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Antibacterial resistance in mice infected with *Mycobacterium lepraemurium*

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SUMMARY

The differences in susceptibility among C57Bl/6, DBA/2 mice and their F_1 hybrids to infections with *M. lepraemurium* were shown to depend upon the route of infection and size of the inoculum. A method was developed to measure the ability of lymphocytes obtained from *M. lepraemurium*-infected donors to effect adoptive immunization of syngeneic naive mice against infection with *M. tuberculosis*. This required sublethal irradiation of recipient mice prior to cell transfer and bacterial challenge. Using this method, it was found that mice infected subcutaneously generated antituberculous immune mechanisms concordantly with the development of delayed-hypersensitivity to antigens of *M. lepraemurium*. In contrast, intravenously infected mice demonstrated only a transient form of delayed hypersensitivity and little or no antimycobacterial immunity in that progression of infection was associated with a rapid decay of both these functions. Moreover, during the terminal stages, *M. lepraemurium*-infected mice lost the ability to control the growth of a sublethal intravenous inoculum of the antigenically unrelated bacterium, *Listeria monocytogenes*.

INTRODUCTION

Human leprosy embraces a wide disease spectrum ranging from subclinical infection to the tuberculoid and lepromatous forms (Ridley & Jopling, 1966). There is a close relationship between various clinical forms of the disease and the state of cell-mediated immunity (CMI) to antigens of *Mycobacterium leprae* (Bullock 1978; Turk & Bryceson, 1971). In an attempt to understand the complexities of the host–parasite interactions in human leprosy more fully, many laboratories have employed rodents infected with *Mycobacterium lepraemurium* (MLM) as an experimental model (Bullock, 1978; Closs, 1975; Lefford *et al.*, 1977; Navalkar, Patel & Kanchana, 1980). Both *M. leprae* and MLM are obligate intracellular parasites which cannot be cultured on any standard laboratory media. This has posed one of the major obstacles to the study of acquired immunity to these pathogens. Measurement of acquired resistance by determining changes in the total number of acid-fast bacteria in various organs is not only a tedious procedure but is also beset with pitfalls because of the variability in the staining properties of mycobacteria. Furthermore, the staining procedures currently used do not readily permit one to distinguish between dead and living organisms (Barksdale & Kim, 1977; Chang, 1977; Nyka, 1971; Rees & Valentine, 1962; Harada, 1967).

In this study, we have employed the acquired capacity of MLM-infected mice to resist infection with *Mycobacterium tuberculosis* R1Rv as an indicator of the generation of protective immunity to *M. lepraemurium*. The rationale for such an approach was based on the knowledge that mice immunized with BCG are resistant to challenge infection with MLM (Hanks & Fernandez, 1956; Lefford, 1975; Robson & Smith, 1960). Moreover, in view of the published reports that MLM

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shares antigens with other mycobacteria that engender CMI and humoral immunity (Closs, Harboe & Wassum, 1975; Lefford, 1980; Navalkar *et al.*, 1980) it was felt that appropriate immunization of mice with the antigens of MLM would result in a state of protective immunity against other mycobacteria. The results presented in this paper provide evidence to support this view. They show that MLM-infected mice develop a state of cross-reactive anti-tuberculosis immunity which, under appropriate conditions, can be passively transferred with lymphocytes. They also show that lymphocytes obtained from the intravenously infected mice lost their ability to effect adoptive transfer of this cross-protective immunity within a short time period. Furthermore, clinical deterioration of the host during the terminal stages of MLM infection was accompanied by a generalized loss of ability of these mice to mount a protective CMI response to an unrelated intracellular pathogen, *Listeria monocytogenes*.

MATERIALS AND METHODS

Mice. Specific pathogen-free C57Bl/6/Tru and DBA/2/Tru mice or their B6D2 (C57Bl/6 × DBA/2)F_1 hybrid were bred at the Trudeau Institute Animal Breeding Facilities. The mice were incorporated into experiments when they were 8–12 weeks of age.

