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Genetic Control of Resistance to *Mycoplasma pulmonis* Infection in Mice

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The differences in susceptibility of various inbred strains of mice to a highly pathogenic strain of *Mycoplasma pulmonis* CT (T2) has been known for some time. We assessed the genetic control of resistance to T2 infection. Tracheolung lavage samples and lungs of mice were assessed for T2 organisms after intratracheal injection of T2. We found that *H-2*^{b} C57BL/6 (B6) and *H-2*^{k} B10.BR mice were resistant, whereas *H-2*^{b} A.By, *H-2*^{k} C3H/Bi, *H-2*^{k} C3H/HeJ (C3H), and *H-2*^{b} BALB.B mice were susceptible. We also typed individual B6C3F2 mice for *H-2* and for resistance to T2 and observed that resistance to T2 infections is controlled by a single dominant gene not linked to *H-2*. Histologic examination revealed severe lung lesions typical of *M. pulmonis* infections in susceptible C3H mice, in contrast to minimal lung lesions in resistant B6 mice. No significant titers of local or systemic antimycoplasma antibodies were detected in either resistant or susceptible mice at 5 days postinfection. Macrophages taken from uninfected B6 or C3H mice failed to inhibit growth of T2 in vitro. However, macrophages from B6 mice did inhibit growth of T2 much better than C3H macrophages when harvested on day 5 of infection. Thus, there is an association between activation of macrophage bactericidal function and genetic resistance to growth of T2 organisms.

Murine respiratory mycoplasmosis caused by *Mycoplasma pulmonis* is one of the most common and important naturally occurring infectious diseases in rodent colonies (4). *M. pulmonis* can cause high morbidity but usually causes a subclinical infection. This organism may cause significant alteration in normal physiology and immunological responses (15, 17), significantly complicating interpretation of experimental data.

It has been reported (19, 25) that mice of various strains differ in susceptibility to infections caused by *M. pulmonis*. ICR mice were more susceptible to the growth of *M. pulmonis* organisms and to the development of gross pneumonic lesions than NIH, CF1, and ddY mice. Subsequently, one group (8) reported that mice of various *H-2* haplotypes, e.g., C57BL/6 (B6) (*H-2*^{b}) and C3H/HeN (*H-2*^{k}) mice, did not differ significantly in the incidence of infection in either the upper or lower respiratory tract or in the severity of upper respiratory tract lesions at 14 days postinfection (p.i.). In striking contrast, B6 mice are significantly more resistant to the development of gross and microscopic lung lesions and death due to pneumonia. There were highly significant differences in the recovery of organisms from the nasal passages, tracheas, and lungs between B6 and C3H/HEJ (C3H) mice after intranasal infection with 5×10^3 CFU. We have infected B6 and C3H mice with 10^7 CFU intratracheally (i.t.). The C3H mice survived 45 ± 5 days, whereas the B6 mice survived indefinitely (unpublished data). Thus, B6 mice are more resistant than C3H mice to the lethal effects of *M. pulmonis* infection.

In this report, we describe studies aimed at assessing genetic resistance to *M. pulmonis* infection in mice by measuring organism growth in the lungs several days after infection. Mice of various strains and different *H-2* types were compared. F_2 progeny of *H-2*^{k} C3H and *H-2*^{b} B6 mice were typed for *H-2* and for resistance to *M. pulmonis* T2. Macrophages from uninfected and infected C3H and B6 mice were tested for the ability to inhibit the replication of strain T2 in vitro. The data presented indicate that an autosomal dominant gene (*MP*^{r}), not linked to *H-2*, confers resistance to early stages of infection before the development of significant humoral or T-cell-mediated responses to *M. pulmonis* organisms. Macrophages from uninfected C3H and B6 mice failed to inhibit the growth of T2. In contrast, macrophages taken from mice on day 5 after infection demonstrated anti-T2 activity, and B6 macrophages were superior to C3H macrophages in inhibiting growth.

