# *Legionella pneumophila* Growth in Macrophages from Susceptible Mice is Genetically Controlled (43207)

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*Abstract.* **Growth of the intracellular opportunistic bacterium *Legionella pneumophila* in macrophages from A/J mice is as vigorous as growth in macrophages from susceptible guinea pigs and human monocytes, whereas growth is inhibited in macrophages from other mouse strains, such as nonpermissive BALB/c mice. Permissiveness versus nonpermissiveness of macrophages from A/J versus BALB/c mice appeared to be controlled by a genetic mechanism dependent upon a single gene or a closely clustered family of genes. Susceptibility versus resistance of macrophages from $F_1$ offspring of these two strains of mice and macrophages from backcrossed mice prepared from $F_1$ hybrids and the original parental strain showed a segregation of permissiveness for growth of Legionella *in vitro*, consistent with genetic control.** [P.S.E.B.M. 1991, Vol 196]

*Legionella pneumophila* is the etiologic agent of pulmonary as well as systemic infections in humans, especially those showing a defect of the immune system (1). This organism is an intracellular opportunistic facultative pathogen that replicates readily in mononuclear phagocytic cells of humans and in macrophages from guinea pigs, which are extremely susceptible to this bacterium and show the characteristic clinical and pathologic features of Legionnaire's disease (2, 3). Studies in a number of laboratories have indicated that mice normally do not support the growth of these organisms and are highly resistant to infection in terms of death (4, 5). However, previous studies in this laboratory showed that A/J mice are much more susceptible to those bacteria than mice of other strains, such as DBA/2, C3H/HeN, C57BL/6, and BALB/c, and that macrophages from A/J mice support the growth of Legionella *in vitro* (6). The permissive nature of A/J mouse macrophages was investigated in the present study to examine whether there is a genetic control for such susceptibility. Growth of Legionella in peritoneal macrophages from this strain was compared with growth in hybrids prepared from susceptible A/J and BALB/c mice that are resistant to Legionella, as well as backcrossed mice from the hybrids and the susceptible (A/J) versus resistant (BALB/c) mice. The results obtained indicate that the trait of permissiveness of mouse macrophages for Legionella growth is controlled by a single gene.

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## Materials and Methods

**Animals.** Inbred A/J and BALB/c mice were used for these studies. They were purchased from The Jackson Laboratory, Bar Harbor, ME. In addition, $F_1$ hybrid mice (ACF1) from mating A/J and BALB/c mice, and $F_2$ hybrids (ACF2), prepared by mating (ACF1 × ACF1) and backcrossed hybrids, (ACF1 × A/J) and (ACF1 × BALB/c), were bred in the animal facilities of this institution. They were 10–14 weeks of age at the time of each experiment.

**Bacteria.** A virulent strain of *L. pneumophila*, Serogroup 1, was obtained at autopsy from a case of fatal legionellosis at Tampa General Hospital and cultured on buffered charcoal yeast extract agar, exactly as described previously (7). The bacteria were grown 2 days on agar plates and harvested into pyrogen-free saline and adjusted to appropriate concentration in tissue culture medium with a spectrophotometer at 620 nm.

**Macrophage Cultures.** Elicited peritoneal macrophages were obtained from individual mice 3–4 days after intraperitoneal injection of thioglycollate medium

exactly as described previously (4). Elicited macrophages were collected with 4.0 ml of RPMI 1640 medium (Gibco Laboratories, Madison, WI) supplemented with 5 units of heparin/ml. The cells were counted by a hemocytometer and allowed to adhere to 24-well tissue culture plates (Mark II; Costar, Cambridge, MA) for 2 hr in an atmosphere of 5% $CO_2$ and 95% air at 37°C. The resulting cell monolayers were washed with Hanks' balanced salt solution (Gibco Laboratories) and used for the experiments.

**Bacterial Growth in Macrophage Cultures.** The fate of Legionella in A/J or BALB/c mice macrophage cultures was determined by the following procedures: the macrophage monolayers (approximately $1 \times 10^6$ cells/well) were infected with $2 \times 10^6$ Legionella for 30 min at 37°C in 5% $CO_2$, washed to remove nonphagocytized bacteria, supplied with RPMI 1640 medium containing 15% heat-inactivated fetal calf serum (Hyclone Laboratories, Logan, UT), and then cultured. The intracellular growth of the bacteria was determined by standard plate count on buffered charcoal yeast extract agar using lysates of the macrophages prepared with sterile distilled water (4). Growth of the Legionella in individual hybrid mouse macrophage cultures was also determined by injecting 0.5 ml of a bacterial suspension containing $2.5 \times 10^6$ Legionella into mice 4 days after injection with 3.0 ml of 3% thioglycollate. After 30 min, the mice were killed and the peritoneal-exudate cells were collected from individual mice in 4.0 ml of RPMI 1640 medium containing 5 units of heparin/ml. The macrophages were then allowed to adhere to 24-well tissue culture plates for 90 min at 37°C, washed to remove nonadherent cells and nonphagocytized bacteria, and incubated with 1.0 ml of medium (15% fetal calf serum-RPMI 1640). The number of viable bacteria in the macrophage lysates was determined 0, 24, and 48 hr after incubation.

