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Specific and Nonspecific Resistance in Mice Immunized with Irradiated *Mycobacterium leprae*

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Following subcutaneous inoculation of irradiated *Mycobacterium leprae* (I-ML) into the left hind footpad of mice, there was increased resistance to *Listeria monocytogenes*, indicative of macrophage activation, at the immunization site. In spite of the high level of localized macrophage activation which was proportioned to the immunizing dose of I-ML, no such activity could be demonstrated systemically in these mice, as evidenced by the absence of increased resistance to an intravenous challenge with *L. monocytogenes*. Under these conditions, I-ML-immunized mice were nonetheless resistant to intravenous infection with either *M. tuberculosis* or *M. bovis* BCG, and this immunity was transferred to normal recipients using spleen or lymph node cells. Neonatal thymectomy completely abolished the development of antimycobacterial immunity after vaccination with I-ML, but immunity was restored by an intraperitoneal infusion of syngeneic thymocytes. Systemic nonspecific resistance could be generated in I-ML-immunized mice by an intravenous injection of disrupted I-ML. This study reveals that, after subcutaneous vaccination with I-ML, there is local accumulation of activated macrophages at the inoculation site and a widespread distribution of lymphocytes which are sensitized to mycobacterial antigens. Nonspecific resistance is mediated by the former cells and specific antimycobacterial immunity by the latter.

In a previous report (14), it was shown that after subcutaneous immunization with 100 μg of irradiated *Mycobacterium leprae* (I-ML) in aqueous suspension, mice expressed responses characteristic of cell-mediated immunity (CMI). Immunized mice exhibited thymus-derived (T)-lymphocyte-dependent macrophage activation at the immunization site, delayed type hypersensitivity to antigens of *M. leprae* and human tuberculin purified protein derivative, and resistance to an intravenous (i.v.) challenge with an attenuated strain of *M. tuberculosis*.

There was some doubt as to whether the observed antituberculosis immunity was a consequence of nonspecific resistance due to systemic macrophage activation or an expression of lymphocytes that were specifically sensitized to antigens shared in common by *M. leprae* and *M. tuberculosis*. This concern was based on the knowledge that, although the induction of macrophage activation during the course of a CMI response is immunologically specific, the enhanced microbicidal power of those cells is expressed against a wide variety of target organisms (7, 9, 23). It is extremely difficult to resolve the specific and nonspecific components of CMI in actively immunized animals, but the presence of specifically sensitized lymphocytes can be established by use of cell transfer studies. This approach has been applied in this study, and it will be shown that immunization with I-ML results in a population of sensitized T-lymphocytes which convey specific resistance to *M. tuberculosis* and in so doing generate nonspecific resistance to *Listeria monocytogenes*.

MATERIALS AND METHODS

Mice. Specific pathogen-free inbred hybrid mice of the B6D2 strain (C57BL/6 by DBA/2)F_1 of either sex were bred at Trudeau Institute and used when 6 to 8 weeks old. T-lymphocyte-depleted mice were neonatally thymectomized (TX) within 24 h of birth. Some of the TX mice were reconstituted (TXR) by infusing 2×10^8 syngeneic thymocytes intraperitoneally when 5 weeks old. At the time of sacrifice, all TX mice were examined macroscopically to ensure that thymectomy had been complete, and those with residual thymic tissue were excluded from the results.

Immunizing antigen. 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.

Cultures. *L. monocytogenes* strain EGD was grown in Trypticase soy broth. An attenuated strain of *M. tuberculosis*, R1Rv (TMC 205), and *M. bovis* 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.

Immunization. I-ML was suspended at a concentration of 2 mg/ml in saline containing 0.05% Tween 80 and exposed briefly to ultrasound to disperse clumped organisms. Of this material, 0.05 ml containing 100 μg of I-ML was injected into the left hind footpad (LHFP). BCG cultures were diluted with saline, and 10^6 viable BCG in 0.04 ml were injected into the LHFP.

Cell transfer. Donor mice were killed by cervical dislocation, and spleens and mesenteric lymph nodes were collected aseptically. The tissues were teased using sterile pointed forceps and compressed through 200-gauge stainless steel wire mesh into Dulbecco phosphate-buffered saline. The cells were washed twice and finally suspended to a desired concentration in phosphate-buffered saline. Viability, as determined by trypan blue exclusion, was in the range of 90 to 95%. The cells were injected i.v. into recipients in a volume of 0.2 ml.