Bacteria. The Hawaiian strain of *M. lepraemurium* was passaged in CB6F_1 mice, periodically harvested from the liver and spleen and purified by a modification of the method of Draper (1971). Portions of bacterial suspensions of known density were stored at −70°C (Lefford *et al.*, 1977). *L. monocytogenes*, strain EGD, was grown in trypticase soy broth. An attenuated strain of *M. tuberculosis* R1Rv (TMC 205) was 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 for inoculation.

MLM infection. Mice were inoculated either subcutaneously into the left hind footpad, or intravenously in the tail vein with a suspension of MLM.

MLM antigen. A suspension of live MLM at a density of $2{\cdot}5 \times 10^9$ bacteria per ml was exposed to ultrasound for 15 min. To elicit a delayed hypersensitivity reaction, mice were inoculated subcutaneously into the right hind footpad with 0·04 ml of this suspension: a volume equivalent to 10^8 MLM (Lefford *et al.*, 1977).

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 infection was expressed in terms of the difference between the left hind and the right hind footpad measurements. In delayed hypersensitivity tests, the right hind footpad was measured immediately before injecting eliciting antigen and 24 hr thereafter, and the increase in footpad thickness expressed as above.

Irradiation. Prior to passive transfer of lymphoid cells and challenge with R1Rv some of the recipient mice and their controls were exposed to 500 rad of total body gamma irradiation (Lefford, 1975).

Cell transfer. Donor mice were killed by cervical dislocation. From normal uninfected controls, popliteal and mesenteric lymph nodes were collected aseptically and pooled. Popliteal lymph nodes from subcutaneously infected and mesenteric lymph nodes from intravenously infected donors were also collected. In addition, spleens were collected from all the donor mice killed. The tissues were teased using sterile pointed forceps, and compressed through 200-gauge stainless wire mesh into RPMI 1640 medium. The cells were washed twice and then processed to remove adherent cells as described in the following section. The non-adherent cells were suspended at the desired concentration in RPMI and infused intravenously into recipients in a total volume of 0·2 ml.

Depletion of adherent cells. To remove adherent cells, 10 ml of the cell suspension were placed in 100-mm-diameter plastic petri dishes (Falcon) at a density of 5×10^7 cells per ml in RPMI 1640 medium containing 10% heat-inactivated normal human serum (DIFCO Laboratories, Detroit, Michigan). The dishes were then incubated at 37°C in 5% CO_2 for 45 min. After incubation, the dishes were gently swirled and all non-adherent cells removed by gentle aspiration. This process was repeated, and the cells pooled, washed once and counted (Patel & Lefford, 1978a). Approximately 60% of the cells were removed by adherence.

Infection with R1Rv. R1Rv cultures were exposed briefly to ultrasound to disperse bacterial clumps, diluted to approximately 5×10^5 viable organisms per ml, and 0·2 ml injected intravenously into mice. Mice were killed 14 days later and the number of viable R1Rv per spleen was determined by plating spleen homogenate on 7H10 agar (DIFCO). After incubation at 37°C for 3 weeks, the numbers of colony-forming units were enumerated. The counts were recorded and converted to $\log_{10}$, and the geometric mean per group calculated. Acquired resistance to R1Rv was expressed as the difference between the number of R1Rv in the spleens of the test group and the number in the spleens of controls.

Listeria infection. A vial of frozen *L. monocytogenes* was quickly thawed at 37°C, and diluted in a standard fashion to obtain $2{\cdot}5 \times 10^4$ organisms per ml in 0·85% sodium chloride. At progressive intervals following infection with MLM, 0·2 ml of this suspension was infused intravenously into mice and into their littermate uninfected controls. The growth of Listeria was followed against time in the spleens for a period of 7 days. Listeria was enumerated by plating 10-fold serial dilutions of spleen homogenates on trypticase soy agar. Colonies were counted after a 24-hr incubation at 37°C. The counts were expressed to the $\log_{10}$, and the geometric mean per group calculated.