MATERIALS AND METHODS

Animals. Specific-pathogen-free age- and sex-matched, 6- to 10-week-old B6, C3H, (B6 × C3H)F_1 (B6C3F_1), and B6C3F_2 mice were purchased from specific-pathogen-free stock bred at the Jackson Laboratories (Bar Harbor, Maine). The mice were housed in groups of five in polycarbonate cages with filter topes in our specific-pathogen-free facility and were allowed free access to rodent diet and water. The inbred strains A.By, BALB.B, C3H/Bi, and B10.BR were bred and maintained in a colony at this university. The mice were stabilized for 1 week after their arrival to preclude any possible effects of stress due to transportation and change in environment on the immune system (1). The mice were free of serum antibodies to various murine viruses (Sendai virus, reovirus 3, Theiler's GD VII [murine encephalomyelitis virus], lymphocytic choriomeningitis virus, and mouse hepatitis virus), free of *M. pulmonis* as determined by oropharyngeal cultures, and free of *M. pulmonis* and *Mycoplasma arthritidis* immunoglobulin G (IgG) antibodies, as determined by an enzyme-linked immunosorbent assay (ELISA) (6).

Inoculations. Mice were inoculated i.t. with 10^5 CFU of *M. pulmonis*; the procedure has been described previously (15). We used the T2 strain, which has been described before (13). Twenty mice (10 female and 10 male) of the B6 and C3H

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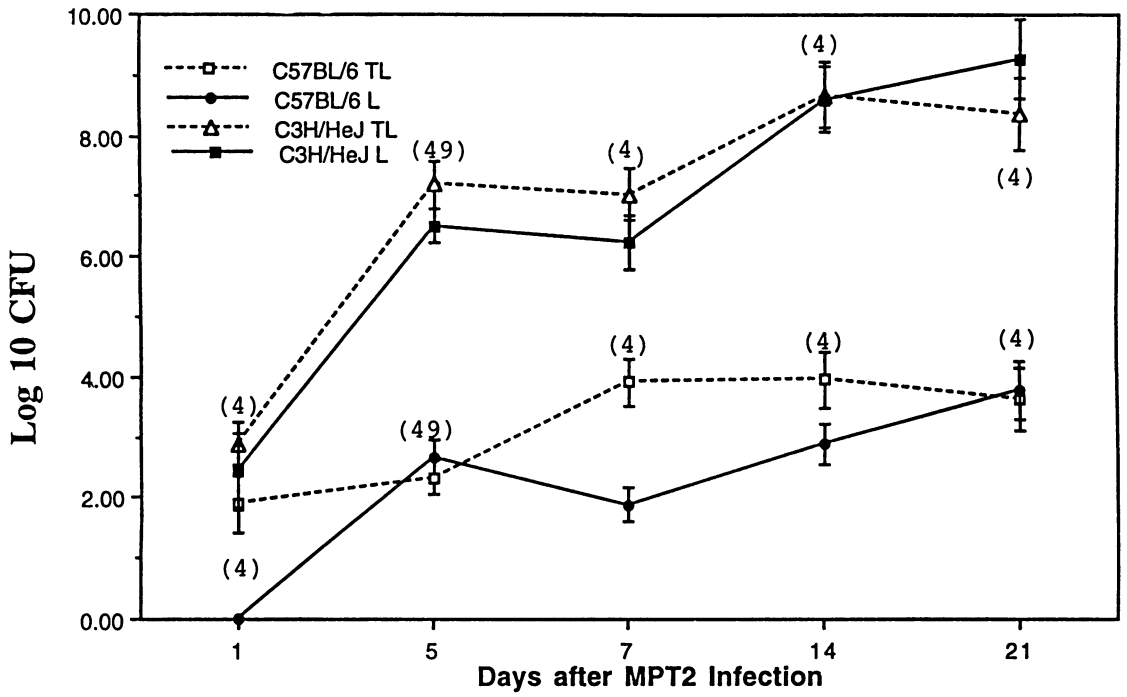

FIG. 1. Kinetics of *M. pulmonis* replication in B6 and C3H mice (4 or 49 mice per point, mean ± SEM). Statistically significant differences in the number of organisms recovered from tracheolung lavage samples (TL) and lung suspensions (L) on days 5, 7, 14, and 21 after infection were observed between these two strains of mice.

strains were anesthetized with ether, and a 30-gauge needle was used to inoculate 10^5 CFU of T2 directly into the trachea. Four mice of each group were killed on days 1, 5, 7, 14, and 21. Quantitative mycoplasma cultures of tracheolung lavage samples and lungs from experimental animals were performed on Chalquest's agar plates.

