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Local immune response to *Mycobacterium lepraemurium* in C3H and C57Bl/6 mice

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SUMMARY

Subcutaneous footpad inoculation of living *M. lepraemurium* (L.MLM) induced, in high responder C57Bl/6 mice, a local granulomatous reaction associated with the production of effector cells which stopped the multiplication of bacilli in the draining popliteal node with the concurrent development of 24–48 hr delayed type hypersensitivity (DTH). The thymus-dependent local reaction did not occur after the injection of heat-killed *M. lepraemurium* (HK.MLM) or after the inoculation of L.MLM in nude mice. However, HK.MLM injection interfered with the onset of the local reaction and enhanced acid-fast bacteria (AFB) counts in the draining node. In low responder C3H mice, L.MLM produced a local and delayed footpad swelling but no restriction of bacilli multiplication in the draining lymph node was observed. This unresponsiveness was not due to an overloading of the inoculum dose since doses ranging from 3×10^4 to 3×10^7 MLM did not produce any granulomatous local reaction as in C57Bl/6 mice. The injection of dead bacilli in the contralateral footpad of subcutaneously (s.c.) infected C3H mice revealed Arthus-like and 18–24 hr delayed reactions.

When 10^6 L.MLM per mouse were injected intravenously (i.v.), systemic infection, measured in the spleen, was found to be less restricted in C57Bl/6 than in C3H mice. Moreover, in C57Bl/6 mice low doses of L.MLM injected i.v. delayed the local reaction at first, then enhanced footpad swelling and AFB counts in the draining nodes, indicating some acquired defect of peripheral immunity.

When a high dose of L.MLM (2×10^8/mouse) was injected i.v., C57Bl/6 mice died sooner than C3H mice, indicating certain discrepancies between local resistance and systemic susceptibility.

INTRODUCTION

T cell-mediated immunity (CMI) is the basis of acquired resistance to a variety of intracellular microorganisms (Mackaness, 1971; North, 1974). But, in a number of chronic infections, the CMI defence mechanism does not operate efficiently (Turk & Bryceson, 1971). Thus, it seems appropriate to use methods of modulating the inadequate immune response so that efficient prophylactic immunization can be achieved. Before using such immunomodulating agents, the first step must be to gain a greater insight into the mechanisms responsible for the defective immune response in the susceptible host. Lepromatous leprosy in humans, for instance, is characterized by a massive microbial population in the infective foci, skin test anergy to lepromin, an absence of reactivity of peripheral lymphocytes to specific antigen and by hyperproduction of specific or non-specific antibodies (Godal *et al.*, 1974). On the other hand, patients with the tuberculoid form of leprosy present a high level of CMI, few viable mycobacteria in the tissues and histopathological features of hypersensitivity granulomata. Several mechanisms have been postulated to explain these two polar forms of leprosy, but no direct evidence is available at present in support of any particular mechanism. Immunomodulating therapy should, therefore, be delayed until the mechanisms are better understood.

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Experimental models of leprosy exist in mice. They present some similarities with the polar forms of human leprosy (Waksman, 1973; Kawaguchi, 1957; Closs & Haugen, 1974). Mice of the C57Bl/6 strain have been shown to be rather resistant to infection with *Mycobacterium lepraemurium* (MLM), whereas C3H mice are highly susceptible and BALB/c are of intermediate susceptibility.

This paper reports an attempt to compare specific immune responses in high and low responder mice after MLM infection, using varying factors for the induction of specific acquired resistance. Specific immunity has been defined as the capacity of the host to limit the growth of mycobacteria. This was correlated with the appearance of a local granulomatous response and the presence of a hypersensitive state to MLM antigens. A dissociation between systemic and local defence mechanisms was also observed which could explain some discrepancies observed in the literature concerning these high and low responder mice.

MATERIALS AND METHODS

Animals. Specific pathogen-free (SPF) inbred mice of either sex of C3H/HeN and C57Bl/6 strains, from the breeding facilities of the Pasteur Institute, were used. They were 6–7 weeks old when the experiments were begun. They were kept in a protected environment and were fed with a vitamin-supplemented diet and sterile water. Inbred congenitally thymusless mice (nu/nu) and their heterozygote (nu/+) litter mates with a C57Bl/6 background were purchased from Dr Friis (Gl. Bomholtgard, 8680 Ry, Denmark). Female Wistar rats were purchased from Iffa-Credo (Laboratoire des Oncins, 69210 St-Germain-sur-l'Arbreole, France).

Mycobacteria. Mycobacterium lepraemurium (MLM). MLM, a murine bacillus, was maintained by repeated 'passages' in susceptible albino rats. Purified suspensions were obtained from non-ulcerated lepromata, 6–7 months after subcutaneous injection of MLM. Bacilli were harvested and purified by differential centrifugation (200–3000 *g*). Inocula of known size were prepared by appropriate dilutions, gradually cooled to − 80°C and stored at this temperature before use. No attempt was made to detect the actual percentage of viable bacilli in each inoculum; they were designated as living MLM. In contrast, heat-killed MLM (HK.MLM) were prepared by heating a suspension of live MLM at 60°C for 60 min.

In some experiments, ^{60}Co irradiated MLM, obtained from Dr R. J. W. Rees (National Institute of Medical Research, Mill Hill, London) were also used.

Experimental infection. C3H and C57Bl/6 mice were infected by subcutaneous (s.c.) injection of 0·04 ml of saline containing an appropriate number of MLM in the left hind footpad with a tuberculin-type glass syringe fitted with a 30 1/2 gauge needle. A volume of 0·2 ml containing appropriate numbers of bacilli was injected intravenously (i.v.) or intraperitoneally (i.p.).

