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HOST FACTORS INFLUENCING OUTCOME OF *LEISHMANIA MEXICANA MEXICANA* INFECTION IN MICE

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Studies were undertaken to determine the influence of several host-related parameters on the course of Leishmania mexicana mexicana *infection in inbred C57Bl/10 (C57) and outbred albino (OA) mice. An important influence of the following variables was demonstrated:* Host strain: *lesions in C57s were significantly less variable in size and outcome than those of OAs under the conditions studied and even when persistent developed at a slower rate.* Host age: *Subcutaneous injection of* 2×10^4 *to* 2×10^6 *amastigotes into the dorsum of the rear paw produced significantly larger lesions which healed more slowly in 2 mo. old C57s than in 4 mo. old mice. Reduced healing ability was observed in older (8 mo. old) female C57s, and low mortality occurred after 15 months of age in infected mice of both sexes.* Lesion site: *Following amastigote infection, lesions in paws of most C57s regress within 15 – 25 wks. In contrast, perinasal lesions produced with the same number of parasites tend to persist for the life of the animal as slowly spreading irregular nodules. In animals infected in both locations, each lesion site behaves similarly to that in singly infected animals of the same age, i.e. regression in the two sites is independent. Our results indicate that while host strain may strongly influence infection outcome, such variables as lesion site and host age play important roles and may explain, in part, reported inter- and intraexperimental variability in responses of murine hosts to a given leishmanial parasite.*

Animal models of cutaneous leishmaniasis have been developed for use in a variety of types of experimental studies, including analyses of immune responses to infection, testing of antileishmanial drugs, and as an aid in species characterization (Lainson & Shaw, 1972; Bjorvatn & Neva, 1979b; Howard, Hale & Chan-Liew, 1980; Trotter, Peters & Robinson, 1980; De Tolla, Scott & Farrell, 1981). Uniformity and reproducibility of results are essential in such studies, both to permit economy in experimental design and to allow interpretation of findings. Therefore, the development of any animal model of leishmaniasis requires the recognition and standardization of all relevent variables influencing the outcome of infection.

Several parasite-related factors have been reported to affect the course of lesions in experimental animals. Thus, when cultured promastigotes are utilized, variation of the number of passages *in vitro*, the stage of the growth cycle, or the medium used for culti-

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vation can result in modification of infectivity of a given parasite strain (Wilson, Dieckmann & Childs, 1979; Keithly & Bienen, 1981; Grimaldi et al, 1982). When amastigotes isolated from lesions constitute the infective inoculum, the presence of host-derived material must be considered. Parasites injected within intact macrophages are more resistant to host attack than extracellular organisms (Poulter, 1980). If the donor and recipient animals are syngeneic, the possible transfer of immunocompetent cells must be considered, while in the case of allogeneic or xenogeneic donors, contamination with histoincompatible tissue antigens could conceivably influence results, especially if repeated injections are given. Lack of control of parasite-related variables would be expected to result in interexperimental variability, but, unless extremely high or low infective inocula were employed, would be unlikely to produce great variability in the response of a group of animals infected from a single parasite preparation. Such intraexperimental variation would be more likely due to host-related parameters.

Since genetically determined factors influence the host response to leishmania (Perez, Labrador & Torrealba, 1979; De Tolla, Scott & Farrell, 1981), it is not surprising that outbred animals injected with a given parasite preparation can demonstrate differences in lesion size or ability to resolve the infection. However, dissimilarity in these parameters has been reported within groups of inbred animals of both resistant and susceptible genotypes, infected with counted doses of promastigotes or amastigotes of several leishmania species (Behin, Mauel & Sordat, 1979; Bjorvatn & Neva, 1979a; Grimaldi, Moriearty & Hoff, 1980). In such circumstances, divergence of ontogenic, phenotypic or local tissue-related characteristics may be influential.

In our laboratories, murine models of *Leishmania mexicana mexicana* infection are employed in investigations of host immune response, pathogenesis and immunoprophylaxis. In standardization of this system, host strain, host age, and lesion site have emerged as critical variables in determining outcome of infection.

MATERIALS AND METHODS

Parasites. Strain 5 of *Leishmania mexicana mexicana* was received from Dr. Z. Brener, Instituto René Rachou, Belo Horizonte, Brazil. The parasite was isolated by P.C.C. Garnham from a case of chiclero ulcer in Belize. The identification as *L. m. mexicana* has been confirmed by analysis of isoenzyme patterns (Grimaldi et al, 1982). Promastigotes are isolated by inoculation of subcutaneous fragments of mouse lesions into biphasic rabbit blood agar medium, with subculture into liquid LIT medium (Gutteridge, Knowler & Coombs, 1969); cultures are maintained at 24 – 26°C in the dark.