Statistics. Ten mice per group were used to determine the susceptibility to MLM. In other cases there were at least five mice in each experimental group. Comparison between group means was made using either Student's *t*-test or test of analysis of variance and *Q*-test, as appropriate (Snedecor & Cochran, 1967).

RESULTS

Susceptibility of mice to MLM

Table 1 shows the effect of varying the dose and the route of infection on the mortality of mice infected with MLM. In agreement with a previous report (Lefford *et al.*, 1977) C57Bl/6 mice exhibited greater susceptibility to infection with MLM than DBA/2 or the F_1 hybrid ($B6D2F_1$). The magnitude of differences in susceptibility was markedly affected by the route and size of the infecting inoculum. For example, the differences in mean survival time between susceptible (C56Bl/6) and resistant (DBA/2 or $B6D2F_1$) strains were 3·5-fold when mice were infected with 10^9 MLM intravenously. Given the same size of inoculum subcutaneously, the differences in mean survival time between the susceptible and the resistant strains were statistically significant, but small (1·25-fold). These results accord well with those reported by Turcotte (1980).

Antimycobacterial resistance in MLM-infected mice

Preliminary experiments were performed, without success, to determine if MLM-infected mice acquire the capacity to generate cross-protective antimycobacterial immunity. It has been shown previously that the passive transfer with lymphoid cells of humoral (Celada, 1966; Dresser, 1961), CMI (Asherson & Zembala, 1970) and, in particular, antimycobacterial immunity (Lefford, 1975;

Table 1. Susceptibility of mouse strains to infection with *M. lepraemurium*

	Survival time in days (mean ± s.d.)		
		Intravenous	
Mouse strain	Subcutaneous 10^9 MLM*	10^8 MLM	10^9 MLM
C57Bl/6	237 ± 20	82 ± 9	38 ± 12
DBA/2	296 ± 25	185 ± 7	133 ± 13
$B6D2F_1$	291 ± 7	201 ± 9	138 ± 11

* Route and dose of infection.

Table 2. Anti-tuberculosis resistance of *M. lepraemurium*-infected mice and its adoptive transfer with lymphoid cells

	Log_{10} R1Rv per spleen	
	No treatment	500 rad*
Control	5·28±0·20	5·57±0·18
10^8 *M. lepraemurium* (week 16)	5·12±0·04	5·06±0·10†
Recipients‡		
Normal lymph node cells	n.d.	5·50±0·15
Immune lymph node cells	5·25±0·05	4·89±0·05†
Normal spleen cells	n.d.	5·45±0·12
Immune spleen cells	5·36±0·15	4·88±0·22†

* Mice were sublethally irradiated prior to cell transfer and/or challenge with *M. tuberculosis*.

† Statistically significant ($P < 0·01$) as compared to controls.

‡ Infused with 10^8 lymphoid cells obtained from uninfected control mice or mice subcutaneously infected with 10^8 *M. lepraemurium* at week 16.

n.d. = Not done.