Local reaction. At varying times after infection, the footpad swelling was measured with dial gauge calipers as described previously (Lagrange, Hurtrel & Ravisse, 1978).

Eliciting of DTH. At varying times after MLM infection, animals were injected s.c. in the right hind footpad with either live MLM or HK.MLM or with purified irradiated MLM. The footpad thickness of the right hind footpad was measured with dial gauge calipers before injection and at varying times thereafter.

Counting of bacilli. The draining lymph nodes, spleen, liver and thymus were homogenized separately, in a total volume of 2 ml or 10 ml of saline, with a teflon pestle in glass homogenizers. Sometimes, the homogenates were concentrated by centrifugation (3000 *g*). The resulting bacillary suspensions were thoroughly mixed with a vortex, diluted in saline and counted by a modified spot-slide method (Hart & Rees, 1960). A small volume (3 μl) of the homogenate and two ten-fold dilutions were spread over a 11 mm diameter circle of a Reich glass slide (Bellco Glass Inc., Vineland, New Jersey, USA). After gentle fixation by heating, the preparations were stained by the auramine fluorescent technique (Vestal, 1973). They were examined using a × 40 objective and a × 10 eye piece in a Leitz Ortholux microscope, equipped with incident illumination for fluorescent microscopy. The number of acid-fast bacilli (AFB) was counted in duplicate spots in one diameter. The number of AFB was calculated using calibration factors for the optical system, the area of the smear and the dilution factor.

RESULTS

Multiplication of live MLM in organs of susceptible C3H mice

C3H mice were injected intraperitoneally with freshly harvested MLM, each mouse receiving an inoculum of 5×10^8 AFB. Each month thereafter for 6 months, a group of mice was killed and the number of AFB per organ calculated. The results of such counts are shown in Table 1. The geometric mean of AFB per organ at varying times was consistent with the multiplication of the bacilli in susceptible mice, and the lack of limitation seemed to indicate an absence of acquired immunity. When survival times were compared after an i.v. injection of 2×10^8 L.MLM in C3H and in C57Bl/6 mice, it was found that

TABLE 1. Sequential counts of AFB in C3H/HeN mice injected intraperitoneally with $3{\cdot}6\times10^8$ *M. lepraemurium*

Organ	Log_{10} AFB/organ Months 1	2	3	4	5	6
Spleen	7·35*	8·03	8·76	9·26	9·42	9·93
Liver	n.t.†	7·60	8·78	8·95	9·93	9·90
Mesenteric lymph node	n.t.	n.t.	n.t.	9·0	9·69	9·92
Thymus	n.t.	n.t.	n.t.	n.t.	n.t.	9·92
Pelvic fat-pad	n.t.	n.t.	n.t.	n.t.	n.t.	10·02

* Geometric mean of $\log_{10}$ AFB count per organ.
† n.t. = Not tested.

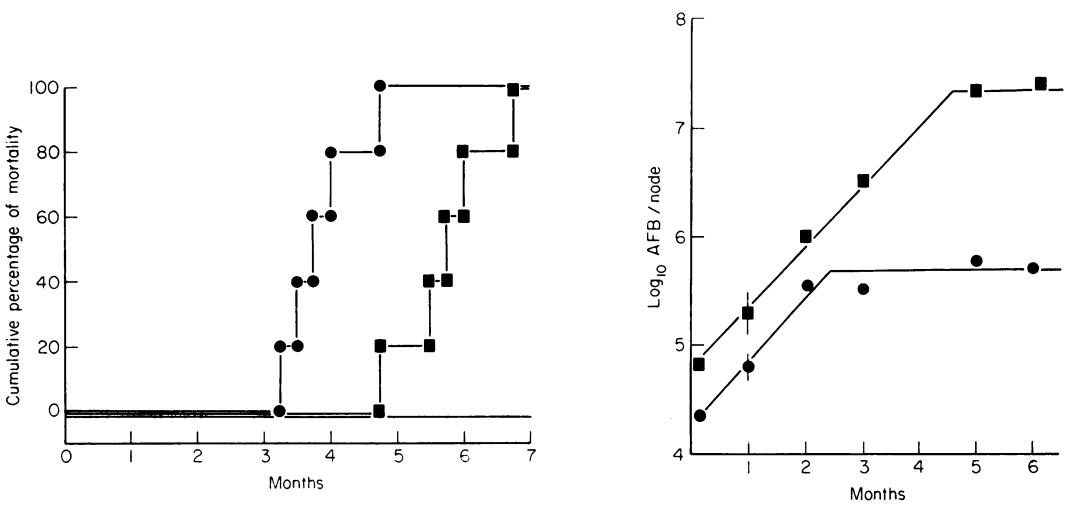

FIG. 1. Cumulative percentage of mortality in C3H (■) or in C57Bl/6 mice (●) after being inoculated with an intravenous injection of 2×10^8 L.MLM. The mean survival time was $192{\cdot}4\pm9{\cdot}5$ and $132{\cdot}2\pm7{\cdot}93$ days, respectively.