Promastigotes for mouse infection are harvested after a maximum of 4 weekly *in vitro* passages. Organisms are separated from LIT medium by centrifugation (1900g, 10 min, 5°C) and washed in phosphate buffered saline pH 7.2 (PBS) or Hank's balanced salt solution (HBSS). Parasites are quantitated by counting a suitable dilution in a Neubauer hemocytometer, and are resuspended at the desired concentration in HBSS. The suspension is sealed in a siliconized penicillin type vial with rubber stopper and metal crimp cap, maintained on ice, and used immediately. Approximately 7×10^8 organisms are obtained per 50 ml of culture medium.

The amastigote form is maintained by periodic subcutaneous inoculation of 10^6 promastigotes or amastigotes into the perinasal region of outbred albino or C57Bl/10 mice. Amastigotes for infection are obtained from non-ulcerated nodular lesions using aseptic technique. The lesion is excised and dissected free of epidermis, then washed in cold tissue culture medium 199 containing antibiotics (1000U penicillin, 1000 μg streptomycin/ml) and transferred to medium without antibiotics. After mincing with fine scissors, the suspension is homogenized in a glass tissue grinder with loose fitting ground glass plunger, then filtered through 2 folded surgical gauze squares into a wide mouthed tube. Homogenization and all subsequent steps are carried out on ice. The suspension is

passed 4 times through a 21g 1" needle and 4 times through a 26g 1/4" needle attached to a disposable plastic syringe, then counted in a hemocytometer, diluted in medium to the desired concentration and bottled as described above. Approximately 2×10^8 parasites are obtained per lesion.

Host. Outbred (Poiley system) albino mice of Swiss Webster origin were obtained from the Central Animal House of the Fundação Oswaldo Cruz and held for at least 3 days before infection to allow adaptation and overcome transfer stress. Female mice, weighing 24 – 28g, were used for experimentation.

Inbred C57B1/10 mice were raised in the departmental animal quarters by sibling matings, from breeding stock originally received from Dr. S. Thales Torres, Laboratório de Imunologia, Instituto Biomedico, Niteroi, Brazil. Young are weaned at 4 weeks and maintained in lots according to month of birth. In describing results, minimum age in the lot is cited, unless otherwise indicated; therefore, ages in a "4 month old" lot are from 4 to 5 months.

All mice are maintained under conventional conditions, housed in plastic cages with metal wire tops and wood shaving bedding. Commercial mouse pellets (Moinho São Cristovão, Rio de Janeiro), whose stated ingredients conform to growing mouse requirements (Sober, 1970), and tap water are provided ad lib.

Infection. Parasite suspensions were held over crushed ice during this procedure. For each individual infection, the vial containing the suspension was gently inverted, a single dose of 0.02 ml was drawn into a 1/4 cc glass syringe (marked in 1/100 ml) fitted with a 26g3/8" needle, and was immediately injected into the recipient. Mice were hand-held or restrained (Moriearty & Deane, 1981), but not anesthetized. For paw injections the needle was inserted subcutaneously (SC) in the dorsum of the left rear foot. Nose injections were made SC into the fleshy area at the base of the whiskers.

Lesion evaluation. Paw lesions were measured with a dial micrometer caliper (Mitutoyo, Brazil) or, in later experiments, with a spring action caliper (Schnelltaster, Germany). Lesion size was expressed as the thickness difference between the infected and contralateral paws. Nose lesion size was expressed as the diameter of the raised nodular lesion. In later phases, due to irregularity in lesion dimensions, no attempt at quantitation was made, but location of nodule, swelling, and erythema was noted.

Histology. At necropsy, fragments of lesions were fixed in buffered 10 per cent formalin, embedded in paraffin and stained with hematoxylin-eosin. Evaluation of slides was carried out as previously described (Grimaldi, Moriearty & Hoff, 1980).

RESULTS

Host strain. Outbred albino mice showed greater variability of lesion size and outcome than inbred C57B1/10 mice injected with the same parasite preparation. Variability first became apparent by 4 – 6 weeks after infection with doses from 2×10^4 to 2×10^6 amastigotes, at which time lesion growth was arrested in some outbreds but continued rapidly in others. Disparity was accentuated as lesions resolved in some mice, while remaining chronic in others. Typical paw measurements are shown in Fig. 1; difference in variability was also observed in nose lesions in the two strains (see below) and occurred after both promastigote and amastigote infection.

