INFECTION AND IMMUNITY, Mar. 1984, p. 925–930
0019-9567/84/030925-06$02.00/0

Vol. 43, No. 3

Resistance to Subcutaneous Infection with *Mycobacterium lepraemurium* Is Controlled by More Than One Gene

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Received 7 July 1983/Accepted 16 November 1983

The resistance of C57BL (high) and BALB/c (low) mice, their F1 hybrids, and the offspring derived from backcrosses of the F1 to both parental strains was assessed at 20 weeks after subcutaneous infection with 10^7 *Mycobacterium lepraemurium* organisms. The numbers of bacilli recovered from the infected foot and draining lymph node indicated that resistance to subcutaneous infection is controlled by more than one non-*H-2*-linked gene of intermediate dominance. In general, female mice were more resistant than males.

Mycobacterium lepraemurium is an obligate intracellular parasite of cells of the mononuclear phagocyte system. Inbred strains of mice vary in their resistance to infection with this organism given by either the subcutaneous (s.c.) (12) or the intravenous (i.v.) (4, 13) route, although the strain distribution of high and low resistance is different from the two routes of infection. We have confined our studies to infection by the s.c. route and have shown that the main difference in resistance between C57BL (high) and BALB/c (low) mice is controlled by a gene or genes unlinked to the *H-2* complex (7). Single non-*H-2*-linked gene control of resistance to other intracellular parasites of macrophages has been reported (3, 5, 9, 11, 17). In several cases the gene appears to control the rate of organism multiplication inside macrophages early in infection (3, 9, 17, 20). However, other genes often modify the response (2, 15).

In this paper we report that the data on the resistance of F1 mice of BALB/c × C57BL origin and of the offspring derived from backcrosses of F1 mice to both parental strains are not consistent with control by a single gene.

MATERIALS AND METHODS

Mice. BALB/c and C57BL mice were bred in the Animal Unit of the Royal College of Surgeons. The latter strain was derived from a breeding nucleus supplied by the Animal Unit of the Imperial Cancer Research Fund, London. This strain has been used by several groups in London for studies on the resistance of mice to infection with *M. lepraemurium* (1, 4, 18). In our hands female C57BL mice were poor mothers, and in all crosses involving this strain C57BL mice were used as the male parent. F1 mice of BALB/c × C57BL origin were used for backcrossing to C57BL and BALB/c mice, and in the latter case reciprocal backcrosses were performed. Male and female mice were studied and they were aged 6 to 10 weeks at the time of infection.

Organisms. *M. lepraemurium* (Douglas strain) organisms were passaged (10^9 organisms i.v.) in outbred Parkes strain mice (experiment 1) or inbred CBA mice (experiment 2) at the National Institute for Medical Research, London. Organisms for infection were prepared by method 2 of Draper (8) from the livers of mice which had been infected for 3 to 5 months and were counted by the method of Hart and Rees (10) with modifications as given below.

Infection of mice. Mice were infected s.c. in the right hind footpad with 10^7 organisms in 0.025 ml of sterile saline.

Assessment of resistance. Infected tissues were homogenized in 0.1% bovine serum albumin in water; footpads, in 2 ml; and lymph nodes, in 1 ml. Previous experiments have shown that there are few organisms in the lymph nodes of C57BL mice at 20 weeks after infection with 10^7 organisms (6). Therefore, the lymph nodes in this strain were homogenized in pools of five or six lymph nodes in 1 ml. Smears were prepared from appropriate dilutions of tissue homogenates and stained with auramine and rhodamine. Acid-fast bacilli were counted by the slide method of Hart and Rees (10). Counts of less than 20 organisms per 40 oil immersion fields were considered too low for accurate assessment of organism numbers. This is equivalent to 4×10^5 ($\log_{10}$ 5.6) organisms per ml.

