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ENHANCEMENT OF *LEISHMANIA AMAZONENSIS* INFECTION IN BCG NON-RESPONDER MICE BY BCG-ANTIGEN SPECIFIC VACCINE

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Different patterns of cutaneous leishmaniasis can be induced when a challenge of alike dose of Leishmania amazonensis *amastigotes in various inbred strains was applied. Two strains of mice, the Balb/c and $C_{57}BL/10J$, showed exceptional susceptibility, and 10^6 amastigotes infective dose lead, to ulcerative progressive lesions with cutaneous metastasis and loss by necrosis of leg on which the footpad primary lesion occurred. Lesions were also progressive but in a lower degree when C_3H/HeN and $C_{57}BL/6$ were infected. Lesions progress slowly in DBA/2 mice presenting lesions which reach a discreet peak after 12 weeks, do not heal but do not uncerate. DBA/2 mice is, therefore, a good model for immunomodulation.*

In attempt to determine the influence of BCG in vaccination schedule using microsomal fraction, DBA/2 became an excellent model, since it is also a non-responder to BCG. Vaccination of DBA/2 mice, receiving the same 10^6 BCG viable dose and 10 μg or 50 μg of protein content of microsomal fraction, lead to a progresive disease with time course similar to those observed in susceptible non-vaccinated $C_{57}BL/10J$ mice after 6 months of observation. An enhancement of infection in BCG non-responder mice suggests that use of BCG as immunostimulant in humans could be critical for both vaccination and immunoprophylactic strategies.

Key words: *Leishmania amazonensis* – inbred mice – vaccination – BCG – microsomal fraction

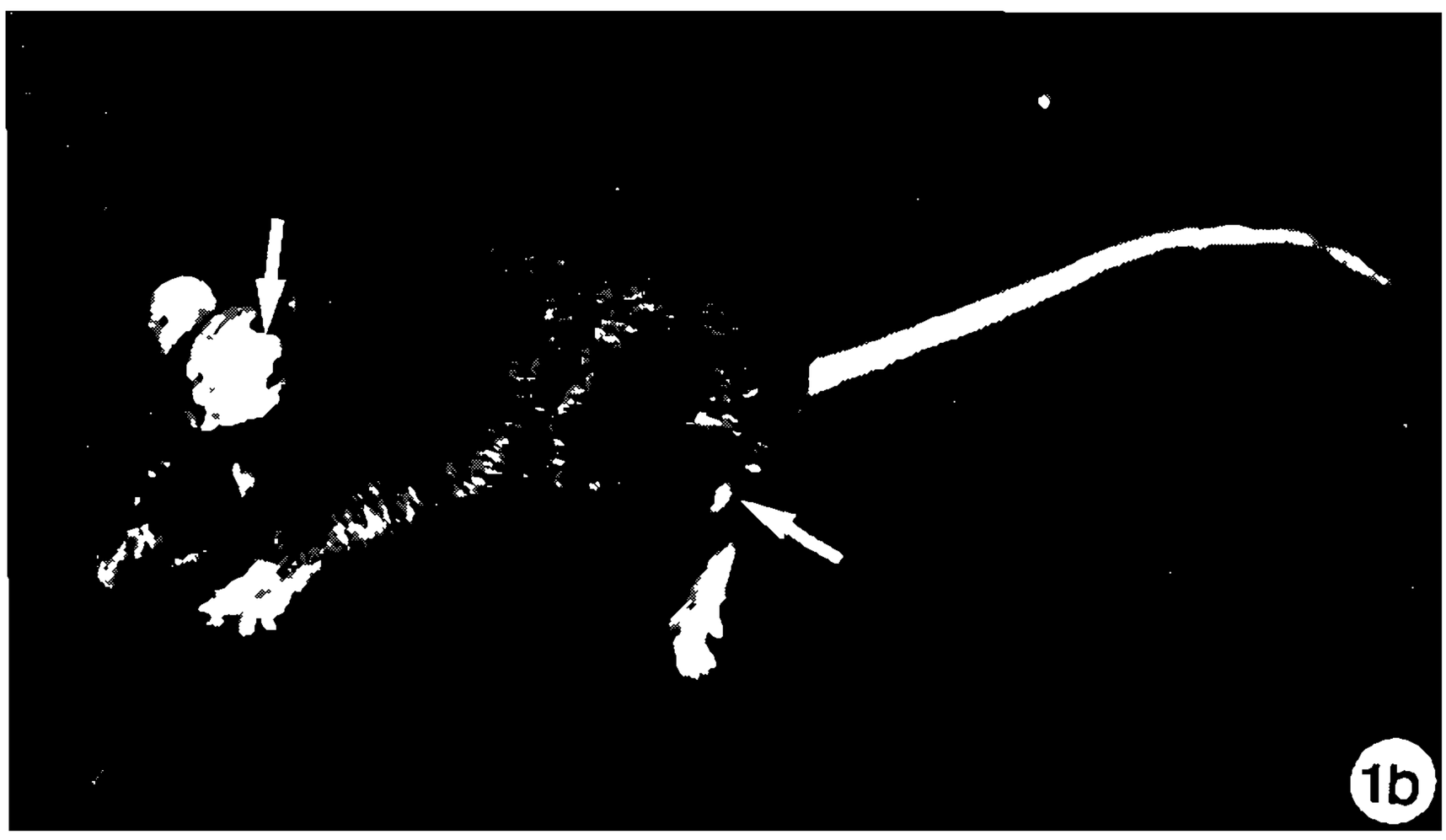
Recently there has been much interest in immunization against tegumentar leishmaniasis (Mayrink et al., 1979; Howard et al., 1982; Handman & Mitchell, 1985; Russel & Alexander, 1988; Frommel et al., 1988). The investigations on the natural susceptibility to leishmania, and its genetical control (Handman et al., 1979; Howard, 1986) opened new perspectives on the availability of experimental murine models.