FIG. 2. Kinetics of the geometric mean of AFB per node indicating a multiplication of *M. lepraemurium* in the draining popliteal lymph nodes of C57Bl/6 mice (●) or in C3H mice (■) after footpad inoculation of 2×10^7 living bacteria in 0·04 ml of saline. Pool of five nodes except on day 28 where s.e.m. are indicated.

susceptible C3H mice lived longer than highly resistant C57Bl/6 mice (Fig. 1). This difference was statistically significant ($P<0{\cdot}005$). Since better immunizations are always achieved with s.c. inoculations (Lefford, 1977), the next experiment was performed to evaluate the growth of MLM after s.c. injection in C3H mice and in C57Bl/6 mice.

Multiplication of live MLM in the draining nodes in C3H or C57Bl/6 mice

Groups of C3H or C57Bl/6 mice were injected with 2×10^7 MLM in the left hind footpad. Five mice per group were killed and AFB counted in the popliteal node, on the third day and every month after injection for 6 months. As shown in Fig. 2, the number of AFB per node increased in both strains during the first 2 months, with both curves having the same slope. The AFB per node in C3H were slightly higher than in C57Bl/6 mice, but the difference was not statistically significant ($P\leqslant0{\cdot}10$). After the eighth week, the growth curve of MLM in C3H differed significantly from that of C57Bl/6. In the former

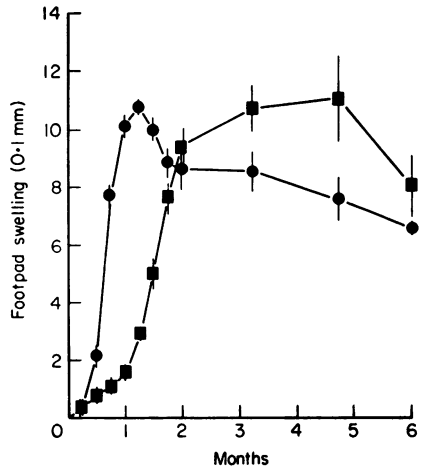

FIG. 3. Kinetics of the arithmetic mean of the increase in footpad thickness in normal C57Bl/6 mice (●) or in normal C3H mice (■) after inoculation in the left-hind footpad with 2×10^7 living bacteria in 0·04 ml of saline. Arithmetic mean of ten mice per time point and s.e.m.

strain MLM multiplied exponentially until the fifth month, when a plateau was reached. On the other hand, in C57Bl/6 mice, no further multiplication of MLM was detected after the eighth week, until the study was ended. This flattening of the growth curve in C57Bl/6 mice strongly suggests an immunological limiting mechanism, as described many years ago in guinea-pigs infected locally with *M. tuberculosis* (Krause, 1926). For this reason, and also because of technical accuracy, the AFB/node was chosen as an index of the presence or absence of acquired resistance which prevents further dissemination of MLM throughout the body after local injection in the footpad.

Local reaction after footpad inoculation with MLM

The increase of footpad thickness after inoculation of 2×10^7 MLM in groups of C3H and C57Bl/6 was measured at weekly intervals during the first 2 months and every month thereafter. The kinetics of the local reactions are shown in Fig. 3. An early swelling, not shown in Fig. 3, occurred during the first 2 days, indicating a non-specific inflammatory reaction induced by mycobacteria in both strains of mice. During the following weeks, a striking difference was observed between the two strains of mice. The local reaction appeared sooner in C57Bl/6 mice than in C3H mice and the maximum difference between the strains occurred 5 weeks after inoculation ($P<0{\cdot}001$). In C57Bl/6 mice, the local reaction peaked at 6 weeks and declined thereafter. In C3H mice, in contrast, footpad swelling was delayed and only reached its peak at 5 months. This swelling was not only delayed but also differed from the swelling in C57Bl/6 mice in some physical characteristics: it was softer, more easily depressed and sometimes white abscesses were observed.

The absence of a local immune reaction in C3H mice could be due to an active paralysis of effector cells, unable to circulate, because of the use of an overloading dose of mycobacteria in these susceptible mice (Rook, 1975). Thus the following experiments were performed to explore the immune reactivity of susceptible or resistant mice to varying doses of antigen.

Kinetics of the local reaction after inoculation of varying doses of MLM

Separate groups of C3H and C57Bl/6 mice were injected in the left-hind footpad with ten-fold dilutions of freshly harvested MLM, the actual count of AFB injected per mouse is expressed in Fig. 4. Footpad swellings were recorded monthly in each group of mice. Again, the kinetics of the local reaction differ between the two strains, whatever the dose considered. In C3H mice, footpad swellings increased slowly and the slopes were directly proportional to the injected dose of MLM. In C57Bl/6 mice, the onset and the magnitude of the local reaction varied with the inoculum. The more bacilli injected, the more

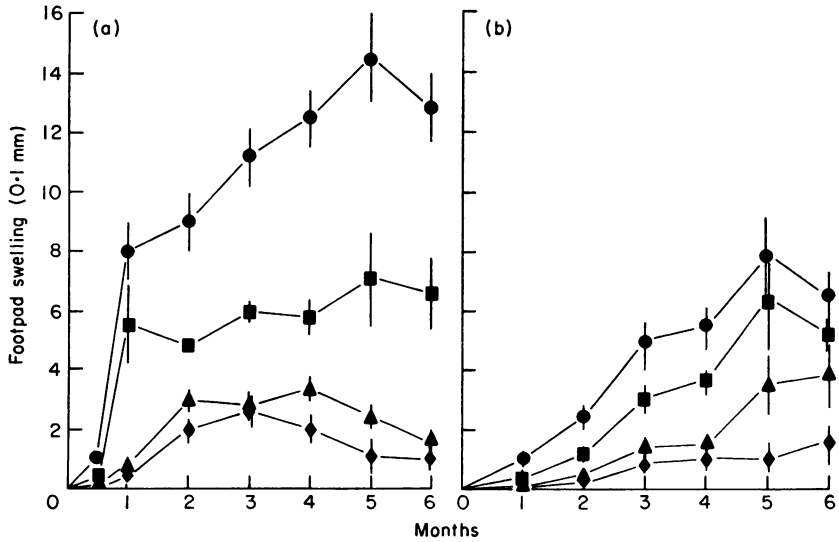

FIG. 4. Kinetics of footpad swelling in C57Bl/6 mice (a) and in C3H mice (b) after injection of 3×10^7 (●), 3×10^6 (■), 3×10^5 (▲) or 3×10^4 (♦) bacteria in 0·04 ml in the left hind footpad. Mean of ten mice ± s.e.m.

