Clin. exp. Immunol. (1980) **41**, 237–242

Leishmania mexicana in C3H mice: BCG and levamisole treatment of established infections

G. F. GRIMALDI,* PAMELA L. MORIEARTY† & R. HOFF *Department of Pathology, and Harvard/Wellcome/PAHO/UFBa Project, Federal University of Bahia, Salvador, Brazil*

(Accepted for publication 20 February 1980)

SUMMARY

The effect of BCG and levamisole on the course of established murine leishmaniasis was examined. C3H mice infected subcutaneously in the perinasal region with 10^5 *L. mexicana* promastigotes produced chronic non-ulcerating, non-healing lesions and demonstrated positive humoral and delayed hypersensitivity responses to leishmanial antigens. Infected animals were treated during months 3–5 of infection with either live BCG or with levamisole. Neither treatment resulted in resolution of lesions or in production of a hyperallergic form of infection; similarly, neither immune responses to leishmanial antigens nor histopathological features of lesions were significantly altered. BCG treatment resulted in accelerated growth of primary leishmanial lesions and in the appearance of metastases in some animals. Levamisole treatment of uninfected animals resulted in low levels of antibodies reacting with promastigote antigens, but not in positive delayed intradermal responses. BCG induced delayed intradermal sensitivity to PPD in both infected and control animals; significantly increased delayed reactions to leishmanial antigens, as well as occasional low levels of cross-reacting antibodies to Leishmania, were observed in treated uninfected mice.

INTRODUCTION

The immune mechanisms which act on Leishmania are not completely understood, but probably involve a complex interplay of humoral and cellular immunity as well as, in the mouse, other non-H-2 locus genetic factors (Preston & Dumonde, 1976; Bradley *et al.*, 1979). We have recently shown that C3H mice, which are susceptible to progressive, non-ulcerating *L. mexicana* lesions, nevertheless develop humoral and cellular immune responses to the parasite (Grimaldi, Moriearty & Hoff, 1980).

The immunostimulants BCG and levamisole have been shown non-specifically to enhance cell-mediated immunity, probably by action on macrophages and subsets of T lymphocytes (Symoens & Rosenthal, 1977; OMS, 1976). The effect of these two substances in Leishmania infection has been variable. BALB/c mice pretreated with BCG and subsequently infected with *L. tropica* had smaller lesions with fewer parasites than controls (Weintraub & Weinbaum, 1977). When *L. donovani*-infected BALB/c mice were treated with BCG 15 to 31 days after infection, the parasite numbers in liver and spleen were reduced (Smrkovski & Larson, 1977a). When BCG and *L.*

* Present address and address for correspondence: Gabriel Grimaldi, MD, MP, Centro de Microscopia Electrônica, Fundação Oswaldo Cruz (Manguinhos), Caixa Postal 926, 21040 Rio de Janeiro, Brazil.

† Present address: Centro de Imunologia Parasitária, Fundação Oswaldo Cruz (Manguinhos), Caixa Postal 926, 21040 Rio de Janeiro, Brazil.

0099-9104/80/0800-0237$02.00

enriettii were inoculated simultaneously into BCG-sensitized guinea-pigs, primary lesions did not develop, but metastatic lesions appeared and resistance to challenge with *L. enriettii* did not develop (Behin, Mauel & Rowe, 1977).

Levamisole treatment accelerated healing of established human *L. tropica* infection in eleven of twelve cases but the treatment was not effective in the early phase of infection (Butler, 1978). This drug was also not effective against the non-healing plateau phase of *M. leprae* infection in mice (Shepard, Van Landingham & Walker, 1977). We have tested the ability of BCG and levamisole to stimulate protective immunity in C3H mice with established *L. mexicana* infection.

