A Simply Prepared Amastigote Leishmanial Antigen for Use in the Indirect Fluorescent Antibody Test for Leishmaniasis

Initially it was the cultural form of Leishmania that was used to demonstrate antibodies in the indirect fluorescent antibody test (IFAT) for both cutaneous and visceral leishmaniasis (Oddo and Cascio, 1963, Rev Ist Sierater Ital 38: 139–143). Subsequently, it was shown that it was possible to stain amastigotes in smears made from infected hamsters and in sections prepared by freeze substitution method (Shaw and Voller, 1964, Trans R Soc Trop Med Hyg 58: 349–352). In more detailed studies it was noted that the test lacked both specificity and sensitivity when promastigotes were used as antigen: when formalin-fixed amastigotes, freed from vero tissue culture cells, were used, however, the test was both specific and sensitive (Walton, Brooks, and Arjona, 1972, Am J Trop Med Hyg 21: 296–299). In parallel work on Chagas’s disease it was also shown that in the IFAT, amastigotes, from Hela tissue culture cells, were a better antigen than epimastigotes from culture (Cerisola, Alvarez, Bock, and Wegerer, 1971, Rev Inst Med Trop São Paulo 13: 162–166).

Clearly the antigen of choice for either leishmaniasis or Chaga’s disease IFAT’s is the amastigote. It is, however, not a simple technique to grow leishmanial amastigotes, especially if large quantities for many serological tests are required. A number of Leishmania strains produce either cutaneous or visceral infections that are rich in parasites, however, thus offering a good source of amastigotes. The present note describes a simple technique to obtain a suspension of amastigotes (Figs. 1, 2) of L. mexicana amazonensis from the cutaneous lesions of hamsters. The strain of L. m. amazonensis (M 3050) used in the present work was isolated from a case of diffuse cutaneous leishmaniasis of man from the Bacabal area of Pará.

The infected hamster is killed in ether and overlying skin is removed before excising the lesion and putting it into cold buffered saline glucose (PSG 4:6). This solution was previously used for the separation of trypanosomes from blood using DEAE cellulose columns (Lanham and Godfrey, 1970, Exp Parasitol 28: 521–534), and we have found parasites remain viable for much longer periods than they do in physiological saline or Locke’s solution. There is also evidence that unbuffered salt solutions alter the surface structure of leishmanial parasites (Pardoe, Han, Jaquet, and Wojnarow, 1975, Z Immun Forsch Exp Ther 150: 225–226). The histiocytoma was cut into small pieces with sterile scissors and homogenized in a Lourde’s electric tissue homogenizer for 5 min. The homogenate was then ground up in a Ten Broek type tissue grinder, held in an ice bath, until the fluid was an even milky consistency. This final homogenate was centrifuged for 2 min in 15-ml glass centrifuge tubes at 100 g (RCF at tip) to remove the larger particles of tissue. The supernatant was then removed and passed through a small column of Balantini glass beads (0.1 mm diameter) supported on a coarse sintaglass support plate (porosity 0) and then through a number 3 fritted glass filter (porosity 20 to 30 μm). The filtered supernatant was washed four times in cold PSG 4:6 by centrifugation in the cold for 15 min at 1300 g (RCF at tip), and the final pellet of amastigotes resuspended in a proportion of 1:9 or more of 1% formalin/PSG 4:6 (v/v) and left for 30 min. This final suspension was centrifuged once more for 15 min at 1300 g and the sediment diluted in fresh 1% formalin/PSG to give an optical density of 0.23 at 550 nm in a Coleman junior spectrophotometer. After fixation and subsequent centrifugation there may be a tendency for some organisms to clump. If this occurs, the suspension should be filtered again through the fritted glass filter before being diluted to the required final concentration.

IFAT slide antigens of the formalin-fixed amastigotes were prepared as follows: eight nail-varnish circles of 5 mm diameter were made on clean slides and 10 μl of parasite suspension (Fig. 2) added to each circle. The excess fluid was then removed and the slides dried at room temperature. They were incubated for 30 min at 50 C and either used
immediately or stored at -20 C in plastic bags containing dry silica gel until needed. The conjugate, Wellcome anti-human IgG (sheep) with a fluorescein/protein ratio of 3.5, was diluted to 1/50 with PBS pH 7.2 containing 1 mg/100 ml Evans blue (Camargo, 1966, Rev Inst Med Trop São Paulo 8: 227-234). At serum dilutions of 1/50 no specific reactions were noted with 50 normal sera, eight Toxoplasma positive sera and the sera from five patients with Chagas's disease, all of whom had positive IFAT titers with T. cruzi antigen. Specific fluorescence was taken as surface fluorescence. The sera of 39 patients suffering from a variety of forms of cutaneous leishmaniasis were also tested. All the cases were from the Lower Amazon Region and all were confirmed by the demonstration of amastigotes in material taken from their lesions. A total of 32 reacted positively with the formalin-fixed L. m. amazonensis antigen at titers ranging from 1/50 to 1/280. No association was noted between the severity of the disease and the antibody titer, except that cases of diffuse cutaneous leishmaniasis, caused by L. m. amazonensis, consistently had high titers. The serum from one such case (M 1132) had a titer of 1/280 and this serum was used to compare different batches of M 3050 amastigote antigen, nor were there any significant differences in the titer of this serum when it was tested against antigens prepared from other isolates of L. m. amazonensis. These preliminary studies indicate that hamster antibodies do not cause inhibition of IFAT reactions most probably because they do not come into contact with the antigen before it is liberated from the cells. A comparison of the results from tests performed with sera from patients with Chagas’s disease, toxoplasmosis, and sera from people considered to be normal with sera from patients with proven leishmanial infections indicates that the formalin-fixed leishmanial antigen prepared from hamsters is both sensitive and specific. The antigen was stored at 4 C and was periodically titered out against our standard L. m. amazonensis antisera (M 1132). We found no significant differences in the titer of this serum when tested against the freshly-made antigen and the same antigen after storage at 4 C for 5 months. These results indicate that the antigen is apparently stable when stored at 4 C for a number of months.

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