Many investigations have been made by assay products and schedules directly in humans as vaccination strategy or as immunotherapy for treatment of localized cutaneous leishmaniasis using killed organisms plus BCG (Convit et al., 1987). Recently we developed a successful immunization schedule using subcellular fractions of promastigotes associated with BCG to induce immunity to *Leishmania amazonensis* in outbred mice (Gonçalves da Costa et al., 1988). The using of BCG in association with specific leishmania antigen brings another questions: how a non-responder mice to BCG is able to control the leishmania infection since in our experimental schedule the mycobacteria were essential?

MATERIALS AND METHODS

Animals – Female DBA/2, C_3H/HeN, $C_{57}BL/6$, $C_{57}BL/10$, Balb/c mice, aged 4 to 6 weeks from Immunobiology Department of Fluminense Federal University; OF_1 outbred mice, 4-6 weeks old, purchased from IFFCREDO (Saint-Germain-Sur-l'Arbresle France) and introduced at Oswaldo Cruz Institut by one of us (S.C.G.C.). Experimental groups consisted of 6 mice.

Parasites – The H_{21} strain of *L. amazonensis* isolated from a patient with diffuse cutaneous leishmaniasis (Schottelius & Gonçalves da Costa, 1982) was maintained *in vivo* by serial mouse passage. Purified suspensions were obtained from non-ulcerated histiocytoma 6 months after subcutaneous injections of the H_{21} strain in female Balb/c mice. Parasites were harvested and purified after rupture of the tissue nodules in a Potter-Elvehjens ho-

