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The effects of protein malnutrition on the course of *Leishmania mexicana* infection in C57Bl/6 mice: nutrition and susceptibility to leishmaniasis

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SUMMARY

The course of cutaneous infection with *Leishmania mexicana* was studied in normally nourished and protein deprived C57Bl/6 mice. Mice fed a normal diet showed self-resolving lesions and produced cellular and humoral responses against the parasite. In contrast, mice fed a protein deficient diet which developed chronic protein calorie deficiency failed to recover from *L. mexicana* infection. Non-healing protein deprived mice had depressed delayed hypersensitivity response (DHR) and *in vitro* lymphocyte reactivity to leishmanial antigen. Responses to PHA and Con A were also suppressed. The possible interaction between malnutrition, impairment of the immune response and chronicity of cutaneous leishmaniasis is discussed.

INTRODUCTION

Clinical and epidemiological evidence suggests that nutritional deficiency leads to increased susceptibility to infections (Chandra, 1976). Malnutrition impairs immunological responses (Edelman, 1977; Chandra, 1977), and the non-specific factors of resistance (Neumann, 1977).

In tropical and subtropical areas of the world nutritional deficiencies may play an important role in the chronicity of some parasitic infections. Cutaneous leishmaniasis is an important public health problem in Central and South America causing considerable morbidity and disfigurement of the affected individuals.

Infection of C57Bl/6 mice with 10^3 amastigotes of *L. mexicana* produced cutaneous lesions which healed within five months, with the appearance of delayed hypersensitivity response and antibodies against the parasite (Pérez, Labrador & Torrealba, 1979). In this study, we have compared the course of cutaneous infection in normally nourished and protein deficient C57Bl/6 mice.

MATERIALS AND METHODS

Animals and diets. Three week female C57Bl/6 mice were purchased from the Jackson Laboratories (Bar Harbor, Maine) and distributed at random into two groups. One group was fed a control diet containing 27% casein and the other group a low protein diet containing 8% casein. These mice were designated normally nourished (N) and protein deprived (D) respectively. Both diets, obtained from Nutritional Biochemical (Illinois, USA), were isocaloric with complete vitamin supplement and administered *ad libitum*.

Parasites. L. mexicana strain AZV was isolated from a human case of cutaneous leishmaniasis in the Carabobo state, Venezuela, by J.W. Torrealba, its maintenance in laboratory conditions has been previously described (Pérez *et al.*, 1979).

Infection. Amastigotes were obtained from infected hamsters as described elsewhere (Pérez *et al.*, 1979). Five week old N and D mice received 10^3 amastigotes subcutaneously (s.c.) injected into the dorsal surface of the right foot. Different groups of mice infected with the same batch of parasites were used to evaluate the course of the infection and the *in vivo*

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and *in vitro* immune responses. Animals were fed their respective diets throughout the experiment. Control uninfected mice of each dietary group were kept simultaneously.

Course of the infection. Ten mice of each group were weighed weekly and examined for the presence of cutaneous metastases. Lesion size was measured with 'Schnelltäster' calipers (Kröplin, Germany). For each mouse, the size of the lesion was calculated from the difference in width between infected and uninfected feet.

Delayed hypersensitivity response. Every four weeks, four infected mice of each dietary group were skin tested with leishmanial antigen obtained from promastigotes of *L. mexicana* (Pérez *et al.*, 1979); 50 μg of antigen were injected s.c. into the left hind footpad. Dermal reactions were recorded as increase of the footpad thickness measured with 'Schnelltäster' calipers at 0 and 24 hr after injection (Preston *et al.*, 1972).

Antibody response to leishmania. Sera were obtained from four mice of each group of experimental animals every four weeks after infection and stored at $-20°C$. Antibody assay was performed by direct agglutination of trypsinized formalinized promastigotes as described by Mattossian-Rogers, Lumsden & Dumonde (1976).

In vitro cultures. Pooled spleen cell suspensions were prepared from four infected and four uninfected mice of each dietary group.