**Statistical Analysis.** Comparisons between experimental groups were performed according to Student's *t* test and were considered significant at the 95% confidence limit.

## Results

As can be seen from Table I, the Legionella replicated vigorously in the macrophages from A/J mice as compared with the macrophages from BALB/c animals. Growth of the bacteria occurred after a lag of several hours. Peak counts were present at 24 and 48 hr of incubation of the macrophages from the A/J mice. However, there was little difference between the low numbers of Legionella in macrophages from A/J versus BALB/c mice early after infection, since initially there was little replication of the bacteria in the cultured cells from either strain.

The growth of Legionella in the macrophages from individual ACF1 mice prepared from a cross between A/J and BALB/c parents was very low and essentially the same as when Legionella were cultured in macrophages from nonpermissive parental BALB/c animals (Fig. 1). However, macrophages from about one-third of the ACF2 mice obtained by brother/sister inbreeding of ACF1 mice showed some permissiveness (approximately 30%) for growth of Legionella, whereas macrophages from other ACF2 mice were not permissive. This suggested a dominance of the phenotype in the $F_2$ mice in terms of nonpermissiveness for growth of Legionella.

**Table I.** Fate of *L. pneumophila* in Elicited Peritoneal Macrophages[a]

| Mouse strain | No. of viable bacteria in macrophages ($\times 10^4$/culture) after infection | | | | |
|---|---|---|---|---|---|
| | 0 hr | 3 hr | 6 hr | 24 hr | 48 hr |
| A/J | 7.6±0.9[b] | 7.1±0.2 | 8.5±0.5 | 290±20 | 870±120 |
| BALB/c | 6.4±0.6 | 6.8±1.0 | 5.9±0.8 | 13.0±0.1 | 12.0±1.0 |

[a] Macrophage monolayers (approximately $1 \times 10^6$ cells/well) were infected with $2 \times 10^7$ bacteria for 30 min at 37°C, washed, supplied with medium, and then incubated for appropriate periods. Macrophage lysates prepared at 0, 3, 6, 24, and 48 hr after infection and the number of viable bacteria were determined by the plate count method.
[b] Data represent the mean ± SD of triplicate macrophage cultures.

As shown in Figure 2, when the ACF1 mice were backcrossed with either parental A/J or BALB/c mice, there was a resegregation of permissiveness of growth of Legionella. For example, macrophages from about half of the (ACF1 × A/J) mice were permissive for Legionella growth (approximately 57%), whereas macrophages from all backcrossed ACF1 and BALB/c mice were nonpermissive.

## Discussion

It is well known that natural resistance of murine hosts to infection with a variety of bacteria such as Listeria, Mycobacterium, and Salmonella is genetically regulated (8). Many resistance genes regulating host susceptibility to infections have been recognized such as *Bcg* (9), *Ity* (10), *Lsh* (11), and *Lr* (12). These genes control host resistance to *Mycobacterium bovis*, *Salmonella typhimurium*, *Leishmania donovani*, and *Listeria monocytogenes*, respectively. Recent studies have shown that the *Bcg* gene either is identical to or closely linked to the *Ity* and *Lsh* genes (13). More recent studies have shown that the *Bcg* gene exerts a major influence on the early host response to infections with a majority of mycobacterial strains (14). On the other hand, genetically determined susceptibility to Listeria infection is thought to link to a poor mobilization of mononuclear phagocytes to the site of infection (15). A/J mice are well known to respond poorly to an inflammatory stimulus and are highly susceptible to Listeria infection (12, 16). A defect in the phagocyte inflammatory re-