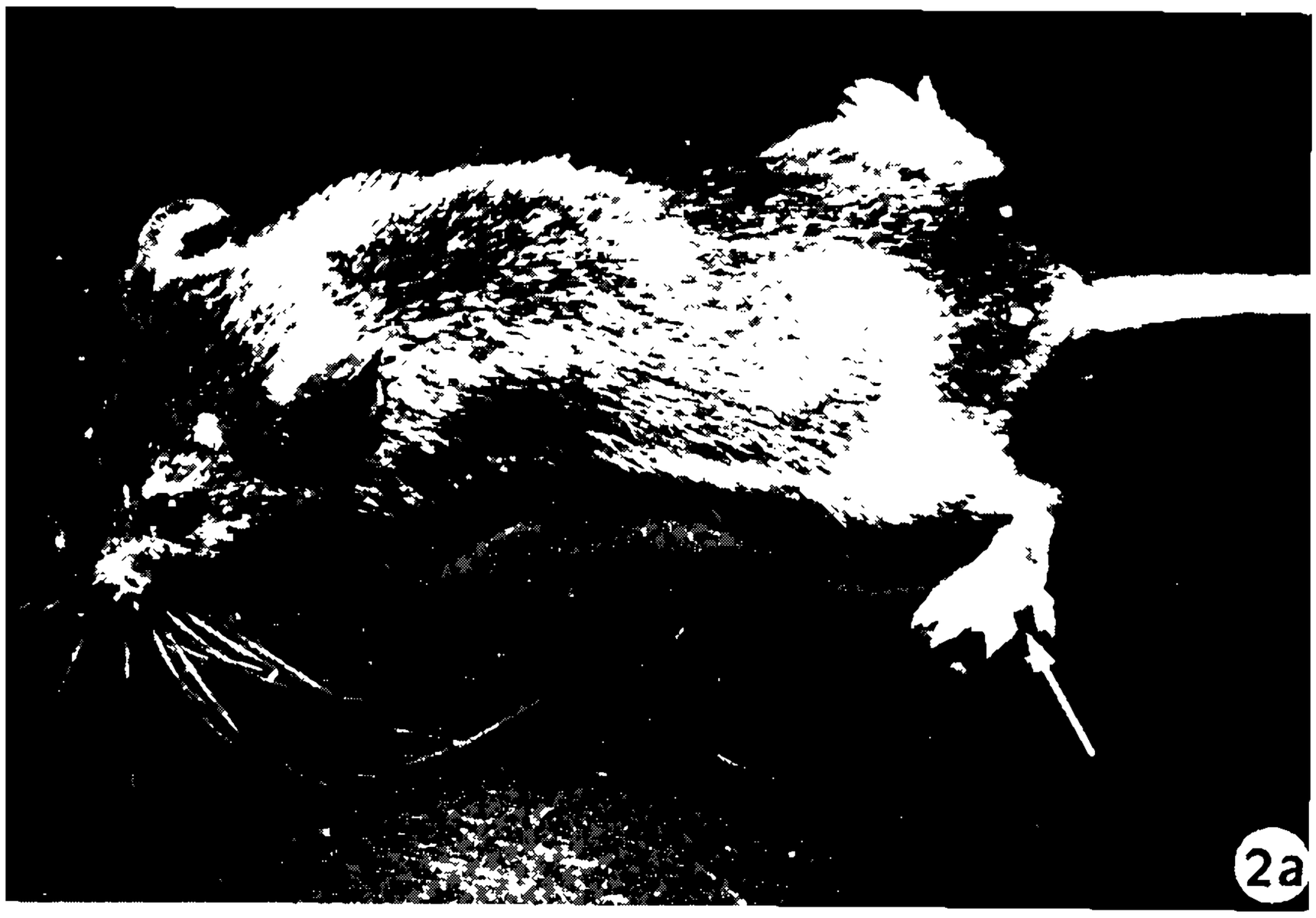


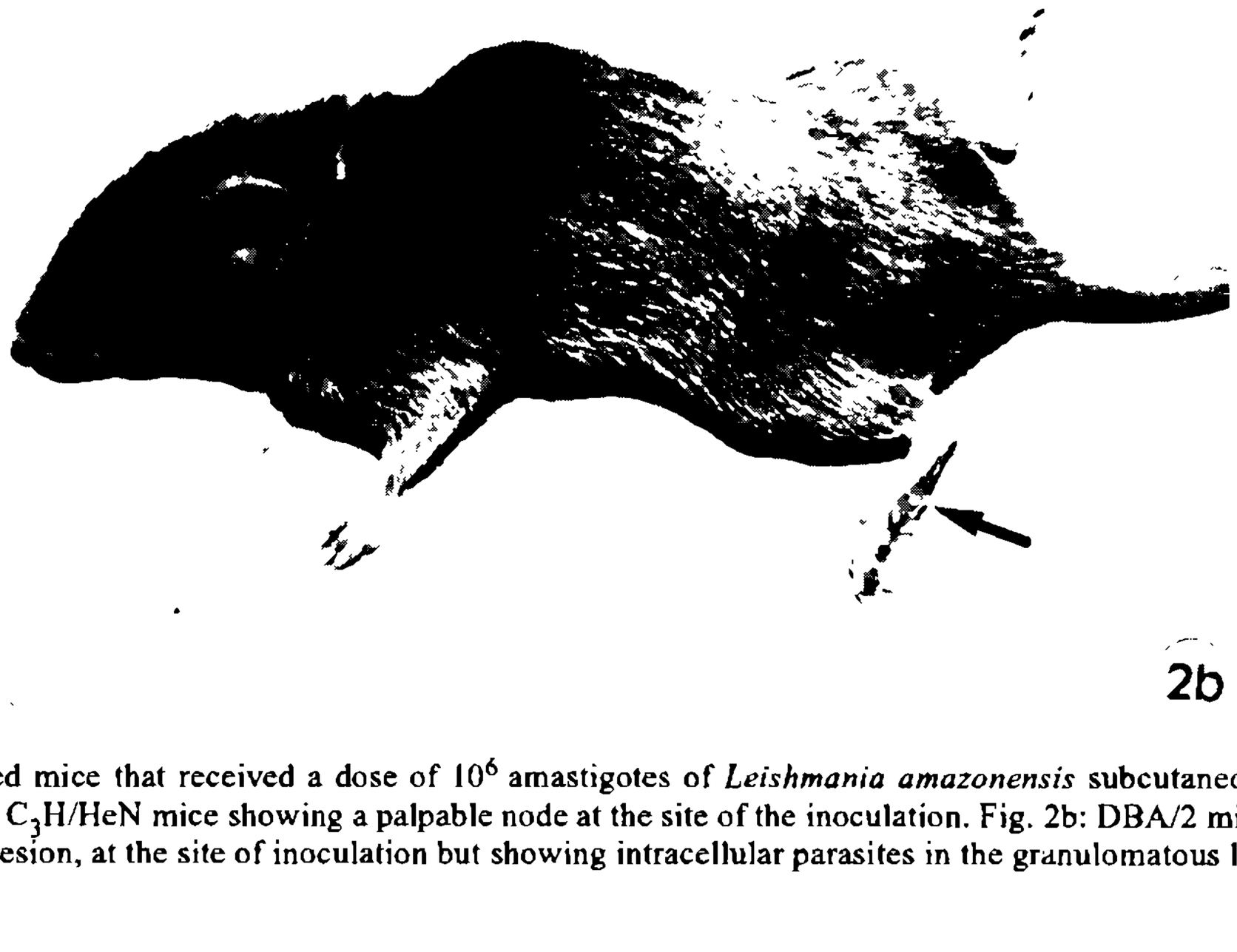
Susceptible mice infected with 10^6 amastigotes of Leishmania amazonensis in the LHFP. Fig. 1a: Balb/c mice showing the loss of number corresponding to the right footpad which received the inoculation dose, as consequence of a progressive necrosis (arrow). Fig. 1b: $C_{57}BL/10$ mice showing the same lesion observed in Balb/c mice with the loss of the leg that received the infective dose, but after 340 days of infection.