Sample collection. Tracheolung lavage samples were collected in 0.3 ml of Chalquest's broth containing ammonium reineckate, as described before (16). Lung suspensions were made after the lungs were aseptically removed and homogenized in 1 ml of Chalquest's broth plus ammonium reineckate in a tissue grinder (Fisher, Pittsburgh, Pa.).

Propagation, isolation, and titration of organisms. Chalquest's agar medium and broth (16, 22) containing ammonium reineckate (100 μg/ml) was used to propagate and isolate *M. pulmonis*. Specimens from tracheolung lavage samples and lung suspensions were inoculated in the agar medium and incubated in a humid atmosphere at 37°C for 7 to 10 days. A 0.1-ml volume of each sample was inoculated onto two agar plates, and triplicates of 10 and 1 μl of each 10-fold dilution of the sample were inoculated onto a Chalquest's agar medium plate (15 by 100 mm), which can accommodate three to four serial dilutions. After incubation at 37°C for 7 to 10 days, the number of colonies was counted and expressed as the mean ± standard error of the mean (SEM) CFU per milliliter.

Flow cytometry for *H-2* typing. Cells taken from the lymph nodes or spleen of each $B6C3F_2$ mouse were washed three times in Hanks' balanced salt solution with 1% fetal calf serum (HBSS–1% FCS). The cells were preincubated with rat anti-mouse FcR antibody 2.4G2 to block nonspecific binding of labeled antibodies to FcR in all samples. Aliquots of 10^6 cells were added to Eppendorf microcentrifuge tubes and pelleted at 5,000 rpm for 1 min. The pelleted cells were resuspended in 0.05 ml of primary monoclonal antibody (MAb) 28-8-6 (anti-H-$2D^b$) or 11-4.1 (anti-H-$2K^k$) (Pharmingen, San Diego, Calif.). The MAbs were optimally diluted in HBSS–1% FCS as determined by previous titration. Negative control samples were resuspended with HBSS–1% FCS only. The samples were incubated for 20 to 40 min at 4°C. After the incubation, the samples were washed with HBSS–1% FCS and pelleted again. Fluorescein isothiocyanate-labeled goat anti-mouse IgG secondary antibodies were added, and the cells were washed after incubation. After the final wash, the samples were analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif.). Percent specific staining is calculated as percent staining in the positive sample minus percent staining in the negative control in the fluorescence channels above a threshold chosen at the inflection point of the negative control.

Macrophage inhibition of organism growth. Groups of five uninfected and infected (day 5) mice of both strains were killed. The peritoneal cavities of the mice were washed with 5 ml of 6 mM EDTA in HBSS. The alveolar macrophages were collected by injecting 1 ml of 6 mM EDTA–phosphate-buffered saline into the trachea and withdrawing fluid, repeating this procedure four times. The peritoneal and pulmonary cells were pelleted by centrifugation and counted. Cells were resuspended in RPMI 1640–10% FCS and plated at 10^6/ml in a 24-well plate. Each well received 1 ml of cell suspension and was incubated at 37°C for 1 h. The nonadherent cells were removed, and the adherent cells were incubated with 1 ml of RPMI 1640–10% FCS and 1 ml of Chalquest's broth containing 10^5 CFU of strain T2 in a humidified atmosphere containing 5% CO_2. The number of

