RESEARCH NOTE

Montenegro Skin Test - Evaluation of the Composition and Stability of the Antigen Preparation

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Skin hypersensitivity tests are used for the diagnosis of various diseases. In cutaneous leishmaniasis, the Montenegro skin test (J Montenegro 1926 *Ann Fac Med Univ São Paulo 1:* 323-330), has been used on a large scale in the New World. The test becomes positive a few weeks after infection, and it has been used for differential diagnosis of deep dermatomycosis. The Montenegro skin test is a decisive method for the diagnosis of older leishmanial lesions and mucosal lesions, when the number of parasites is low and therefore, difficult to detect. The test is also useful for follow-up in vaccination programs (W Mayrink et al. 1979 *Trans R Soc Trop Med Hyg 73:* 385-387), and as a parameter for evaluation and of the development of immune protection (CMF Antunes et al. 1986 *Int J Epidemiol 15:* 572-580).

Despite its use for many years, some points have been constantly questioned, one of which refers to the antigen composition when the majority of laboratories use complex parasite extracts (PEC Manson-Bahr et al. 1959 *Trans R Soc Trop Med Hyg 53:* 380-383, MCS Guimarães et al. 1974 *Rev Inst Med Trop São Paulo 16:* 145-148, JJ Shaw & R Lainson 1975 *Trans R Soc Trop Med Hyg 69:* 323-335, RA Neal & RA Miles 1976 *J Trop Med Hyg 79:* 32-37, SG Reed et al. 1986 *Am J Trop Med Hyg 35:* 79-85). Another point refers to the stability of the antigen preparation used, since these antigens are stocked for long periods, specially in epidemiologic surveys, and protein degradation of leishmanial antigenic preparations have been reported (R Badaró et al. 1990 *Trans R Soc Trop Med Hyg 84:* 226-227).

In Brazil, the most used antigen to estimate the cutaneous hypersensibility during the *Leishmania* infection has been produced in Instituto de Ciências Biológicas - Universidade Federal de Minas Gerais (ICB-UFMG), employing the methodology standardized by MN Melo et al. (1977 *Rev Inst Med Trop São Paulo 19*: 161-164). However, it was made of promastigote mixture of *L. (Leishmania) mexicana*, *L. (Leishmania) amazonensis* and *L. (Viannia) guyanensis*. Its stability has not yet been evaluated.

This work aims at contributing to the standardization of antigenic extracts used for the Montenegro skin test. We carried out a comparative study on proved cutaneous leishmaniasis patients using either the multiple strain antigen of ICB-UFMG and a single strain antigen produced in a similar way but stocked for different periods of time. The single strain antigen was prepared from *L. amazonensis* promastigotes (IFLA/BR/67/PH8) grown in LIT culture medium for seven days (EP Camargo 1964 *Rev Inst Med Trop São Paulo 6:* 13-100). Parasites were washed three times with saline, resuspended in saline containing 1/10000 merthiolate and sonicated. The concentration was adjusted to 40 µg of total nitrogen per ml, as described by Melo *(loc. cit.)*. The stability studies were performed with antigen stored for different time periods. A group of 24 patients with cutaneous leishmaniasis was injected in the forearms with the following antigenic preparations: Ag1: single strain antigen stored for 14 months at 4°C, Ag2: freshly prepared single strain antigen, Ag3: freshly prepared single strain antigen autoclaved at 120°C for 20 min, Ag4: single strain antigen stored for three months at 4°C, Ag5: freshly prepared multiple strain. All patients also received 0.1 ml of diluent as control for non-specific reactions. Readings were made after 48 hr, and results were expressed as the mean of two perpendicular diameters, in mm.

Twenty-two (91.6%) of the 24 patients were positive for the five different antigens. The average nodule sizes observed were: Ag1 (21,6 ±13 5,8), Ag2 (20,5 ± 4,6), Ag3 (21,1 ± 5,4), Ag4 (22,5

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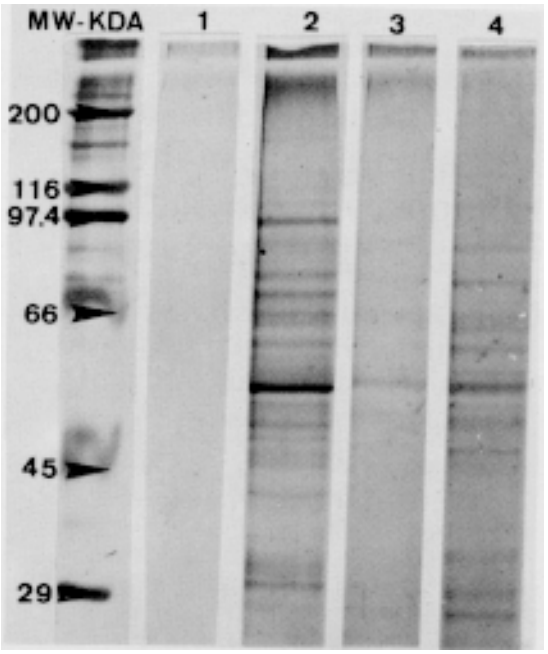
± 6,1) and Ag5 (22,3 ± 5,6). No significant differences among the antigenic preparations were detected. None of the patients presented non-specific reactions to the diluent. The two patients that did not react to the antigens presented recent lesions, with a clinical evolution of less than 30 days.

Our results with patient carriers of tegumentar leishmaniasis in activity sustain the data of W Mayrink et al. 1993 *Mem Inst Oswaldo Cruz 88* (suppl): 228, employing a clone of *L. (Leishmania) amazonensis*. Similar results were also obtained by MCA Marzochi et al. 1995 *Rev Soc Bras Med Trop 28* (suppl): 135, when they studied patients without symptoms of cutaneous leishmaniasis.

The different antigen preparations were submitted to polyacrylamide gel electrophoresis followed by silver staining (Fig.). Qualitative and quantitative differences among the profiles were found. These differences were more evident in Ag1 (14 months after prepared) and Ag3 (subjected to autoclaving); these profiles have fewer bands than seen in recently prepared antigen (Ag2).

Protein degradation, in both cases, was evident, despite the use of merthiolate, a well known inhibitor of proteases present in promastigote extracts. Similar data were obtained with a soluble antigenic preparation used for skin tests for visceral leishmaniasis stored for different periods of time (Badaró *loc. cit.*).



Polyacrylamide gel electrophoresis (10% gel) of the single strain skin test antigen preparations, stained by silver nitrate. Lane 1, antigen prepared 14 months before, lane 2, antigen freshly prepared, lane 3, antigen freshly prepared and autoclaved, lane 4, antigen prepared 3 months before.

Despite of the great advances of molecular biology, the production of molecularly defined skin test antigens is not yet possible. The identification of the components responsible for antigenic activity is undoubtly the starting point to reach this goal. Therefore, we believe our results are useful since they indicate that elements responsible for antigenic activity are present in different *Leishmania* strains, and that there is no need to work with complex extracts from more than one strain. Moreover, the maintenance of biologically active antigenic extracts for a long time, in spite of intense proteolytic activity or even the drastic treatment by autoclaving, suggests the possibility that elements responsible for antigenic activity are not protein or, in case they are, they would act as small peptidic units. Roberto Naiffi, from the Instituto Nacional de Pesquisas da Amazônia, described a great reactivity of the Montenegro antigen ICB-UFMG in patients with American cutaneous leishmaniasis, caused by *L. guyanensis* in Manaus, Brazil, even after 10 years of storage at 4°C (personal communication). These data are in accordance with Badaró observations (*loc. cit.*) with *L. chagasi.*