MATERIALS AND METHODS

Parasite and host. L. mexicana, strain 5, obtained from Dr Z. Brener, Rene Rachou Research Center, Fiocruz, Belo Horizonte, Brazil, was used for infections and for preparation of antigens. Promastigotes were obtained by culturing cutaneous lesions from mice in NNN blood agar medium with an overlay of Hanks' balanced salt solution, then subculturing in modified LIT liquid medium (Gutteridge, Knowler & Coombes, 1969). Young adult inbred C3H mice of both sexes were inoculated subcutaneously in the perinasal area with 10^5 washed promastigotes. Designated groups were killed after 3, 5 and 8 months of infection.

Treatment. Mice were treated with BCG or levamisole regimens beginning 3 months after *L. mexicana* infection. Lyophilized BCG (Fundação Ataulpho de Paiva, Rio de Janeiro, Brazil) was obtained from cultures (Moreau–Rio de Janeiro isolate, Lot 681) maintained by the seed-lot system of the Statens Seruminstitute, Copenhagen. The mice first received an intraperitoneal injection of 5×10^8 BCG (Hoff, 1975), followed after 10 days by eight weekly applications of 6×10^5 mycobacteria directly to the lesions with scarification in grid pattern with a hypodermic needle. Non-infected control mice were treated with BCG in the same manner.

Levamisole HCl (Lot 2230; Johnson & Johnson of Brazil) was dissolved in PBS, pH 7·3, and injected intraperitoneally in three series of treatments lasting 2 weeks each, with a 2-week rest between each series (Fidler & Spitler, 1975). For the first series 12 mg/kg was given every 2 days. For the second and third series, 5 mg/kg was given on two consecutive days of each week.

Immunological responses. Methods for the detection of delayed skin hypersensitivity responses and serum antibodies to *L. mexicana* antigens were described elsewhere (Grimaldi *et al.*, 1980). Negative (pooled normal mouse serum) and positive controls were included in each test run.

BCG sensitization was tested by intradermal injection of 0·7 UT PPD (Rt 23, with 0·005% Tween 80) in 0·03 ml into the dorsal surface of the ear. Histology of the antigen and control skin test sites were studied for all skin tests.

Pathology. The size and appearance of leishmanial lesions and metastases were evaluated at weekly intervals. The lesions were classified according to diameter into four categories: large = greater than 1 cm; medium = 0·6 to 1 cm; small = 0·2 to 0·5 cm and minimal = less than 0·2 cm. At killing, primary lesions and any metastases were entirely removed and fixed in Bouin's solution. Paraffin sections (5 μm) were prepared from central and peripheral zones of the lesion and stained with haematoxylin–eosin, Masson trichrome, and Dominici stain (Litt modification) (Litt, 1963). The sections were examined in blind fashion by two observers and evaluated for nine features of dermal pathology (presence of histiocytes, vacuolated macrophages, macrophages with parasites, necrosis, neutrophils, eosinophils, lymphocytes, plasma cells and fibroblasts with fibrosis) scored on a scale of 0 (no abnormality) to 3. Perinasal tissue from treated and normal controls was included in the series and sections of lymph nodes, spleen and liver were examined for animals presenting cutaneous metastases.

RESULTS

Histological and immunological features of *L. mexicana* infection in C3H mice are described in detail elsewhere (Grimaldi *et al.*, 1980). Although the leishmanial lesions varied in size, all animals

had histological and immunological evidence of infection at the time of death. The lesions had diffuse infiltrations of histiocytes, lymphocytes and plasma cells. Neutrophils and eosinophils were also encountered in some cases. Cutaneous metastases were not observed in untreated animals during the 8 months of study. The *L. mexicana*-infected mice developed positive intradermal delayed-type hypersensitivity and specific IgG, IgM, and indirect haemagglutinating antibodies to leishmanial antigens when tested 3, 5 and 8 months after infection (Figs 1–4).

