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Induction of Cell-Mediated Immunity to *Mycobacterium leprae* in Mice

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The immune response of mice to armadillo-derived, irradiation-killed *Mycobacterium leprae* (I-ML) was investigated. Following injection of 100 μg of I-ML into the left hind footpads of mice, a state of cell-mediated immunity (CMI) was engendered to antigens of *M. leprae*. The evidence for CMI was as follows: (i) development of delayed-type hypersensitivity to both human tuberculin purified protein derivative and soluble *M. leprae* antigens; (ii) T-lymphocyte-dependent macrophage activation at the inoculation site; (iii) specific systemic resistance to the cross-reactive species *M. tuberculosis;* and (iv) immunopotentiation of the delayed-type hypersensitivity response to an unrelated antigen. The CMI induced by I-ML in aqueous suspension was greater than that obtained with the same antigen in water-in-oil emulsion, even though the latter generated a more severe reaction at the site of immunization. I-ML also induced a stronger CMI response than the corresponding dose of heat-killed BCG.

The protective immune response to mycobacterial infections, including leprosy, appears to be cell mediated in nature. In human leprosy, resistance to infection is associated with delayed-type hypersensitivity (DTH) reactions to *Mycobacterium leprae* antigens, and the histology of lesions in tuberculoid leprosy is that of hypersensitivity granulomas (20). Studies of *M. leprae* infection in rodents also suggest that cell-mediated immunity (CMI) may be instrumental in controlling the infection. Thus, the multiplication of *M. leprae* in the footpads of mice and rats is enhanced in T-lymphocyte-depleted animals (3, 13). Such evidence for CMI is not conclusive because it has not been shown whether the T-lymphocytes in this system function as mediators of CMI or antibody-helper cells.

In the present study, a number of correlates of CMI have been measured in mice immunized with *M. leprae* antigens. These correlates included DTH, macrophage activation, adjuvant properties, and resistance to infection with *M. tuberculosis*. The immunizing antigen was irradiation-killed *M. leprae* (I-ML), a material which has been shown to effectively immunize mice against challenge with live *M. leprae* (15).

MATERIALS AND METHODS

Animals. In most experiments, inbred hybrid mice of the B6D2 strain (C57BL/6 by DBA/2)F_1 of either sex were used when 6 to 8 weeks old. T-lymphocyte-depleted mice were prepared by thymectomy of random-bred ICR mice within 24 h of birth. All mice were bred at the Trudeau Institute.

Immunizing antigens. Lyophilized I-ML was obtained from R. J. W. Rees, National Institute of Medical Research, London, England, through the World Health Organization Immunology of Leprosy Program. The *M. leprae* had been purified from tissues of armadillos that had been infected with human leprosy bacilli. Heat-killed BCG (HK-BCG) was prepared by heating to 100°C for 20 min a culture of BCG Pasteur (TMC 1011, Trudeau Mycobacterial Culture Collection, Saranac Lake, N.Y.) that had been grown in Proskauer and Beck medium containing glycerol and Tween 80.

Living organisms. *Listeria monocytogenes* strain EGD was grown in Trypticase soy broth. An attenuated strain of *M. tuberculosis* (RIRv, TMC 205) and BCG Pasteur (TMC 1011) were grown in Proskauer and Beck medium containing glycerol and Tween 80. All the cultures were distributed in vials, stored at −70°C, thawed immediately before use, and diluted appropriately.

SRBC. Sheep red blood cells (SRBC) were obtained from the Animal Blood Center, Syracuse, N.Y., in Alsever solution. Prior to use, they were washed three times in sterile saline and then suspended in saline at known density. For purposes of immunization and elicitation, 10^7 and 10^8 SRBC were injected into the left (LHFP) and right (RHFP) hind footpads, respectively.

Tuberculin. Lyophilized human tuberculin purified protein derivative (PPD; National Institute of Allergy and Infectious Diseases, Bethesda, Md.) was diluted in sterile phosphate-buffered saline containing 0.05% Tween 80 to a concentration of 125 μg/ml, and 5 μg (0.04 ml) was injected into the RHFP.