Irradiation. Prior to challenge with R1Rv and cell transfer, all recipient mice and their controls were exposed to 500 rads of total body irradiation (5).

Disrupted I-ML. This material was prepared by exposing an aqueous suspension of I-ML (2 mg/ml) to ultrasound for 15 min with an ultrasonic disintegrator (Bronwill Biosonic IV, VWR Scientific) set at 80% of maximum power. Microscopic examination indicated almost complete disintegration of the bacilli.

Lymphocyte transformation. The method used for this assay has been described in detail elsewhere (16). Dissociated spleen cells from groups of three to five mice were obtained as described above and pooled. A total of 4×10^6 to 6×10^6 cells were cultured in 2 ml of RPMI 1640 containing glutamin and supplemented with 5% deactivated normal human serum (lyophilized, Difco) and antibiotics. Either phytohemagglutinin-P (PHA; Difco) or lipopolysaccharide (LPS, prepared from *E. coli* and supplied by B. M. Sultzer) (20) was added to the cultures, which were incubated for a total period of 72 h with a terminal 20-h pulse of 1 μCi of tritiated thymidine per ml (5 Ci/mmol). The cells were then washed twice with saline, precipitated with cold 5% trichloroacetic acid, and counted in a liquid scintillation spectrophotometer. The results were expressed as counts per minute.

Challenge infections. A dose of 10^4 *L. monocytogenes* in 0.04 ml was injected into the LHFP of immunized and control mice. After 24 h, 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 24 h at 37°C, then colony counts were made. R1Rv and BCG cultures were exposed briefly to ultrasound to disperse bacterial clumps and were diluted to contain approximately 5×10^5 viable organisms per ml, and 0.2 ml was injected i.v. into mice. Mice were killed 14 days later, and viable counts were made from each spleen (5). These 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.

Statistics. There were at least five animals in each experimental group. Comparisons between group means with respect to bacterial counts were made using either Student's *t* test or analysis of variance and Q test, as appropriate (21).

RESULTS

Effect of the dose of I-ML on the development of nonspecific resistance. It was known that a dose of 100 μg of I-ML induced local nonspecific resistance to *L. monocytogenes* (14); this experiment was performed to determine the minimum effective dose of I-ML. Mice were inoculated in the LHFP with doubling concentrations of I-ML from 12.5 to 100 μg in aqueous suspension. Other mice were similarly injected with 10^6 living BCG or diluent. At 2-week intervals, five mice from each group were challenged with 10^4 *L. monocytogenes* injected into the LHFP, and viable counts were performed 24 h later (Fig. 1). Mice that were immunized with 10^6 living BCG developed significant levels of nonspecific resistance to listeria most rapidly ($P < 0.01$ at 2 weeks). An inverse relationship was observed between the length of time required to induce resistance to listeria and the immunizing dose of I-ML in the range of 25 to 100 μg. Thus, significant levels ($P < 0.01$) of resistance to listeria were first detected at weeks 4, 8, and 16 with I-ML doses of 100, 50, and 25 μg, respectively. The early peak of resistance observed at week 4 in mice immunized with 25 μg of I-ML was statistically nonsignificant ($P > 0.05$). The lowest dose of I-ML, 12.5 μg, was totally inert in this assay (Fig. 1). Although the maximum resistance to listeria was reached at different times in mice immunized with graded doses of I-ML, the peak values were log linearly related to those doses (Fig. 2). It was also notable that closely similar levels of nonspecific resistance to listeria were induced by 100 μg of I-ML and 10^6 living BCG (Fig. 1 and 2).

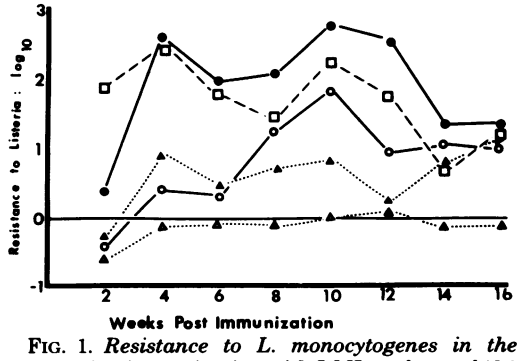

FIG. 1. *Resistance to L. monocytogenes in the LHFP after immunization with I-ML at doses of 12.5 (△), 25 (▲), 50 (○), and 100 (●) μg and 10^6 living BCG (□).*

