Induction and modulation of the immune response to *Leishmania* by Montenegro's skin test

Maria D. S. B. Nascimento[1], Neuza M. Alcântara-Neves[2,3], Maria Edna B. Muniz[1], Samia F. Nunes[1], Moacir Paranhos[2,4] and Lain C. Pontes de Carvalho[4] [1]*Programa de Pesquisa e Pós-Graduaçao em Imunologia, Departamento de Patologia, Universidade Federal do Maranhao, 65000 Sao Luis, Brazil;* [2]*Departmento de Bioagressao, ICS, Universidade Federal da Bahia, 40000 Salvador, Brazil;* [3]*Laboratório Central do Estado da Bahia, 41945 Salvador, Brazil;* [4]*Laboratório Avançado de Saúde Pública, Centro de Pesquisas Gonçalo Moniz, FIOCRUZ, 41945 Salvador, Brazil*

Abstract

The intradermal inoculation in naive or in previously sensitized individuals of small amounts of *Leishmania* extract (Montenegro's skin test) induced or modulated, respectively, the immune response to *Leishmania*, as assessed by subsequent Montenegro's skin tests. These phenomena could hinder the interpretation of Montenegro's skin tests in a population already subjected to the test in the past and, in addition, could affect in an unknown way the development of mucosal lesions in people infected with *L. braziliensis* or *L. amazonensis*, since those lesions have been associated with hypersensitivity to *Leishmania* antigens. Anti-*Leishmania* antibody responses, assessed by enzyme-linked immunosorbent assay, were not induced in naive individuals by Montenegro's skin tests, but tended to become more intense following these tests in previously sensitized individuals.

Introduction

Mucocutaneous leishmaniasis, caused in the 'New World' by *Leishmania (Viannia) braziliensis* or *L. (Leishmania) amazonensis* (reviewed by MARSDEN, 1986; BARRAL *et al.*, 1991), is a serious form of leishmaniasis, due to its relative refractoriness to antimonial therapy (MARSDEN, 1986; FRANKE *et al.*, 1990) and its progressively mutilating character (reviewed by MARSDEN, 1986). Since mucosal lesions have been associated with hypersensitivity to *Leishmania* antigens, either in terms of skin reactivity (SHAW & LAINSON, 1975; CASTES *et al.*, 1983), *in vitro* lymphoproliferative responses (CASTES *et al.*, 1984; CARVALHO *et al.*, 1985), or frequency of specific T lymphocytes in the lesion (CONCEIÇAO-SILVA *et al.*, 1990), procedures that may influence the immune response to *Leishmania* in people from endemic areas should be the object of specific studies.

The elicitation of delayed type hypersensitivity by the intradermal inoculation of small amounts of *Leishmania* extracts, Montenegro's skin test (MST), is routinely used as an aid in the diagnosis of leishmaniases (MONTENEGRO, 1926; MELO *et al.*, 1977). In the present paper the possible effect of this test on the *Leishmania*-specific immune response of non-sensitized and previously sensitized individuals has been investigated.

Material and Methods

Individuals studied

These were (i) 20 individuals cured of cutaneous leishmaniasis, (ii) 5 individuals without past history of leishmaniasis, with negative MST results and with no exposure to sandfly vectors during the duration of the study, and (iii) 6 individuals without past history of leishmaniasis but with positive MSTs.

Montenegro's skin test

Induration diameters were measured 48 h after the intradermal inoculation of 0·1 ml of leishmanin (40 µg protein nitrogen/ml) in the ventral face of the forearm. The leishmanin preparation, kindly supplied by Dr W. Mayrink (Universidade Federal de Minas Gerais, Belo Horizonte, Brazil), was prepared from a mixture of equal numbers of *L. braziliensis*, *L. amazonensis* and *L. guyanensis* promastigotes (MELO *et al.*, 1977) and kept at 4°C before use. Skin induration borders were defined by their interference with the movement of a ball-point pen.

Three tests were performed in each individual at intervals of 2 weeks.

Address for correspondence and offprint requests: L. C. Pontes de Carvalho, Department of Pathology, New York University Medical Center, 550 First Avenue, New York, New York 10016, USA.