rapidly and more pronounced the reactions appeared. When the dose-response was examined at 7 weeks, a linear relationship was observed in C57Bl/6 but not in C3H mice (Fig. 5). At the end of the experiment, local reactions were measured, the mice killed and AFB per draining node counted. The dose relationships for the local reaction and for MLM found in the nodes are shown in Fig. 6. There are striking homologies between the regression lines for the two parameters. There was only a small difference between the highest and the lowest injected dose in C3H ($P>0{\cdot}05$). In contrast, in C57Bl/6 mice, highly significant

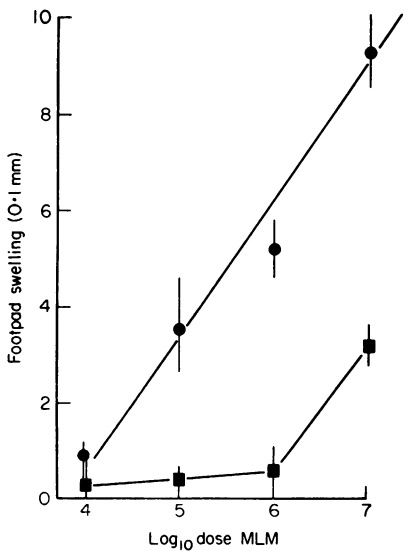

FIG. 5

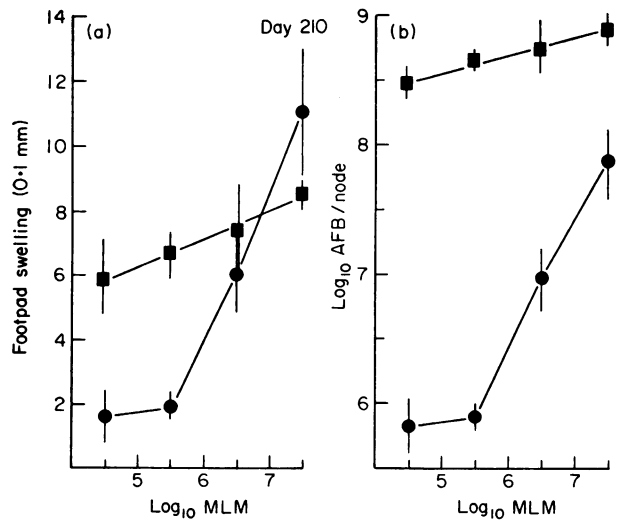

FIG. 6

FIG. 5. Dose relationship for footpad swelling in C57Bl/6 mice (●) and in C3H mice (■) inoculated 49 days previously in the left hind footpad in a volume of 0·04 ml of saline. Mean of ten mice ± s.e.m.

FIG. 6. Dose relationship for footpad swelling (a) or for geometric mean of the AFB per popliteal node (b) in C57Bl/6 mice (●) or in C3H mice (■) inoculated 210 days previously in the left hind footpad. Mean of five mice ± s.e.m.

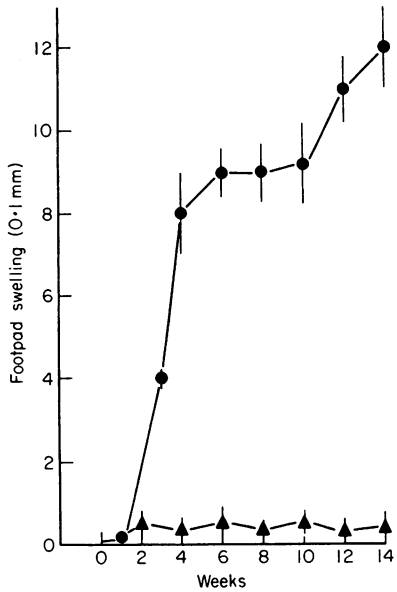

FIG. 7. Kinetics of footpad swelling in groups of C57Bl/6 mice injected in the left hind footpad with 2×10^7 living *M. lepraemurium* (L.MLM) (●) or with 2×10^7 heat-killed *M. lepraemurium* (HK.MLM) (▲). Mean of five mice ± s.e.m.

differences were noticed ($P<0{\cdot}005$). This seems to indicate that small doses of MLM are well controlled in C57Bl/6 mice, but larger ones are not, as others have also reported (Closs, 1975). This again is in favour of a local immune mechanism which performs well in C57Bl/6 mice but seems to be defective in C3H mice, in which no limitation of local multiplication seems to occur.

It has been shown with other mycobacteria, and particularly after BCG vaccination in guinea-pigs (Jensen, 1946) or in mice (Lagrange *et al.*, 1978), that local reactions do not develop when dead bacilli are injected. But in the Mitsuda reaction, heat-killed *Mycobacterium leprae* can induce local granulomata in humans and could possibly induce immunity in mice (Shepard, 1975). Thus, it was of interest to test this possibility with HK.MLM in high responder mice.

