

Induction of Cell-Mediated Immunity to *Mycobacterium lepraemurium* in Susceptible Mice

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Received for publication 16 May 1977

A mouse strain (CB6) that is highly susceptible to *Mycobacterium lepraemurium* was infected with 10^8 bacilli into the hind footpad. These mice developed cell-mediated immunity to *M. lepraemurium*, as expressed by the development of a granulomatous lesion at the site of inoculation in normal but not in T-lymphocyte-depleted mice, a proliferative response in the paracortical zone of the draining lymph node, delayed-type hypersensitivity to a sonic extract of *M. lepraemurium*, and immunopotentialization of the delayed hypersensitivity response to sheep erythrocytes. Resistance to a second challenge infection with *M. lepraemurium* was not demonstrated.

Although the susceptibility of mouse strains varies considerably, all mice that are infected with *Mycobacterium lepraemurium* (MLM) eventually die of that infection. Failure to combat this organism effectively can be attributed either to an inability to develop any response towards the parasite, or to the induction of immunological tolerance, or to the generation of a potentially effective immunological response that is insufficient or becomes subverted. Since mycobacterial infections are in general characterized by the induction of cell-mediated immunity (CMI), it was hypothesized that during the course of infection with MLM a state of CMI was engendered, but that this state was eventually subverted by blocking factors such as excess antigen, antibodies, or immune complexes (1), or by suppressor T-cells (8). Since intradermal/subcutaneous inoculation favors the induction of CMI as compared with intraperitoneal/intravenous injection (11), the immunological response of mice to subcutaneous footpad (FP) infection with MLM has been studied. Evidence is presented that even mice of a highly susceptible strain generate a CMI response to MLM.

MATERIALS AND METHODS

Mice. Female mice of a variety of strains, both randomly bred and inbred, were examined. The random-bred CD-1 and CFW strains were obtained from Charles River Breeding Labs, Wellington, Mass. The inbred parental strains A/J, BALB/cJ, C57BL/6J, C3H/HeJ, and DBA/2J and their hybrid crosses (Table 1) were raised at Trudeau Institute from stocks obtained from Jackson Laboratory, Bar Harbor, Me. In one experiment (Table 1, experiment 2), inbred mice from commercial suppliers were used directly.

MLM. The Hawaii strain of MLM was provided in

CFW passage mice by Norman Morrison, Leprosy Research Laboratory, Leonard Wood Memorial, Baltimore, Md. The strain has since been maintained in susceptible mice by passage. Purified suspensions of MLM were prepared from the livers and spleens of passage mice by the method of Draper (7), and only these suspensions were used for animal inoculations. Inocula of known size were prepared by appropriate dilution of the stock suspension after counts of acid-fast bacilli in stained smears were made (18, 20).

Organ counts of MLM. Each spleen was homogenized in 5 ml of saline with a motor-driven Teflon pestle. The hind foot was severed at the ankle, cut up, and homogenized in 5 ml of saline with a Virtis grinder. Each homogenate was centrifuged for 5 min at $500 \times g$, and appropriate dilutions in saline of the supernatant were used to make 1-cm² smears on Reich slides (18). These were stained with auramine-rhodamine and counted with a fluorescence microscope (20). The number of stainable bacteria per organ and the geometric mean per group of five mice were calculated.

Radioactive labeling. A 20- μ Ci amount of [³H]-thymidine was injected subcutaneously; 1 h later, the mice were killed, and the left popliteal lymph node (LPLN) was homogenized in 5% cold trichloroacetic acid. After sequential extraction with cold and hot (90°C) trichloroacetic acid, the [³H]thymidine incorporated into deoxyribonucleic acid was measured in a liquid scintillation counter.

Organ size. The thickness of the hind feet was measured with a dial-gauge caliper. The difference in thickness between the hind feet was expressed in 0.1-mm units, and the arithmetic mean difference per group was calculated. The popliteal lymph nodes were carefully removed and trimmed of adherent fat, and their length in millimeters was measured on a dissecting microscope with a calibrated eyepiece. Spleen size was determined by weighing.

T-cell depletion. Inbred mice were thymectomized at 6 weeks of age. One week later, they were exposed to 800 rads of total body irradiation from a ¹³⁷Cs source and reconstituted with 10^6 syngeneic bone marrow

cells. The mice were allowed to recover for 2 months before being admitted to an experiment.