Cell suspensions were adjusted to a concentration of 5×10^6 cell per ml in RPMI 1640 medium supplemented with 2 mM L-glutamine, penicillin 100 U/ml, streptomycin 100 μ/ml and 5% foetal calf serum. 5×10^5 spleen cells were delivered into the wells of disposable microculture trays (Linbro-IS-FB-96-TC, International Scientific Instruments, Illinois, USA). Concanavalin A (Con A) (Sigma Chemical, Missouri, USA); phytohaemagglutinin (PHA) (Reagent grade; HA15, Wellcome Research Laboratories, Beckenham, England); lipopolysaccharide B (LPS) from *E. coli* 017 B:8 (Difco, Michigan, USA) and leishmanial antigen were diluted to the desired concentration in complete medium and added to each well in a 0·1 ml volume. Culture trays were incubated for 72 hr at 37°C in a humid atmosphere of 5% CO_2 in air. To assess DNA synthesis 1 μCi of ^{3}H-thymidine (specific activity 5 Ci/mM, Amersham/Searle, Illinois, USA) was added to each well for the final 16 hr incubation of culture. Therefore, cells were collected by suction on glass-fibre filters, using a multiple automated sample harvester (Mash II, Microbiological Associates, Maryland, USA). The radioactivity was measured in a liquid scintillation spectrometer and the results expressed as the arithmetic mean of triplicate cultures.

RESULTS

Effect of diet on the body weight of L. mexicana infected mice

The growth rates of *L. mexicana* infected mice fed 27% and 8% protein diets are shown in Fig. 1. At four weeks of treatment (seven weeks of age) body weights of D infected mice were significantly less ($P<0·001$) than those from N infected mice. D mice put on weight very slowly and the difference between the whole body weight of D and N animals remained statistically significant throughout the observation period.

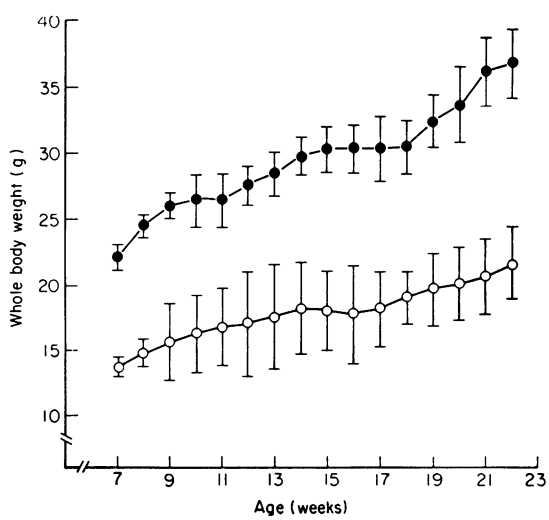

FIG. 1. Body weight of normally nourished (●) and protein deprived (○) mice infected with *L. mexicana*. Mean ± s.e.

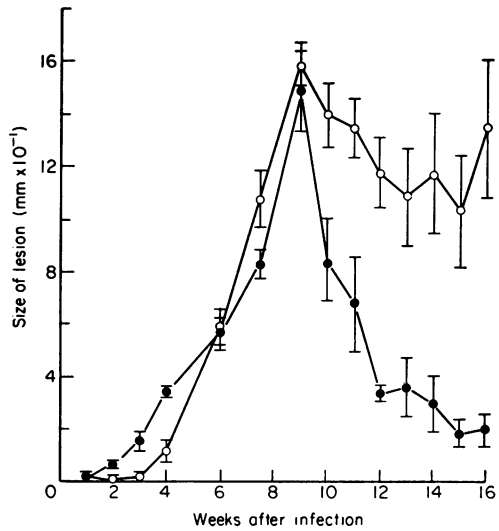

FIG. 2. Size of lesions of normally nourished (●) and protein deprived mice (○) infected with *L. mexicana*. Mean ± s.e.