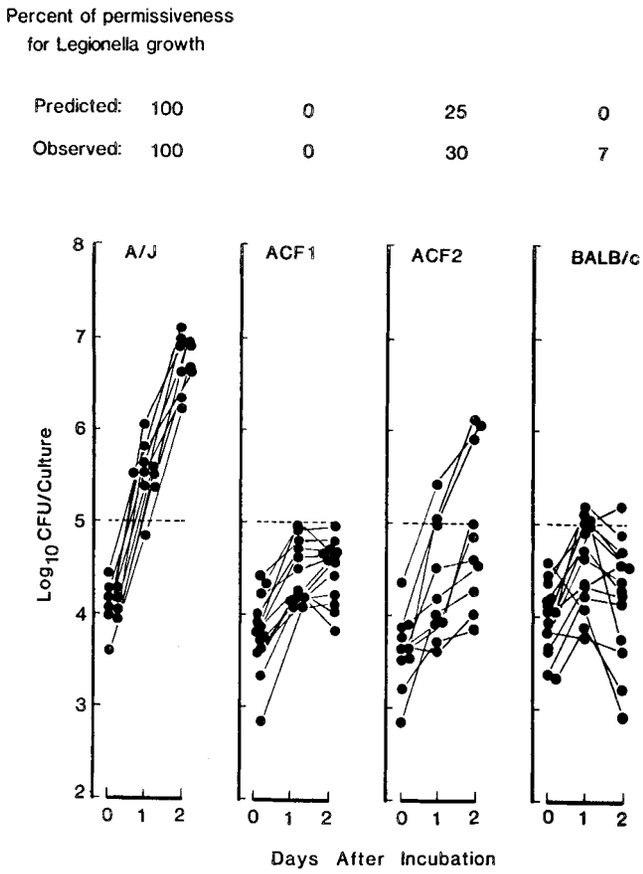


**Figure 1.** Fate of *L. pneumophila* in thioglycollate-induced macrophages from individual A/J, BALB/c, ACF1 (A/J × BALB/c), and ACF2 (ACF1 × ACF1) mice. Each mouse was injected intraperitoneally with 3.0 ml of thioglycollate 4 days prior to infection of macrophages with Legionella. After phagocytosis *in vivo* during 30 min following intraperitoneal infection with Legionella, peritoneal exudate cells were collected from individual mice and allowed to adhere to tissue culture plates (24-well plates), washed to remove nonadherent cells and nonphagocytized bacteria, and incubated with medium for 1 to 2 days. The starting point of this incubation is 0 time. Each point represents the results obtained from an individual mouse. The dotted line shows 95% confidence limit for typing individual animals as permissiveness (above line) or nonpermissiveness (below line) of macrophages to Legionella growth. Predicted percentages of permissiveness of individuals among segregating progeny are those for a trait under monogenic control.

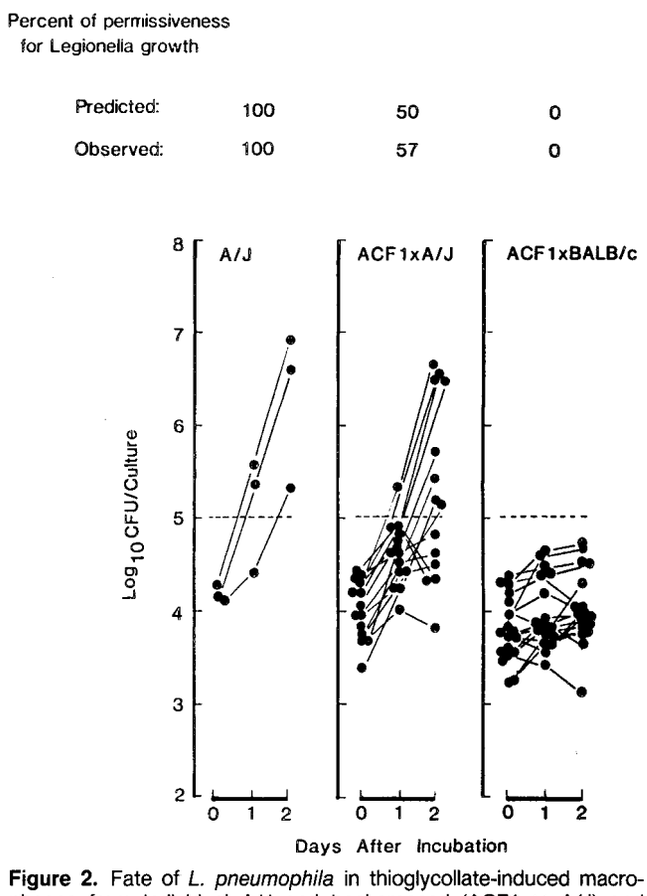


**Figure 2.** Fate of *L. pneumophila* in thioglycollate-induced macrophages from individual A/J and backcrossed (ACF1 × A/J) and (ACF1 × BALB/c) mice. See the legend of Figure 1.

sponse caused by the C5 component has been shown to be a major reason for susceptibility of this mouse strain to Listeria infection (17, 18). However, most studies on host resistance of A/J mice did not consider mechanisms at the cellular level such as antimicrobial activity of phagocytic cells. Thus, the study reported here was designed to examine genetic mechanisms at the cellular level, i.e., whether the permissive nature of macrophages for the growth of Legionella is controlled by a possible genetic mechanism.