***M. leprae*-soluble antigen.** Lot LR.A8 of antigen was provided by R. J. W. Rees at a concentration of 610 μg of protein per ml. It was appropriately diluted

in saline to contain 2.5 to 10 μg of protein in 0.04 ml, which was injected into the RHFP.

Immunization. I-ML and HK-BCG were suspended at known concentrations in saline containing 0.05% Tween 80 and exposed to ultrasound to disperse clumped organisms. Each suspension was then diluted with an equal volume of Tween-saline or emulsified with an equal volume of light mineral oil (Drakeol 6-VR), and 0.04 ml was injected into the LHFP.

Footpad measurements. The thickness of the hind feet was measured with dial-gauge calipers and expressed in 0.1-mm units. The response at the site of immunization was expressed in terms of the difference between the LHFP and RHFP measurements. In DTH tests, the RHFP was measured immediately before eliciting antigen was injected and at intervals thereafter. The difference in measurement before and after antigen injection was an estimate of the local inflammatory reaction.

Challenge infections. A total of 10^4 *L. monocytogenes* in 0.04 ml was injected into the LHFP of immunized and control mice. Twenty-four hours later, the mice were killed, the feet were placed in sterile saline and homogenized with a Virtis 45 tissue grinder (Virtis Co., Gardiner, N.Y.), and appropriate dilutions were plated on phenylether alcohol agar. The plates were incubated for 48 h at 37°C, and colonies were enumerated.

A total of 10^5 RIRv was inoculated intravenously into immunized and control mice. Fourteen days later the mice were killed, and viable counts of RIRv were made by plating spleen homogenates on 7H-10 agar and counting the colonies after 21 days of incubation at 37°C (7).

Viable counts were expressed to the $\log_{10}$, and the geometric mean per group was calculated. "Resistance" was estimated by subtracting the mean viable count of a test group from that of the control.

Radiometry. Mice were given 20 μCi of tritiated thymidine (specific activity, 3.0 Ci/mmol) intravenously. Thirty minutes later they were killed, the left popliteal lymph node (LPLN) was removed, and the radioactivity incorporated into deoxyribonucleic acid was measured (12). The results were expressed as counts per minute per LPLN.

Histology. Mouse feet were fixed in buffered Formalin, decalcified, and embedded in paraffin wax. The sections were stained with hematoxylin-eosin and by the Ziehl-Neelsen method for acid-fast bacilli.

Statistics. Comparisons between group means with respect to bacterial counts and DTH were made following analysis of variance and application of the Q test (17). It was found that the increases in footpad thickness were not normally distributed. However, transformation of the measurements into square roots did normalize the data, and, consequently, all the footpad results are presented in terms of square-root units.

RESULTS

Granuloma formation at the site of immunization. Initially, it was uncertain what dose of I-ML would induce CMI in mice, whether an adjuvant would be necessary, or, indeed, if it would be at all possible to engender CMI. The first experiment was therefore designed to discover what dose of I-ML as an aqueous suspension or water-in-oil emulsion would induce a granuloma at the inoculation site. Doubling concentrations of I-ML from 4 to 1,000 μg, either in aqueous suspension or water-in-oil emulsion, were injected into the LHFP of groups of 10 mice. Swelling of the inoculation site was measured weekly for 10 weeks. The results show that all but the very lowest doses of antigen induced footpad swelling regardless of whether the menstruum was aqueous or the water-in-oil emulsion (Fig. 1). The degree of swelling was approximately proportional to the dose of antigen and was greater in mice immunized with antigen in water-in-oil emulsion.