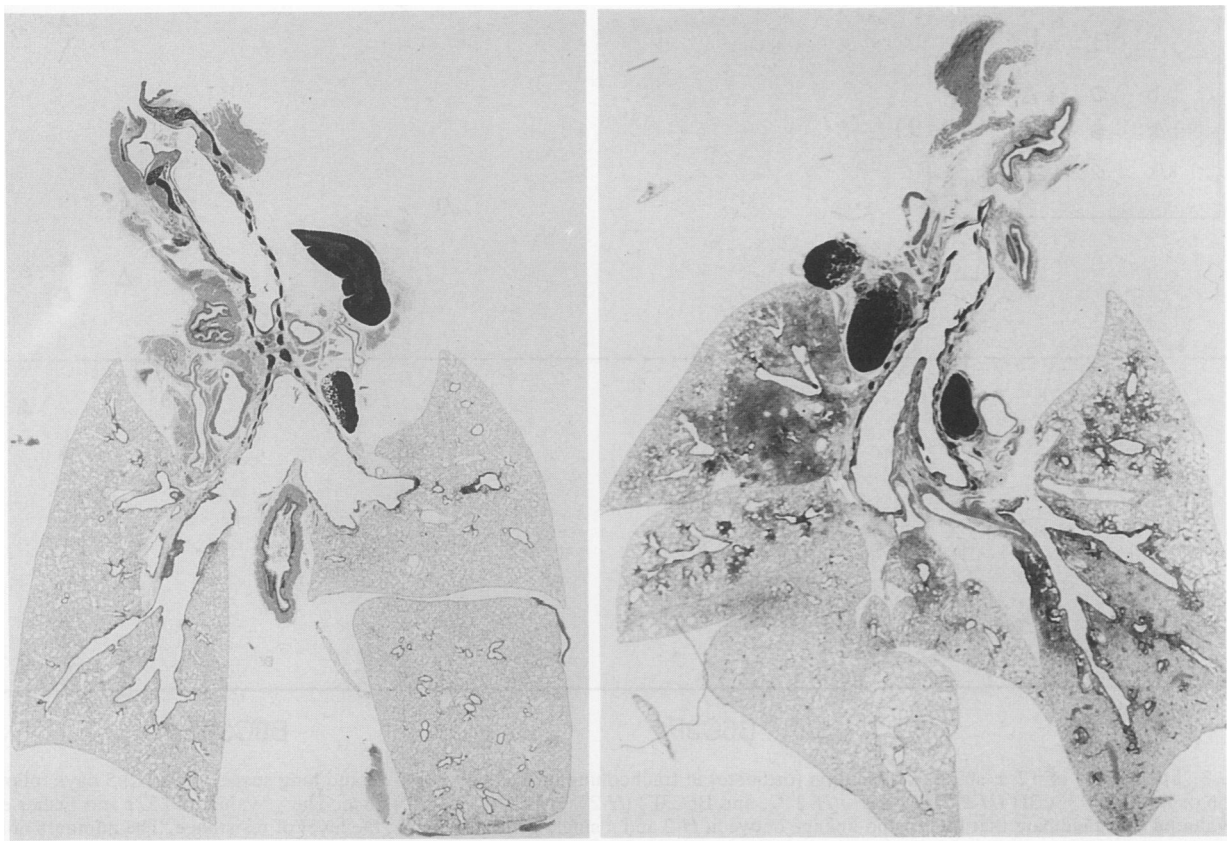

FIG. 2. Lung sections from representative mice taken 5 days after infection with 10^5 CFU of strain T2. Moderate to severe peribronchial and perivascular infiltration by lymphoid cells was observed in C3H lungs (right), whereas minimal histopathological changes were found in B6 lungs (left). Magnification, ×10.

M. pulmonis organisms present in the supernatant was determined onto Chalquest's agar plates at 3, 24, and 48 h.

Histopathologic studies. Whole lungs and tracheas from both uninfected and infected (5 days earlier) B6 and C3H mice were inflated with 10% neutral buffered Formalin and embedded in paraffin. Multiple longitudinal sections 5 μm thick were cut through the trachea and lungs and stained with hematoxylin and eosin for examination of microscopic lesions. To determine the severity of pneumonia, histologic sections were identified by code and scored from + to +++ according to the severity of the lesion (11). This score represents the individual animal lesion index. For comparison of group scores, the sum of scores for the organ from individual animals was divided by the sum of maximal scores possible given the lesion index for the organ. A lesion index of 1.0 is the most severe change possible for an experimental group.

Immune responses. Serum was collected from each mouse on days 5 and 21 p.i. and analyzed for anti-*M. pulmonis* IgM and IgG antibodies by ELISA. Tracheolung lavage samples were analyzed for anti-*M. pulmonis* IgG and IgA antibodies by a modified ELISA (10). The lymphocyte transformation test for measurement of cell-mediated immunity was conducted as described before (15). Values are expressed as stimulation indices, calculated as [^{3}H]thymidine incorporation (cpm) by spleen cells with *M. pulmonis* antigen divided by incorporation by spleen cells without *M. pulmonis* antigen.

Statistical analysis. Parameter and nonparameter analyses for CFU in lavage samples and lung preparations and histopathology scores were performed as described before (11, 13). Chi square analysis was used to determine whether the gene(s) for resistance to *M. pulmonis* infection is linked to *H-2*. A value of $P < 0.05$ was considered significant.