Histology. Tissues were fixed in buffered Formalin. Lymph nodes were embedded in glycol methacrylate, and 1- μ m sections were cut and stained with methyl green-pyronin. Mouse feet were decalcified and embedded in paraffin wax, and 5- μ m sections were cut and stained, either with hematoxylin and eosin or by the Ziehl-Neelsen method for acid-fast bacilli.

SRBC. Sheep erythrocytes (SRBC) were obtained in Alsever solution. Immediately before use, the cells were washed three times in saline and suspended in saline to a density of 2×10^8 /ml. A quantity (0.05 ml) of this suspension, containing 10^8 SRBC, was injected into the left hind footpad (LHFP) for immunization and the right hind footpad (RHFP) to elicit hypersensitivity.

MLM antigen. A suspension of live MLM in tris(hydroxymethyl)aminomethane buffer containing 0.05% Tween 80, at a density of 2.5×10^9 bacteria per ml, was exposed to ultrasound for 15 min, at which point the organisms were almost totally disrupted. The RHFP received an injection of 0.04 ml of this suspension, equivalent to 10^8 MLM.

DTH. The thickness of the RHFP was measured with dial-gauge calipers immediately before and 24 h after the injection of antigen. Delayed-type hypersensitivity (DTH) was denoted by a statistically significant increase of FP thickness in MLM-infected mice compared with normal controls.

RESULTS

Susceptibility of mice to MLM. There is a substantial body of evidence that random-bred and inbred mouse strains vary in their susceptibility to MLM (4, 5, 10). Accordingly, all the mouse strains maintained at Trudeau Institute were compared with a known susceptible strain, CFW. Doses of MLM in 10-fold increments from 10^6 to 10^{10} were injected intravenously into groups of 10 CFW mice, and the 10^8 dose was also similarly inoculated into groups of 10 mice of the strains shown in Table 1, experiment 1. The results were expressed as the 50% survival time. The data reveal that the inbred parental strains BALB/cJ and C57BL/6J were as susceptible as the index strain CFW. The F₁ hybrid of these susceptible inbred strains (CB6) was also susceptible. The remaining strains were much more resistant, surviving substantially longer than CFW mice given a 100-fold-smaller inoculum. F₁ hybrids of susceptible and resistant parental strains (AB6 and B6D2) were uniformly resistant.

These results were disturbing, in that other workers had shown the C57BL/6 strain to be resistant to MLM and the C3H strain to be susceptible (5, 10). To eliminate the possibility that subline differentiation in the Trudeau-bred mice accounted for this anomaly, C57BL/6J and C3H/HeJ were obtained from the supplier and tested directly (Table 1, experiment 2). The

results confirmed the susceptibility of the C57BL/6 strain and revealed that, contrary to expectation, the C3H strain was resistant. Similar results have been obtained repeatedly.

The major objective of the study was to determine whether a highly susceptible mouse strain could nonetheless mount a CMI response against MLM infection. Consequently, the succeeding experiments were performed in the CB6 hybrid inbred strain.

Kinetics of the immune response to MLM. Mice were inoculated with 10^8 MLM into the LHFP. At 2-week intervals, the thicknesses of the RHFP and LHFP were measured, as were the length of the LPLN and the incorporation of [³H]thymidine into deoxyribonucleic acid within that node (Fig. 1). At each time point, additional mice were killed to obtain popliteal lymph node and FP tissue for histological examination. Commencing 10 days after infection, there was a concomitant increase in LHFP thickness and LPLN radioactivity (Fig. 1A) and a corresponding enlargement of LPLN (Fig. 1B). The general features of the response shown in Fig. 1 are reproducible apart from the latent period, which varies between 10 and 24 days, doubtless depending on the viability and infectivity of a given inoculum of MLM. At the time that the LPLN first became enlarged, proliferation of lymphocytes in the paracortical area of the node was evident histologically. There were also discrete granulomata within the LPLN, comprising lymphocytes and macrophages, some of which contained MLM. These lesions

TABLE 1. Susceptibility of mouse strains to MLM

Mouse strain	Time (weeks) to 50% mortality				
	10 ¹⁰ ^a	10 ⁹	10 ⁸	10 ⁷	10 ⁶
Expt 1					
Random-bred					
CFW	11	13	16	18	20
CD-1		32			
Inbred parental					
A/J		25			
BALB/cJ		16			
C57BL/6J		17			
DBA/2J		29			
Inbred hybrid (F₁)					
A/J × BALB/c (AB6)		29			
A/J × DBA/2J (AD2)		31			
C57BL/6J × DBA/2J (B6D2)		32			
BALB/cJ × C57BL/6J (CB6)		18			
Expt 2					
CFW	15	16			
C3H/HeJ	19	24			
C57BL/6J	14	16			

^a Intravenous dose of MLM.