mogenizer with a teflon pestle in PBS. Cells were then filtered through gauze using a 10 ml syringe. The filtered suspension was generally free of intact host cells. Evaluation of the percentage of damaged cells was made using erythrosin B stain, as described elsewhere (Hodgkinson et al., 1980).

Antigen – Log-phase promastigotes on the 4th day of cultivation in LIBHIT medium (Gonçalves da Costa & Lagrange, 1981) supplemented with 5% fetal calf serum (LIBHIT – FCS) were harvested and washed three times in phosphate-buffered saline with 2% glucose (PBS-G). The pellets (3 to 4g) were suspended in hypotonic medium for swelling and then disrupted in a Dounce-type homogenizer (Kontes Glass, Vineland, NJ) with a tight-fitting pestle (type B), in presence of the non-ionic detergent Lubrol PX. Fractionation procedure for obtain the microsomal fraction antigen (Mic.-Ag.) has been described in detail (Gonçalves da Costa et al., 1988).

BCG vaccine – The Pasteur strain of *Mycobacterium bovis* var. BCG (Bacille Calmette-Guérin) was obtained from Mrs Gheorghiu, BCG Production Unit, Institut Pasteur. The organism was grown in dispersed culture as described elsewhere (Lagrange et al., 1978). After 6 days





Resistant inbred mice that received a dose of 10^6 amastigotes of *Leishmania amazonensis* subcutaneously in the LHFP. Fig. 2a: C_3H/HeN mice showing a palpable node at the site of the inoculation. Fig. 2b: DBA/2 mice showing a very discret lesion, at the site of inoculation but showing intracellular parasites in the granulomatous lesions after 40 weeks.

of incubation, the culture were frozen slowly to 70 °C and were stored at this temperature until use. Dosage was based upon viable counts performed by plating dilutions on Middlebrook 7H 10 medium. BCG (2 x 10^6 BCG/mouse) was injected subcutaneously (Sc) into the left hind footpad (LNFP) 14 days before specific immunization with Mic.-Ag. (Group A).

Immunization and challenge – Normal and BCG-pretreated mice were injected with Mic.-Ag. fraction antigen (20 µg of protein/mouse in a volume of 0.04 ml pyrogen-free saline into the LHFP. Other groups of normal and BCG-pretreated mice were left unimmunized and served as controls. Six days later all mice were inoculated in the right hind