Host age. Subcutaneous injection of 2×10^4 or 2×10^6 amastigotes into the rear paw produced significantly larger lesions which healed more slowly in 6 – 8 wk old than in 4 month old C57 mice. For each dose, age differences only became apparent after 4 wks, although mean lesion size was dose dependent at this time. Variability in lesion

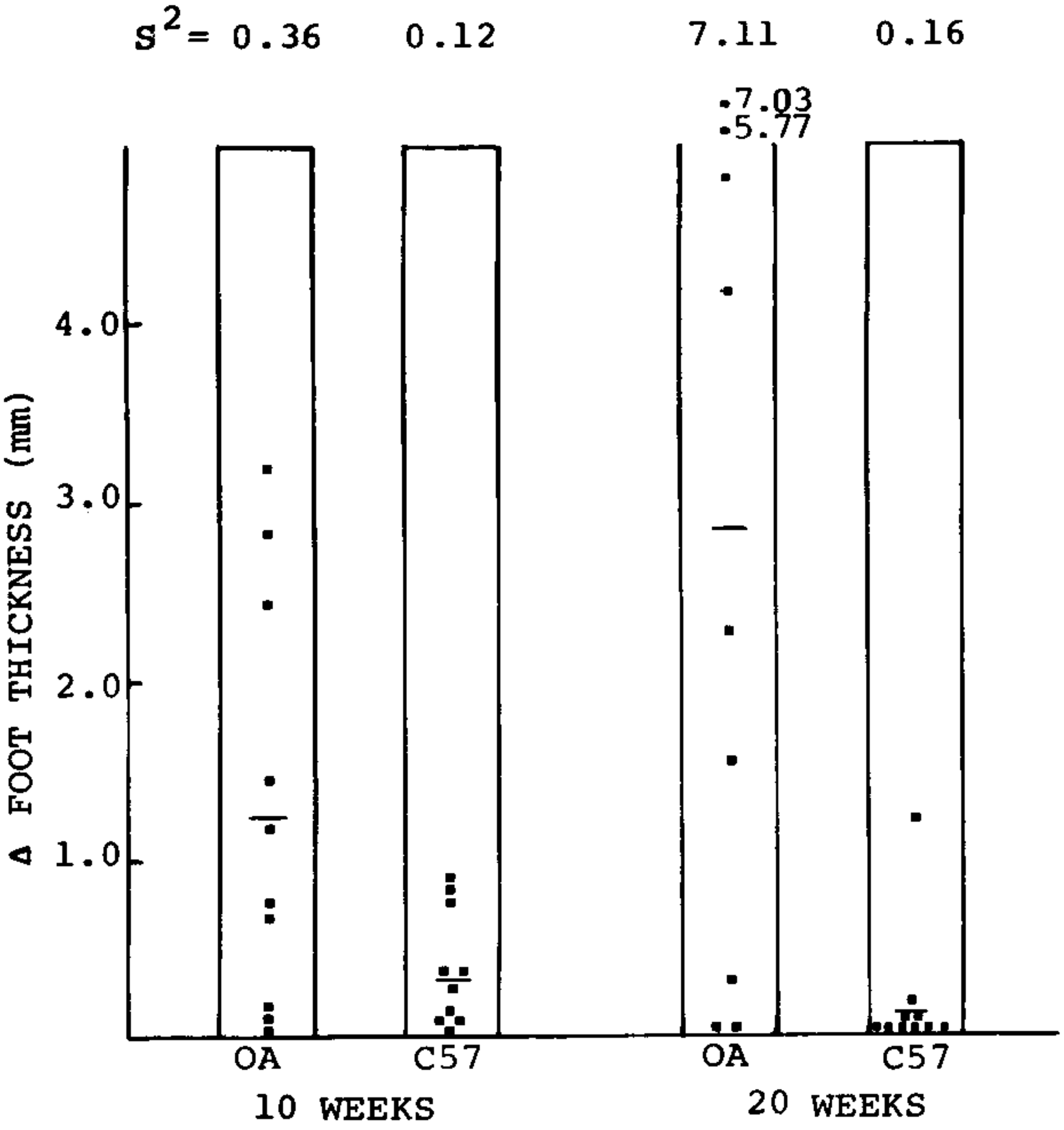

Fig. 1 – Lesion size as measured by increased thickness of infected paws (foot thickness) is compared for individual OA and C57 mice 10 and 20 wks after subcutaneous injection of 7×10^5 amastigotes from the same preparation of *L. mexicana*. Variance (S^2) is significantly greater in OAs by 20 wks post infection ($F = 44.4$, $p < 0.01$), at which time lesions have resolved in most C57s. Horizontal bars represent mean for each group.

size was greater in younger animals, especially when infected with the higher dose (Fig. 2). These results were reproduced in another experiment (infective dose, 10^6 amastigotes), in which 10 month old male mice responded similarly to 4 month olds (Fig. 3).