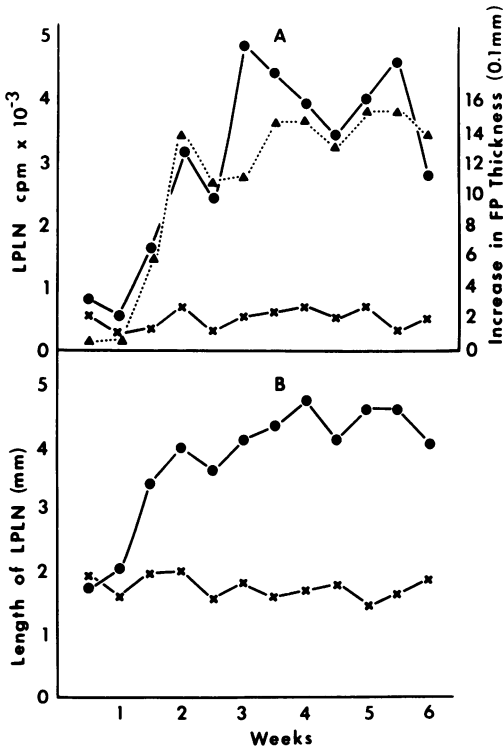


FIG. 1. FP and draining LPLN response after immunization with 10^6 live MLM. (A) Incorporation of [3 H]thymidine into deoxyribonucleic acid in normal (x) and stimulated (●) LPLN; ▲, increase in FP thickness of immunized mice. (B) Length of LPLN in normal (x) and immunized (●) mice.

closely resembled those seen in mouse tuberculosis. Three days after infection, the inoculated MLM were located within a dense band of mononuclear cells in the FP, but there was no surrounding inflammatory reaction. When the feet began to swell rapidly, the zone of MLM-laden cells was surrounded by and infiltrated with macrophages. The mycobacteria that had been concentrated at high density in relatively few cells were now distributed at much lower density in a much larger number of macrophages. Discrete granulomata of the type present in the LPLN were not seen in the FP. This appearance did not alter notably between days 17 and 42, when the experiment ended.

DTH to MLM. Mice were infected with 10^8 MLM in the LHFP and tested for DTH to sonically treated MLM at weekly intervals for 8 weeks, together with normal controls (Fig. 2). At each time point, the 24-h FP reactions of infected mice were significantly greater than those of the corresponding controls ($P = <0.05$ to <0.01). The week-to-week fluctuations in the

magnitude of the FP reactions in both infected and normal mice were not statistically significant and are a reflection of experimental variation. The data indicate that a state of DTH to homologous antigen was established within a week of MLM infection and was sustained for at least 2 months.

Effect of T-lymphocyte depletion. Mice that had been depleted of T-lymphocytes, together with normal controls, were challenged with 10^8 MLM into the LHFP, and the hind feet were measured weekly (Fig. 3). The LHFP of normal mice began to thicken at week 3, reached a peak at week 6, and then plateaued. In contrast, the LHFP of T-cell-depleted mice did not start to enlarge until week 6, but then continued to swell progressively until they were bigger than those of the control-infected mice.

Immunopotential of DTH to SRBC. It has long been known that inoculation of an unrelated antigen into the site of an ongoing mycobacterial infection promotes the induction of DTH to that antigen (6). It has been suggested that such immunomodulation or immunopotential depends on the induction of a powerful T-lymphocyte-proliferative response by the ad-

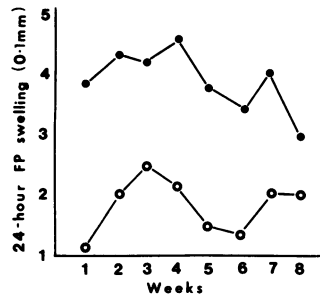


FIG. 2. DTH FP reactions to sonically treated MLM in normal (O) and MLM-infected (●) mice.