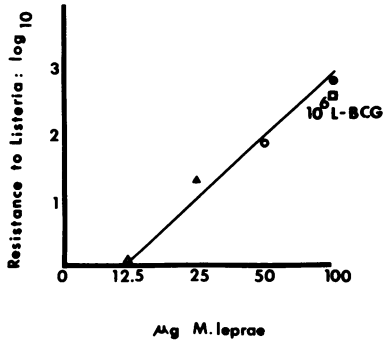

FIG. 2. *Relationship between the immunizing dose of I-ML and maximum resistance to L. monocytogenes in the LHFP. Symbols as in Fig. 1.*

Challenge of actively immunized mice with R1Rv and BCG. Mice were immunized with 100 μg of I-ML in aqueous suspension. Six weeks later, these mice and appropriate controls were challenged i.v. with 10^5 R1Rv. Viable counts of mycobacteria per spleen were carried out 14 days later. Mice which were immunized with I-ML showed a highly significant level ($P < 0.01$) of resistance to R1Rv (Table 1). Since mice (19) have been successfully immunized against *M. leprae* with *M. bovis* BCG, we wished to learn whether mice immunized with I-ML were also resistant to BCG. The above experiment was therefore repeated, substituting an i.v. challenge with 10^5 BCG for R1Rv. The immunized mice were significantly resistant to BCG (Table 1), and the levels of resistance to BCG and R1Rv were closely similar in the two experiments.

Transfer of adoptive immunity to R1Rv. Since immunization of mice with I-ML produced nonspecific resistance due to macrophage activation at the inoculation site, it was unclear whether the observed systemic resistance to BCG and R1Rv was a consequence of nonspecific resistance resulting from systemic macrophage activation or due to specific cross-reactivity between *M. bovis/M. tuberculosis* and *M. leprae*. Accordingly, an experiment was designed to segregate the nonspecific and specific components of the immune response to I-ML.

A large panel of mice was immunized with 100 μg of I-ML into the LHFP, and matched normal controls and recipients were set aside. These mice were used for three different kinds of assay. (i) At weeks 5 and 7 after immunization, groups of mice were challenged with 10^4 *L. monocytogenes* either into the LHFP or i.v. Viable counts of *L. monocytogenes* in the LHFP and spleen, respectively, were carried out 24 h later. (ii) Other groups of vaccinated and normal mice were challenged i.v. with 10^5 R1Rv, 5 weeks after immunization. Fourteen days later, these mice were sacrificed, and viable counts of R1Rv per spleen were performed. (iii) Still another group of immunized mice were used as donors of spleen or mesenteric lymph node cells, which were infused into irradiated syngeneic recipients that were challenged i.v. with 10^5 R1Rv.

As expected, substantial levels of nonspecific resistance to listeria ($P < 0.01$) were observed at the inoculation site of I-ML (Table 2). However, there was no difference between the immunized mice and normal controls with respect to systemic resistance to *L. monocytogenes* either at week 5, the time of R1Rv challenge, or at week 7, when the R1Rv infection was terminated ($P > 0.05$). Nonetheless, a significant level of antituberculous immunity ($P < 0.01$) was expressed by the actively immunized mice and was transferred to normal recipients with either spleen or mesenteric lymph node cells.

Induction of systemic resistance to *L. monocytogenes*. The above results reveal that, in spite of high levels of macrophage activation at the I-ML immunization site, no such activity could be detected systemically in these mice. However, it was conjectured that systemic mac-

TABLE 1. *Systemic resistance to M. tuberculosis R1Rv and M. bovis BCG of mice immunized by footpad injection with I-ML*

M. tuberculosis strain	Viable bacilli/spleen (log_{10})			*P*
	Control (A)	I-ML immune (B)	Resistance (A − B)	
R1Rv	5.33	4.81	0.52	<0.01
BCG	4.89	4.34	0.55	<0.01

TABLE 2. *Resistance to L. monocytogenes and R1Rv in mice immunized with I-ML*

Challenge	Viable counts (log_{10})			*P*
	Normal (A)	Immune (B)	Resistance (A − B)	
L. monocytogenes				
Donors, week 5				
LHFP	5.81	4.25	1.56	<0.01
Spleen	5.11	5.16	−0.05	NS[a]
Donors, week 7				
LHFP	6.02	4.40	1.62	<0.01
Spleen	5.22	5.36	−0.16	NS
R1Rv				
Donors, week 5				
Spleen	5.23	4.68	0.55	<0.01
Recipients, week 5				
Spleen[b]	5.62	4.71	0.91	<0.01
Spleen[c]	5.62	5.16	0.46	<0.01