Local reaction after injection of HK.MLM

Two groups of C57Bl/6 mice were injected in the left-hind footpad with an equivalent dose of HK.MLM or living MLM and the footpad swelling was recorded at weekly intervals (Fig. 7). No reaction was detected in the mice injected with HKMLM. This is not due to an accelerated disappearance of non-living antigens, because HKMLM can be detected in the footpad or in the draining node for a long time (Brown & Krenzien, 1976). Specific resistance and classical delayed type hypersensitivity (DTH) to mycobacteria or other intracellular parasites develops when bacilli multiply in the phagocytes (Mackaness, Auclair & Lagrange, 1973). However, HK.MLM can interfere with the induction of the immune response by living bacilli, when the dead bacteria are injected simultaneously by the same or a different route. Varying proportions of HK.MLM and living MLM—the total count being constant—were inoculated s.c. in the left-hind footpad of C57Bl/6 mice and the local reactions were measured at various times thereafter. Small numbers of HK.MLM (20%) in the inoculum enhanced the local reaction (Fig. 8a). A high proportion (80%) delayed the local reaction and no limitation of the swelling was detected (Fig. 8b). In both cases, the AFB per node were greater than calculated when taking into account the sums of L.MLM and HK.MLM found in separated groups. Since specific acquired resistance to intracellular bacteria is the result of production and circulation of effector cells which are known to be thymus-dependent (North, 1974), the next experiment was performed to evaluate the production of the local reaction in thymusless nude mice.

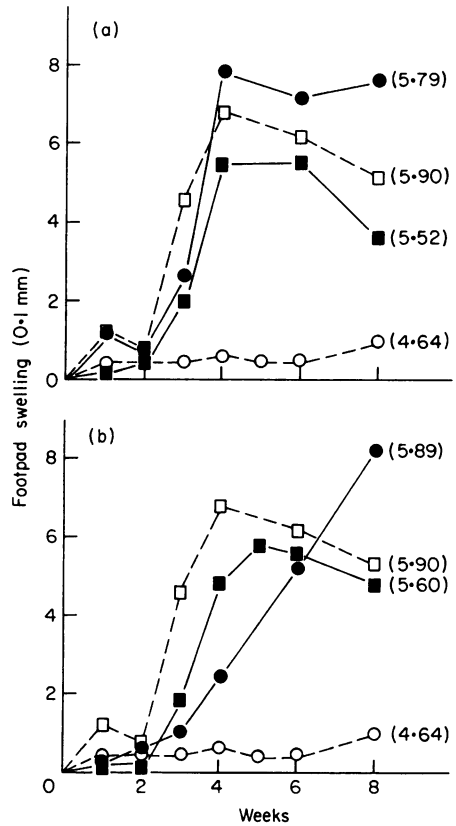

FIG. 8. (a) Kinetics of footpad swelling in groups of C57Bl/6 mice inoculated with 2×10^7 living *M. lepraemurium* (□——□), with 2×10^7 HK *M. lepraemurium* (○ - - ○), with a total count of 2×10^7 AFB containing 20% of HK.MLM (●——●) or with $1{\cdot}6\times10^7$ living *M. lepraemurium* (■——■) in the left-hind footpad in a volume of 0·04 ml. The numbers in brackets indicate geometric mean ($\log_{10}$) of AFB per node counted at the end of the experiment in respective groups of mice (mean of five mice). (b) Kinetics of footpad swelling in groups of C57Bl/6 mice injected in the left-hind footpad with 2×10^7 L.MLM (□ - - - □), with 2×10^7 HK.MLM (○ - - - ○), with a total count of 2×10^7 AFB containing 80% of HK.MLM (●——●) or with $0{\cdot}4\times10^7$ L.MLM (■——■) in a volume of 0·04 ml. The numbers in brackets indicate the geometric mean ($\log_{10}$) of the AFB per node counted at the end of the experiment in respective groups (mean of five mice).

Local reaction and AFB/node in nude mice

Homozygote thymusless nude mice (nu/nu) of a C57Bl/6 background and their litter mates (nu/+) were injected with 2×10^7 MLM in the left-hind footpad and local reaction measured thereafter. As recorded in Fig. 9, no granulomatous reaction was detected in nude mice during the experiment. The differences between these reactions and those observed in heterozygotes were highly significant after the second week ($P<0{\cdot}001$). When the AFB per node were counted 1, 2 or 3 months after infection, a diminution of AFB count was seen only in mice which produced local immune reactions. However, in nude mice, no multiplication occurred until the twelfth week, and the level of MLM found in the node was lower at 1 month than in the control group, indicating a higher natural resistance to MLM in nude mice, as described previously for other intracellular parasites (Fauve & Hevin, 1974).

Specific acquired resistance to intracellular bacteria often develops in association with the appearance of a DTH to related antigens (Collins & Mackaness, 1970). Thus, it was of interest to measure hypersensitivity reactions in high or low responder mice after s.c. inoculation of MLM.