Experimental design. The mice were infected with 10^7 *M. lepraemurium* organisms given s.c. into the right hind footpad. They were killed at 20 weeks after infection, and resistance was assessed by counting the numbers of acid-fast bacilli recovered from the infected footpad and the draining popliteal lymph node. This criterion has been used to assess the differential resistance of mice of BALB and C57BL strains in our previous experiments on the genetic control of resistance to s.c. infection with *M. lepraemurium* (7). Studies on the time of death of mice infected with 10^7 organisms s.c. indicate that organism multiplication and dissemination as assessed by organism counts at the infection site and in the draining lymph node at 20 weeks correlate with time to death with disseminated infection in BALB/c and C57BL mice (time of death 61.0 ± 1.4 weeks [n = 60] in BALB/c mice and 80.6 ± 1.8 weeks [n = 52] in C57BL/6 mice). In the first experiment the resistance of F1 (BALB/c × C57BL), C57BL, BALB/c, and backcross (F1 × C57BL) mice were compared. In the second experiment F1 (BALB/c × C57BL), BALB/c, F1 × BALB/c, and BALB/c × F1 mice were studied. The reciprocal F1 (C57BL × BALB/c) cross was also studied in the second experiment, but only 10 mice were obtained from this cross using six pairs of parents compared with 40 offspring from the cross of six BALB/c females with six C57BL males.

Statistics. Organism numbers per footpad in parental and F1 mice were compared by Student's t test for nonpaired data (Table 1). Organism numbers per lymph node in these mice were compared by Wilcoxon's two-sample test for unpaired ranked observations because lymph node counts included values of <5.6 and so means could not be calculated (Table 2). Organism counts in both the lymph nodes and feet of backcross offspring were compared by Wilcoxon's test because these data are not expected to be normally distributed. The χ^2 test was used to compare observed and expected data in the backcross analysis.

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TABLE 1. Log_{10} of the number of organisms per footpad at 20 weeks in parental and F1 mice in experiments 1 and 2

Mice	Log_{10} no. of organisms per footpad (mean ± SE)		Male vs Female
	Male	Female	
Expt 1			
BALB/c		9.01 ± 0.13 (24)[a]	
		$P < 0.001$	
F1 (BALB/c × C57BL)	8.54 ± 0.11 (21)	8.20 ± 0.09 (21)	$P < 0.05$
	$P < 0.001$	$P < 0.001$	
C57BL	7.36 ± 0.09 (26)	7.39 ± 0.01 (20)	NS[b]
Expt 2			
BALB/c	9.96 ± 0.07 (20)	9.05 ± 0.16 (20)	$P < 0.001$
	$P < 0.001$	$P < 0.001$	
F1 (BALB/c × C57BL)	8.59 ± 0.15 (20)	7.87 ± 0.12 (20)	$P < 0.001$
	$P < 0.05$	NS	
F1 (C57BL × BALB/c)	7.95 ± 0.11 (6)	7.40 ± 0.09 (4)	$P < 0.02$

[a] Number in parentheses is number of mice tested.
[b] NS, Not significant.

RESULTS

Organism numbers in the feet and draining lymph nodes of parental and F1 mice at 20 weeks. Organism counts in the infected feet at 20 weeks indicated that the gene(s) controlling resistance is of intermediate dominance; the F1 (BALB/c × C57BL) mice had significantly more organisms per footpad than C57BL mice and significantly fewer organisms per footpad than BALB/c mice (Table 1). Organism counts in the lymph nodes showed that significantly fewer organisms were recovered from the lymph nodes of F1 mice than from those of BALB/c mice (Table 2). Organism numbers per lymph node were low in C57BL and F1 mice and there was a barely significant difference between these two categories (Table 2). In BALB/c and F1 mice, female mice were significantly more resistant than male mice, but there was no difference between the sexes in the resistance of C57BL mice (Tables 1 and 2). There was a significant difference ($P < 0.05$) between the number of organisms per footpad between male mice from the two reciprocal F1 crosses (Table 1), but only six males of C57BL × BALB/c origin were studied.

TABLE 2. Log_{10} of the number of organisms per draining lymph node at 20 weeks in parental and F1 mice in experiments 1 and 2

Mice	Median log_{10} no. of organisms per lymph node (range)		Male vs Female
	Male	Female	
Expt 1			
BALB/c		7.16 (6.15–8.03)	
		$P < 0.001$	
F1 (BALB/c × C57BL)	6.29 (5.6–6.82)	5.95 (5.6–6.74)	NS[a]
	$P < 0.05$	NS	
C57BL[b]	5.84 (5.74–6.11)	5.75 (5.44–5.96)	NS
Expt 2			
BALB/c	7.94 (5.71–8.73)	7.38 (6.44–8.39)	$P < 0.001$
	$P < 0.001$	$P < 0.001$	
F1 (BALB/c × C57BL)	6.44 (5.90–7.43)	5.70 (<5.6–6.32)	$P < 0.001$
	NS	NS	
F1 (C57BL × BALB/c)	6.17 (6.06–6.47)	<5.6 (<5.6–5.78)	$P < 0.02$

[a] NS, Not significant.
[b] Results refer to five pools of lymph nodes from C57BL males and four pools of lymph nodes from C57BL females.