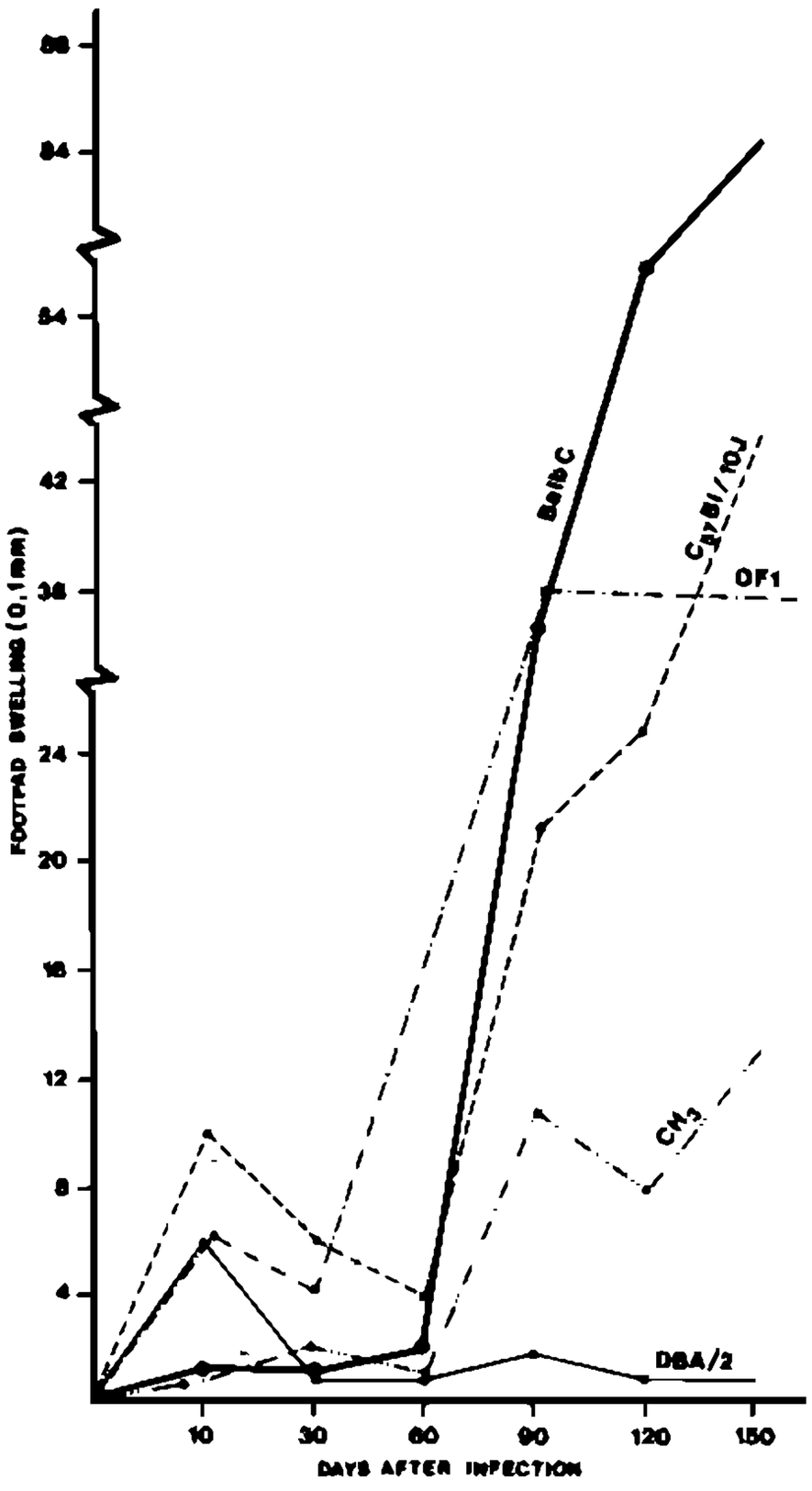


Fig. 3: time course of primary lesions in mice Swiss outbred strains infected with 10^6 amastigotes of *Leishamania amazonensis* H21 strain in the footpad.

footpad (RHFP) with 1 x 10^6 purified amastigotes of H21 strain.

Delayed-type hypersensitivity (DTH) – DTH reaction to living amastigote cells was measured at various times after challenge with a dial gauger caliper (Schnelltaster, H. C. Kröplin, GRBH, Hessen, FRG). DTH was expressed as especific footpad thickness after subtracting the background swelling in non-immunizing mice.

Lesion kinetic – Infective amastigotes (1 x 10^6 parasites/mouse) were injected into a granuloma-free RHFP of vaccinated mice and lesions were measured at various times after infection using the dial gauge caliper. They were expressed as the difference in millimeters between the infected footpad and average of 15 normal footpads taken from a group of non-infected mice maintained during the course of infection.

The same dose of infective amastigotes were used in preliminary experiments using different inbred strains of mice to know the best model of resitant mice to *L. amazonensis* that are non-responder to BCG.

Statistical analysis – Results are expressed as the arithmetic mean and standard error of the mean (SEM).