Course of the infection

N infected mice developed palpable nodes at the site of inoculation three weeks after infection (Fig. 2). These lesions progressed rapidly and by eight weeks they had ulcerated. The sizes of these ulcerated lesions were maximal at nine weeks when they were covered with a crust. In D mice, lesions developed more slowly than in N mice during the first four weeks of infection. Thus at four weeks, the mean size of lesions (11·5 ± 6·85) was significantly less than in N mice (34·5 ± 6·85) as revealed by Student's *t*-test

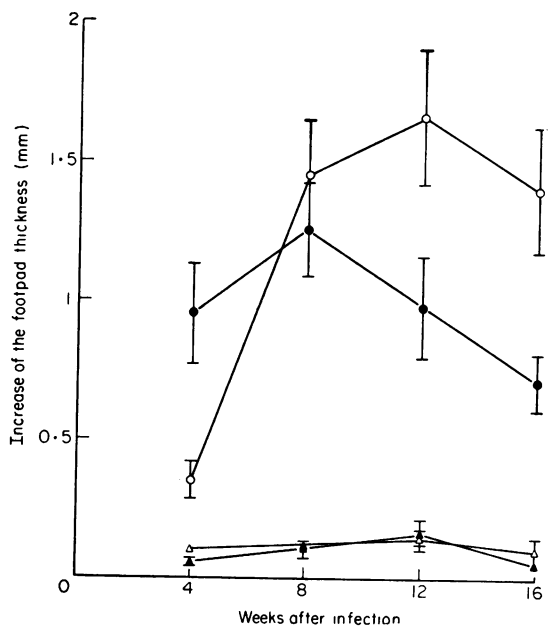

FIG. 3. Development of delayed hypersensitivity response to 50 μg of leishmanial antigen in normally nourished (N) and protein deprived (D) mice after infection with *L. mexicana*. N infected (○), N uninfected (△), D infected (●) and D uninfected (▲) mice. Mean ± s.e.

($P<0{\cdot}001$). However, once lesions established they progressed rapidly and six weeks after infection all D mice had ulcerated lesions.

At twelve weeks, lesions in N mice had started to heal and by sixteen weeks these mice had recovered from infection. By contrast, twelve weeks after the infection D mice still had large ulcerated lesions which persisted until the end of the observation period (sixteen weeks) (Fig. 2).

Delayed hypersensitivity responses

Fig. 3 shows the development of DHR to leishmanial antigen in N and D *L. mexicana* infected mice. Four weeks after infection, D mice showed a significantly increased DHR ($P<0{\cdot}01$) as compared to that of four weeks N infected mice. At eight weeks, N mice showed greater DHR than did D mice but differences were not significant. A suppressed DHR ($P<0{\cdot}05$) was observed in D as compared to normally nourished mice twelve weeks after infection. Thereafter, DHR in D mice remained suppressed and by sixteen weeks, it represented about 50% of the response observed in N recovered mice.

Antibody response to leishmania

Table 1 shows the agglutination titres of sera from N and D mice taken at regular intervals after the infection. Both N and D *L. mexicana* infected mice developed agglutinating antibodies against the parasite. These antibodies were detectable at four weeks and persisted throughout the experiment. Control sera from N and D uninfected mice did not show agglutination at the starting serum dilution of 1:2.

In vitro spleen cell responses to mitogens

The proliferative response to PHA and Con A of spleen cells from non-infected D mice was moderately increased as compared to that of N non-infected animals in tests performed at three and eight weeks. Nevertheless, at the end of the experiment (week 16) the responses of spleen cells from D and N animals were similar, although a depression of the response of D spleen cells became evident upon stimulation with suboptimal doses of mitogen (Tables 2 and 3). The response to PHA and Con A of spleen cells from infected D mice was significantly depressed at the time lesions reached their maximal size whereas responses of spleen cells from infected N mice were enhanced at similar time. Sixteen weeks after infection spleen cells from N and D infected mice showed similar responses to mitogens (Tables 2 and 3).