[a] NS, Not significant ($P > 0.05$).
[b] Recipients of 2.5×10^8 spleen cells.
[c] Recipients of 10^8 mesenteric lymph node cells.

rophage activation would be induced in I-ML-sensitized mice by the systemic administration of the homologous antigen. Accordingly, a group of mice was immunized with 100 μg of I-ML into the LHFP, and equal numbers of mice were similarly injected with diluent. Six weeks later, these mice were divided into subgroups of five mice, which were injected i.v. with graded doses of disrupted I-ML. The next day, these mice were challenged i.v. with 10^5 *L. monocytogenes*, and viable counts of listeria per spleen were made after 24 h had elapsed. It is evident from Table 3 that the only mice to exhibit significant nonspecific resistance were those which had been immunized with I-ML into the LHFP and injected i.v. with disrupted I-ML. At the doses tested, these levels of induced nonspecific resistance were independent of the amount of disrupted I-ML injected.

Confirmation of T-lymphocyte depletion. Mice were TX or TXR. To ensure that these procedures had wrought appropriate changes in the lymphocyte population, the in vitro reactivity of spleen cells to known T- and B-lymphocyte mitogens was tested. Spleen cells were obtained from TX, TXR, and intact mice and cultured in the presence of either 1 μl of PHA or 100 μg of LPS, after which the blastogenic response to these mitogens was measured. Neonatal thymectomy completely eliminated the in vitro responsiveness of spleen cells to PHA, a T-lymphocyte mitogen (Table 4). This reactivity was, however, reconstituted by infusion of TX mice with syngeneic thymocytes. The blastogenic response to LPS, a B-lymphocyte mitogen, was of similar degree in cells from all three groups of mice.

T-cell dependence of antimycobacterial immunity. Groups of TX, TXR, and age- and sex-matched intact mice were inoculated into the LHFP with either 100 μg of I-ML or diluent. Six weeks later, when increased resistance to R1Rv was anticipated in the intact immunized mice, all the mice were challenged i.v. with 10^5 R1Rv. Viable R1Rv counts per spleen were made 14 days later. No greater resistance ($P > 0.05$) to R1Rv was observed in TX mice immunized with I-ML than in the three groups of unimmunized mice (Table 5), but highly significant ($P < 0.01$) resistance to R1Rv was expressed by TXR and intact mice immunized with I-ML. The apparent difference in the level of resistance between these latter two groups was statistically insignificant ($P > 0.05$).

TABLE 3. *Induction of systemic resistance to L. monocytogenes in mice first immunized by footpad injection of M. leprae and then injected i.v. with disrupted M. leprae*

Disrupted I-ML (μg)	Viable *L. monocytogenes* per spleen (log_{10})			*P*
	Control (A)	I-ML immunized (B)	Resistance (A − B)	
0 (Diluent)	6.23	5.83	0.40	NS[a]
1	6.21	4.97	1.24	<0.01
10	6.22	5.02	1.20	<0.01
25	6.12	4.78	1.34	<0.01

[a] NS, Not significant ($P > 0.05$).

TABLE 4. *In vitro lymphocyte transformation of splenic cells cultured with either 1 μl of PHA or 100 μg of LPS*

Source of cells	cpm[c]		
	Nil	PHA	LPS
TX	1,387 ± 82	1,336 ± 190	17,773 ± 2,129
TXR	4,480 ± 264	19,020 ± 2,290	24,136 ± 700
Intact	3,080 ± 410	36,540 ± 5,318	18,967 ± 1,120

[a] Average of triplicate cultures ± standard deviation.

TABLE 5. *T-cell dependence of antituberculosis immunity in I-ML-immunized mice*

Treatment	R1Rv/spleen (log_{10})			*P*
	Control (A)	I-ML immunized (B)	Resistance (A − B)	
TX	5.16	5.08	0.08	NS[a]
TXR	5.17	4.62	0.55	<0.01
Intact	5.24	4.48	0.76	<0.01

[a] NS, Not significant ($P > 0.05$).

DISCUSSION

M. leprae is a human intracellular pathogen, and by analogy with other mycobacterial intracellular parasites, immunity against *M. leprae* would be expected to be mediated by cellular rather than humoral defense mechanisms (23). This concept accords well with the close correlation between the intensity of CMI against *M. leprae* and the clinical course of human leprosy (10, 11).