RESULTS

Comparative time-course of infection in different inbred strains of mice – The time course of infection by *L. amazonensis* presents various patterns and characteristic clinical manifestations in different mouse strains. Within 20 days Balb/c mice have a measurable palpable lesion at the inoculated footpad. The lesions are progressive and after 20 weeks become ulcerated and metastatic lesions begin to appear at different feet, tail and ear. Frequently, the last event is the loss of the member corresponding to the inoculated footpad by progressive necrosis (Fig. 1a). Visceralization has been observed in the spleen. The same tendence to form metastatic lesions and loss of the footpad by necrosis has been observed in C_{57}BL/10J mice (Fig. 1b), however only after 240 days this occurs and had been less dramatic, since the mice do not present the cachexia which is the final clinical feature of the infection in Balb/c mice. A slower time course lesions was observed in C_{57}BL/6J and C_3H/HeN mice (Fig. 2a) than those seen in Balb/c and C_{57}BL/10J mouse strains, and in outbred OF_1 mice as well (Fig. 3). In outbred OF_1 mice, the progressive lesions reach extraordinary levels without loss of the leg (Fig. 4a) but frequently metastatic lesions occur in the tail, ear, nose and feet (Fig. 4b). Mortality rate begins earlier in Balb/c mice, about 5 months, while in C_{57}BL/10J and C_3H/HeN death occured only after 13 months of infection (Fig. 5). In DBA/2 (Fig. 2b), infection developed very slowly, lesions being palpable later, 12 weeks after infection and persisting for 12 months. All DBA/2 infected mice remained alive until 15 months when we stopped the protocol.

Vaccination in BCG-immune and normal mice – The development of DTH was test in groups of OF_1 outbred mice and DBA/2 mice immunized with 20 μg of microsomal fraction antigen with or without the influence of BCG priming. Six days after, DTH to a challenge dose of 10^6 amastigotes was measured by the mean levels of increase in corresponding foot-

Adult outbred OF_1 mice that received a dose of 10^6 amastigotes of *Leishmania amazonensis* subcutaneously in the LHFP. Fig. 4a: mouse presenting necrosis of an ulcerated primary lesion that reached extraordinary level. Fig. 4b: appearance of metastatic lesions that occured in the tail, ear, nose and all footpad.

pad swelling. No great difference was observed in skin test against leishmania antigen between BCG-primed and normal control mice infected with *L. amazonensis*.

Time course infection of vaccinated mice, that received a challenge dose of 10^6 purified amastigotes of *L. amazonensis* H21 strain 6 days after microsomal fraction immunization, was measured regularly thereafter. Thirty days after, the vaccinated group present a palpable node at the site of the inoculation in contrast with the control groups in which insignificant footpad sweeling was observed. After 6 and 12 months post-infection, the lesions of vaccinated group were twice and three times larger than in the control groups (Fig. 6). The skin of the primary lesion showed the squamous epithelium cornified and the dermis infiltrated with macrophages colonized by leishmania (Fig. 7).

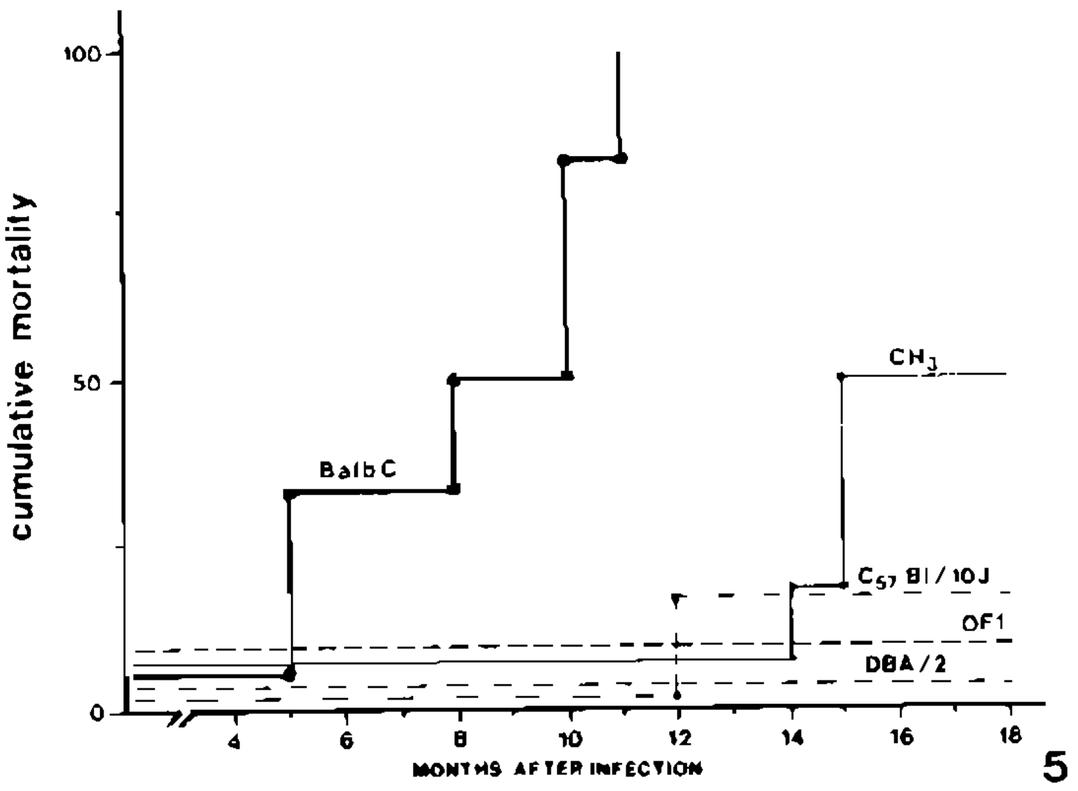


Fig. 5: cumulative mortality rate in inbred mice infected with 10^6 amastigotes of *Leishmania amazonensis*.