BCG treatment. BCG treatment failed to cure the lesions or alter their histopathology. In the BCG-treated animals, the leishmanial lesions appeared to grow more rapidly than those of untreated or levamisole-treated animals, and three of eight BCG-treated mice presented distant cutaneous metastases after 8 months of infection. Visceralization was not observed.

Delayed hypersensitivity to PPD. Normal and *L. mexicana*-infected mice had negative PPD reactions before BCG treatment. Compared to untreated animals, significantly increased PPD reactions were seen after treatment in controls (rank sum test, $P<0{\cdot}05$) (Snedecor, 1956) and *L.*

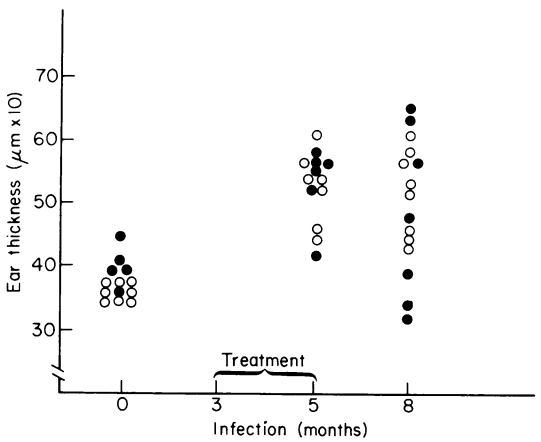

Fig. 1. Individual intradermal reactions (48 hr) to promastigote antigen in C3H mice infected with *L. mexicana*. (o) Untreated animals, (•) animals with lesions treated with BCG. BCG-treated and normal control mice are shown at 0 months of infection.

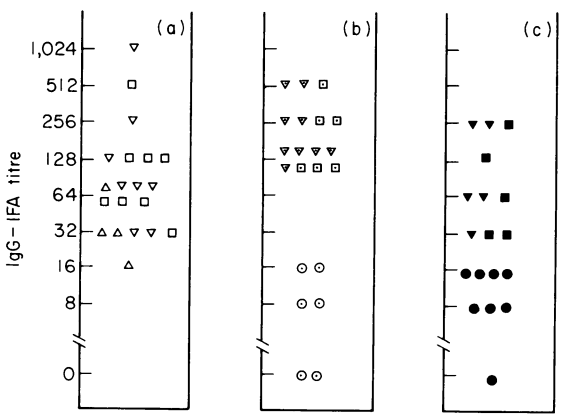

Fig. 2. Individual IgG immunofluorescence titres to promastigote antigen (IgG-IFA) in C3H mice infected with *L. mexicana* and treated with levamisole or BCG. Treatment was from months 3–5 of infection. (o) Uninfected, (△) infected 3 months, (▽) infected 5 months, (□) infected 8 months; (a) untreated (▽); (b) treated with BCG (▼); (c) treated with levamisole (▼). Pooled normal C3H mouse serum was negative in this test.

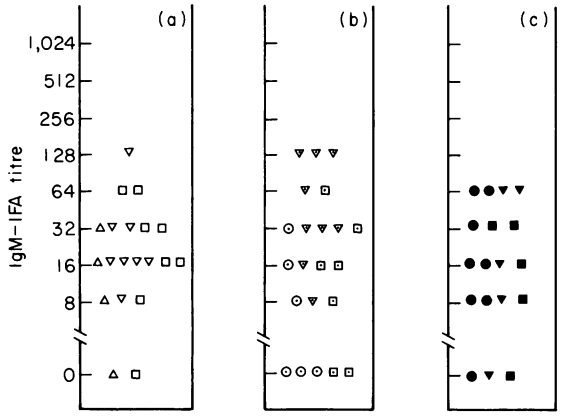

Fig. 3. Individual IgM immunofluorescence titres to promastigote antigen (IgM-IFA) in C3H mice infected with *L. mexicana* and treated with levamisole or BCG. Treatment was from months 3–5 of infection. (o) Uninfected, (▵) infected 3 months, (▿) infected 5 months, (□) infected 8 months; (a) untreated (▿); (b) treated with BCG (▾); (c) treated with levamisole (▾). Pooled normal C3H mouse serum was negative in this test.