There is substantial evidence indicating that sublethal infection with facultative intracellular parasites results in the generation of specifically sensitized T-lymphocytes (3, 8, 12). These T-cells, however, do not possess the ability to kill the homologous organism in the challenge directly; instead, when exposed to the homologous antigen, they influence the behavior of mononuclear phagocytes to give them the capacity to destroy either the homologous parasite or a number of antigenically and phylogenetically unrelated intracellular parasites (7, 8, 12). This process is thought to involve soluble mediators secreted by the sensitized T-cells upon encoun-

ter with the specific antigen (3, 8, 16, 20) and has been termed macrophage activation. The process of macrophage activation is usually localized in nature in that it occurs at the site of interaction of sensitized T-cells and homologous antigen in the tissues (2, 8). Only under conditions of exceptionally high and disseminated antigenic stimulation is there generalized macrophage activation (1, 7, 8). In keeping with this view, nonspecific resistance could be demonstrated at the I-ML immunization site but not systemically.

Throughout this study, macrophage activation was measured in terms of increased microbicidal activity, as expressed against the facultative intracellular parasite, *L. monocytogenes*. This agent has been widely used as a tool for measuring macrophage activation (1, 13). The larger the immunizing dose of I-ML, the more rapidly was nonspecific resistance induced and the higher the level of such resistance, in conformity with other studies of inoculum effects in CMI (4). The minimum effective immunizing dose lay between 12.5 and 25 μg. A similar conclusion was reached in earlier studies, in which other criteria of immunity were used (14). It might be argued that macrophage activation at the inoculation site of I-ML was caused by the direct interaction of these cells with mycobacterial cell wall components without the intercession of lymphocytes. Such is not the case, because the induction of macrophage activation after I-ML vaccination is dependent on T-lymphocytes (14) and is therefore an immunological event.

There is evidence from experimental studies using rodents that vaccination with *M. tuberculosis* (17) or *M. bovis* BCG (19) is effective against infection with *M. leprae*, presumably as a result of cross-reactive specific immunity. It was postulated that such cross-reactivity would be reciprocal in that appropriate immunization with antigens of *M. leprae* would be protective against *M. tuberculosis* and BCG. Some support for this notion was obtained earlier (14) and has been amply confirmed by the present study.

It has been shown elsewhere that antituberculosis immunity is mediated by specifically sensitized T-lymphocytes which belong to the recirculating pool and are widely distributed throughout the lymphoid system, including the spleen (5, 6). If the same were true for the lymphocytes sensitized to I-ML, one would expect that immunized mice would be resistant to systemic challenge with *M. leprae* and antigenically related organisms, despite the lack of systemic resistance to antigenically unrelated organisms. In support of this view, there was significant systemic resistance to BCG and R1Rv, but not to *L. monocytogenes* in I-ML-vaccinated mice. The specificity of systemic resistance to *M. tuberculosis* in I-ML-vaccinated mice was further substantiated by the transfer of immunity to syngenic recipients with spleen and mesenteric lymph node cells from donors that were lacking systemic nonspecific resistance. The generation of these mediator lymphocytes was ablated by neonatal thymectomy, supporting previous data that antimycobacterial immunity is mediated by T-lymphocytes (5, 12). The selective depletion of T-lymphocytes by neonatal thymectomy was verified by the ability of spleen cells from these mice to respond normally to LPS but not PHA.

These experiments proved that there was a specifically sensitized population of lymphocytes in the spleens of I-ML-vaccinated mice. In conformity with other studies, it was anticipated that these sensitized cells would be stimulated by an i.v. injection of homologous antigen to produce lymphokines with consequent macrophage activation (1, 8). This prediction was satisfied in that i.v. inoculation of disrupted I-ML produced systemic nonspecific resistance in immunized but not normal mice. This response was highly sensitive, since 1 μg of antigen generated a maximal level of nonspecific resistance. This system also appears to possess a great potential for studying the cross-reactivity of mycobacterial antigens at the CMI level.

Results obtained from this and previous (14) studies provide convincing evidence that vaccination of mice with I-ML generates specific CMI which is effective against infections with *M. tuberculosis* and *M. bovis*. There is also evidence that vaccination of mice with I-ML protects them against *M. leprae* infection (18). In light of these findings, it appears that I-ML may prove to be a valuable immunoprophylactic agent in human leprosy.

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