Backcross analysis. Because of the higher resistance of female mice the results for the two sexes of backcross offspring have been analyzed separately. Since there was only a small difference in the numbers of organisms per lymph node between C57BL and F1 mice (Table 2), the organisms in the lymph nodes of the offspring of the F1 × C57BL backcross were not counted. The data on organisms per footpad from the two experiments (Fig. 1 and 2) or per lymph node (Fig. 3) do not show the pattern expected if control of resistance were by a single gene. Inconsistency with the single-gene hypothesis is proved by transforming the data to a histogram, e.g., organisms per footpad in the male offspring of F1 × C57BL origin (Fig. 4). On the single-gene hypothesis the expected number in each bar of the histogram is obtained by averaging the proportions in the corresponding categories of the F1 and the parental types and multiplying by the total number of backcross progeny (Table 3). A comparison of the observed and single-gene expectation for the backcross in Table 3 gave a χ^2 (5 df) of 14.104 ($P < 0.05$). A test was also carried out of the simplest possible multigene model, i.e., two additive genes of equal activity. The assumption was made that the phenotypic variance of those carrying only one of these resistance genes was equal to the mean of the F1 and parental variances. In the case shown in Fig. 4 the observed distribution was consistent with this particular multigene hypothesis. Of course, it would also be consistent with a range of other multigene hypotheses. Similar tests were applied to the other five data sets (footpad and lymph nodes, male and female). The expected distributions on the single-gene hypothesis were generally bimodal, but in the case of the female backcross offspring of F1 × C57BL origin, where the F1 and parental modes were very close, the backcross expectation was unimodal. In this case the observed data did not differ significantly from either the single-gene or the two equal additive gene model, but in the other four cases there were significant deviations ($P < 0.05$ to $P < 0.001$) between the observed distributions and either of these two possible models.

There were no significant differences between the reciprocal backcrosses of either sex in the numbers of organisms

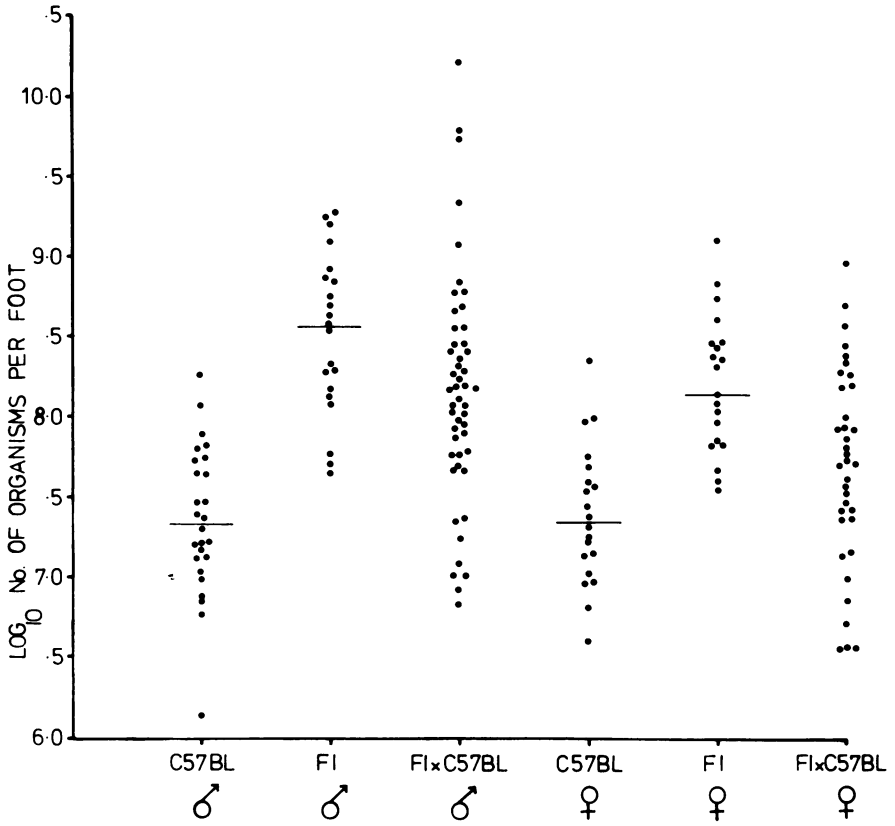

FIG. 1. Number of organisms recovered from the footpads of male and female C57BL, (BALB/c × C57BL) F1, and F1 × C57BL mice at 20 weeks after infection. Horizontal lines indicate the medians.

