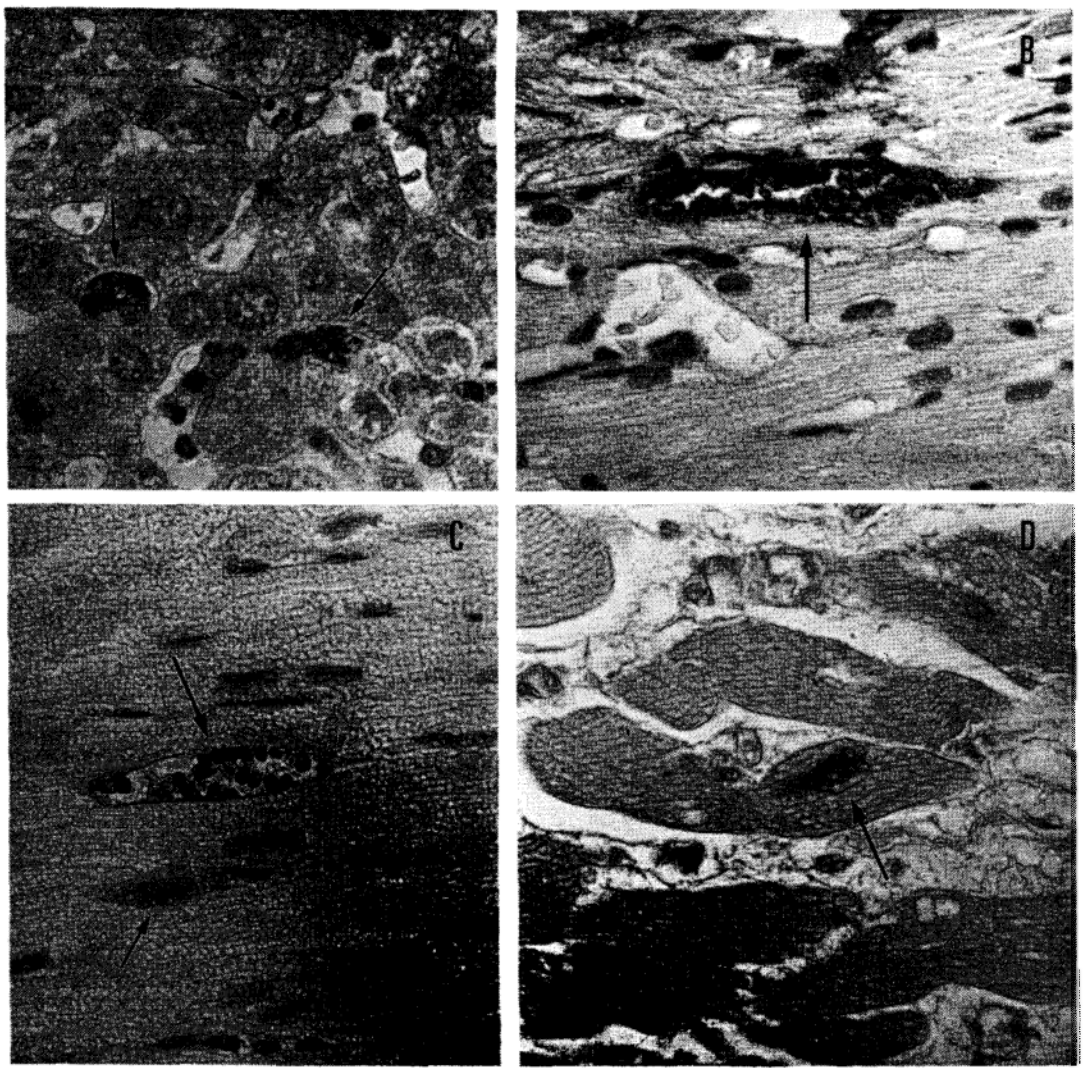

Figure 2 – Histological sections stained by the PAP method for demonstrating T. cruzi *amastigotes in cardiac muscle fiber from a man with chronic Chagas' disease (D) and in liver and myocardium tissues from mice in the acute phase of experimental trypanosomiasis (A, B, C) using species-specific and heterologous antibodies. The parasites appear as dark-bodies, isolated or forming conspicuous nests (arrows). The animal tissue, antigen, antiserum, and antiserum dilutions were, respectively: A – liver of mouse,* T. cruzi/*Y strain, serum anti-T. cruzi/Y strain, 1:2,000; B – myocardium of mouse.* T. cruzi/*Y strain, serum anti-*L. mexicana amazonensis, *1:1,000; C – myocardium of mouse.* T.cruzi/*Y strain, serum anti-*L. braziliensis guyanensis, 1:2,000; D – human myocardium, T. cruzi/*natural infection, serum anti-L. braziliensis guyanensis, 1:2,000. A, B, C, D: Hematoxylin counterstain, original magnification x500.*

DISCUSSION

The specific staining of amastigotes by the immunoperoxidase techniques has considerable potential for research and routine diagnostic in trypanosomiasis. The high degree of contrast between the amastigotes and the host tissue allows a quick assesment of the infection. However, in the case of leishmaniasis the low ratio of the specific staining signal to background interferes with the quality of the preparations. Our present results show that *T. cruzi* amastigotes are detected by homologous (anti-*T. cruzi*) or heterologous (anti-*Leishmania*) sera at high dilutions (up to 4,000). However, detection of *Leishmania* species *in situ* was only possible at titres excessively low for application in immunoperoxidase methods.

