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KINETICS AND HISTOPATHOLOGY OF THE EAR THICKNESS TEST FOR DELAYED HYPERSENSITIVITY IN MURINE LEISHMANIASIS

Gabriel GRIMALDI FILHO (1) and Pamela Lane MORIEARTY (2)

SUMMARY

C3H mice, infected for 3, 5 and 8 months with **Leishmania mexicana**, were evaluated by the ear thickness test for delayed-type hypersensitivity to leishmanial promastigote antigen. Ear thickness increase, which peaked at 48 hr after antigen injection, was significantly greater in infected mice than in uninfected controls. Histopathology of skin test sites showed significantly greater intensity of mononuclear cell infiltration, vascular congestion, and edema in infected mice than in controls. There was a significant correlation between a composite score assessing intensity of these three histopathological features and macroscopic ear thickness measurements of infected mice. There was no correlation between ear thickness and titers in any one of three serological tests using leishmanial antigens. Advantages of the ear thickness test over other **in vivo** measures of delayed-type hypersensitivity in mice are discussed.

INTRODUCTION

Because the development of cellular immunity as a critical factor in determining the outcome of infection with organisms such as **Mycobacteria** and **Leishmania**, delayed-type hypersensitivity (DTH) skin testing is frequently employed in experimental investigations of these infections. Footpad DTH testing of mice infected with **Leishmania** [10,12,15] and **Mycobacteria** [1,3] has been described.

The ear thickness test is more sensitive than the footpad swelling test for measuring DTH responses to protein antigens in artificially immunized animals [13,14] but, to our knowledge, use of the ear thickness test in infected animals has not been previously reported. In addition, available descriptions of histopathological features of these DTH reactions in mice are limited, making comparisons of results of different investigators difficult.

In a recent study of **L. mexicana** infection in C3H mice [6] we selected the ear thickness test for assessment of DTH to leishmanial antigens. We report here the time course and a systematic semiquantitative histopathological analysis of this test, which indicated that it is reliable measure of DTH.

MATERIALS AND METHODS

Young adult C3H mice of both sexes were infected by subcutaneous inoculation in the perinasal region with 10^5 washed promastigotes of **L. mexicana** grown in LIT medium. Sera obtained at sacrifice after 3, 5 and 8 months of infection were tested individually for IgG and IgM fluorescent antibodies and indirect hemagglutinating antibodies to promastigote antigen. Details of parasite cultivation, serological tests, and course of **L. mexicana** infection in C3H mice are reported elsewhere [6].

Skin testing — L. mexicana promastigotes were grown in NNN medium with 7.5% rabbit

(1) Centro de Microscopia Eletrônica. Fundação Oswaldo Cruz. Caixa Postal 926. 21040 Rio de Janeiro, Brazil
(2) Departamento de Imunologia. Fundação Oswaldo Cruz. Caixa Postal 926. 21040 Rio de Janeiro, Brazil

blood and Hank's balanced salt solution (HBSS) overlay. Promastigotes were collected, washed three times in HBSS, four times in phosphate buffered saline, 0.15M pH 7.2 (PBS), then resuspended in PBS and heat-killed at 50°C [11]. The suspension was adjusted to $6x10^4$ organisms/0.03 ml, with 0.4% phenol preservative. The PBS diluent with 0.4% phenol (PPBS) was used as a control.

Using a 1 ml disposable syringe and 27 g 1/2 in needle, 0.03 ml of parasite suspension was injected intradermally into the dorsal surface of the ear. Ear thickness was measured with a Starrett micrometer caliper; then mean of three successive measurements of the same ear was taken as the ear thickness.

A group of 8 mice were tested after 3 months of leishmanial infection with PPD (RT 23, with 0.005% Tween 80, National Tuberculosis Division, Brazilian Ministry of Health). A dose of 0.03 ml containing 0.7 UT was tested as for leishmanial antigen.

