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Genetic Control of Resistance to *Mycobacterium intracellulare* Infection in Mice

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The susceptibilities of various strains of mice to a highly pathogenic strain of *Mycobacterium intracellulare*, the Mino strain, were determined by intravenous injection of 5×10^6 bacteria. CFU were counted on days 1 and 21 of infection. Among 10 strains of mice, C57BL/6, C57BL/10, BALB/c, B10.BR, B10.A, and B10.D2 were susceptible, whereas DBA/2, A/J, CBA, and C3H/He were resistant. In the susceptible mouse strains, the number of bacteria increased during 21 days of infection, whereas no bacterial growth was observed in the resistant strains. Susceptible mice showed weak but positive delayed-type hypersensitivity to *M. intracellulare* purified protein derivative 20 days after injection of bacteria. Resistant mice developed no delayed-type hypersensitivity. Histological examination showed severe granulomatous lesions in livers or spleens of the susceptible mice after *M. intracellulare* injection. Analysis of F1 hybrids of susceptible and resistant strains and of F2 and backcross mice showed that the resistance to *M. intracellulare* seems to be controlled genetically by a single dominant gene. The pattern of distribution of resistance to *M. intracellulare* among the mouse strains was consistent with that of natural resistance to *Mycobacterium bovis* to BCG. Thus, resistance to *M. intracellulare* infection may be regulated by a gene linked to the *Bcg* gene on chromosome 1.

Mycobacterium intracellulare is found widely in the natural environment and sometimes causes pulmonary disease in human adults and localized lymphadenitis in pigs (21). Clinically, most strains of *M. intracellulare* are resistant to antituberculosis drugs, which makes chemotherapy difficult. There is no experimental model system for *M. intracellulare* infection so far in spite of its need for determination of the virulence of clinically isolated bacteria or the effect of chemotherapy against *M. intracellulare* infection. Because most experimental animals are resistant to infection with *M. intracellulare*, the pathogenicity of this organism itself has been questionable.

Mice have also been considered to be resistant to *M. intracellulare* and therefore inadequate for an experimental model. However, many reports have shown that there are differences among mouse strains in sensitivities to some bacterial (4, 5, 6, 11), viral (3, 16), fungal (13), and protozoal (1, 24) infections. These differences are influenced by the virulence of the parasite and the genetic background of the host. Therefore, we compared the virulences of various strains to *M. intracellulare* for a given mouse strain and also compared the sensitivities of various mouse strains to infection with a given strain of *M. intracellulare* to find an adequate host-bacteria combination for the establishment of an experimental model system for *M. intracellulare* infection.

In the present study, 16 strains of *M. intracellulare* were examined for virulence for CF1 mice. A freshly isolated *M. intracellulare* strain, Mino, which is highly virulent for CF1 mice, was used to determine the susceptibilities of various mouse strains to *M. intracellulare*. A marked difference was observed in bacterial growth among inbred strains of mice, and the possible genetic control of resistance to *M. intracellulare* infection is discussed.

MATERIALS AND METHODS

Mice. Male and female C57BL/6 (B6), C57BL/10 (B10), C3H/He (C3H), BALB/c, DBA/2, B10.A, B10.D2, B10.BR, and (B6 × C3H)F1 (B6C3F1) mice were obtained from Shizuoka Experimental Animals, Hamamatsu, Japan. A/J, CBA, and outbred CF1 mice were bred in our laboratory. All of the mice were conventional and were fed laboratory chew and tap water. They were used at 5 to 6 weeks of age.