Lesion site. In the perinasal region, as in the foot, lesion size and outcome vary in outbred mice. In a typical experiment, 150 mice were infected perinasally with 5×10^6 promastigotes (2 wks *in vitro*). After 10 weeks, 46 per cent showed no sign of infection, 32 per cent had small ($\leqslant 0.5$ cm diam) lesions, while 22 per cent presented larger (0.6 – 1.0 cm) nodules. These large nodules indicate the pathogenetic potential of this strain of leishmania (Fig. 4). In contrast, we have never observed macroscopically similar bulky parasite-laden "leishmanioma" lesions in C57s; however, the ability of the latter to resolve the disease depends on the site of parasite inoculation.

Table I shows results in 4 different experiments in which varying numbers of amastigotes from different sources were injected into different lots of C57 mice. All animals produced detectable lesions by 8 weeks post-infection. Whereas in most experiments 80 – 100 per cent of paw lesions regressed by 20 weeks, with no signs of re-activation

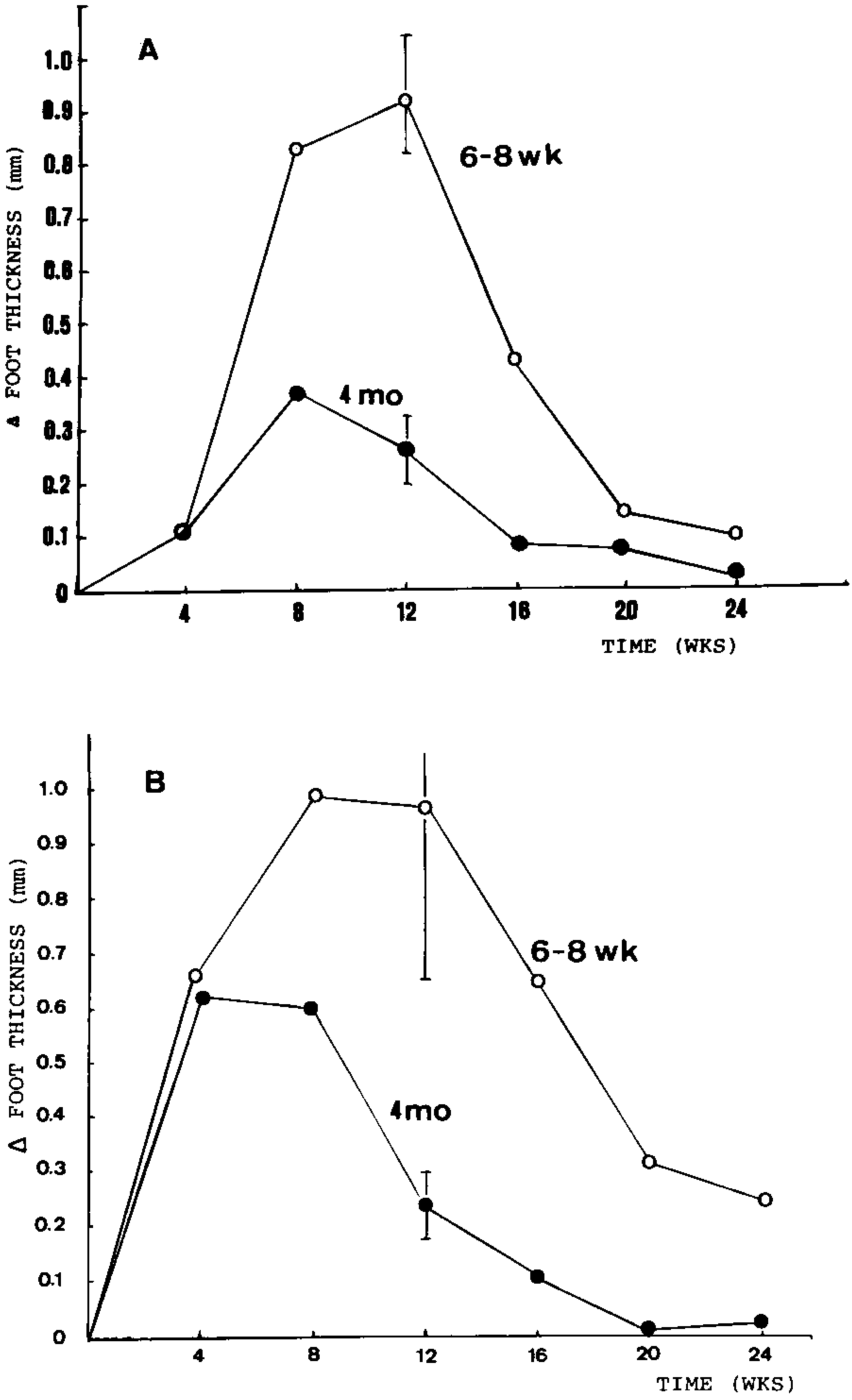

Fig. 2 – C57 mice 6 to 8 wks old at the time of infection produce larger lesions with greater size variability than 4 mo old C57s. A) Paw lesions, 2×10^4 amastigotes. Lesions are significantly larger in younger mice at 12 wks (rank sum test, $p < 0.01$). B) Paw lesions, 2×10^6 amastigotes. Note that for each dose, age-related differences only become apparent after 4 wks of infection, though mean lesion size at this time is dose dependent. Thickness of uninfected paws is similar in these two age groups. All points represent mean $\pm$ SEM of groups of 5 to 10 mice.

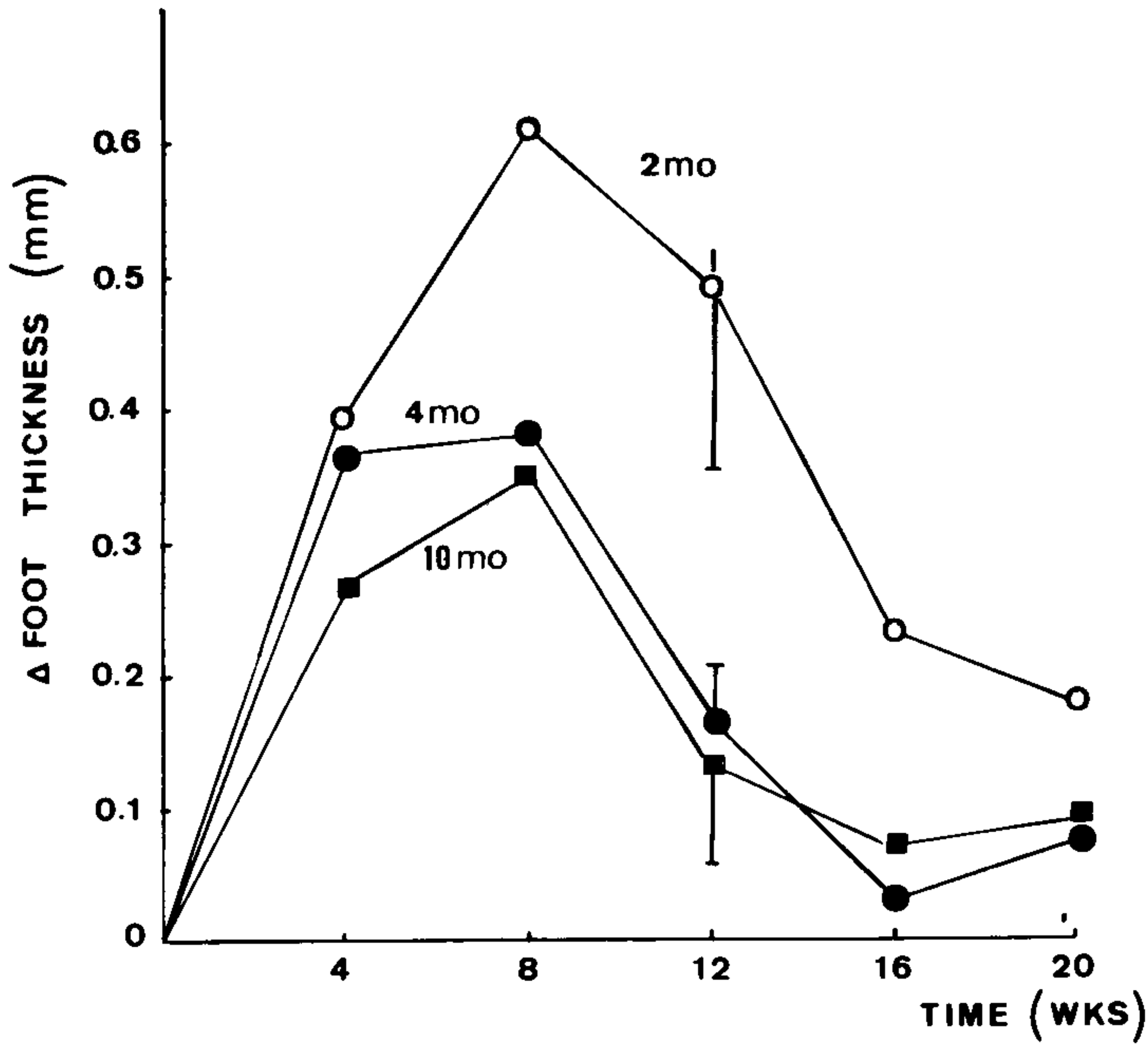

Fig. 3 – Age dependence of lesion size was confirmed in another experiment (infective dose = 10^6 amastigotes), in which 10 mo old male C57s responded like 4 mo olds (mean ± SEM of groups of 5 – 10 mice).

