ARTIGOS

IMMUNOCYTOCHEMICAL IDENTIFICATION OF LEISHMANIA AND TRYpanosoma CRUZI AMASTIGOTES IN SITU WITH HOMOLOGOUS AND HETEROLOGOUS POLyclonal ANTIBODIES


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 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 against Leishmania species, when used for immunocytochemical detection of these parasites in situ, react more strongly with T. cruzi amastigotes than with the homologous amastigotes.


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 antigens1 2 4 24 and (b) the unlabelled antibody peroxidase-antiperoxidase (PAP) method for T. cruzi amastigotes1 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 unlabelled antibody PAP method in comparison to the labeled antibody immunoperoxidase technique is well known3 18 21 and could be an explanation for the differences of the antibody titres used. Concerning this, Croker & Kuhn5 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 purpose4 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 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.

**Antiseras 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 vapoour of either benziquinone 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 choorioamiontioites 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 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 peroxide 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 tryptomastigote-sepharose affinity column as previously reported. The anti-*Leishmania* sera were adsorbed by incubation overnight with promastigotes of *L. braziliensis guyanensis*.

Aliquots 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 heterophile antibodies to the surface membrane of *T. cruzi*. Sera obtained from rabbits after immunization against *Leishmania/T. cruzi*-unrelated antigens, using the same immunization schedule as described above for *Leishmania* 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).

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 formaldehyde and Bouin’s fluid fixed tissue sections. PAP method.

<table>
<thead>
<tr>
<th>Antisera to</th>
<th>Leishmania antigens*</th>
<th>T. cruzi antigens**</th>
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<tr>
<td></td>
<td>80</td>
<td>160</td>
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<tr>
<td><em>T. cruzi</em></td>
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<td>CL</td>
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<td>Y</td>
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<td><em>Leishmania</em></td>
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<td>Lma</td>
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* 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. Antiseras 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 antisera 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 assessment 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. The specific staining of amastigotes by the peroxidase technique in comparison to the PAP method. High titres of antibodies is the high degree of cross-reactivity, as observed in relation to other species and sub-species of Leishmania and T. cruzi. 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 stain 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.

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. On the other hand, immunity to leishmaniasis is dominated by cell-mediated reactions although antibodies are formed. 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. Whether this protection results or not from cross-reactive antibodies is yet to be investigated.

RESUMO

O método de peroxidase-antiperoxidase foi utilizado para estudar as propriedades immunoquímicas 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 anti-soros 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 anti-soros do 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 anti-soros 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 imunocitoquimica.
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