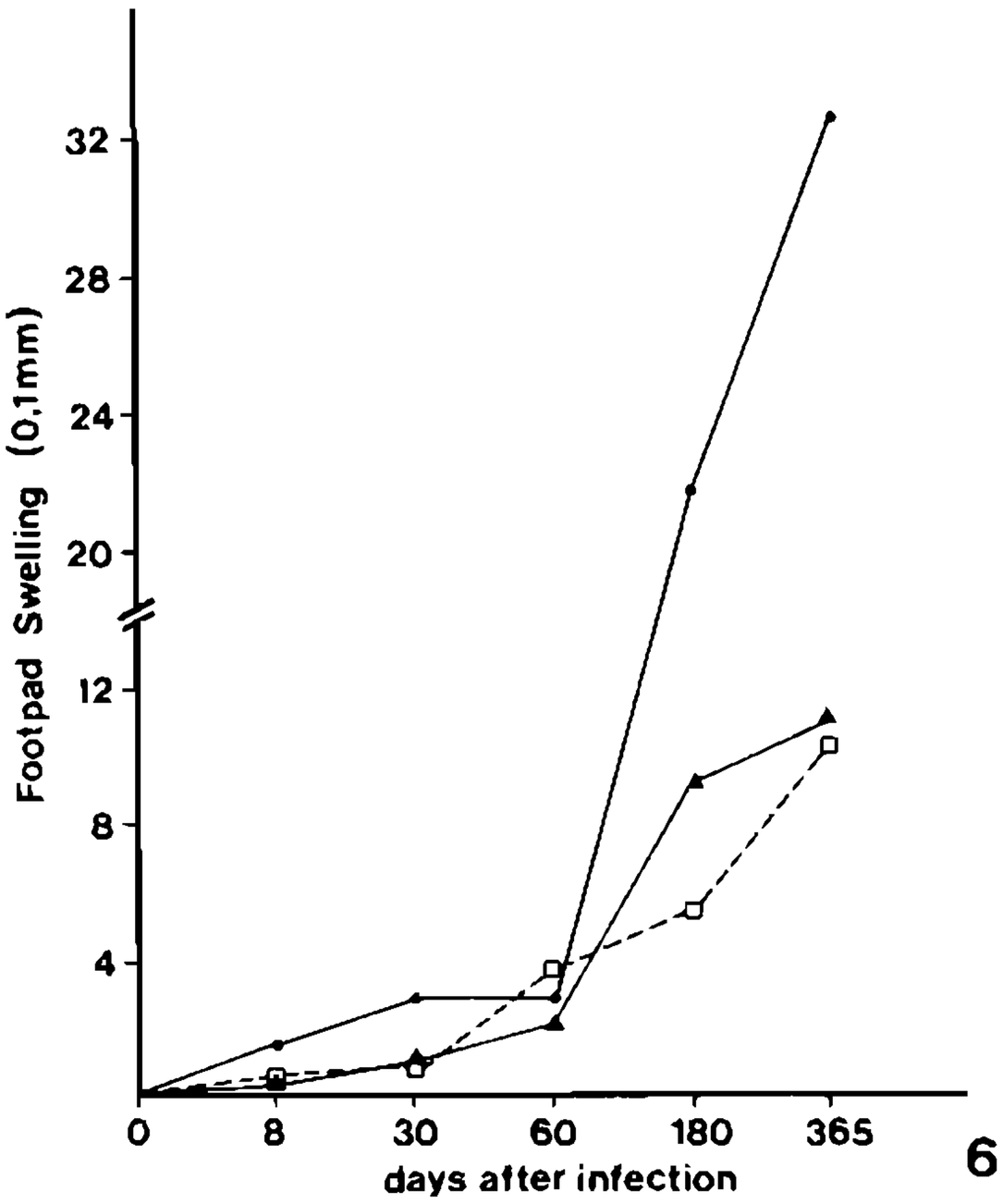


Fig. 6: time course of lesions in DBA/2 mice infected with 10^6 amastigotes of *Leishmania amazonensis*. (● – ●) immunized with 10^6 BCG and treated with microsomal fraction 20ug; (▲ – ▲) only BCG immunized and (□ – □) control group receiving the infective dose of parasite.