Two anti-*leishmania* sera were used, (anti-*L. mexicana amazonensis* and anti-*L. braziliensis guyanensis*), both presenting antibodies which bound amastigotes of different kinetoplastid species. Indeed, many antigens are shared by different species and evolutionary stages of *Leishmania* and *T. cruzi*, consequently cross-immunity reactions between these parasites are expected[1 8 9 10 13 14 16 17 19 20 22 24].

The polyclonal antibodies that we used in PAP technique to identify *Leishmania* and *T. cruzi* amastigotes *in situ* demonstrate by examination of all sections stained that antibodies against *Leishmania* react more strongly with *T. cruzi* amastigotes than with the homologous amastigotes. On the other hand, antibodies against *T. cruzi* did not react efficiently with leishmanial antigens. Also, the anti-*Leishmania* serum react with *Leishmania* at low titres reducing its application in immunoperoxidase methods because the weak staining signal yelded and the high degree of nonspecific background. Thus, the low titres of the anti-*Leishmania* sera in detecting homologous amastigotes, previously reported in literature[16 24] was not a result of a lower sensitivity of the labelled-antibody peroxidase technique in comparison to the PAP method. In addition, deleterious effect of fixatives on leishmanial antigens, as observed in relation to other type of cells[27], was rulled out because different fixatives in liquid and vapour phases as well as freeze-dried unfixed tissues were used. On the other hand, the presence of heterophyle antibodies in the rabbit anti-*Leishmania* sera, which could bind to *T. cruzi* amastigotes[15], was discarded by pre-adsorptions with eritrocyte of various origens.

The anti-*Leishmania* sera stained well the *T. cruzi* amastigotes in tissue sections with a stronger affinity for *T. cruzi* than for *Leishmania* antigens. There is no clear explanation for this immunocytochemical "reverse-monodirectional" cross-reactivity phenomenon. This could be due to intrinsic properties of the leishmanial surface antigens. *T. cruzi* amastigote epitopes recognized by anti-*Leishmania* antibodies may have different positions and a lower density in *Leishmania* surface. Also, specific host-parasite chemical interactions could mask the leishmanial epitopes presentation. In favour of these hypothesis, is the recent report that antisera from mice infected and/or immunized with *Leishmania* did not stained leishmanial amastigotes in tissue sections using the immunoperoxidase technique. High titres of antibodies in these sera were, however, detected by ELISA against various species and sub-species of *Leishmania* and *T. cruzi*[17].

Immunologic similarities between *Leishmania* and *T. cruzi* are important in regions of the world where both trypanosomiasis and leishmaniasis are endemic. The immunologic diagnosis could give a false positive result of Chagas disease in patients with leishmaniasis. The possibility that immunization against leishmaniasis would be effective against Chagas' disease should be investigated based on our demonstration of cross-reactive intra-specific antigens. In the case of *T. cruzi,* protective antibodies from experimental and from human Chagas' disease were shown to destroy trypomastigotes *in vitro* and *in vivo*[11 12]. On the other hand, immunity to leishmaniasis is dominated by cell-mediated reactions although antibodies are formed[30]. These antibodies are not thought to be of decisive importance in protection against leishmaniasis. In favour of a possible increased resistance against *T. cruzi* by anti-*Leishmania* antibodies is the high degree of cross-protection against fatal murine Chagas' disease in animals previously infected with *Leishmania braziliensis panamensis*[29]. Whether this protection results or not from cross-reactive antibodies is yet to be investigated.

RESUMO

O método da peroxidase-antiperoxidase foi utilizado para estudar as propriedades imunocitoquímicas de Leishmanias *e de amastigotas do* Trypanosoma cruzi, *in situ, após os tecidos terem sido submetidos a diferentes tipos de fixação. Anti-soros foram obtidos de coelhos cronicamente infectados com três cepas de* T. cruzi *ou imunizados com* L. mexicana amazonensis *e* L. braziliensis guyanensis *e aplicados nos cortes histológicos de 5 μm de espessura. Os antígenos de* T. cruzi *foram corados muito bem pelos três soros anti-*T. cruzi *e pelos dois soros anti-*Leishmania *com diluições entre 1:1.000 e 1:2.000. Diferentemente, os antígenos de* Leishmania *foram revelados pelos soros anti-*Leishmania *somente em baixas diluições, ou seja, entre 1:60 e 1:160 enquanto que os soros anti-*T. cruzi, *mesmo nestas diluições baixas, proporcionaram colorações fracas e irregulares quando usados para revelar* Leishmania. *Embora não haja explicação clara para esta reação imunocitoquí-*

mica cruzada "reversa-monodirecional" entre Leishmania *e amastigotas de* T. cruzi *os resultados do presente trabalho mostram que anticorpos policlonais contra diferentes espécies de* Leishmania, *quando usados para detecção imunocitoquímica de* Leishmania *e* T. cruzi in situ, *reagem mais fortemente com amastigotas de* T. cruzi *do que com espécies homólogas.*

Palavras-chaves: Leishmania. Trypanosoma cruzi. *Antigenicidade de amastigotas. Método peroxidase-anti-peroxidase.*

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