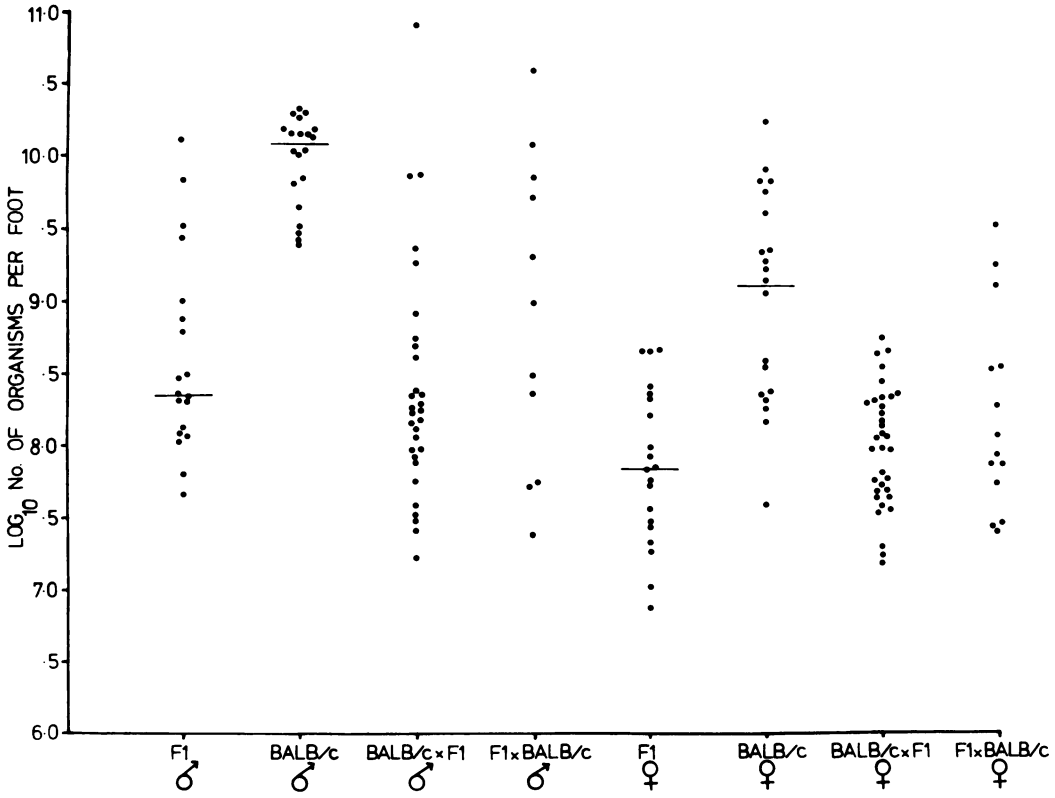

FIG. 2. Number of organisms recovered from the footpads of male and female (BALB/c × C57BL) F1, BALB/c, BALB/c × F1, and F1 × BALB/c mice at 20 weeks after infection. Horizontal lines indicate the medians.