RESULTS

Kinetics of replication in vivo. B6 and C3H mice were chosen for these studies because our mortality tests (unpub-

TABLE 1. Recovery of *M. pulmonis* organisms from tracheolung lavage samples and lung suspensions from six inbred strains of mice[a]

Strain	No. of mice	Mean $\log_{10}$ CFU/sample ± SEM	
		Lavage	Lung
A.By (*H-2*^{b})	5	7.22 ± 0.26	7.35 ± 0.42
BALB.B (*H-2*^{b})	5	6.32 ± 0.30	6.77 ± 0.45
B6 (*H-2*^{b})	6	2.74 ± 0.80[b]	3.47 ± 0.18[b]
C3H/Bi (*H-2*^{k})	5	6.75 ± 0.53	7.00 ± 0.52
B10.BR (*H-2*^{k})	6	3.34 ± 0.97[b]	1.95 ± 1.12[b]
C3H/HeJ (*H-2*^{k})	6	6.53 ± 0.66	6.47 ± 0.68

[a] Mice were inoculated i.t. with 10^5 CFU of strain T2 5 days before the assay.

[b] Mean values significantly ($P < 0.01$) different from values for A.By, BALB.B, C3H/Bi, or C3H/HeJ mice.

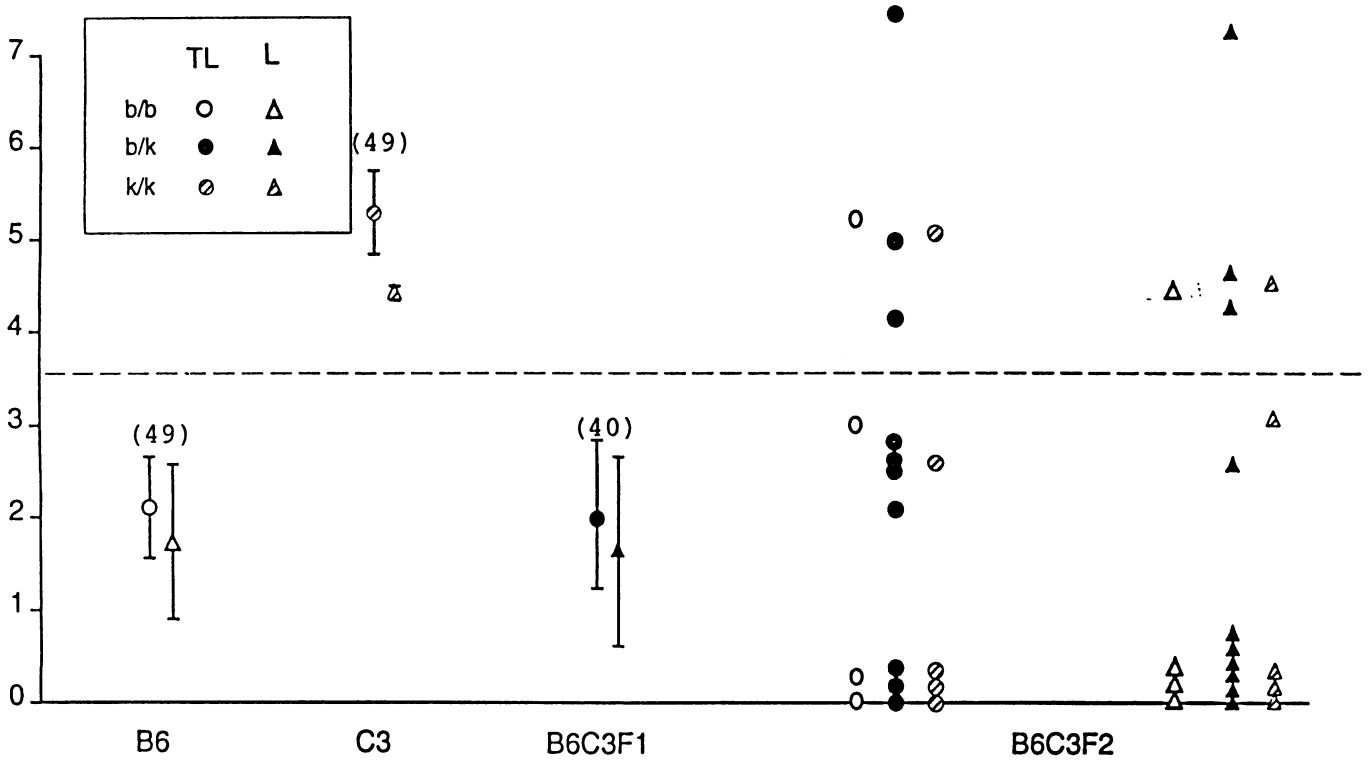

FIG. 3. Mean CFU of T2 ± standard deviation (ordinate) in tracheolung lavage samples (TL) and lung suspensions (L) 5 days following infection of B6 ($H\text{-}2^{b/b}$), C3H ($H\text{-}2^{k/k}$), B6C3F$_1$ ($H\text{-}2^{b/k}$), and B6C3F$_2$ ($H\text{-}2^{b/b}$, $H\text{-}2^{b/k}$, or $H\text{-}2^{k/k}$) mice. The χ^2 value of 0.527 and Fisher exact P test value of 0.482 indicate that there is no linkage between *H-2* and a single gene controlling the level of resistance. The numbers of mice tested are in parentheses.

lished data) and those reported before (8, 23) show that B6 mice are more resistant than C3H mice to the development of gross and microscopic lung lesions and death due to pneumonia after *M. pulmonis* infection. Statistically significant differences in CFU between the two mouse strains were first observed on day 5 after *M. pulmonis* infection. There were also statistically significant differences in the number of organisms harvested from the tracheolung lavage samples and lung suspensions on days 7, 14, and 21 after infection (Fig. 1). The sex of the mice had no significant effect on the number of organisms recovered from either mouse strain. The data suggest that the genetic control of *M. pulmonis* growth is expressed at early stages of infection.

Resistant B6 mice had 2.10 and 1.81 $\log_{10}$ CFU/ml in lavage and lung samples, respectively. In subsequent studies, strains with $\leq$3.46 and $\leq$3.49 $\log_{10}$ CFU/ml in lavage and lung samples, respectively (two standard deviations above the mean for the resistant B6 strain), were considered resistant.