at later times, nose lesions typically persisted for the life of the animal. By 6 – 7 months the central area of the lesion site showed depressed scarring, with peripheral erythema and nodular swelling spreading to reach the lip, nose, or opposite perinasal region (Fig. 4). Mortality only appeared in mice of advanced age (> 15 mo.).

In Experiment 1 (Table I), macroscopic observations of differences in nose and paw lesions were confirmed by histopathological analysis of Groups b, c, e and f. Normal-appearing paws of Groups b and c typically showed moderate fibrosis and occasional epithelioid granulomas but no parasites. In 9/10 mice in Groups e and f (nose lesions), low numbers of parasitized macrophages were observed, along with epithelioid cell granulomas, a mononuclear infiltrate including lymphocytes and plasma cells, granulocyte infiltration, and mild to moderate fibrosis.

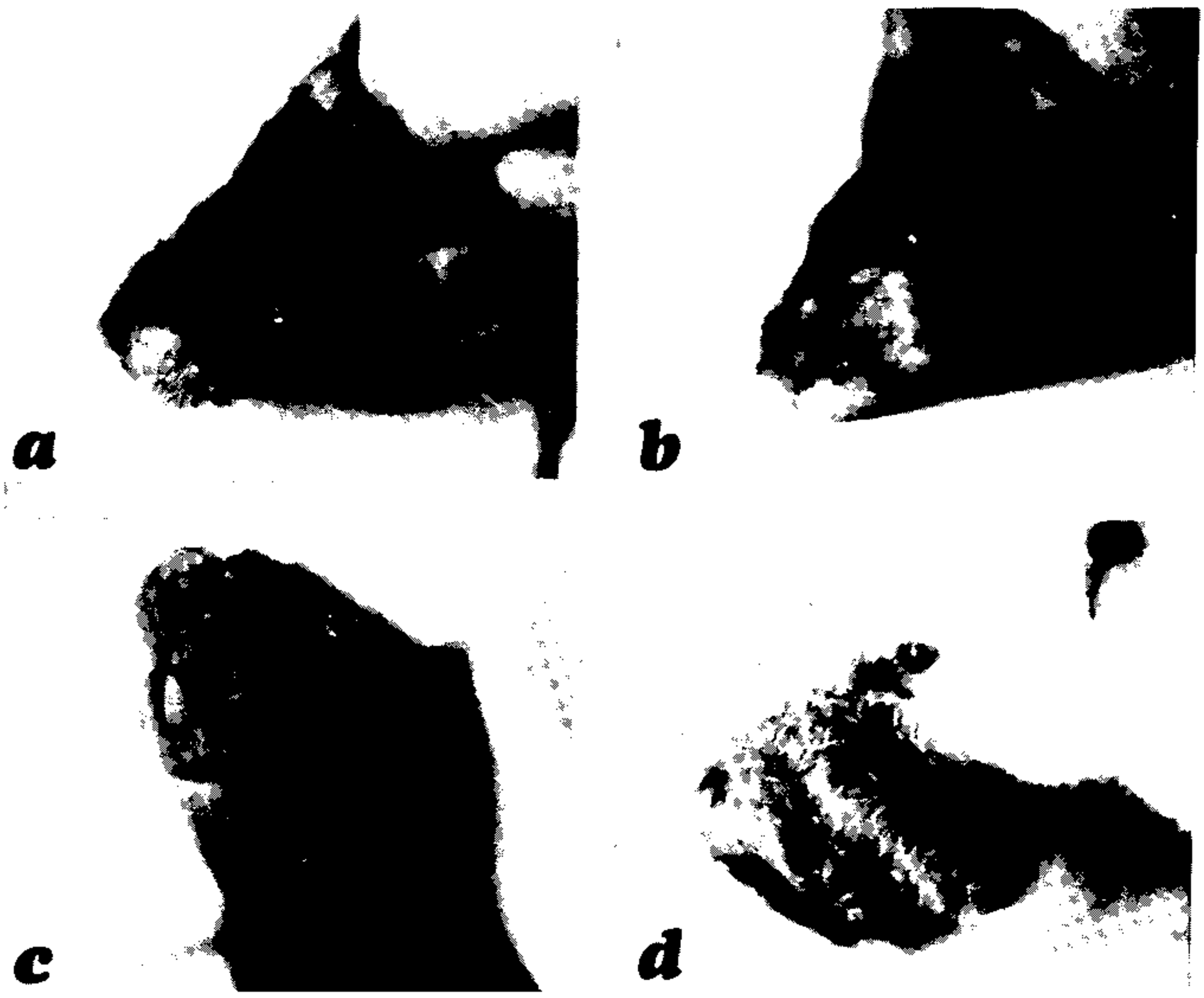

Fig. 4 – Unlike paw lesions in C57s, nose lesions typically become chronic. a) Nodule 3 mo after infection with 2×10^6 amastigotes. b) Central scarring is present at 7 mo. c) At 14 mo, the original lesion site shows scarring, while the active nodular area has spread to the lip, nose and contralateral perinasal area. d) In contrast to C57s, some albinos develop massive parasite-laden "histiocytoma" lesions (7 mo infection).

To evaluate the influence on cure rate of lesions in both nose and paw, 2 groups of female C57s were infected with a preparation of 2×10^6 amastigotes in one or both sites. In doubly infected animals, cure rate in each site was similar to that in singly-infected mice of the same group (Tabl II). An increased susceptibility in older females (c.f. Table I, Group 3a) was also demonstrated in this experiment.

In Experiment 1 (Table I) other groups of 5, 5, and 10 C57 mice (4 mo old) were infected with 2×10^4, 2×10^6 and 4×10^6 amastigotes SC in the lateral aspect of the tail-body junction. While 80 per cent in all groups developed detectable lesions, cure rate at 25 wks (40, 25 and 16 per cent respectively) was low. Metastases to nose or paws, not seen in other groups, were present in these mice (20, 25 and 50 per cent).