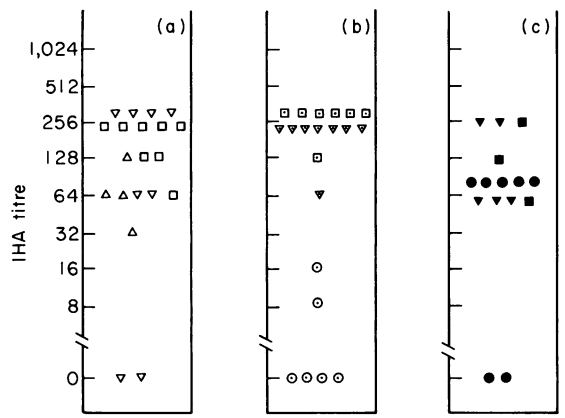

Fig. 4. Individual indirect haemagglutination titres to promastigote extract antigen (IHA) in C3H mice infected with *L. mexicana* and treated with levamisole or BCG. Treatment was from months 3–5 of infection. (o) Uninfected, (▵) infected 3 months, (▿) infected 5 months, (□) infected 8 months; (a) untreated (▿); (b) treated with BCG (▾); (c) treated with levamisole (▾). Pooled normal C3H mouse serum was negative in this test.

mexicana-infected animals (rank sum test, $P<0{\cdot}01$). The size of PPD reactions did not differ significantly between uninfected and *L. mexicana*-infected mice inoculated with BCG.

Delayed hypersensitivity to leishmanial antigens. Uninfected control mice inoculated with BCG had significantly larger reactions to leishmanial antigen than normal non-treated mice (rank sum test, $P<0{\cdot}05$) (Fig. 1). BCG treatment had no effect on leishmanial skin reaction size in *L. mexicana*-infected mice (Fig. 1).

Antibodies to leishmanial antigens. Four of six control mice treated with BCG developed low levels of antibodies that reacted with leishmanial antigens (Figs. 2–4). BCG treatment did not affect the anti-leishmanial IgM or haemagglutinating antibody titres in *L. Mexicana*-infected animals. Anti-leishmanial IgG titres in BCG-inoculated, *L. mexicana*-infected mice were found to be related to the greater prevalence of larger lesions in this group; animals with large lesions, independently of treatment, had significantly higher IgG titres (median 1:256) than animals with medium (median 1:128), small (median 1:128) or minimal (median 1:64) lesions (rank sum test, $P<0{\cdot}01$).

Levamisole treatment. Levamisole failed to alter the course and histopathological characteristics of the leishmanial lesions. Seven of eight uninfected animals treated with levamisole developed low levels of antibodies that cross-reacted with leishmanial antigens. Antibody titres and intradermal reactivity to leishmanial antigen in the *L. mexicana*-infected mice were not altered by levamisole treatment (Figs 2–4).

DISCUSSION

Inbred strains of mice vary in susceptibility to *L. mexicana* infection (Perez, Labrador & Torrealba, 1979). In one group, represented by BALB/c, the lesions develop rapidly, with metastasis, and the mice fail to develop delayed-type hypersensitivity (DTH). In a second group, represented by C3H (Grimaldi *et al.*, 1980), DBA/2 and NMRI the lesion growth eventually slows, but the infection persists despite DTH and humoral responses to leishmanial antigens. In a third group, represented by AKR and C57Bl/6, initial growth of the lesion is followed by regression and healing within 20–25 weeks, accompanied by DTH and humoral responses to parasite antigens.