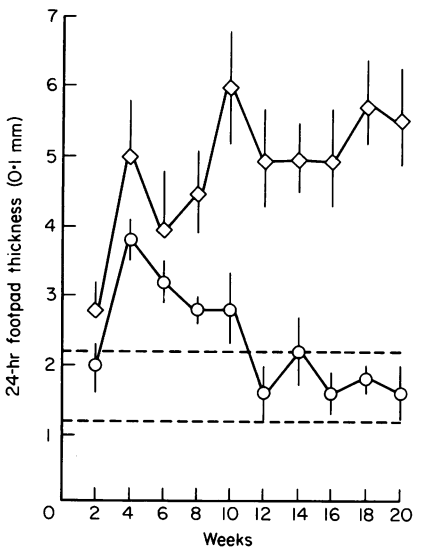

Fig. 1

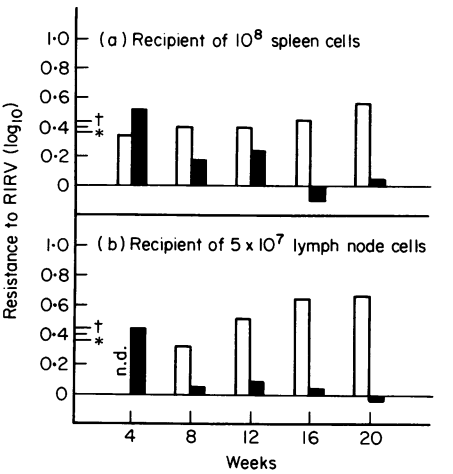

Fig. 2

Fig. 1. Development in mice infected either intravenously (o——o) or subcutaneously (◆——◆) with 10^8 *M. lepraemurium* of the capacity to exhibit delayed hypersensitive reactivity after challenge with 10^8 equivalents of sonicated *M. lepraemurium*. Means of five mice ± standard deviation. The horizontal lines represent the range of the swelling seen in normal mice challenged with an identical dose of *M. lepraemurium* sonicate.

Fig. 2. Generation and loss of ability in *M. lepraemurium*-infected mice to effect adoptive immunization of syngeneic recipients against *M. tuberculosis*. Adherent cell-depleted 10^8 spleen (a) or 5×10^7 lymph node (b) cells obtained at progressive time intervals from donor mice that were infected by either subcutaneous (□) or intravenous (■) inoculation of 10^8 *M. lepraemurium* were infused into sublethally irradiated recipients, and their ability to confer resistance against a standard R1Rv challenge was determined. * Level of statistical significance at 5%. † Level of statistical significance at 1%. n.d. = Not done.

Alexander, 1979) is facilitated by employing X-irradiated recipients. It was of interest, therefore, to investigate the effect of sublethal irradiation on the expression of antimycobacterial immunity in MLM-infected mice, and on the adoptive transfer of this immunity to syngeneic recipients. The results of such an experiment are presented in Table 2. It can be seen that mice infected subcutaneously with 10^8 MLM did not differ significantly from normal controls in their ability to control the growth of an intravenous R1Rv challenge. However, sublethal irradiation of mice prior to challenge with R1Rv resulted in the expression of a statistically significant antimycobacterial immunity. Moreover, lymphocytes harvested from the spleens and lymph nodes of MLM-infected mice failed to transfer protective immunity to syngeneic recipients, unless the recipients were sublethally irradiated before cell transfer.

Generation and loss of antibacterial immunity in MLM-infected mice

Having established suitable conditions for measuring antimycobacterial immunity, the kinetics of development of delayed hypersensitivity and protective immunity were measured in $B6D2F_1$ mice infected by subcutaneous or intravenous route with 10^8 MLM. In addition, a number of mice were sampled from the same populations to determine the effect of progressive MLM infection on the host's ability to mount cell-mediated antibacterial resistance to the antigenically unrelated intracellular pathogen, *L. monocytogenes*.

Fig. 1 shows that subcutaneous infection with MLM induced a measurable level of delayed hypersensitivity to MLM sonicate within 2 weeks of infection. Delayed hypersensitivity reached a peak at week 10 and remained at almost the same level until week 20 when the experiment was terminated. In contrast to this, intravenously infected mice did not develop a measurable level of delayed hypersensitivity until week 4. Moreover, delayed hypersensitivity then underwent progressive decay until week 12 at which time the mice were totally anergic.