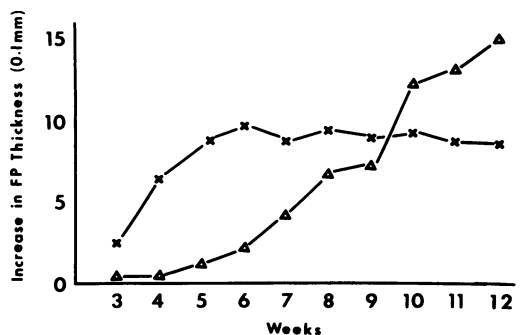


FIG. 3. Increase in thickness of LHFP after inoculation of 10^8 live MLM into normal (x) and T-cell-depleted mice (Δ).

juvant (14). Hence, the potentiation of DTH to an unrelated antigen may be regarded as indirect evidence that the potentiating agent is in itself an inducer of CMI. Accordingly, an experiment was performed to evaluate the influence of MLM infection on the CMI response to SRBC.

Mice were infected with 10^8 MLM into the LHFP and normal controls were set aside. Groups of five infected and normal mice were immunized with SRBC into the LHFP at intervals, commencing on the day of infection. Tests for DTH to SRBC were made 6 days after immunization (Fig. 4). Infected mice displayed significant ($P = <0.05$ to <0.01) increases in the level of DTH to SRBC, beginning at week 1, peaking at week 3, and disappearing by week 6. Maximum immunopotential was associated with a phase of rapid swelling at the site of infection, which was shown to correspond with cellular proliferation in the draining lymph node (Fig. 1).

Protective immunity in mice immunized with MLM. A large panel of mice was infected with 10^8 live MLM into the LHFP, and matched normal controls were set aside. At 2-week intervals, 10 immunized and 10 normal mice were challenged intravenously with 10^9 MLM, and their survival times were compared. Mice challenged at intervals from week 0 to week 12 relative to immunization survived challenge no better than normal controls. This failure to de-

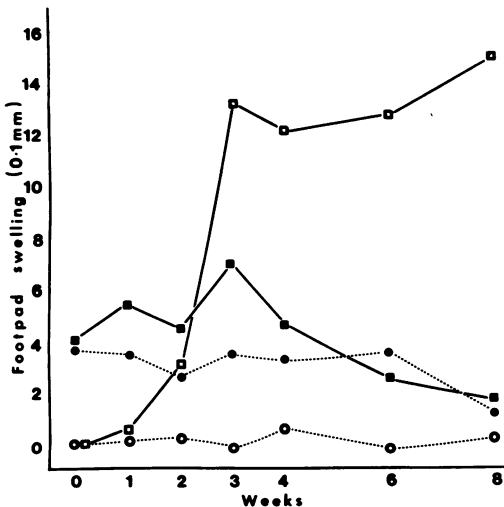


FIG. 4. Immunopotential of the DTH response to SRBC in FP of mice infected with MLM. Increase in thickness of the LHFP after injection of either diluent control (○) or 10^8 MLM (□) before immunization with 10^8 SRBC. Increase in RHFP thickness was measured 24 h after injection of 10^8 SRBC-eliciting antigen into diluent control (●) and MLM-infected (■) mice.

fect protective immunity provoked a change in the design of this experiment, as follows.

At 20 weeks after primary LHFP infection, groups of 10 infected and 10 normal mice were challenged with 10^8 live MLM into the RHFP. The mice infected at week 0 began to die at 32 weeks (12 weeks postchallenge), regardless of whether or not they had been challenged, so the experiment was terminated at 36 weeks and counts of MLM were then made from the RHFP. The mean values were 8.53 and 8.84 \log_{10} units of MLM in the RHFP of previously infected and uninfected mice, respectively, an insignificant difference. At the same time, four mice that had survived primary infection but had never been challenged were killed, their spleens were weighed, and counts of MLM were made from the LHFP and spleen. The results (Table 2) clearly show that these mice had disseminated infection and were carrying large populations of MLM in the organs examined.