The mechanisms whereby *L. mexicana* persists in C3H mice despite seemingly adequate antibody and DTH responses remains to be elucidated. Behin, Mauel & Sordat (1979) found that macrophages from inbred strains of mice which fail to heal *L. tropica* lesions were less efficient in destroying the parasites *in vitro* than macrophages from resistant inbred strains. Alexander & Vickerman (1975) have shown that *L. mexicana* survived lysosomal enzymes in the vacuoles of peritoneal macrophages of NIH strain mice. However, the experiments of Alexander & Phillips (1978a), showing that inoculation of *L. mexicana*-infected mice with *L. tropica* healed the lesions caused by both Leishmania species, indicated that macrophages in susceptible mouse strains have the potential to destroy *L. mexicana*. Thus, the immune response in *L. mexicana* infection appears to be regulated in some way that allows the parasites to evade otherwise functional killing mechanisms of the host. Whatever the defect in susceptible mouse strains, non-specific immunostimulation with BCG and levamisole in our experiments failed to diminish the *L. mexicana* lesions.

BCG treatment actually complicated the disease by stimulating metastatic spread of the lesions. Metastasis may result from seeding by greater numbers of infected macrophages leaving BCG-treated lesions (Gaafar & Turk, 1970). Increased metastases also occur when *L. enriettii* is injected together with BCG into BCG-sensitized guinea-pigs (Behin *et al.*, 1977). The slowing of leishmanial lesion growth by concurrent infection by other parasites (Alexander & Phillips, 1978b), by irradiation (Shaw & Voller, 1968) and by anti-macrophage serum (Bryceson *et al.*, 1972) may be a function of the decreased number of host macrophages available for infection.

Both levamisole and BCG stimulated immune responses to leishmanial antigens in uninfected mice. In the case of BCG these responses may be due to cross-reacting antigens shared by the two organisms (Smrkovski & Larson, 1977b). The stimulation of low levels of cross-reacting antibodies to leishmanial antigens by levamisole may indicate that *L. mexicana* also shares antigens with host tissue or with other micro-organisms.

Because non-specific immunotherapy of human cutaneous leishmaniasis would likely be applied in established infections, we gave BCG and levamisole treatment 3 months after infection. Our results indicate that non-specific immunostimulation is ineffective against the chronic non-healing type of leishmaniasis in which the host has humoral and DTH immune responses to the parasites. Furthermore, BCG treatment may be detrimental by causing metastatic spread of infection. The outcome of BCG or levamisole treatment in anergic diffuse leishmaniasis or in lesions destined eventually to heal spontaneously must be evaluated in other host-parasite combinations.

REFERENCES

ALEXANDER, J. & PHILLIPS, R.S. (1978a) *Leishmania tropica* and *Leishmania mexicana*: cross immunity in mice. *Exp. Parasitol.* **44**, 93.

ALEXANDER, J. & PHILLIPS, R.S. (1978b) *Leishmania mexicana* and *L. tropica*: inhibition of growth in mice by concurrent infections of *Trypanosoma brucei*. *Exp. Parasitol.* **44**, 136.

ALEXANDER, J. & VICKERMAN, K. (1975) Fusion of

host cell secondary lysosomes with the parasitophorous vacuoles of *Leishmania mexicana*-infected macrophages. *J. Protozool.* **22,** 502.

BEHIN, R., MAUEL, J. & ROWE, D.S. (1977) Mechanisms of protective immunity in experimental cutaneous leishmaniasis of the guinea-pig. III. Inhibition of leishmanial lesion in the guinea-pig by delayed hypersensitivity reaction to unrelated antigens. *Clin. exp. Immunol.* **29,** 320.

BEHIN, R., MAUEL, J. & SORDAT, B. (1979) *Leishmania tropica*: pathogenicity and *in vitro* macrophage function in strains of inbred mice. *Exp. Parasitol.* **48,** 81.

BRADLEY, D.J., TAYLOR, B.A., BLACKWELL, J., EVANS, E.P. & FREEMAN, J. (1979) Regulation of *Leishmania* populations within the host. III. Mapping of the locus controlling susceptibility to visceral leishmaniasis in the mouse. *Clin. exp. Immunol.* **37,** 7.