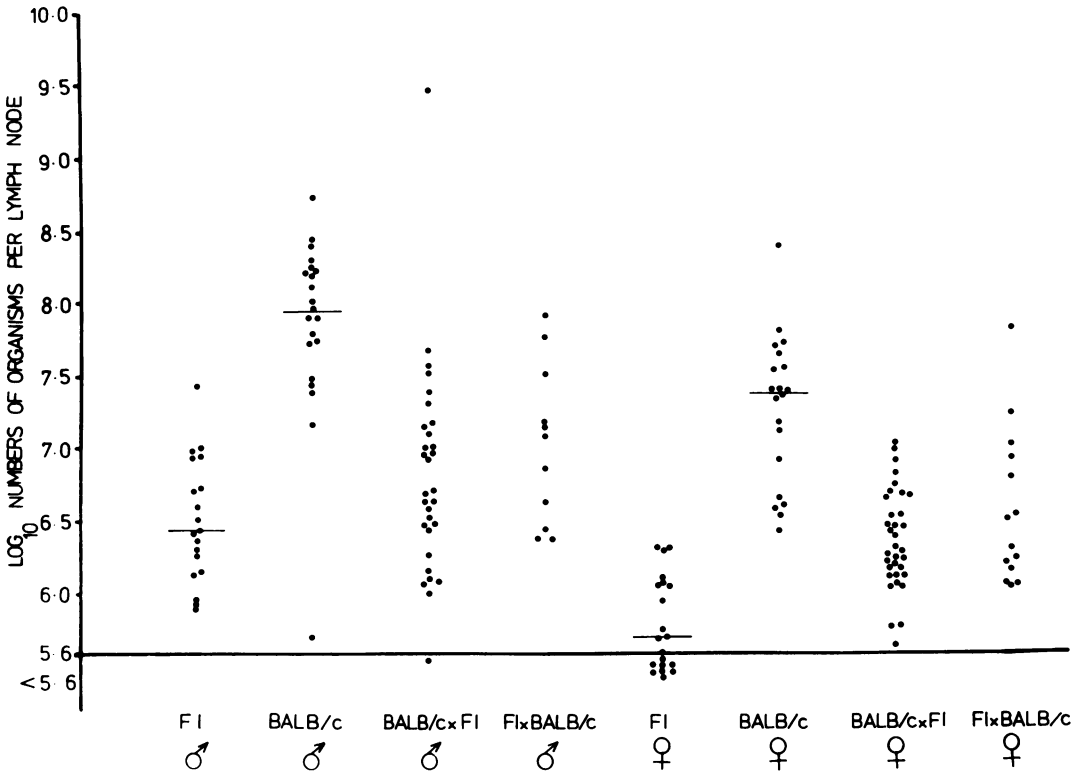

FIG. 3. Number of organisms recovered from the right popliteal lymph nodes of male and female (BALB/c × C57BL) F1, BALB/c, BALB/c × F1, and F1 × BALB/c mice at 20 weeks after infection. Horizontal lines indicate the medians.

per lymph node or per foot. In the first experiment female backcross offspring (F1 × C57BL) had fewer organisms per foot than male backcross offspring but in experiment 2 there were no differences between the sexes of either of the two reciprocal backcrosses in organism numbers per footpad. Female backcross offspring of BALB/c × F1 origin had fewer organisms per lymph node than males ($P < 0.01$), and female offspring of F1 × BALB/c origin had fewer organisms per lymph node than males ($P < 0.05$).

TABLE 3. Analysis of data from F1 × C57BL backcross males for control by a single gene

Log_{10} no. of organisms per footpad	C57BL	F1	F1 × C57BL		
			Observed	Expected	
<7.0	5[a]	0	2	$\frac{1}{2}\left(\frac{5}{26}\right) \times 49$	= 4.71
7.01–7.5	12	0	6	$\frac{1}{2}\left(\frac{12}{26}\right) \times 49$	= 11.31
7.51–8.0	7	3	11	$\frac{1}{2}\left(\frac{7}{26}+\frac{3}{21}\right) \times 49$	= 10.10
8.01–8.5	2	6	18	$\frac{1}{2}\left(\frac{2}{26}+\frac{6}{21}\right) \times 49$	= 8.88
8.51–9.0	0	8	7	$\frac{1}{2}\left(\frac{8}{21}\right) \times 49$	= 9.33
>9.0	0	4	5	$\frac{1}{2}\left(\frac{4}{21}\right) \times 49$	= 4.67
Total	26	21	49		49.0

[a] Number of mice.

DISCUSSION

When a single gene is involved in the control of a characteristic, the offspring obtained from a backcross would segregate in a 1:1 ratio of F1 and parental types. Control by more than one gene (in the absence of linkage and epistasis) would result in a binomial distribution of phenotypes among the offspring of the backcross, with a mode intermediate between those of the F1 and the parental strains. Other workers studying the genetic control of resistance or delayed hypersensitivity in experimental infections have analyzed their data by using a cutoff point, usually the 95% confidence limit of the distribution of the data of the parental strain. A 1:1 ratio on either side of this cutoff point has often been found and taken as proof of single-gene control. However, the cutoff point is often roughly half-way between the means of the parental and F1 distributions, and if the data from the backcross offspring are binomially distributed, 50% will be above and 50% will be below the cutoff point regardless of how many genes are involved in the control of the parameter being studied. In those cases where raw data showing the distribution of phenotypes among the backcross offspring have been published, the fallaciousness of the single-gene conclusion is sometimes obvious (14, 21).