Histopathological changes. The *M. pulmonis*-induced pneumonic lesions in the B6 mice were quite different from those in the C3H mice (Fig. 2). Minimal lung lesions were observed in infected B6 mice. However, a severe typical peribronchial and perivascular lymphocytic cuffing, a pathognomonic lesion of murine respiratory mycoplasmosis, was observed in the lungs of C3H mice only 5 days after infection. Atelectasis and consolidation of the parenchyma were most severe adjacent to airways in C3H mice. Lung lesion ratios were compared between the two strains (n = 10) which had received equal doses of *M. pulmonis* (10^5 CFU per animal). The mean lung lesion score was 0.25 ± 0.01 for B6 and 0.75 ± 0.20 for C3H mice ($P < 0.05$).

Recovery of strain T2 from tracheolung lavage samples and lung suspensions of various strains of mice. Mice of six different inbred strains were inoculated i.t. with 10^5 CFU of T2, and the number of organisms recovered from lavage and lung samples was estimated on day 5 p.i. (Table 1). Two sharply distinct patterns of response emerged. Mice of the BALB.B, C3H/Bi, A.By, and C3H/HeJ strains appeared to be susceptible, whereas B6 and B10.BR mice were resistant. As many as 10^3- to 10^4-fold more organisms were recovered from T2 from susceptible hosts as from resistant hosts. The data also indicate that there is no linkage between *H-2* haplotype and resistance or susceptibility to *M. pulmonis*, as assessed by the number of organisms recovered at 5 days p.i.

Single, dominant gene appears to mediate resistance in mice. We performed a Mendelian segregation analysis of susceptibility (MP^s) and resistance (MP^r) to *M. pulmonis* infection 5 days after i.t. inoculation, using B6C3F$_2$ mice. Groups of 20 B6, C3H, B6C3F$_1$, and B6C3F$_2$ mice were infected with 10^5 CFU of T2. Forty mice from each group were analyzed from two experiments (Fig. 3). B6, B6C3F1, and 29 of 40 B6C3F$_2$ mice were resistant, whereas C3H and 11 of 40 C3B6F$_2$ mice were susceptible ($P < 0.001$). The 3:1 ratio of resistant to susceptible (MP^r/MP^s) mice among the F$_2$ progeny indicates that a "major" gene responsible for resistance is segregating and that the resistance phenotype is determined by its dominant allele (MP^r).