Pathology — Segments from skin test sites were fixed in buffered 10% formalin, pH 7.0, embedded in paraffin, and sections were stained with haematoxylin and eosin (HE). Sections were examined blind and scored on a scale of 0 — not observed; 1 — slight; 2 — moderate; 3 — marked; 4 — intense, for the following features: epidermis — hyperkeratosis, necrosis, hyperplasia, intercellular edema, bulla or vesicles, cellular infiltrate; dermisperivascular mononuclear cells, intervascular mononuclear cells, granulocytes, plasma cells, vascular congestion, erythrocyte extravasation, edema, and fibrosis.

Statistical analysis — Ear thickness differences were analyzed by the nonparametric rank sum test [16]. Mean histopathology scores were compared by Student's t test [16], and correlation of ear thickness and histopathology scores by the correlation coefficient, r [2].

RESULTS

Injection of PPBS caused a slight but significant ($p < 0.01$) increase above the normal ear thickness of C3H mice. There was no difference between ear thickness after PPBS injection and after injection of leishmanial antigen in uninfected mice (Fig. 1). Four mice tested after 3 months of leishmanial infection had significantly increased ear thickness responses which peaked at 48 hr ($p < 0.01$) and remained elevated to 96 hr (Fig. 1). Significantly increased ear thickness at 48 hr was also observed in 7 mice tested after 5 months of infection (0.528 ± 0.053mm, $p < 0.01$) and 8 mice tested after 8 months of infection (0.514 ± 0.065 mm, $p < 0.01$).

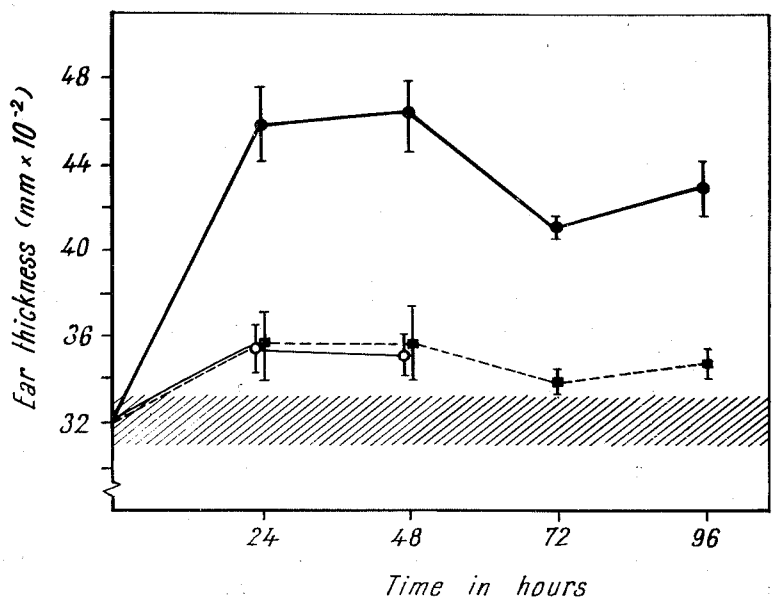

Fig. 1 — Ear thickness (mean ± standard deviation) as a function of time after antigen injection. (●——●) — infected mice injected with leishmanial antigen (N = 4); (□- - - -□) — infected mice injected with PPBS (N = 4); (○——○), uninfected mice injected with leishmanial antigen (N = 6); hatched area represents mean ± standard deviation of ear thickness of normal C3H mice (N=11).

Eight animals infected for 3 months with leishmania were tested with PPD. Ear thickness at 48 hr of infected mice (0.320 ± 0.016 mm) did not differ significantly from that of similarly tested normal mice (0.320 ± 0.029 mm).

Rarely the bleb formed by antigen injection was not resorbed, and the ear retained a fluid-filled hematoma at 48 hr. This was observed in both infected and uninfected animals, after injection of both PPBS and antigen, in approximately 4% of tests performed. Such test sites were excluded from the ear thickness and histological analyses.

Histological alterations of ear thickness test sites at 48 hr were restricted to the dermis and included presence of intervascular mononuclear cells, granulocytes, vascular congestion, edema, and extravasation of erythrocytes (Table I, Fig. 2). The group mean score of infected mice tested with leishmanial antigen was significantly greater than the group mean score of uninfected animals tested with the same antigen for three features: intervascular mononuclear cells, congestion and edema. Mean total histopathological scores and total scores for these three features also differed significantly between the two groups (Table I). Histological results at 96 hr for 4 mice infected 3 months were similar to those of 48 hr; intervascular mononuclear cells, congestion and edema remained prominent, but granulocyte infiltration had diminished (mean total score = 5.5 ± 1.0).