In vitro response to L. mexicana leishmanial antigen

Sixteen weeks after the infection D mice showed an impaired capability to control *L. mexicana* infection as compared to N mice (Fig. 2). Failure to control the infection was accompanied by a depressed

TABLE 1. Agglutinating antibody titres* in sera from normally nourished (N) and protein deprived (D) mice infected with *L. mexicana*

Weeks after infection	N mice	D mice
4	32	32
8	16	32
12	16	32
16	16	32
Controls	< 2	< 2

* Titres are expressed as the reciprocal of highest serum dilution given agglutination. Pooled sera from four mice of each group.

TABLE 2. *In vitro* spleen cell responses of normally nourished (N) and protein deprived (D) mice infected with *L. mexicana* to optimal and suboptimal doses of PHA

Weeks after infection	N mice		D mice	
	Uninfected	Infected	Uninfected	Infected
	Optimal dose (4 μl/ml)			
3	53,749 (2607)*	61,871 (1221)	76,564 (1102)	92,277 (1901)
5	42,027 (6306)	117,205 (7347)	75,319 (4724)	116,620 (563)
8	94,208 (10,109)	178,380 (7515)	165,034 (10,102)	51,939 (13,366)
16	129,067 (10,921)	99,264 (4183)	96,799 (3370)	110,930 (2239)
	Suboptimal dose (1 μl/ml)			
3	11,895 (734)	16,274 (849)	18,392 (2195)	29,236 (1154)
5	28,995 (2661)	70,906 (5794)	39,281 (652)	28,752 (3796)
8	n.d.†	n.d.	n.d.	n.d.
16	35,182 (3510)	35,630 (3771)	12,770 (1264)	15,177 (670)

* cpm mean ± s.e. of triplicate cultures of pooled spleen cells from four mice of each group.
† n.d. = Not done.

TABLE 3. *In vitro* spleen cell responses of normally nourished (N) and protein deprived (D) mice infected with *L. mexicana* to optimal and suboptimal doses of Con A

Weeks after infection	N mice		D mice	
	Uninfected	Infected	Uninfected	Infected
	Optimal dose (2·5 μg/ml)			
3	122,903 (6231)*	118,788 (2543)	154,402 (6912)	184,954 (12,094)
5	31,674 (3377)	123,810 (11,740)	87,361 (15,666)	25,394 (536)
8	143,082 (2194)	257,216 (10,124)	216,810 (10,109)	66,633 (13,688)
16	126,243 (18,474)	88,021 (3574)	100,051 (9040)	96,938 (7294)
	Suboptimal dose (0·5 μg/ml)			
3	14,556 (1345)	10,418 (1188)	18,315 (1339)	20,882 (1055)
5	26,317 (595)	72,725 (5466)	41,752 (3961)	28,662 (2958)
8	n.d.†	n.d.	n.d.	n.d.
16	31,687 (708)	26,313 (658)	16,233 (957)	40,619 (3010)

* cpm, mean ± s.e. of triplicate cultures of pooled spleen cells from four mice of each group.
† n.d. = Not done.

TABLE 4. Proliferative responses of spleen cells from normally nourished (N) and protein deprived (D) mice sixteen weeks after infection with *L. mexicana* to leishmanial antigen

	Δ cpm	
	N mice	D mice
Uninfected	1184 (129)*	895 (38)
Infected	2188 (107)	922 (106)

* Mean ± s.e. of triplicate cultures of pooled spleen cells from four mice of each group.