The results of the experiments reported here indicate that the resistance of mice to s.c. infection with *M. lepraemurium*

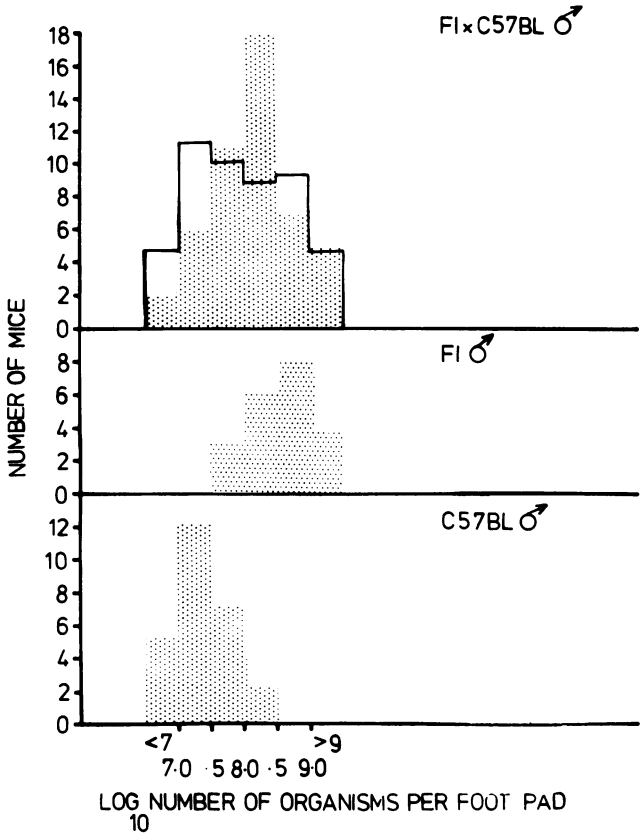

FIG. 4. Frequency of organism numbers per footpad of male C57BL, (BALB/c × C57BL) F1, and F1 × C57BL mice at 20 weeks after infection. Solid outline indicates frequency distribution expected if trait is controlled by a single gene.

is controlled by more than one gene. Our analysis of the data cannot establish the exact mode of inheritance of this resistance and further breeding tests could perhaps elucidate this. The F1 of BALB/c × C57BL origin has intermediate resistance, indicating intermediate dominance of the genes involved in control at the dose of organisms used for infection (10^7 organisms per mouse). It was also found that, in general, female mice were more resistant to infection than male mice. The phenomenon of sex limitation of the immune response to infection has been found by other groups (22, 23).

A study of the resistance of *H-2* congenic mice of C57BL/10 and BALB background to s.c. infection with *M. lepraemurium* has indicated that resistance is controlled mainly by a non-*H-2*-linked gene(s) but that resistance is modified by a gene(s) in the *H-2* complex (7). Since C57BL and BALB/c mice have different *H-2* haplotypes ($H\text{-}2^b$ and $H\text{-}2^d$, respectively), both sets of genes would be segregating in the offspring of crosses derived from these strains. However, we found that significant differences in organism numbers in the footpads of $H\text{-}2^b$ and $H\text{-}2^d$ mice on either background and in the lymph nodes on the C57BL background were not detectable at 20 weeks after infection (7). Thus, it is possible that the *H-2*-linked gene(s) may have little influence on the differences in organism numbers in the feet or lymph nodes found in the current experiments and that we are measuring differences controlled by non-*H-2*-linked genes.

A study of the resistance of different inbred strains to i.v. infection with *M. lepraemurium* has shown that the classification of high- and low-resistance strains is the same as that for resistance to *Leishmania donovani*, *Salmonella typhimurium*, and *M. bovis* BCG (4), which appear to be controlled by the same gene on chromosome 1 (16, 19). This gene can be playing no role in the differences in resistance to s.c. infection with *M. lepraemurium* since BALB/c and C57BL mice are both of the low-resistance genotype (3, 17). After i.v. infection with *M. lepraemurium*, significant differences in time of death were found between strains within both the high- and low-resistance groups (4), indicating that other genes are also modifying resistance to infection by this route. It cannot be ruled out that these genes may also influence the resistance to s.c. infection since C57BL mice were significantly more resistant than BALB/c mice after i.v. infection when resistance was assessed by both time of death and the doubling time of organisms in the bone marrow (4).

ACKNOWLEDGMENTS

We thank Hannah Adu, Diana Griffiths, and P. Pappasavva for their assistance and C. F. Curtis for discussions of the method of genetic analysis.

This work was supported by grants from LEPRA, the British Leprosy Relief Association.

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