At this point it remained unclear whether the induced swelling was an expression of a hypersensitivity granuloma or a nonspecific inflammation. Accordingly, each group of ten mice was divided into two subgroups of five mice. One series of subgroups was tuberculin tested, and the results obtained are shown in Fig. 2. Statis-

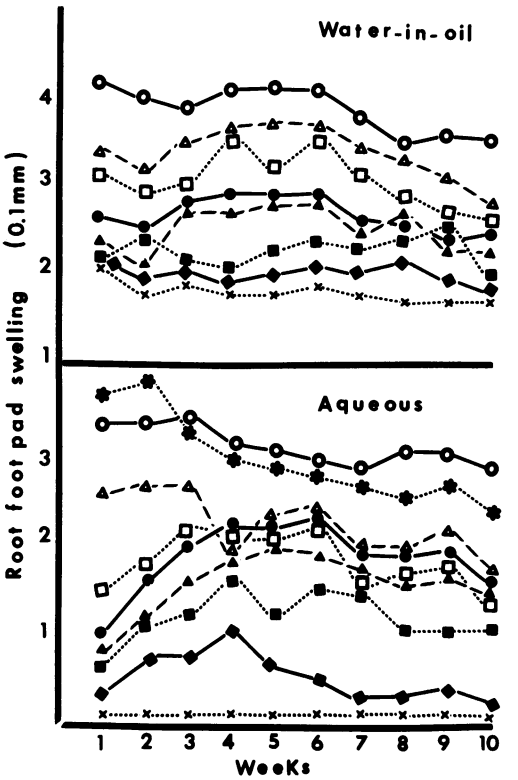

FIG. 1. *Footpad swelling following inoculation of I-ML at doses of 1,000 (✱), 500 (○), 250 (△), 125 (□), 62 (●), 31 (▲), 16 (■), 8 (◆), and 0 (×) μg. The 4-μg dose is not shown because the swelling of the footpad did not exceed that of the diluent control.*

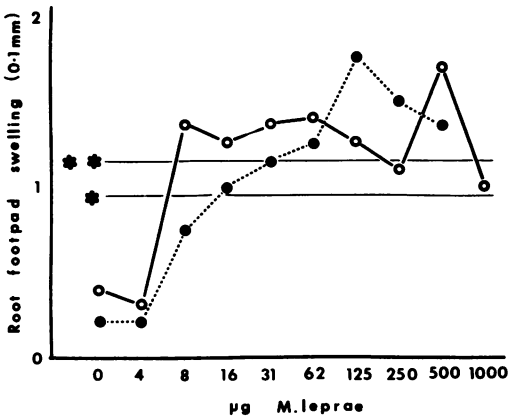

FIG. 2. *DTH footpad reactions to 5 μg of PPD in mice immunized with I-ML. Symbols:* (○) *antigen in saline suspension;* (●) *antigen in water-in-oil emulsion. Horizontal lines indicate the level of significance* (✱, $P < 0.05$; ✱✱, $P < 0.01$).

tically significant reactions were obtained in mice immunized with 16 μg or more of I-ML. These mice were killed, and the left hind feet were removed and examined histologically. The plantar subcutaneous tissues were found to be densely infiltrated with mononuclear cells (Fig. 3). There were no special features such as perivascular cuffing, tubercles, or giant cells. The Ziehl-Neelsen stain revealed that acid-fast bacilli had persisted in the tissues for the 10 weeks since immunization.

The positive DTH reactions elicited by human tuberculin PPD suggested that I-ML-immunized mice might be resistant to *M. tuberculosis*. To test this hypothesis, the remaining mice were challenged with RIRv. There was significant and consistent inhibition of growth of RIRv in the spleens of mice immunized with 16 μg or more of I-ML in aqueous suspension (Fig. 4). The response of mice immunized with I-ML in adjuvant was less uniform.

These experiments indicated that the minimal immunogenic dose of I-ML was 16 μg, but there was no clear-cut optimal dose. It was decided, arbitrarily, to adopt 100 μg of I-ML as the standard dose for further experiments.