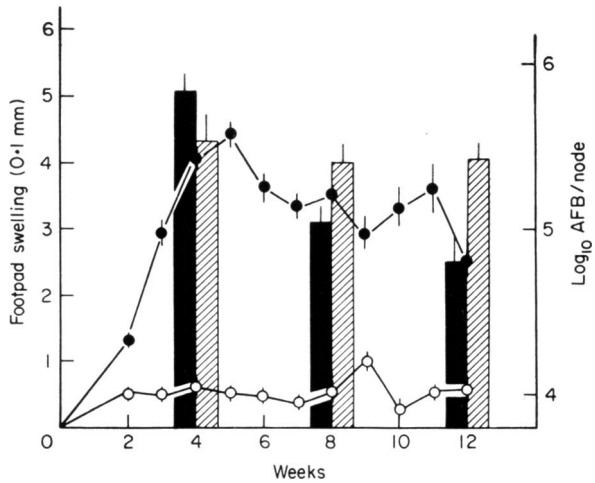

FIG. 9. Kinetics of the footpad swelling in groups of nude mice (nu/nu) (○) (▨) or in their litter mates (nu/+) (●) (■) after injection of 2×10^7 L.MLM in the left hind footpad in a volume of 0·04 ml. The bars indicate the geometric mean of AFB per node counted monthly in the respective groups of five mice. Mean of five mice ± s.e.m.

DTH to MLM in C3H and C57Bl/6 mice after local infection.

Groups of C3H and C57Bl/6 mice were inoculated in the left-hind footpad with 2×10^7 MLM. At weekly intervals, after measuring the thickness of the left-hind and the right-hind footpads, five mice per group were injected with 0·04 ml of saline containing 4×10^6 AFB/ml of ^{60}Co irradiated purified MLM in the right-hind footpad. Footpad swellings were recorded after 4, 18, 24, 48 and 72 hr. As shown in Fig. 10, the occurrence of the local granulomatous reaction at the infection site developed concurrently with a 24–48 hr DTH reaction in C57Bl/6 mice. On the other hand, no local reaction and no 48 hr DTH occurred in C3H mice. On day 28, it was interesting to note a larger 4 hr reaction in the C3H mice than in the C57Bl/6 mice. In both strains, there was an 18 hr reaction and the 24 hr swelling was always less in C3H than in C57Bl/6 mice. These types of delayed reactions need further investigation. Thus, C3H mice produce an early immune response after being inoculated with MLM, but it seems to be inadequate

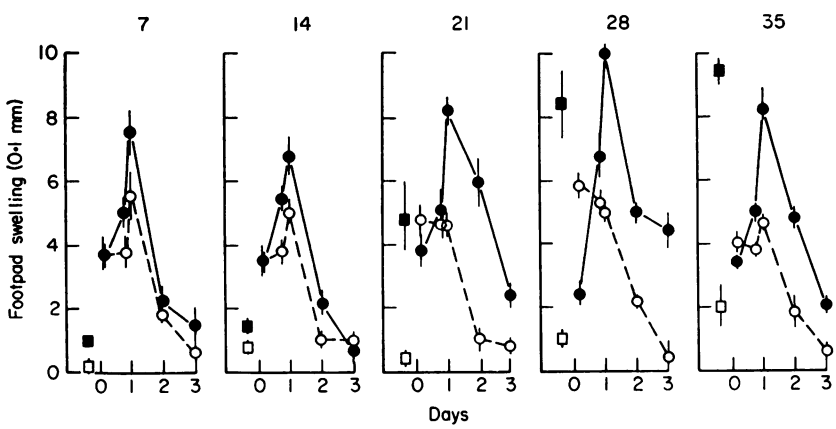

FIG. 10. Kinetics of footpad swelling in groups of C57Bl/6 mice (●——●) or in C3H mice (○ – – ○) injected with 4×10^6 irradiated purified *M. lepraemurium* in the right hind footpad at varying times after injection of 2×10^7 living *M. lepraemurium* in the left hind footpad. Levels of local reaction of left hind footpad at respective times are represented in C57Bl/6 mice (■) and in C3H (□). Mean of five mice ± s.e.m.

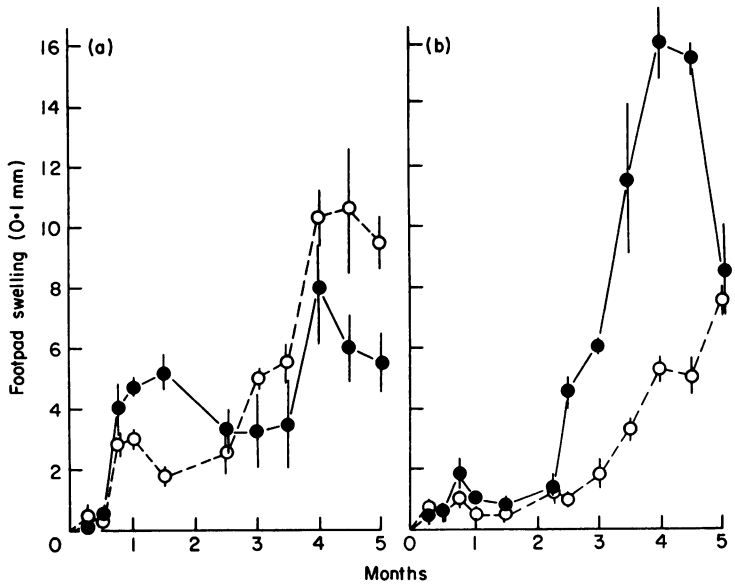

FIG. 11. Kinetics of footpad swelling in groups of C57Bl/6 mice (a) or in C3H (b) inoculated in the left-hind footpad with 2×10^7 living *M. lepraemurium* 3 weeks after i.v. injection of 1×10^6 L.MLM (○ – – ○) or after saline (●——●). Mean of ten mice ± s.e.m.

and possibly can interfere with the circulation of mediators of DTH and effectors for acquired resistance. Thus, the next question was to compare systemic or local acquired resistance in C3H and C57Bl/6 mice after s.c. or i.v. inoculation with living MLM.