DISCUSSION

In the present experiments, C57B1/10 mice proved to be relatively resistant to the strain of *Leishmania mexicana mexicana* used. Results were similar to those of Perez (1980) using C57B1/6 mice and *Leishmania mexicana;* however, we never observed extensive ulceration of either primary or metastatic lesions. The variation in response of outbred

TABLE I

Outcome of Perinasal and Paw Infection of C57B1/10 Mice Injected with Amastigotes of *Leishmania mexicana mexicana*

			Host			*Parasite*		*Outcome*	
Lesion site	*Experiment*	*Group*	*Sex*	*Age*	*No*	*Donor*	*No*	*Observatn Period*	*No Lesions Regressed*
Rear Paw	1	a	F	6-8 wk	7	albino	2×10^4	9 mo	6/7
		b	F	4 mo	5	"	"	"	4/5
		c	M	6-8 wk	9	"	2×10^6	"	7/9
		d	F	4 mo	5	"	"	"	5/5
	2	a	M	5 mo	8	C57B1/10	1×10^6	6 mo	7/8 (9 mo 3/4)[a]
		b	M	5 mo	8	"	"	5 mo	8/9 (9 mo 4/4)[a]
		c	M	2 mo	9	"	"	5 mo	9/9
		d	M	10 mo	6	"	"	5 mo	6/6
	3	a	F	8 mo	10	C57B1/10	1×10^6	6 mo	3/10 (11 mo 4/8)[b]
	4	a	F	4 mo	10	albino	7×10^5	7 mo	9/10
Perinasal	1	e	F	4 mo	5	albino	2×10^4	9 mo	0/5
		f	F	4 mo	5	"	2×10^6	9 mo	1/5
	2	e	M	3 mo	6	C57B1/10	1×10^6	5 mo	0/6 (14 mo 0/4)[b]
	3	b	F	8 mo	10	C57B1/10	1×10^6	7 mo	0/10 (11 mo 0/8)[b]

a – remaining cured mice sacrificed
b – remaining mice died

TABLE II

Persistence of Nose and Paw Lesions in C57B1/10 Mice Ten Months after Infection with 2×10^6 Amastigotes of *Leishmania mexicana mexicana* / Site

Group	*Age at Infection*	*Mortality*	*No. with lesions after infection of*		
			Nose + Paw	*Nose*	*Paw*
1	3 mo	0	nose – 6 nose + paw – 2 neither – 2	8/12	3/12
2	8 mo	20%[a]	nose – 2 nose + paw – 5 neither – 1	8/8	5/8

a – 2 mice died in each group

mice, apparent only after the first month of infection, limits the utility of such animals for certain types of experimentation. Crucial immunological events probably take place during the first four weeks of infection; by the time the host can be classified macroscopically, secondary effects, such as immunosuppression due to high antigen levels, may be superimposed on the initial response in susceptible individuals.