Fig. 2 shows the results of an attempt to effect passive transfer of antimycobacterial resistance with 10^8 non-adherent spleen cells or 5×10^7 lymph node cells harvested at progressive times of MLM infection. This experiment employed irradiated syngeneic recipients. It can be seen that spleen and lymph node lymphocytes obtained at week 8 from subcutaneously infected donors

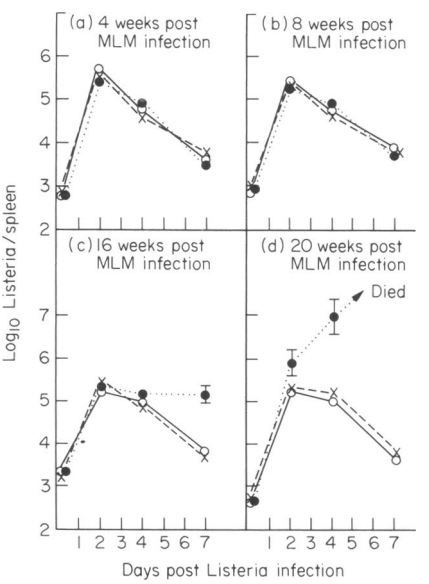

Fig. 3. Comparison of growth kinetics of Listeria in the spleens of normal mice (×– – –×) and mice that were infected with 10^8 *M. lepraemurium* by either subcutaneous (o——o) or intravenous route (•· · · ·•). A number of mice from each of three groups were inoculated intravenously with $2–5 \times 10^3$ Listeria at progressive time intervals of MLM infection, and numbers of Listeria present in the spleens at different time points were determined for up to 7 days.

transferred a significant level of protection against R1Rv and that the level of protection transferred increased slightly with increasing time of infection over a period of 20 weeks. Lymphocytes obtained from intravenously infected donors, on the other hand, transferred a significant level of antituberculous immunity only when harvested at week 4.

Fig. 3 compares growth kinetics of Listeria in the spleens of normal mice and mice that were infected with 10^8 MLM by either the subcutaneous or intravenous route. A number of mice from each of the three groups were inoculated intravenously with an immunizing dose of Listeria at progressive time intervals of MLM infection, and numbers of Listeria present in the spleens were determined at different time points for up to 7 days. Listeria counts within 30 min of inoculation showed that all the mice received an identical inoculum. Four- and 8-week MLM-infected mice showed Listeria growth kinetics that were identical to those of normal mice. Similar results were obtained at week 12 of MLM infection (not shown). However, at week 16, intravenously MLM-infected mice showed higher Listeria counts at day 7 as compared to normal and subcutaneously MLM-infected mice. By 20 weeks, the intravenously MLM-infected mice lost the ability to resist an immunizing infection of Listeria and all the mice died of listeriosis within 6 days.

DISCUSSION

There is little doubt that susceptibility of different mouse strains to infection with MLM varies considerably (Closs, 1975; Lefford *et al.*, 1977; Turcotte, 1980). However, the magnitude of these differences in susceptibility among various mouse strains appears to be dependent upon the size and the route of the infecting inoculum. There is evidence that even the most susceptible strains of mice acquire a state of CMI, as indicated by both the nature of local granulomatous response at the site of infection (Closs & Haugen, 1974; Lefford *et al.*, 1977), and by development of delayed hypersensitivity to antigens of MLM (Lefford *et al.*, 1977; Poulter & Lefford, 1977). Although a correlation has been shown to exist between delayed hypersensitivity and the progression of the infection in that delayed hypersensitivity reactions can be elicited at a time when the multiplication of the micro-organisms was being restricted, this relationship is not absolute (Poulter & Lefford, 1978). Furthermore, attempts to demonstrate resistance to a second challenge infection with MLM in such putatively immunized mice have not been successful (Lefford *et al.*, 1977).