TABLE I

Group mean scores for histopathological features of ear thickness reactions

Group[1]	A. Control + Infected (12)	B. Control (6)	C. Infected (15)	B vs C
Injection	PPBS	Leish[2]	Leish	
Ear Thickness[3]	0.349 ± .026 mm	0.352 ± .020	0.521 ± .058	$p < 0.01$[4]
Histopathological Feature		Group Mean Score[5]		
1. Intervascular mononuclear cells	0	0.25 ± 0.42	2.33 ± 0.62	$p < 0.005$[6]
2. Vascular congestion	0.50 ± 0.67	0.83 ± 0.75	1.73 ± 0.59	$p < 0.005$
3. Edema	0.83 ± 0.83	1.17 ± 0.98	2.00 ± 0.93	$p < 0.05$
4. Granulocytes	0.25 ± 0.45	1.00 ± 1.26	1.80 ± 0.86	NS
5. RBC Extravasation	0.17 ± 0.57	0.17 ± 0.41	1.00 ± 1.36	NS
Total Score	1.75 ± 1.66	3.42 ± 3.17	8.87 ± 3.25	$p < 0.005$
Total of Features 1,2,3	1.33 ± 1.44	2.25 ± 1.84	6.07 ± 1.67	$p < 0.005$

1. Number of mice in parentheses. Group C mice infected 5 or 8 months with **L. mexicana**
2. Leishmanial promastigote antigen
3. All values are mean ± standard deviation
4. Rank sum test
5. For scoring system, see text
6. Student's t test

Among mice infected for 5 and 8 months there was a moderate but significant correlation ($r = 0.555$, $p < 0.05$) between macroscopic ear thickness and total histopathological score for mononuclear infiltration, congestion and edema (Fig. 3). There was no correlation in infected animals between ear thickness at 48 hr and titers (log 2) of IgG fluorescent antibody ($r = 0.126$, $p > 0.05$), IgM fluorescent antibody ($r = 0.239$, $p > 0.05$) or indirect hemagglutination antibody ($r = 0.049$, $p > 0.05$) to leishmanial antigen. There was no apparent relation between ear thickness and size of leishmanial lesion.

DISCUSSION

In evaluating a test as an indicator of delayed-type hypersensitivity, several factors must be considered, including specificity, time course of the response, and histopathology of the reaction site. Mice differ in several respects from other species, including guinea pigs and humans, in their delayed hypersensitivity responses. Thus, in mice induration may be less pronounced, and granulocytes are often a prominent histopathological feature, especially at 24 hr[3,5].