Development of nonspecific resistance to *L. monocytogenes*. One of the hallmarks of CMI is the induction of macrophage activation at the site of interaction of sensitized lymphocytes and homologous antigen (8). It was postulated that such a situation would arise at the immunization site when CMI was induced and that macrophage activation would be detectable as increased microbicidal activity against an unrelated organism such as *L. monocytogenes*. Accordingly, 100 μg of I-ML or HK-BCG suspended in Tween-saline or water-in-oil emulsion was injected into the LHFP of mice. Control animals were similarly inoculated with the antigen vehicle. At weekly intervals, hind-feet measurements were made on five mice per group, after which *L. monocytogenes* was injected into the LHFP, and viable counts were made 24 h later.

The results (Fig. 5) reveal that, although the water-in-oil emulsion by itself produced chronic footpad swelling whereas the aqueous vehicle did not, the increment of chronic footpad swelling was greater when the antigen was administered in saline. Resistance to *L. monocytogenes* at the immunization site was first detected 2 to 3 weeks after inoculation (Fig. 5, Table 1). Resistance was significantly greater ($P < 0.01$) in feet injected with the immunogen in aqueous suspension than in water-in-oil emulsion (Table 1). Moreover, between weeks 3 and 7, feet inoculated with I-ML in aqueous suspension were more resistant ($P < 0.01$) to listeria than those given HK-BCG (Table 1). The week 3 results appear to be anomalous in this regard but do not invalidate the statistical analysis. The re-

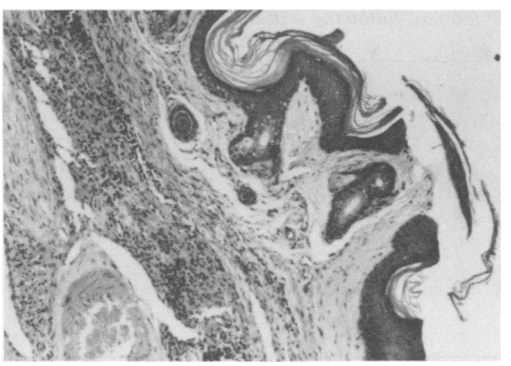

FIG. 3. *The histological appearance (hematoxylin and eosin) of the footpad granulomatous lesion 10 weeks after immunization with 125 μg of I-ML in aqueous suspension.*

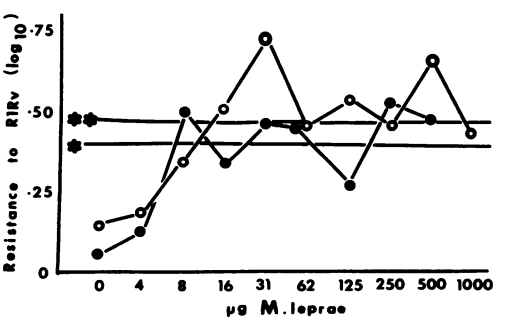

FIG. 4. *Systemic resistance to M. tuberculosis RIRv in mice immunized with I-ML. Symbols as in Fig. 2.*

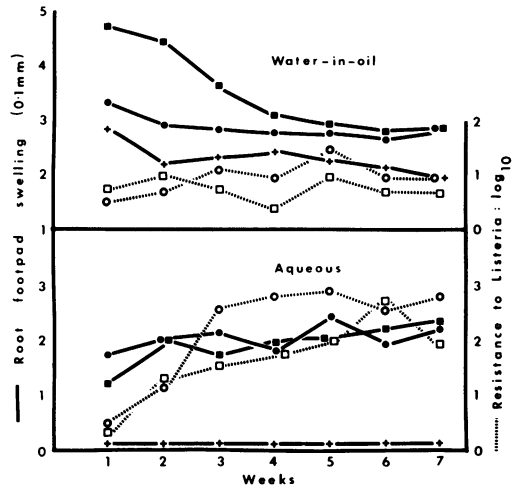

FIG. 5. *Footpad swelling (closed symbols) and local resistance to L. monocytogenes (open symbols) in mice immunized with diluent (crosses), HK-BCG (squares), and I-ML (circles) in either aqueous suspension or water-in-oil emulsion.*