Results presented in this study show that MLM-infected mice generate mechanisms that protect them against the antigenically related *M. tuberculosis*, R1Rv. This form of cross-protective immunity allows one to use R1Rv to detect the generation and loss of protective immunity to antigens of MLM, much in the same way that it has been used to study the induction of a cell-mediated immune response to the antigens of *M. leprae* (Patel & Lefford, 1978a). It should be emphasized, however, that lymphocytes obtained from either MLM or *M. leprae* immune donors confer markedly lower levels of antituberculous immunity than those conferred by equivalent numbers of cells obtained from BCG-immune donors (Lefford, 1975; Patel & Lefford, 1978b). It was found by using this experimental model that successful adoptive transfer of resistance to R1Rv with lymphoid cells from *M. lepraemurium*-infected donors required that recipient mice be subjected to sublethal irradiation prior to cell transfer. This is also a requirement for the passive transfer of anti-tuberculosis immunity with lymphocytes from BCG-immune donors (Lefford, 1975), and transfer of resistance to MLM using T cell-enriched spleen cells from MLM-infected donors (Alexander, 1979). The reason why sublethal irradiation facilitates the expression of adoptive immunity is poorly understood at this time. It may be relevant to note that similar facilitation of the expression of adoptive antituberculous immunity can also be achieved by injecting recipients with cyclophosphamide 2 days prior to cell transfer (Patel, unpublished observations) in a dose that has recently been shown to be inhibitory to the induction of suppressor T cells (Nakamura & Tokienaga, 1980).

There is ample precendent to justify the statement that the dose of antigen and the route it is presented are of prime importance in the type of immune response generated. For example, it has been shown that intravenous administration of relatively large doses of antigen favours the induction of suppressor mechanisms, whereas the same dose of antigen given subcutaneously

favours the generation of protective T cells (Green & Bach, 1979; Lagrange, Mackaness & Miller, 1974). In agreement with this published evidence, the results presented here show that intravenously infected mice demonstrated only a transient form of delayed hypersensitivity to antigens of MLM and little or no antimycobacterial immunity. Rapid progression of infection was associated with a decay of both delayed hypersensitivity and protective immunity, presumably because of the influence of an infection-induced suppressor mechanism. In contrast, subcutaneously infected mice generated antimycobacterial immune mechanisms concordantly with the development of delayed hypersensitivity to MLM antigens. It is important to stress, however, that none of these mice generated a high enough level of immunity to stop the proliferation of MLM, as evidenced by the eventual fatal outcome of the infection. This situation relates to patients with borderline leprosy where the patient displays a weak and unstable resistance to his infection.

During the terminal stages of intravenous MLM infection, mice lost the ability to control the growth of a sublethal inoculum of Listeria. It is not clear whether the unrestricted growth of Listeria in these mice was due to the host's inability to generate anti-Listeria-specific immunity or to express it. It may be relevant to note, however, that differences in Listeria growth were observed after 4 days of inoculation. This time corresponds with the time of generation of anti-Listeria-specific immune defence mechanisms in normal mice (Mackaness, 1969; North, 1973a, 1973b). The resolution of this question would require, in MLM-infected mice, a detailed study of production of T cells that mediate anti-Listeria immunity as well as a measure of the bactericidal activity of macrophages that are implicated in the ultimate killing of Listeria. The generalized disability to mount a protective antibacterial resistance clearly appears to be the sequel of an overwhelming MLM infection, rapidly resulting from the intravenous route of infection. Secondary deficiencies involving both humoral and cell-mediated immune responses to unrelated antigens in experimental murine leprosy have been reported earlier by other authors (Bullock, 1978; Ptak *et al.*, 1970; Turk & Bryceson, 1971). The evolution of these deficiencies appears to coincide with the appearance of suppressor T cells initially in the spleen and later in the peripheral lymph nodes (Bullock, Evans & Filomeno, 1977; Bullock, Carlson & Gershon, 1978).

Finally, the demonstration that mice infected with MLM generate immunity to the antigenically related R1Rv provides evidence that antimycobacterial immunity is indeed generated. The ability to effect adoptive transfer of this immunity to normal mice with lymphoid cells allows one to determine the immunological status of the host at any time of the MLM infection. It also should prove to be of immense value in analysing the mechanisms of antibacterial resistance in terms of the types of sensitized cells that mediate and regulate the immunity.

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