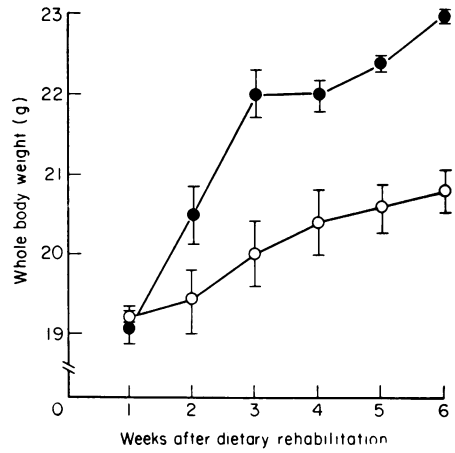

FIG. 4. Effect of dietary rehabilitation on the body weight of protein deprived (D) C57Bl/6 mice infected with *L. mexicana*. D mice rehabilitated with a normal protein diet (●), non rehabilitated controls (○). Mean ± s.e.

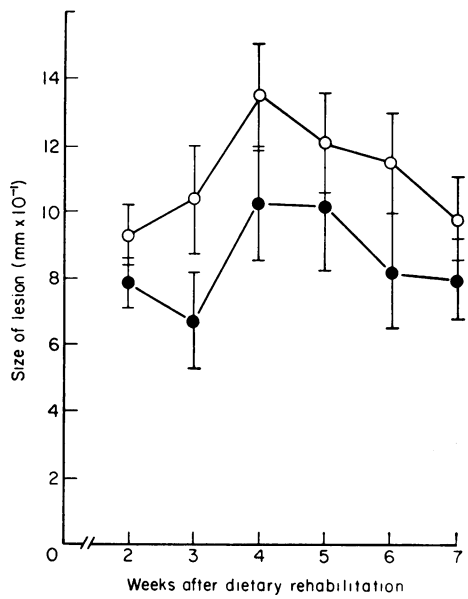

FIG. 5. Effect of dietary rehabilitation on the course of infection with *L. mexicana* in protein deprived (D) C57Bl/6 mice. Size of lesions in D infected mice after dietary rehabilitation (●) and in non-rehabilitated controls (○). Mean ± s.e.

DHR to leishmanial antigen (Fig. 3). Therefore, experiments were carried out to investigate whether lymphoid cells from D mice were able to respond to leishmanial antigen *in vitro*. Results showed in Table 4 indicated that lymphoid cells from D non-recovering mice were unable to respond to leishmanial antigen *in vitro* as compared to those of N recovered mice.

Effect of dietary rehabilitation on the course of L. mexicana in protein deprived mice

Since D mice infected with *L. mexicana* developed non-resolving lesions it was of interest to examine whether transfer to a normal protein diet would affect the course of infection. For this purpose D mice which had been infected for seventeen weeks were divided into two groups of seven mice each. Group A

was changed to a 27% protein diet and group B was maintained in the low 8% protein diet. Six weeks after dietary rehabilitation mice in group A showed a significant increase in their body weight ($P<0{\cdot}001$) (Fig. 4). However, these mice did not show significant differences in the size of their cutaneous lesions as compared to those in group B (Fig. 5).

DISCUSSION

The object of this study was to gain more information regarding those factors affecting the spectrum of clinical manifestations of cutaneous leishmaniasis. Dostrovsky (1934) studying several cases of oriental sore in Palestine drew attention to the association between poor nourishment and chronic leishmaniasis.

Results reported in the present work show that malnutrition has a profound and long term effect on the ability of C57Bl/6 mice to control *Leishmania* infection and that once both chronic protein deprivation and infection were established, dietary rehabilitation, capable of producing a partial recovery in the body weight, did not affect the course of the infection.

The delay of lesion growth observed in D mice during the first four weeks of infection (five weeks of protein malnutrition) suggested a direct effect of protein restriction on the parasite multiplication, an enhancement of the host resistance to the parasite or both. Since after four weeks of infection N and D infected mice did not show significant differences in the size of their cutaneous lesions it appears unlikely that a diminished availability of nutrients affected the multiplication of parasites. Nevertheless, progressive adaptation of the parasite to the nutrient restriction cannot be discarded. Comparison of DHR to leishmanial antigen and *in vitro* responses to T cell mitogens in N and D mice revealed that at the time lesion growth was delayed, D mice had enhanced responses, suggesting increased capability to develop cell mediated immune responses at the early stage of protein deprivation. Evidence supporting this idea comes from previous works which have demonstrated that during the first weeks of dietary restriction mice show enhanced graft versus host reactivity (Bell & Hazell, 1976; Malavé, Németh & Blanca, 1978), accelerated rejection of skin allografts (Cooper, Mariani & Good, 1974) and increased resistance to syngenic tumour (Blanca & Malavé, 1978).