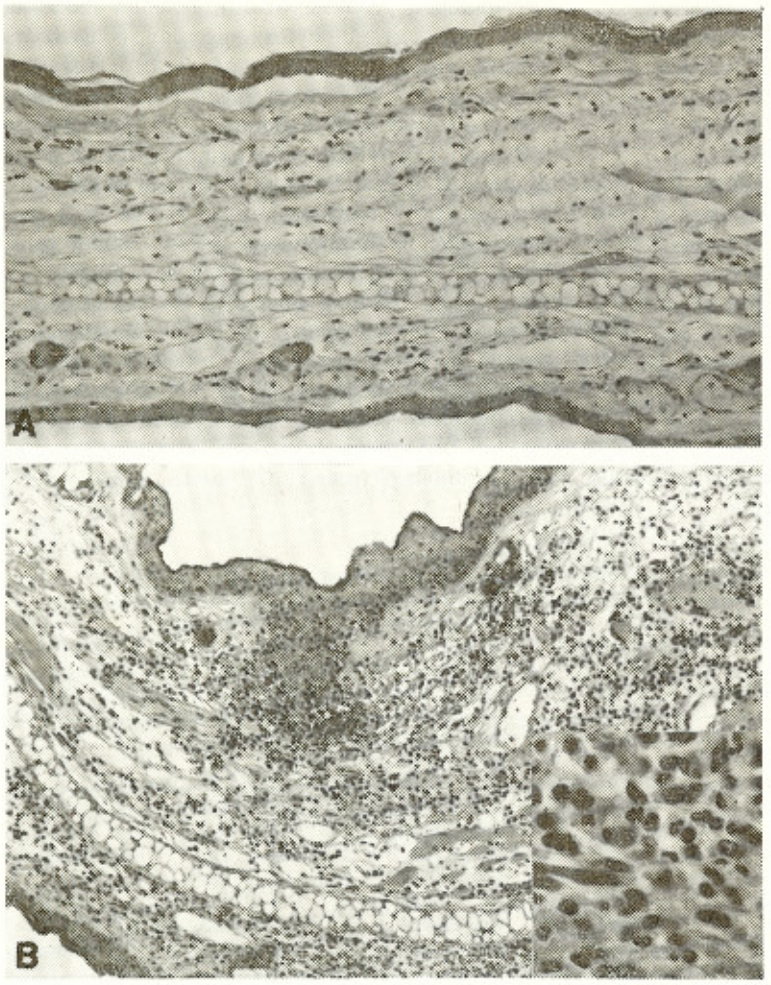

Fig. 2 — A) Ear of uninfected control C3H mouse 48 hours after injection of leishmanial antigen. Macroscopic ear thickness was 0.375 mm. Histologically the section was scored as: intervascular mononuclear infiltration — 0; granulocyte infiltration — 1; vascular congestion — 1; extravasation of RBC — 0; edema —2. (H.E., 500 ×). B) Ear of C3H mouse infected for 8 months with **Leishmania mexicana**, 48 hours after injection of leishmanial antigen. Macroscopic ear thickness was 0.577 mm. The section was scored as: intervascular mononuclear infiltration — 3; granulocytes — 3; congestion — 2; RBC extravasation — 1; edema — 3. (H.E. 500 ×). Inset: Detail of mononuclear infiltration (2,000 ×)

The increase in ear thickness in **L. mexicana** — infected mice tested with leishmanial antigen is apparently specific, since there was no difference between responses to PPBS and to antigen in uninfected animals. Also the presence of leishmanial infection in itself caused no significant response to another antigen, PPD. The lack of correlation between ear thickness and titers in any one of three tests for humoral immunity indicates that increased ear thickness at 48 hr is not likely to be due to Arthus-type reactions. Increased thickness persisted to 96 hr without evidence of necrosis.

Kinetics of ear thickness tests were also consistent with a T-cell mediated response. Results of footpad swelling tests are frequently reported for 24 hr measurements. However ALEXANDRE & CURTIS [1] have noted that relatively **M. lepraemurium** resistant C57Bl mice can be distinguished from susceptible Balb/c mice only when intradermal tests are read at 48 hr. Measurements at 24 hr are similar in the 2 strains. Antibody-dependent forms of late-onset hypersensitivity usually peak at 24 hr or earlier [5]. In mice 24 hr reactions usually contain more neutrophils, while mononuclear cells increase in prominence at 48 hr [3]. Test readings and histopathological analysis at 48 hr are thus more likely to reflect T-cell mediated DTH than results at 24 hr.

The histological features of the ear reaction were also consistent with a cell-mediated response. The mere presence of mononuclear cells in a skin test site is not assurance of a specific immune response, since they may be present in reaction sites of control animals [8]. We therefore adopted a "blind" intensity scoring system to overcome, albeit somewhat arti-