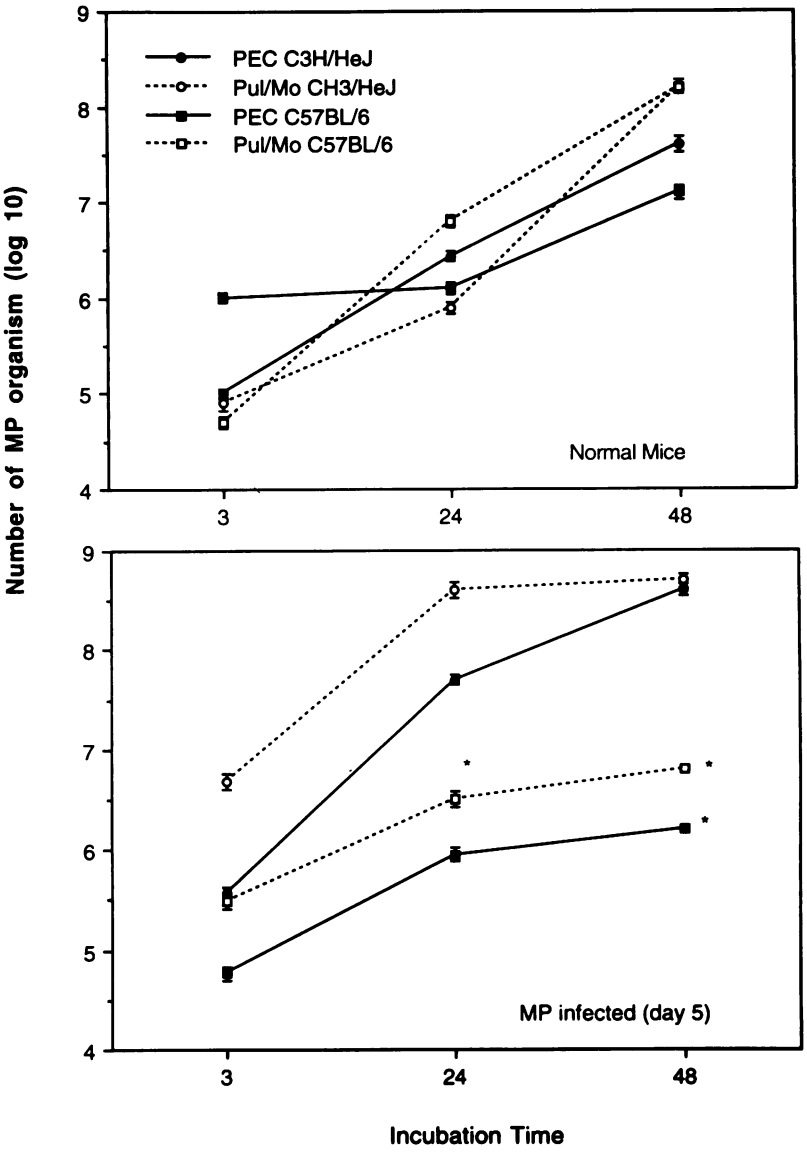

FIG. 4. *M. pulmonis* (MP) CFU in supernatants of cultured pulmonary macrophages (Pul/Mo) and peritoneal exudate cells (PEC). Cells were obtained from uninfected and infected (5 days p.i.) C3H and B6 mice. * Fewer organisms ($P < 0.05$) in B6 cells.

***H-2* type does not correlate with susceptibility.** To determine the *H-2* genotype of individual B6C3F_2 mice in an attempt to evaluate linkage between *H-2* and resistance to *M. pulmonis*, we serotyped 20 F_2 mice (Fig. 3). The ratio of the *H-2* genotypes *b/b*, *b/k*, and *k/k* was 4:10:5, close to the expected 1:2:1 ratio. Since four of the five *k/k* mice were resistant and mice of all three *H-2* genotypes had susceptible and resistant individuals, no linkage between the locus under study and *H-2* was observed. The chi square value of 0.527 and the Fisher exact test value of 0.482 confirmed this conclusion.

Inhibition of growth by macrophages in vitro. The number of organisms recovered from the supernatants of cultures of normal peritoneal or pulmonary B6 and C3H macrophages at 3 and 24 h was equal (Fig. 4). At 48 h, there were fewer organisms in cultures of B6 peritoneal macrophages, but this difference was not significant. In contrast, the numbers of organisms recovered from peritoneal or pulmonary macrophages from B6 mice was less (10 to 100 times) than the number recovered from macrophages from C3H mice at 5 days after infection. Significant differences were obtained when B6 and C3H macrophages were compared at 24 and 48 h of culture.