Enzyme-linked immunosorbent assay

This assay (ELISA) was carried out as described by VOLLER *et al.* (1976), using microtitre plate wells sensitized with *L. chagasi* extract, sera obtained immediately before the first, and 15 d after the third, MST (diluted 1:100), and polyspecific rabbit antibodies against human immunoglobulins conjugated to peroxidase (Sigma Chemical Co., St Louis, Missouri, USA).

Statistical analysis

Variations in induration diameters < 2 mm were not taken into consideration to allow for possible measurement imprecisions. The statistical significance of the variation within groups, and of frequencies of patterns of variation among groups, was determined by the signed-rank sum test for paired observations or by Fisher's exact probability test, as indicated in the text.

Results

Skin indurations were induced by the second and third MST in 4 of the 5 individuals in whom no skin reaction was observed after the first test (Fig. 1a). In one of them
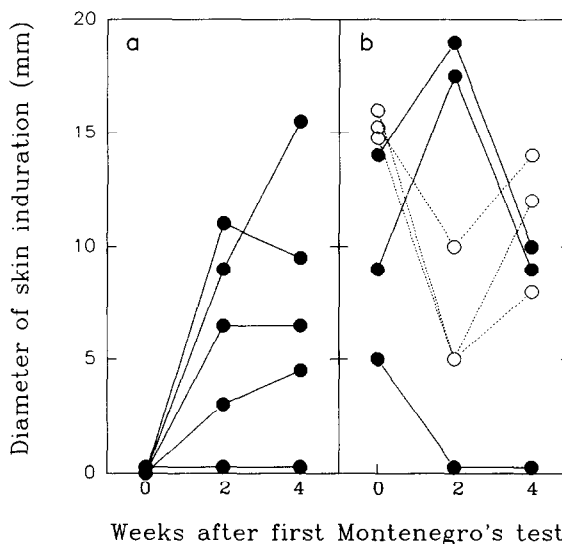

Weeks after first Montenegro's test

Fig. 1. Skin induration diameters following Montenegro's skin test (MST) in individuals without past history of *Leishmania* infection. Each line represents data obtained from a single individual subjected to MST at weeks 0, 2 and 4 (abscissa). a. Individuals with a negative MST at week 0; b. Individuals with a positive MST at week 0. Note that in 3 individuals there was a pattern of decrease followed by an increase in skin induration diameters (broken lines).

the diameter of the induration in the third MST was smaller than that in the second.

Induration diameters in the third test were significantly smaller than in the second test ($P<0.01$, signed-rank sum test for paired observations) in the group of 20
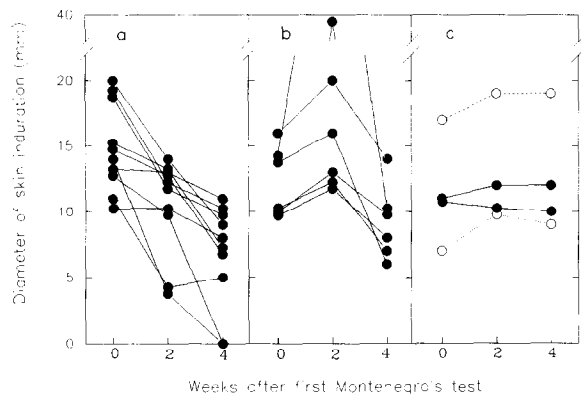

Fig. 2. Skin induration diameters following Montenegro's skin test (MST) in individuals with past history of *Leishmania* infection. Each line represents data obtained from a single individual subjected to MST at weeks 0, 2 and 4 (abscissa). Note different patterns of induration diameters in a, b and c. Cases with induration diameters larger in week 4 than in week 0 are represented by broken lines.