A clear-cut effect of host age on response was also observed, infection being more severe in animals under 4 months of age and, at least in females, after 8 months. Though some facets of the murine immunological response, such as resistance to viral infections (Hirsch, Zisman & Allison, 1970) and response to immunization with SRBC (Mosier & Johnson, 1975) reach maximal levels by 4 to 6 weeks of age, this does not appear to be true of acquired resistance to leishmania. De Souza (1978) reported age-related differences in susceptibility of outbred mice infected with *Leishmania mexicana amazonensis;* with a dose of 2×10^6 amastigotes, lesions developed in 100 per cent of 2 mo olds, with 70 per cent mortality, while the same dose caused lesions in only 30 per cent of 3 mo olds. Similarly, resistance to *Listeria monocytogenes* continues to increase up to 8 mo in A/Tru/C57B1/6 F1 mice, followed by a gradual decline as the host ages. These differences in response to *Listeria* have been traced to events occurring after the first 48 hr of infection, and T cell-mediated immune responses have been implicated (Patel, 1981). In the present study, age-related differences only became apparent after one month, suggesting that acquired responses were responsible. Thus, pooling of mice of varied ages, especially in the range of 6 – 16 wk, may result in intraexperimental variability, both in lesion size and in rate of resolution of the lesions. Failure to control this variable, or use of very young mice, may account for some reported variability in response to leishmania in inbred mice.

Lesion site was another factor influencing outcome of infection in the present study. In human infections, the tendency of *L. mexicana* to produce non-healing lesions in certain sites, especially the ear, has been documented (Lainson & Strangways-Dixon, 1963). In other leishmanial model systems, site-related differences occur in lesion "take" (Wilson, Dieckmann & Childs, 1979) or tendency to metastasize (Poulter, 1979), but site-related chronicity has not been reported.

Chronicity following perinasal infection is apparently due to a local defect, rather than a systemic failure of resistance, since simultaneous lesions in the paw can regress while nose lesions remain active, and since perinasally infected mice can resist challenge in other sites (Moriearty & Grimaldi, manuscript in preparation). The perinasal tissue does not seem to represent a priviledged site inaccessible to the immune response; the chronic lesion is characterized by an intense infiltrate of lymphocytes, plasma cells and activated macrophages, and considerable parasite destruction occurs. That this destruction is not optimally efficient may be due to parasitization of a local subpopulation of cells with limited capacity to kill the amastigote and/or to present antigens; alternatively, local microenvironmental conditions may impede killing mechanisms. Since macrophages are less capable of killing leishmania when deprived of glucose (Murray, 1981), we postulated that tissue glucose levels might be low due to peculiarities of microvasculature or fluid exchange in the region. However, repeated injections, during 1 month, of 5 per cent glucose solution into chronic lesions did not induce regression (Moriearty, unpublished results). Further studies are currently under way in our laboratories to investigate possible causes of chronicity in this model.

RESUMO

Estudando a influência de alguns parâmetros relacionados com o hospedeiro no curso da infecção com *Leishmania mexicana mexicana* em camundongos singênicos C57B1/10 (C57) e não singênicos albinos (OA) demonstramos o papel importante das variáveis enumeradas a seguir. *Cepa do hospedeiro:* o desenvolvimento e tamanho das lesões variaram significativamente menos nos camundongos C57 do que nos OA. *Idade do hospedeiro:* inóculos subcutâneos de 2×10^4 a 2×10^6 amastigotas no dorso da pata traseira produziram lesões significativamente maiores e que regrediram mais lentamente nos camundongos C57 de 2 meses de idade do que nos de 4 meses. Enquanto uma redução da capacidade de curar as lesões foi também observada nas fêmeas C57 mais idosas (8 meses), uma mortalidade baixa ocorreu nos animais infectados de 15 meses de idade, de ambos os sexos. *Local da lesão:* as lesões de pata de C57 infectados com amastigotas regrediram entre 15 a 25 semanas, mas os da região perinasal, produzidas nesses animais com idêntico inóculo parasitário, foram persistentes e progressivos, formando nódulos irregulares. Nos animais infectados simultaneamente em ambas as regiões, cada uma das lesões apresentou uma evolução semelhante àquela observada nos animais da mesma idade infectados em uma das regiões isoladamente, demonstrando que as involuções do processo nesses locais são fenômenos independentes. Assim, esses resultados indicam que enquanto a cepa do hospedeiro pode influir grandemente no curso da infecção, tais variáveis como o sítio da lesão e a idade do animal também desempenham papéis importantes, podendo explicar, pelo menos em parte, a variabilidade nas respostas do hospedeiro face à infecção com uma determinada *Leishmania*.

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