TABLE 1. *Resistance to L. monocytogenes in the footpad following immunization with I-ML or HK-BCG*

Week	Resistance to *L. monocytogenes* ($\log_{10}$)			
	Aqueous		Water in oil	
	HK-BCG	I-ML	HK-BCG	I-ML
1	0.40	0.45	0.76	0.43
2	1.25	1.16	1.05	0.66
3	1.50	2.59	0.75	1.09
4	1.81	2.73	0.38	0.96
5	2.00	2.78	0.95	1.44
6	2.64	2.55	0.66	0.97
7	1.91	2.76	0.68	0.91

sults obtained with HK-BCG and I-ML in water-in-oil emulsion (Table 1) revealed no significant difference ($P > 0.05$) between those immunogens.

Effect of T-lymphocyte depletion. Mice which had been neonatally thymectomized and normal controls were inoculated in the LHFP with either an aqueous suspension of 100 μg of I-ML or diluent. Four weeks later, when increased resistance to listeria was anticipated in the normal immunized mice, five mice from each group were challenged with 10^4 live *L. monocytogenes* into both hind feet. Twenty-four hours later, viable counts of listeria per footpad were made. Significant ($P < 0.01$) resistance to listeria was found only at the site of I-ML immunization in intact mice (Table 2).

DTH to *M. leprae* soluble antigens. Mice were immunized with 100 μg of I-ML into the LHFP. Four weeks later, these mice and normal controls were tested in the RHFP with either 5 μg of PPD or 0.6, 1.25, 2.5, 5.0, or 10.0 μg of *M. leprae* soluble antigen. RHFP swellings were monitored for 48 h. During the first 6 h there was substantial nonspecific swelling amounting to 1 to 2 root units in both normal and immunized mice (not shown), but by 12 h the swelling in normal mice had subsided to less than 1 root unit, and by 24 h it had entirely disappeared. In general, the reactions in immunized mice reached a peak at 24 h and were diminished but still present at 48 h (Fig. 6). However, the largest dose of *M. leprae* soluble antigen elicited a response maximal at 12 h. The kinetics of the

TABLE 2. *T-cell dependence of resistance to L. monocytogenes at the immunization site*

Mice	Immunization	Resistance to listeria ($\log_{10}$)[a]	*P*
Intact	Diluent	0.16	NS[b]
Intact	I-ML	1.70	<0.01
Thymectomized	Diluent	−0.28	NS
Thymectomized	I-ML	0.52	NS

[a] Obtained by subtracting the geometric mean of viable listeria in the LHFP from that in RHFP.
[b] NS, Not significant: $P > 0.05$.

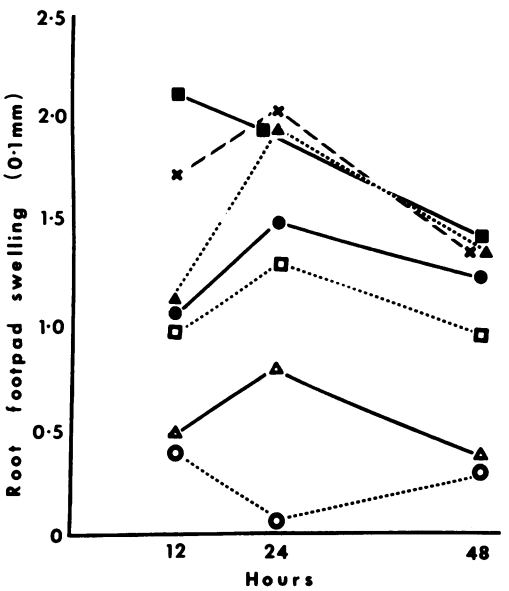

FIG. 6. *DTH footpad reactions to graded doses of M. leprae soluble antigen and 5 μg of PPD in mice immunized with I-ML in aqueous suspension. Symbols: (■) 10, (▲) 5, (●) 2.5, (□) 1.25, and (△) 0.6 μg of M. leprae soluble antigen; (×) 5 μg of human tuberculin PPD; (○) diluent control.*