BRYCESON, A.D.M., PRESTON, P.M., BRAY, R.S. & DUMONDE, D.C. (1972) Experimental cutaneous leishmaniasis. II. Effects of immunosuppression and antigenic competition on the course of infection with *Leishmania enriettii* in the guinea-pig. *Clin. exp. Immunol.* **10,** 305.

BUTLER, P.G. (1978) Levamisole therapy of chronic *Leishmania tropica*. *J. Trop. Med. Hyg.* **81,** 221.

FIDLER, I.J. & SPITLER, L.E. (1975) Effects of levamisole on *in vivo* and *in vitro* murine host response to syngenic transplantable tumor. *J. Natl. Cancer. Inst.* **55,** 1107.

GAAFAR, S.M. & TURK, J.L. (1970) Granuloma formation in lymphnodes. *J. Pathol.* **100,** 9.

GRIMALDI, G.F., MORIEARTY, P.L. & HOFF, R. (1980) *Leishmania mexicana*: immunology and histopathology in C3H mice. *Exp. Parasitol.* **49.** (In press.)

GUTTERIDGE, W.E., KNOWLER, J. & COOMBES, J.D. (1969) Growth of *Trypanosoma cruzi* in human heart tissue cells and effects of aminonucleoside of puromycin, trypacidin and aminopterin. *J. Protozool.* **16,** 521.

HOFF, R. (1975) Killing *in vitro* of *Trypanosoma cruzi* by macrophages from mice immunized with *T. cruzi* or BCG and absence of cross-immunity on challenge *in vivo*. *J. exp. Med.* **142,** 299.

LITT, M. (1963) Studies in experimental eosinophilia. V. Eosinophils in lymph nodes of guinea pigs following primary antigenic stimulation. *Am. J. Pathol.* **42,** 529.

ORGANIZACIÓN MUNDIAL DE LA SALUD (1976) *Coadyuvantes Inmunológicos*. Informes Tecnicos, 595. Ginebra.

PEREZ, H., LABRADOR, F. & TORREALBA, J.W. (1979) Variations in the response of five strains of mice to *Leishmania mexicana*. *Int. J. Parasitol.* **9,** 27.

PRESTON, P.M. & DUMONDE, D.C. (1976) Immunology of clinical and experimental leishmaniasis. In *Immunology of Parasitic Infections* (ed. by S. Cohen and E. Sadun), pp. 167–202. Blackwell, Oxford.

SHAW, J.J. & VOLLER, A. (1968) Observations on the susceptibility of white mice to infection with *Leishmania mexicana* following whole-body X-irradiation. *Ann. Trop. Med. Parasitol.* **62,** 174.

SHEPARD, C.C., VAN LANDINGHAM, R. & WALKER, L.L. (1977) Effect of levamisole on *Mycobacterium leprae* in mice. *Infect. Immun.* **16,** 564.

SMRKOVSKI, L.L. & LARSON, C.L. (1977a) Effect of treatment with BCG on the course of visceral leishmaniasis in BALB/c mice. *Infect. Immun.* **16,** 249.

SMRKOVSKI, L.L. & LARSON, C.L. (1977b) Antigenic cross-reactivity between *Mycobacterium bovis* (BCG) and *Leishmania donovani*. *Infect. Immun.* **18,** 561.

SNEDECOR, G.W. (1956) *Statistical Methods* 5th edn. Iowa State University Press, Ames.

SYMOENS, J. & ROSENTHAL, M. (1977) Levamisole in the modulation of the immune response: the current experimental and clinical state. *J. Reticuloendothel. Soc.* **21,** 175.

WEINTRAUB, J. & WEINBAUM, F.I. (1977) The effect of BCG on experimental cutaneous leishmaniasis in mice. *J. Immunol.* **118,** 2288.