The results of this study indicate that susceptibility of macrophages from A/J mice for growth of Legionella *in vitro*, as compared with resistance of macrophages from BALB/c mice (as well as probably most, if not all, other mouse strains (5, 6)) is under genetic control. Legionella did not grow in macrophages from BALB/c mice and were essentially noninfectious for these animals. However, these bacteria replicated readily in macrophages from A/J mice and can cause infection in these animals (6). Although the pathogenicity of Legionella for this susceptible mouse strain is somewhat lower than the greater level of infectivity for guinea pigs, *in vitro* growth of the bacteria in macrophages from A/J mice versus guinea pigs is essentially the same.

ACF1, prepared by crossing BALB/c and A/J mice, were essentially negative for permissiveness for Legionella growth since macrophages from these animals did not replicate the organisms. In contrast, ACF2 prepared by brother-sister inbreeding of ACF1 mice resulted in macrophages from approximately 30% of the animals showing permissiveness for Legionella growth, whereas macrophages from the remainder of the mice were nonpermissive. Backcrossed animals prepared by breeding BALB/c mice with $F_1$ animals from BALB/c and A/J mice were essentially nonpermissive. Furthermore, when $F_1$ animals were backcrossed with A/J mice, approximately 57% of the animals showed permissiveness for Legionella growth in their macrophages. These results indicate that the permissive nature of macrophages for Legionella growth is a recessive

feature of the animals and may be controlled by a single gene.

In other studies in this laboratory, it was found there is no relationship between the number of inflammatory cells in the peritoneal cavity of A/J versus BALB/c mice and permissiveness versus nonpermissiveness (unpublished data). Furthermore, the killing activities of macrophages for the temperature-sensitive mutant of *S. typhimurium* were variable and did not correlate with permissiveness versus nonpermissiveness for growth of Legionella in the various hybrid and backcrossed mice (unpublished data). Thus, the results of the present study support the concept that macrophage regulation of susceptibility versus resistance to Legionella infection is controlled by a single gene or closely related gene family. This view is supported by the segregation into distinct groups of mice by backcrossing studies.

It is important to note that the Legionella bacteria do not proliferate in medium used for the culture of the macrophages and only intracellular growth occurred *in vitro* (19). Thus, the studies in our laboratory, as well as in other studies, support the conclusion that growth of Legionella in macrophage cultures reflects permissiveness versus nonpermissiveness. It is also important to note that macrophages from A/J mice have been reported to be deficient in the killing capacity of intracellular microorganisms such as Chlamydia (20), as well as tumor cell killing (21). The lack of ability of the macrophages from these animals to evince cytolytic activity against targets as diverse as Chlamydia and tumor cells suggest that there may be a defect in the functional activity of macrophages from these animals.

As shown in the present study, the replication of Legionella in macrophages from A/J mice, but not from other mouse strains such as BALB/c, suggests that these cells have the ability to be infected by and replicate an important intracellular opportunistic pathogen. However, Legionella has been shown to grow intracellularly in a wide variety of mammalian cells (22), as well as in algae (23) and Protozoa (24, 25). It should be noted that studies in this laboratory have shown that cytokines, such as interferon-$\gamma$, can activate macrophages from A/J mice to evince killing of Legionella and inhibit the growth of these bacteria (26). Thus, the defect in the killing and growth-inhibition activity can be overcome by activation of macrophages from A/J mice.

The same immunostimulators have the ability to activate macrophages from other mouse strains and, furthermore, even treatment of nonpermissive macrophages with $\gamma$-interferon inhibits the lower ability of the cells to replicate these bacteria for the first 24 hr (unpublished data). Thus, the genetic control of resistance versus susceptibility of macrophages for growth of Legionella *in vitro* can be abrogated by activation with a cytokine. The nature and mechanism of control of resistance versus susceptibility of macrophages from A/J mice in terms of Legionella growth *in vitro* are being further studied in additional experiments in this laboratory.

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