reaction is consistent with a DTH response. The magnitude of the reactions was proportional to the dose of eliciting antigen in the range of 0.6 to 5.0 μg. From a practical viewpoint, it appeared that 5 μg was the most satisfactory dose. It is notable that 5-μg doses of *M. leprae* antigen and PPD yielded closely similar reactions.

Adjuvant properties of I-ML. Mice were immunized into the LHFP with either 100 μg of I-ML (aqueous) or 10^6 live BCG. A control group of mice received diluent in the LHFP. At weekly intervals, five mice from each group were immunized with 10^7 SRBC into the site of I-ML inoculation in the LHFP. Tests for DTH to SRBC were made 4 days later by injecting 10^8 SRBC into the RHFP and measuring footpad swelling after an interval of 24 h.

A small, uniform level of DTH to SRBC was observed at each time point in mice that had been immunized with SRBC alone (Fig. 7A), and a much higher level of sensitivity was exhibited by BCG-infected mice. For the first 4 weeks, the mice immunized with I-ML behaved similarly to normal mice, but much higher levels of sensitivity, similar to those in BCG-infected mice, were witnessed at weeks 5 and 6.

The proliferative response in the LPLN was monitored at weekly intervals in separate groups of mice as part of the above experiment. There was a striking increase in deoxyribonucleic acid synthesis in the LPLN of BCG-infected mice beginning at week 2, peaking at week 4, and declining somewhat by week 6 (Fig. 7B). Proliferation in nodes of mice immunized with I-ML did not exceed normal until week 5, when it rose sharply. Since the experiment was terminated at week 6, it is impossible to say whether the response had yet reached its peak. There was a striking association between LPLN proliferation and induction of enhanced DTH responsiveness to SRBC in I-ML-immunized mice, but not in BCG-infected animals (Fig. 7).

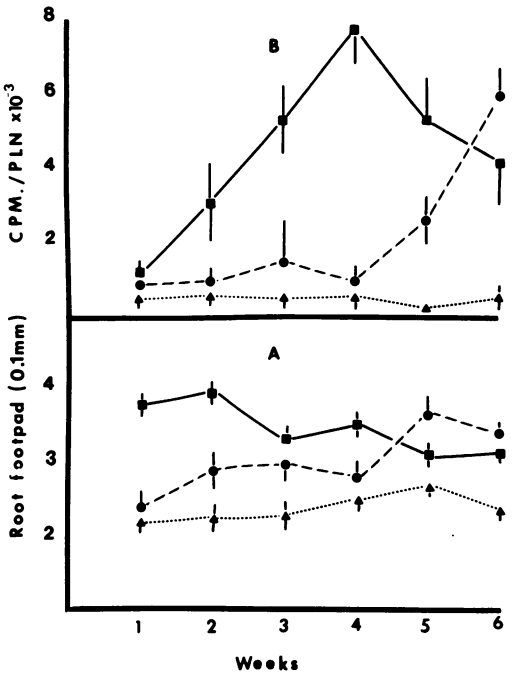

FIG. 7. *(A) Potentiation of the DTH response to SRBC in mice immunized with I-ML. Twenty-four-hour increase in RHFP thickness following injection of eliciting dose of SRBC into diluent control (▲), I-ML-immunized (●) and BCG-infected (■) mice. Mean of five mice ± standard deviation. (B) Incorporation of tritiated thymidine in draining lymph node in normal (▲), I-ML-immunized (●), and BCG-infected (■) mice. Mean of five mice ± standard deviation.*

DISCUSSION

The purpose of this study was to determine whether CMI to *M. leprae* antigens could be induced in mice. Cellular rather than humoral immunity was examined because of prior evidence that the former was implicated in the antibacterial response to *M. leprae* in man and rodents (3, 13, 20). Evidence of CMI was sought in terms of the histopathology of the lesion at the site of immunization; DTH to tuberculin PPD and *M. leprae* soluble antigens; nonspecific resistance to listeria; specific cross-resistance to another mycobacterial pathogen, *M. tuberculosis*; and the ability to adjuvant the CMI response to an unrelated antigen, SRBC (9).