individuals with past history of leishmaniasis (Fig. 2a and b). This was observed in 15 individuals of this group (75%), whereas in no case was the induration in the third test larger than in the second. In 2 cases there was no measurable induration after the third test, in another case the second test would usually be considered as negative (induration diameter <5 mm; MELO *et al.*, 1977). Two main patterns of variation in induration diameters were seen in this group, both involving reduction of the diameters and accounting for 80% of the cases: (i) reduction in induration diameters with the second and/or third tests (10 cases; Fig. 2a); (ii) increase in induration diameters in the second test and reduction in the third (6 cases; Fig. 2b). Only 2 additional patterns were observed
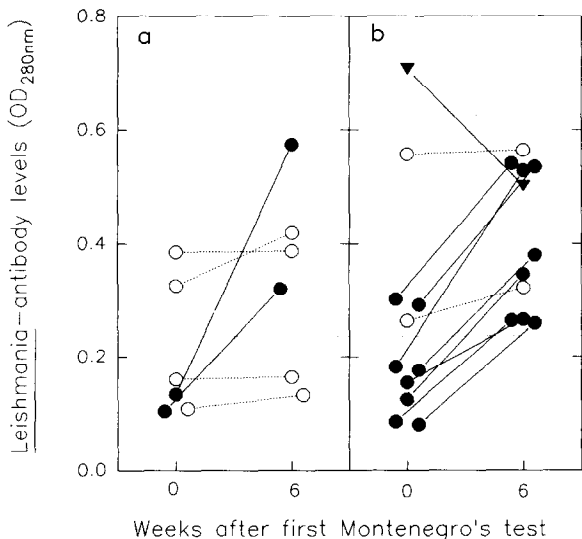

Fig. 3. Anti-*Leishmania* antibody levels in individuals with a positive reaction to the first Montenegro's skin test (MST), as detected by enzyme-linked immunosorbent assay. a. Individuals without past history of leishmaniasis. b. Individuals with past history of leishmaniasis. Each line represents data obtained from serum samples collected at weeks 0 and 6 (abscissa) from a single individual subjected to MST at weeks 0, 2 and 4. The mean plus 2 standard deviations of the results from 9 healthy individuals with negative MST and without past history of leishmaniasis is represented by the horizontal line. Eight individuals with past history of leishmaniasis had circulating *Leishmania* antibody levels below this value. For the sake of clarity, their data were not included in the figure.

in the group: (i) increase in induration diameters in the second and no change in the third (2 cases; Fig. 2c) and (ii) no change in any subsequent test (2 cases, Fig. 2c).

In 3 of the 6 previously sensitized individuals without past history of leishmaniasis, a pattern not seen in the group of individuals with past history of leishmaniasis was seen: a decrease in induration diameter in the second test and an increase in the third (Fig. 1b).

Anti-Leishmania antibodies

None of the 5 previously non-sensitized individuals had detectable amounts of anti-*Leishmania* antibodies in the serum as assessed by ELISA, either before or after the MST (data not shown). In 9 of the 19 tested individuals with past history of leishmaniasis there was an increase in these antibody levels after the third MST (Fig. 3b). Reductions in antibody levels were seen in only one individual. A similar increase was observed in 2 of the 6 previously sensitized individuals without past history of leishmaniasis (Fig. 3a).

Discussion

The present results demonstrate that the amount of antigen utilized in the MST, aiming at eliciting an immune reaction for diagnostic purposes, is in fact capable of inducing a new, or modifying a pre-existing, immune response to *Leishmania*. This would hinder the interpretation of MST results in people previously subjected to the test, not only when it is carried out for diagnostic purposes but also when used as a criterion for inclusion into vaccination or immunological studies. As a consequence, information on possible past MST should be sought from the people being tested.

There was a tendency for a reduction in skin induration diameters in the last MST. For instance, skin indurations in the third MST were smaller than in the first test in 20 of 26 previously sensitized individuals (Figs 1b, 2a and 2b), whereas they were larger in only 2 of 26 (Fig. 2c). The probability of this being due to chance is less than 0.01 (signed-rank sum test for paired observations). This finding would be compatible with a stimulation by the MST of mechanism(s) modulating a *Leishmania*-specific cellular reaction in previously sensitized individuals. The possibility that the changes in induration diameters were due to a possible decrease in the antigenicity of the leishmanin preparation in the 30 d period during which it was used can be discounted because of (i) the observation of a pattern of increasing skin induration sizes, mainly at the second test in relation to the first (in 14 of the total number of 31 individuals studied) and (ii) the careful maintenance of the leishmanin preparation at 4°C, at which temperature it is stable for months.