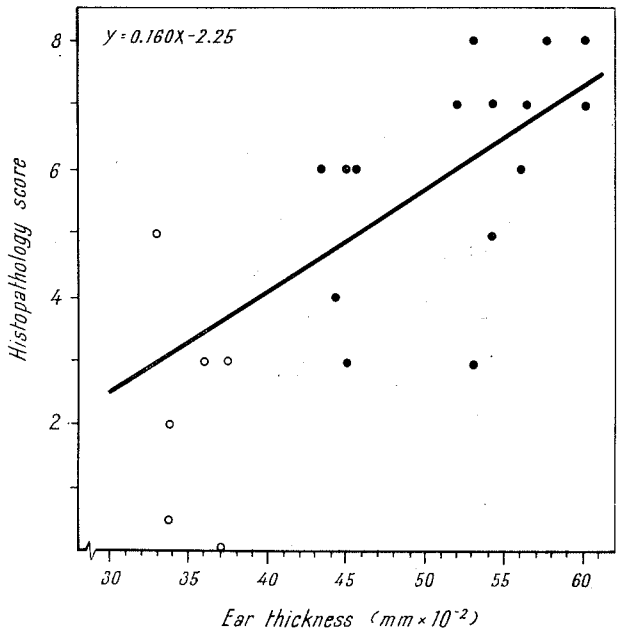

Fig. 3 — Correlation ($r = 0.555$, $p < 0.05$) between ear thickness and total histopathological score for mononuclear cell infiltration, vascular congestion and edema in leishmanial antigen intradermal tests of mice infected 5 or 8 months with **Leishmania mexicana** (•). Values for uninfected control mice (0), which fall mostly below the line, are included for comparison. For histopathological scoring system, see text.

ficially, the subjectiveness inherent in histological analysis and to permit assessment of the contribution of several factors to the response. In the present case the intensity of three different features associated with cellular immune responses [3,4], mononuclear cell infiltration, vascular congestion and edema, was significantly greater in infected animals than in uninfected controls. In addition, a composite score assessing the intensity of these three features correlated with ear thickness.

The ear thickness test is only one of several techniques for measuring DTH in mice, but it has several advantages over other procedures. Accurate measurements are facilitated by the large flat surface and lack of flexibility of the ear compared to the paw. The test site can be divided easily into several representative samples for multiple fixation techniques, and histological processing need not included decalcification steps. Advantages over radioisotope arrival techniques [7,13] include greater simplicity, reduced cost and absence of necessity to sacrifice the animal to perform the test. Some workers prefer the rear paw as a site for leishmanial infection. Experimental procedures involving front paws are not recommended because the animal uses these to manipulate food. The ear offers an alternative site for skin testing in such cases. The main disadvantage of test lies in the greater difficulty of injecting antigen accurately into the mouse ear, than into the footpad. However, with some practice and with the help of an assistant to immobilize the animal, a high rate of successful placement of antigen can be obtained. Inadvertent incorrect (i.e. subcutaneous) placement of antigen is virtually impossible since such tissue is minimal in the ear. The use of anesthetics, though they may facilitate the operation, is not strictly necessary, and they were not used in the present study.

Because of the large variability of immune responses in different mouse strains [9,14], adaptation of the ear thickness technique to testing with other host-parasite combinations would obviously require standardization of the system. Nevertheless, the sensitivity and ease of histological analysis of this test should make it widely applicable.

RESUMO

Cinética e histopatologia do teste de espessamento de orelha para sensibilidade tardia na leishmaniose murina

Camundongos C3H, infectados com **Leishmania mexicana** durante 3,5 e 8 meses, foram avaliados, através do teste de espessamento da

orelha, para a reação de hipersensibilidade retardada aos antígenos de formas promastigotas do parasita. O aumento da espessura da orelha, o qual teve seu pico 48 horas após a injeção antigênica, foi significantemente maior nos animais infectados do que nos controles não infectados. A histopatologia do sítio do teste intradérmico mostrou intensidade significativamente maior da infiltração de células mononucleares, da congestão vascular e do edema intersticial nos animais infectados, quando comparados com os controles. Houve, também, uma correlação significativa entre a intensidade desses três achados histológicos, analisados no seu conjunto, e as medidas macroscópicas da espessura das orelhas dos animais infectados. Entretanto, não houve nenhuma correlação entre o teste do espessamento das orelhas e os títulos em qualquer um dos três testes sorológicos efetuados, utilizando-se antígenos específicos. São discutidas as possíveis vantagens deste teste de espessamento da orelha, em relação aos outros testes **in vivo**, de avaliação de hipersensibilidade retardada.

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