The characteristic pathology of hypersensitivity granulomas, namely, discrete tuberculoid lesions with giant and epithelioid cells, was not found at the site of immunization. However, the observed accumulation of macrophages and lymphocytes is similar to that found at the site of inoculation of BCG cell walls in oil (4) and *M. lepraemurium* following the induction of CMI (2). It is notable that the I-ML retained its acid-fast staining property for as long as 10 weeks after inoculation.

Immunization with I-ML engendered a state of DTH to human tuberculin PPD and homologous *M. leprae* soluble antigen. Since mediation of DTH is known to be a T-lymphocyte function that does not involve antibodies (21), this result provides unequivocal evidence for the induction of CMI. It is arguable that the DTH reaction to *M. leprae* antigen might be attributable to contaminating armadillo tissue antigens that may be present in both the immunizing and eliciting material. Although theoretically

possible, this does not occur in practice (R. J. W. Rees, personal communication). In any case, this objection does not apply to the observed reactivity to human tuberculin PPD.

Nonspecific resistance to listeria infection is a less direct measurement of CMI. It has been shown in numerous studies that macrophage activation accompanies CMI responses (1, 8, 11), although it is admitted that certain agents such as endotoxin can produce similar effects (14). In earlier studies, nonspecific resistance was measured following intravenous or peritoneal challenge in vivo (1, 8, 11) or after infection of macrophage monolayers in vitro (5). Intralesional challenge has not been used until fairly recently, when it has been applied in tumor systems to detect both enhanced and impaired resistance at the site of tumor growth (18). In this study, resistance to listeria has proved to be a highly sensitive and consistent indicator of events at the inoculation site. There was a good association between nonspecific resistance and unequivocal manifestations of CMI such as DTH. Moreover, resistance to listeria was absent in I-ML-immunized mice that had been depleted of T-lymphocytes.

When SRBC are injected into the site of an ongoing BCG infection, the antibody and DTH responses to the former antigen are enhanced (9). The magnitude of those effects depends on the interval between the injections of BCG and SRBC which, in turn, correlates with lymphoproliferative response to BCG in the draining lymph node (10). Similarly, the adjuvant action of I-ML was found to coincide with the lymphoproliferative response in the draining LPLN.

This series of experiments provides compelling, cumulative evidence that I-ML, injected into the footpad, engenders a state of CMI to *M. leprae* and other mycobacterial antigens. Two additional observations of considerable importance emerge from this study. First, the immunogenicity of I-ML is very high in that CMI was induced by as little as 16 μg of material, which is equivalent to approximately 1×10^7 to 2×10^7 bacilli. The immunogenicity of I-ML was not increased by incorporation in a water-in-oil emulsion and was more impressive than that engendered by HK-BCG. The latter point must be interpreted with caution in that the I-ML was derived from tissue-grown organisms that had been killed by gamma irradiation, whereas the BCG was grown in vitro and heat killed. The second point of interest is the high degree of cross-reactivity at the T-lymphocyte level between *M. leprae* and *M. tuberculosis*. This was manifest in the DTH tests and, more importantly, as cross-protection. This result is consistent with some earlier work (16) which indicated that immunization with *M. tuberculosis* protected mice against infection with *M. leprae*. More recent taxonomic studies have shown that the two species are not closely related serologically in that they share only those antigens which are common to all mycobacteria (6, 19).

Finally, this study provides encouragement for the view that it may prove possible to develop a protective vaccine against human leprosy using armadillo-derived I-ML.

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