The reduction in the cellular immune response elicited by the MST, however, was not paralleled by a similar reduction in the humoral immune response to *Leishmania*. If anything, this response, as assessed by ELISA, was exacerbated after the third MST in the group of individuals with past history of leishmaniasis. There was no correlation between variations of antibody levels and variations in skin reaction intensities (data not shown).

It is also of note that in 3 pre-sensitized individuals without history of leishmaniasis there was a pattern of reduction followed by increase in skin induration diameters with subsequent tests. The frequency of this pattern in this group of individuals (50%) was significantly higher than its frequency (0%) in the group of 20 individuals with cured leishmaniasis ($P=0.0077$, Fisher's exact probability test). This finding would be consistent with the data from the other groups if the 3 individuals were primarily sensitized to a cross-reactive antigen, such as those from *Trypanosoma cruzi*, for instance, rather than to *Leishmania*: a first MST would tend to suppress the cross-reactive response (as happened for the total *Leishmania*-specific response in individuals with cured leishmaniasis), while additional tests would stimulate a non-cross-reactive response (as occurred for

the total anti-*Leishmania* response in non-sensitized individuals).

In conclusion, the present results show that the MST is not only able to induce a localized immune reaction but also to initiate or interfere with the *Leishmania*-specific immune response. Whether this would influence the development of mucosal lesions in people living in areas endemic for *L. (V.) braziliensis* or *L. (L.) amazonensis* is open to investigation.

References
Barral, A., Pedral-Sampaio, D., Grimaldi, G., jr, Momen, H., McMahon-Pratt, D., Ribeiro-de-Jesus, A., Almeida, R., Badaró, R., Barral-Netto, M. & Carvalho, E. M. (1991). Leishmaniasis in Bahia, Brazil: evidence that *Leishmania amazonensis* produces a wide spectrum of clinical disease. *American Journal of Tropical Medicine and Hygiene*, **44**, 536–546.

Carvalho, E. M., Johnson, W. D., Barreto, E., Marsden, P. D., Costa, J. L. M., Reed, S. & Rocha, H. (1985). Cell-mediated immunity in American cutaneous and mucocutaneous leishmaniasis. *Journal of Immunology*, **135**, 4144–4149.

Castes, M., Angelli, A., Verde, O. & Rondon, A. J. (1983). Characterization of the cellular immune response in American cutaneous leishmaniasis. *Clinical Immunology and Immunopathology*, **27**, 176–186.

Castes, M., Angelli, A. & Rondon, A. J. (1984). Mechanisms associated with immunoregulation in human American cutaneous leishmaniasis. *Clinical and Experimental Immunology*, 57, 279–286.

Conceiçao-Silva, F., Dórea, R. C. C., Pirmez, C., Schubach, A. & Coutinho, S. G. (1990). Quantitative study of *Leishmania braziliensis braziliensis* reactive T cells in peripheral blood and in the lesions of patients with American cutaneous leishmaniasis. *Clinical and Experimental Immunology*, **79**, 221–226.

Franke, E. D., Wignall, F. S., Cruz, M. E., Rosales, E., Tovar, A. A., Lucas, C. M., Lannos-Cuentas, A. & Berman, J. D. (1990). Efficacy and toxicity of sodium stibogluconate for mucosal leishmaniasis. *Annals of Internal Medicine*, **113**, 934–949.

Marsden, P. D. (1986). Mucosal leishmaniasis ('espundia' Escomel, 1911). *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **80**, 859–876.

Melo, M. N., Mayrink, W., da Costa, C. A., Magalhaes, P. A., Dias, M., Williams, P., Araujo, F. G., Coelho, M. V. & Batista, S. M. (1977). Standardization of the Montenegro antigen. *Revista do Instituto de Medicina Tropical de São Paulo*, **19**, 161–164.

Montenegro, J. (1926). Cutaneous reactions in leishmaniasis. *Archives in Dermatology and Syphilis*, **13**, 187–194.

Shaw, J. J. & Lainson, R. (1975). Leishmaniasis in Brazil. X. Some observations on intradermal reactions to different trypanosomatid antigens of patients suffering from cutaneous and mucocutaneous leishmaniasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **69**, 323–335.

Voller, A., Bartlett, A. & Bidwell, D. E. (1976). Enzyme immunoassays with special reference to ELISA techniques. *Journal of Clinical Pathology*, **31**, 507–520.