DISCUSSION

The purpose of this study was to determine whether mice of a genetically susceptible strain could mount a CMI response to MLM. Evidence of CMI was sought in terms of the nature of the lesion at the site of infection, lymphoproliferative and pathological changes in the draining lymph nodes, the requirement for T-lymphocytes, development of DTH to MLM antigens, potentiation of the DTH response to an unrelated antigen, and resistance to infection with the homologous organism. It is recognized that intact organisms present a variety of antigenic determinants to the host, and the measured responses may have been excited by different antigens. This problem is intrinsic to all studies of CMI in host-parasite situations in which the protective antigens cannot be isolated or identified with certainty. It is assumed that the antigens that engender protective immunity may be related to those that induce other expressions of CMI (12). There would otherwise be no justification for examining the latter.

In general, the response of CB6 mice to FP infection with MLM conformed to the criteria of CMI. This is notable, in that others have

TABLE 2. Bacterial load in mice surviving MLM FP infection for 9 months

Increase in FP thickness (x0.1 mm)	MLM/FP (\log_{10})	Spleen wt (mg)	MLM/spleen (\log_{10})
13	10.08	685	9.60
21	10.10	521	8.54
25	10.37	674	9.32
37	10.47	771	9.70
Mean 24	10.26	663	9.29
Standard deviation 10	0.20	104	0.53

detected CMI to MLM only in a resistant strain of mice (3) and have implied that genetically susceptible mice are immunologically unresponsive to MLM infection (9). The CMI response to MLM in CB6 mice is weak in some respects. For example, the potentiation of the DTH response to SRBC by live MLM is modest as compared with that observed with live BCG (14), although the latter observations pertain to mouse strains that are resistant to MLM.

Failure to demonstrate immunity to reinfection with MLM in putatively immunized mice was a severe disappointment, but may be ascribed to inadequate procedures. The initial intravenous challenge with 10^9 MLM may have been too heavy. There is a parallel here to immunity in tuberculosis, which can be overwhelmed by too heavy a challenge with virulent organisms (21). The less severe challenge also failed to demonstrate immunity, but it was given 20 weeks after primary infection, at a time when preexisting immunity may have been exhausted. This is analogous to the failure of progressor tumor-bearing mice to control a secondary challenge (2). In retrospect, it would have been preferable to reinfect with a small dose of MLM at an earlier stage of the primary infection, when DTH, lymphoproliferation, and immunopotentiality were all maximal.

The genetic aspects of the immune response to MLM deserve further comment. Of the inbred parental strains, C57BL/6J and BALB/cJ were susceptible and others, including C3H/HeJ, were resistant. Since it has been reported that C3H mice are more susceptible to MLM than C57BL mice (5, 10), the experiments were repeated in animals obtained directly from Jackson Laboratories; the results were similar to those obtained previously. The discrepancy between these results and those of Closs and Haugen (5, 9) may be attributable to Closs and Haugen's use of C3H/An mice rather than our C3H/He strain, since Kawaguchi has reported that C3H/He mice are more resistant to MLM than C3H (?C3H/An) mice (10). The varying susceptibility of C57BL/6J mice in different laboratories is inexplicable.

Susceptibility to MLM was not linked to H-2 genes, and the F₁ hybrids of susceptible and resistant parents were uniformly resistant. The susceptibility of mouse strains to MLM (Table 1) may not be peculiar to that organism, since the C57BL strain has also been reported to be highly susceptible to *M. tuberculosis* (15, 22) and similar patterns of response have been described for *Salmonella typhimurium* infection (16) and in humoral antibody systems (13, 19). It may transpire that in some strains of mice there is a widespread deficiency in the expres-

sion of CMI and the production of certain classes of antibody.

The ability of even a highly susceptible mouse strain to mount a CMI response to MLM infection is of some importance, because it relates to patients with borderline leprosy. Both the mice and human subjects possess a weak and unstable resistance to their infection due to the fact that CMI is not sustained in the face of mechanisms that tend to undermine it. These include blocking factors such as circulating antigen, antibodies, or immune complexes (1), or suppressor T-cells (8, 17). The fact that these hosts are not genetically unresponsive to these infections but suffer a specific acquired suppression of CMI offers the real possibility of reversing the process by means of immunotherapy.

ACKNOWLEDGMENTS

We thank Robert Miller, Dwight Milne, and Sandra Warner for excellent technical assistance.

This work was supported by Public Health Service grant AI-07015 from the National Institute of Allergy and Infectious Diseases and a grant from the World Health Organization IMMLEP Program.

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