Immune responses. The presence of specific antibodies in tracheolung lavage samples and serum obtained on days 5 and 21 p.i. were assessed by ELISA with *M. pulmonis* lysate as the antigen. All samples from day 5 p.i. were negative for IgG and IgM (serum samples) and IgG and IgA (lavage samples). However, the levels of antibodies were higher in C3H than in B6 mice at day 21 p.i. (Table 2). Splenocyte proliferative responses to the presence of *M. pulmonis* antigens were weak in both strains at day 5 but strong in both strains at day 21. No significant differences between mouse strains were observed.

TABLE 2. Immune responses to *M. pulmonis*[a]

Strain	Day p.i.	Mean ELISA titers ± SEM				SI[b]
		Serum samples		Tracheolung lavage samples		
		IgG	IgM	IgG	IgA	
C3H	5	0	0	0	0	1.1 ± 0.5
	21	3,000 ± 260[c]	800 ± 64[c]	350 ± 35[c]	245 ± 33[c]	9.4 ± 2.7
B6	5	0	0	0	0	1.5 ± 0.3
	21	2,000 ± 316	500 ± 45	150 ± 33	145 ± 17	17.5 ± 2.3

[a] See Table 1, footnote *a*. Assays were done on days 5 and 21 p.i.
[b] SI, stimulation index. Mean values not significantly different between C3H and B6 mice on day 5 or 21.
[c] Mean values significantly ($P < 0.05$) different from those for B6 mice.

DISCUSSION

The data presented confirm and extend earlier findings (8, 19, 25) indicating mouse strain differences in susceptibility to infection with *M. pulmonis*. The data in Fig. 1 and 2 indicate that B6 mice better resist replication of *M. pulmonis* in the lung and better resist the histopathological effects of *M. pulmonis* infection than C3H mice. The data in Table 1 indicate that mice of the narrow ancestral C57BL background, i.e., B6 and B10.BR, are more resistant to growth of *M. pulmonis* in the lung than A.BY, C3H/Bi, C3H/HeJ, or BALB.B mice. The data in Table 1 suggest that *H-2* is unlikely to be a major genetic factor (3). The data in Fig. 3 indicate that a single, dominant gene, not linked to *H-2*, confers resistance to *M. pulmonis* growth in the lungs. A single resistance gene on chromosome 1 for resistance to *Mycobacterium bovis* BCG infection has been cloned and sequenced (31). This gene, which is probably identical to the gene for resistance to *Salmonella typhimurium* and *Leishmania donovani* (5, 24, 26, 27), primarily affects macrophage functions (20, 21).

We are using recombinant inbred mice to map the MP^r gene. Preliminary findings suggest that chromosome 1 is not involved and that chromosome 4 may contain the gene. The data in Fig. 4 suggest that the MP^r gene function is not manifested in macrophages of uninfected mice, presumably "resting macrophages." This result suggests that the MP^r gene differs in function from the *Ity*, *Lsh*, and *Bcg* genes. However, macrophages obtained 5 days after infection did manifest genetic differences in the capacity to control the replication of *M. pulmonis* organisms in vitro (Fig. 4). How might this be explained? The differences in macrophage function 5 days after infection could reflect (i) the relative production of cytokines, e.g., gamma interferon, by T or natural killer cells or (ii) the ability of macrophages to respond to equal cytokine secretions. Experiments to distinguish between these possibilities are under way.

The lack of humoral antibody and T-cell proliferative responses to *M. pulmonis* on day 5 (Table 2) indicate that the MP^r gene acts on lymphoid cells early in infection, before the appearance of significant acquired immunity to *M. pulmonis* (29, 30). Whereas genetic resistance to various organisms appears to be a property expressed by resting macrophages (2, 7, 9, 18, 28), we failed to observe such resistance in lung or peritoneal macrophages taken from uninfected donors (Fig. 4). We have observed that gamma interferon and natural killer cells are involved in resistance to *M. pulmonis* infection (12, 14), but it remains to be determined whether C3H and B6 mice differ in the ability to stimulate macrophages or in the ability of macrophages to respond to stimulation during *M. pulmonis* infection.

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