DISCUSSION

Attempts to immunize against leishmaniasis frequently use the potencialization of poor immunogen by using adjuvant. BCG has been used as immunopotentiator associated with semi-purified fractions in an experimental mouse model (Gonçalves da Costa et al., 1988) or even with whole dead promastigotes with sucess in man (Convit et al., 1987). As these attempts did not take into account if animals or humans were able to responder to BCG it was necessary to investigate the effect of this vaccine in BCG non-responder mice. The present data show that the same protocol developed by us in outbred mice in which 10^6 BCG colony-forming units were injected subcutaneously two weeks before immunization with promastigotes microsomal fraction from *L. amazonensis* at the same site, inducing an important protection against a challenge of viable amastigote cells, failed to protect DBA/2 mice. This negative result may be discussed regarding two independent variables such as the susceptibility or resistance to BCG and to *L. amazonensis*. Experimental studies indicate that immunopotentiation is correlated with BCG specific cell mediate immunity and this is directly related to the level of BCG load in the lymphoid tissues which in turn is dependent on BCG strain and dose, as well the host's genetic control against BCG. Thus DBA/2 being naturally resistant to BCG present innate resistance to intravenous low dose but is unable to mount specific CMI and protection to BCG (Lagrange & Hurtrel, 1989). On the other hand analyzing the mouse strain distribution of response to different species of cutaneous leishmaniasis on can observe, in the case of DBA/2 mice, a large spectrum of responses reported by different authors. It was considered susceptible to *L. major* infection by several authors (Behin et al., 1979; Handman et al., 1979; Howard et al., 1980), intermediate by Kellina (1973) and resistant by De Tolla et al. (1980a, b). Working with *L. mexicana*, Peres et al. (1979) showed that DBA/2 was relatively resistant while Alexander & Blackell (1986) found this strain of mice to be strikingly resistant. In our experiments C_3H/HeN and DBA/2 developed persistent lesions that remain stationary with a small palpable nodule without clearence of the infection, but DBA/2 lesions were smaller than those of C_3H/HeN. These results coincide with those observations of Perez et al. (1979), who found DBA/2 relatively resistant (intermediate), since although the lesion ceases to grow it persists and fails to heal after one year of observation. The difference in natural resistance observed by various authors seems to be a consequence of the use of various inoculum doses, different stage of parasite development, and confusing results due to the use of different species and strains of leishmania. So DBA/2 mouse be-

Fig. 7: histopathology of primary lesion 6 months after infection with 10^6 amastigotes. Heavy colonization of macrophages by *Leishmania amazonensis* and some inflamatory cells. Haematoxylin and eosin stain; 400X.

came interesting to our purpose of investigating the effect of BCG in modulating protection induced by a leishmania vaccine in a BCG non-responder host. The most remarkable result obtained by us deals with DBA/2 mouse, in which the BCG-leishmania microsomal fraction vaccine induced an enhancement to the infection in animals challenge with 10^6 vaible amastigotes. This contrasts with the promissing results obtained in outbred mice using the same preparation and schedule (Gonçalves da Costa et al., 1988). Thus, there is an urgent need for further studies in this area to evaluate the different variables concerning the host genetic factors, interval between vaccination schedule, vaccination route and dose, in order to establish the effectiveness and risk of vaccines preparations before any attempt to vaccinate humans.

The methodology used in the experiments of the present paper is based on the induction of DTH to leishmania antigen under modulation of BCG. Most of DTH obtained using subcellular fractions of trypanosomatids to sensitize mice induces the Jones-Mote like hypersensitivity and frequently this sensitization leads to an enhancement of infection. This was true when we immunized mice with purified flagellar fraction of *T. cruzi*, but the picture was reverted by pre-immunization of mice with fresh-frozen BCG (Gonçalves da Costa & Lagrange, 1981). The different DTH, classical tuberculin or Jones-Mote like response associated with different modulatory agents over mice resistance to parasite infection may be related to the inducements of distinct subsets of CD_4^+ T cells as proposed by Mosmann et al. (1986). TH1 been kindreb to host protective response and TH2 with disease enhancement, which has been studied in leishmania system (Scott, 1989). The strategy of using BCG in modulate parasite antigen seems at least in part to induce CD_4^+ T cell sub-population Th1 related to host protective response and in our experience this was achieved in two different models, such experimental Chagas' disease using flagellar fraction and in cutaneous leishmaniasis using microsomal fraction from promastigotes. The present report shows, however, that the response of mice strain to BCG is critical, and studies are being carried on to explain the mechanismes of enhancement of infection in BCG immune DBA/2 vaccinated mice.

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