Local or systemic infection in high or low responder mice.

Different groups of C3H and C57Bl/6 mice were injected i.v. with 1×10^6 living MLM or with saline and 3 weeks later all mice were challenged with 2×10^7 MLM in the left hind footpad. Local reactions were then measured and AFB in the draining nodes and the spleens were counted at varying times after the challenge. The results of such an experiment are shown in Fig. 11. Local reactions in C57Bl/6 mice were delayed at first but were eventually greater in magnitude than in the controls. Since histological examinations were not performed here, this increase in footpad swelling needs further exploration. In C3H mice, local reactions were delayed and in the fifth month reached the levels observed in controls. As shown in Table 2, in controls when the AFB per node were considered, significantly higher counts were observed in C3H than in C57Bl/6 mice at 63 days ($P < 0{\cdot}02$) or at 140 days ($P < 0{\cdot}05$) after challenge. When AFB counts were calculated in pre-treated mice, there were no differences between C3H and C57Bl/6 mice. In the former, pre-treatment seemed to have no effect upon the AFB in the nodes ($P > 0{\cdot}10$), but in the latter a significant difference was seen after i.v. pre-treatment ($P < 0{\cdot}05$). In addition, lower counts of AFB/spleen were found in C3H than in C57Bl/6 at both 63 days ($P < 0{\cdot}02$) and 140 days ($P < 0{\cdot}05$) after i.v. pre-treatment with living MLM.

DISCUSSION

The local reaction after subcutaneous injection of mycobacteria in guinea-pigs has been used for the determination of the virulence of saprophytic, acid-fast strains (Lester, 1939). For the control of BCG vaccines, it was introduced and described by Jensen (1942). In mice, Shepard (1960) used the injection of human *Mycobacterium leprae* in the footpad to evaluate the viability of the injected bacilli. Several other authors have described the local response of different strains of mice after subcutaneous injection of MLM in the footpad (Kawaguchi, 1957; Waksman, 1973; Closs & Haugen, 1974; Closs, 1975; Poulter

TABLE 2. Geometric mean ± s.e.m. of $\log_{10}$ AFB counts in draining lymph node and spleen from C57Bl/6 of C3H mice treated i.v. 3 weeks previously with saline or with 1×10^6 living *M. lepraemurium* and inoculated in the left-hind footpad with 2×10^7 living *M. lepraemurium*

		Node		Spleen	
		Day 63	Day 140	Day 63	Day 140
C57Bl/6	10^6 MLM	6·10 ± 0·07	7.61 ± 0.06	6.24 ± 0.17	7·37 ± 0·25
		$P < 0·05$	$P < 0.0005$		
	Saline	5·92 ± 0·01	6·24 ± 0·13	6·14 ± 0·13	5·56 ± 0·24
		$P < 0·02$	$P < 0·05$	$P < 0·02$	$P < 0·05$
C3H	10^6 MLM	6·19 ± 0·19	7·68 ± 0·15	5·11 ± 0·09	6·76 ± 0·09
		$P > 0·10$	$P > 0·10$		
	Saline	6·20 ± 0·10	7·14 ± 0·31	< 4·97	5·78 ± 0·03

& Lefford, 1977). This delayed local granulomatous reaction is also produced after BCG inoculation in the footpads of mice (Lagrange *et al.*, 1978) and its magnitude and onset are related to the specific and non-specific immune responses which develop after BCG vaccination (Lagrange & Hurtrel, 1978). Thus, granulomata formation permits the evaluation of the virulence of mycobacteria, the actual number of viable bacteria in an unknown inoculum and also the capacity of the host to mount an immune response after local infection.

MLM causes granulomatous reactions only in high responder C57Bl/6 mice. This reaction is defined as a nodular granulomatous infiltrate (Closs & Haugen, 1975b), showing numerous lymphocytes surrounding activated macrophages (Haugen, Skjørten & Closs, 1975). A cellular immune response has been detected in T cell areas of the draining nodes where enlargement, epithelioid cell granuloma formation and proliferation of pyroninophilic blast cells were seen (Haugen & Closs, 1975). On the other hand, no such reactions develop in the C3H susceptible mice. In the high responder mice, along with the development of the local reaction, a specific immune mechanism occurs which tends to limit the local multiplication and spread of infective bacilli (Closs & Haugen, 1975a). Acid-fast bacilli were shown in the draining popliteal node soon after footpad inoculation. Multiplication proceeded for 8 weeks and then was stopped when the local reaction occurred in the footpad (Figs 2 & 3). Exponential multiplication continued in C3H mice until the fifth month and the injected footpad swelled but the swelling was delayed and macroscopically the local reaction was softer and had white abscesses. Histologically there is an infiltrate of non-activated macrophages which is overwhelmed by large quantities of living MLM in the C3H lesions (Haugen *et al.*, 1975). This was also described as occurring in thymus-deprived mice (Kawaguchi *et al.*, 1976), in which no limitation of multiplication of bacilli occurred and, as described here, no local reaction was seen in 3 months (Fig. 9). When dead MLM alone were injected, no local reaction developed in C57Bl/6 mice. Thus, dead MLM acted quite differently when compared with dead *M. leprae* in mouse footpads, where an immune response was observed (Shepard, 1975). Moreover, when dead MLM were injected together with, or before, living MLM, they induced a modified immune response in C57Bl/6 mice. This was found after the systemic injection of HK.MLM (Leffort & Mackaness, 1977), or after administering HK.MLM together with living MLM in the footpad (Fig. 8). The local reaction was always delayed and subsequently its magnitude increased. AFB counts per node were also augmented. Thus HK.MLM seemed to induce an inadequate immune response which can interfere with the action of immune effector cells, perhaps by preventing them from circulating and reaching infective foci (Bullock, 1976a, b). Since after i.v. immunization with high doses of a non-replicating antigen, effector cells are trapped in the spleen (Lagrange & Mackaness, 1978), Lefford used splenectomized mice to modulate the immune response to HK.MLM but failed to restore the local immune reactivity of paralysed mice (Lefford & Mackaness, 1977). Other sites or other mechanisms have to be found to explain the immune unresponsiveness in low responder mice.