The nature of the immune response leading to recovery from the *Leishmania* infection is not well understood but it seems to require participation of T cells (Preston *et al.*, 1972), macrophages (Zuckerman, 1975; Handman & Spira, 1977) and may well additionally involve antibody activity (Mauel & Behin, 1974). Analysis of the interdependence of these components is difficult to assess in chronically protein deprived mice. Evidence for a role of T cells in the recovery from *Leishmania* infection comes from work on *L. tropica* and *L. enrietii* experimental infections which have demonstrated that T cell deprivation leads to suppression of DHR to parasite antigens and persistence of cutaneous lesions (Preston *et al.*, 1972; Bryceson *et al.*, 1972). It was of interest that in spite of the decreased DHR and *in vitro* response to leishmanial antigen, malnourished mice bearing non-healing lesions produced normal levels of agglutinating antibodies, a humoral response which is apparently thymus independent (Preston *et al.*, 1972). This observation gives further support to the idea that T dependent responses play a crucial role in the recovering from *Leishmania* infection. On the sixteenth week of experiment D mice showed depressed responses to suboptimal doses of PHA and Con A. Furthermore, persistence of cutaneous lesions in D infected mice was accompanied by suppressed *in vivo* and *in vitro* cellular responses to leishmanial antigen. Altogether it suggests that depression of T cell responses after long term protein malnutrition was largely responsible for the chronicity of the infection. However, there are contradictory observations on the influence of chronic malnutrition on T cell responses and, in fact, an enhancement of several immune responses involving T lymphocytes has been reported. For instance, both a significantly increased graft versus host reaction and an acceleration of skin graft rejection abrogated by thymectomy have been observed in mice chronically deprived of protein (reviewed by Good *et al.*, 1977).

Interestingly, *Leishmania* infection had a contrasting influence on the response of spleen lymphocytes from D and N mice to the T cell mitogens during the phase of progressive growth of the lesion. Spleen cells from D infected mice showed significantly depressed responses to PHA and Con A whereas spleen cells from N infected mice displayed enhanced responses to these mitogens. It could be speculated that the decreased body weight of D mice could result in a relatively greater antigenic load in these animals

and thus, favour the activation of suppressor cells (Gershon, 1974). These cells may inhibit the proliferation and action of effector cells that can cause the elimination of the parasite.

Participation of macrophages in immunity to *Leishmania* via an inhibitory effect on the parasite multiplication or an antibody dependent cytotoxic mechanism have been suggested by several workers (Mauel & Behin, 1974; Zuckerman, 1975; Handman & Spira, 1977). Depressed number and/or metabolic activity of macrophages reported in chronic protein deprivation (Passwell, Steward & Soothill, 1974; Coovadia & Soothill, 1976) could impair immune mechanisms controlling the parasite.

The persistence of the cutaneous lesions in nutritional rehabilitated mice is difficult to understand. Cell populations participating in the control of the infection could be permanently affected after long term protein restriction. Alternatively, a longer period may be required to allow full recovery of immune function after dietary rehabilitation.

It seems likely, therefore, that malnutrition interferes with several important functions of the immune system resulting in an impaired capability to control *Leishmania* infection. This suggests that in addition to other factors such as the host genetic constitution and the antigenic load (Preston & Dumonde, 1976; Pérez *et al.*, 1979), the nutritional status of the host should be carefully considered when dealing with chronic leishmaniasis.

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