Overloading doses of mycobacteria have been shown to induce an anergic state in mice (Rook, 1975). Thus, varying doses of living MLM were injected into high or low responder mice but, whatever the injected dose, no local granulomatous reaction occurred in C3H mice (Fig. 4). These mice seemed to behave as thymusless mice.

When hypersensitivity reactions were tested in the contralateral footpad at varying times after local infection with living MLM, a small 24–48 hr delayed-type reaction and Arthus-like reactions were noticed at 4 and 5 weeks in C3H mice. Moreover, a local swelling at 18 hr was also seen. On the other hand, only the tuberculin-type reactions (24–48 hr) were observed in C57Bl/6 mice. Thus, strain differences between these mice could consist of a different type of immune response induced by MLM infections. When C3H mice were injected with BCG vaccine, a very weak specific immune response occurred, but when Freund's complete adjuvant containing heat-killed *M. tuberculosis* was used, these mice developed tuberculin reactivity (Lagrange, unpublished results). This seems to indicate that the immune unresponsiveness to mycobacteria in C3H mice is not related to a genetic restriction linked to the histocompatibility complex.

C3H mice are more capable of mounting a B cell immune response to MLM antigens (Waksman, 1973; Haugen *et al.*, 1975), which perhaps can interfere with T cell effector mechanisms, either by pre-

venting their production, or suppressing their function (Geffard & Orbach-Arbouys, 1976), or by altering their circulation, specific committed T cells being sequestered in central organs, as occurs after desensitization (Schlossman *et al.*, 1971). When a small inoculum (10^6 MLM) was injected i.v., it did not interfere with the local infection, but centrally, in the spleen, lower AFB counts were found than in C57Bl/6 mice (Table 2). Thus, C3H mice present a higher natural resistance to mycobacteria in general and to MLM specifically, but also are perhaps more able to mount a systemic humoral immune response (only measured in the Arthus-like reaction) which seems to be inadequate for the limitation of local, peripheral infection. This also appeared to be true when immune responsiveness to unrelated antigens was studied: T cell responses were impaired but not serum antibody levels (Ptak *et al.*, 1970; Bullock, Evans & Filomeno, 1977). Some contradictory results about the effectiveness of suppressive effects of serum antibody have been described previously (Waksman, 1973; Closs & Haugen, 1974).

In C3H or C57Bl/6 mice, several mechanisms could be involved in the suppression or absence of peripheral reactivity, which occurs naturally or after HK.MLM inoculation, and different stages of the disease have to be studied in order to detect such mechanisms. Sequestration, for instance, of antigen-reactive cells could be the primary cause in C3H mice and only the consequence of severe disseminated disease in C57Bl/6 mice.

In C57Bl/6 mice, lower natural immunity seems to exist and this is associated with the intracellular multiplication of mycobacteria, which induces specific immune resistance without producing high antibody titres: specific mediator cells are produced which in turn activate macrophages which limit local growth of mycobacteria. When HK.MLM are inoculated i.v. or s.c. another type of immune response is induced which interferes with the specific local immune reaction.

Since a form of DTH has been detected in C3H mice, it would be of great interest to study the nature of this DTH reaction (Rook, 1978), which seems to have no positive correlation with immune acquired resistance. But, when C57Bl/6 mice are injected with living MLM, they develop a tuberculin-type DTH that can be deleterious when systemic infection occurs. The survival time of C57Bl/6 mice after i.v. injection of 2×10^8 MLM has been found to be shorter than in C3H mice (Fig. 1). Thus, high responder mice, after local infection, are more susceptible after systemic injection (Lefford *et al.*, 1977). Moreover, as described by Pierce, Dubos & Middlebrook (1947) and Youmans & Youmans (1972) this high susceptibility of C57Bl/6 mice can be explained on the basis of a superimposition of the tuberculin-like DTH inflammatory reaction on an overloading dose of antigen. Thus, attempts at immunorestoration with mycobacterial adjuvants, such as BCG, after immune paralysis in highly resistant C57Bl/6 mice, could be harmful and probably ineffective.

There are at least three concomitant related phenomena which need to be considered for explaining the strain difference between C3H and C57Bl/6 mice. One is the natural resistance to mycobacterial infection, which more or less rapidly inactivates the bacilli. The second is the very long persistence in cells of the reticuloendothelial system (Brown & Krenzien, 1976) of dead bacilli, which are still immunogenic. The third is the nature and the level of the immune response that develops after contact with dead or living mycobacteria in C3H or in C57Bl/6 mice. These three phenomena are currently being tested in Biozzi mice (Biozzi *et al.*, 1976) which have been selected for their capacity to produce high or low levels of antibody to sheep red blood cells (Lagrange, Hurtrel & Thickstun, 1979).

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