Bacteria and infection. Sixteen strains of *M. intracellulare* were used. They were isolated from either atypical mycobacteriosis patients or pigs. These strains of *M. intracellulare* were cultured on Ogawa egg medium (Ogawa medium) or Ogawa medium containing 1% Tween 80 (T-Ogawa medium) at 37°C for 21 days and then subcultured on T-Ogawa medium for 7 days. The bacteria were suspended in distilled water and injected into CF1 mice intravenously to evaluate in vivo growth. For further experiments, the Mino strain was selected for use, and large numbers of this strain were cultured on Dubos Tween 80-albumin medium for 5 days and stored at −70°C in separate tubes. To determine the susceptibility of mice to the Mino strain, we thawed the frozen bacteria at room temperature, homogenized them in a glass homogenizer, diluted them in sterile distilled water, and injected them into mice via the lateral tail vein. The viability of the inoculum was checked by plating samples of 10-fold dilutions of the bacterial suspension on T-Ogawa medium. The colonies were counted after 3 weeks of incubation at 37°C.

Bacterial growth in vivo. The mice were killed at various intervals after injection, and the spleens were removed aseptically, weighed, and homogenized separately in sterile distilled water. Each sample was diluted appropriately, and 0.1 ml of the suspension was inoculated on T-Ogawa medium. Colonies were counted after 3 weeks of incubation. The growth rate was expressed by the mean number of bacteria in the spleen on day 21 divided by the mean number on day 1.

PPD-I. To obtain *M. intracellulare* purified protein derivative (PPD-I), *M. intracellulare* (Tasaka strain) was cultured in Sauton liquid medium at 37°C for 6 weeks and killed by heating at 121°C for 15 min. The bacteria were removed by centrifugation at 10,000 rpm for 30 min, and the supernatant fluid was passed through a 0.45-μm (pore size) Millipore

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filter. Ammonium sulfate was added to 80% final concentration. The pH was adjusted to 7.8 with NH_4OH. The precipitate was harvested by centrifugation and dissolved in a small amount of 0.05 M phosphate buffer (pH 7.8). After dialysis against 0.05 M phosphate buffer overnight, this protein-rich suspension was eluted through a Sephadex G-25 column in 0.05 M phosphate buffer. The optical density at 280 nm of the eluted fractions was measured by the Lowry method (14).

Mouse footpad reaction and macrophage disappearance test. Delayed-type hypersensitivity (DTH) to PPD-I was measured in mice infected with *M. intracellulare* by injecting 10 μg of PPD-I in 0.05 ml of phosphate-buffered saline (PBS) into one hind footpad, and the amount of footpad swelling was measured 24 and 48 h later with a dial gauge (Peacock; Ozaki Co., Tokyo, Japan). For the macrophage disappearance test, the mice received an intraperitoneal injection of 10% Proteose Peptone (Difco Laboratories) at time zero and 50 μg of PPD-I in 0.5 ml of PBS at 72 h. The control mice received Proteose Peptone and PBS alone at 72 h later. At 6 h after PPD-I or PBS injection, the mice were killed, and the peritoneal exudate cells were collected in 5 ml of PBS. The total numbers of cells and macrophages were counted. The presence of macrophages was determined morphologically by Giemsa stain.

Agglutinin antibody. Serum was taken from each of the *M. intracellulare*-infected mice and inactivated at 56°C for 30 min before use. *M. intracellulare* Mino was cultured on Middlebrook 7H10 agar slants at 37°C for 2 weeks. The bacteria used as the antigen were suspended in PBS containing 0.5% phenol to be adjusted to the optical density of 0.3 in a spectrophotometer with a green filter. For the agglutination reaction, 0.5-ml quantities of dilutions of the mouse sera were mixed with equal volumes of the antigen suspension, and the mixtures were incubated at 37°C for 3 h. The agglutinin antibody titer was determined by reading the agglutination endpoint where the bacteria had settled on the bottom of the tube, and the supernatant was perfectly clear.

Histopathology. Specimens for histological examination were fixed in 10% Formalin in PBS, dehydrated in alcohol, and embedded in paraffin. Sections (4 μm thick) were made and stained with hematoxylin and eosin. Some sections were stained by the Ziehl-Neelsen method.

RESULTS

In vivo growth of *M. intracellulare* in CF1 mice. Sixteen strains of *M. intracellulare* were examined for pathogenicity for CF1 mice which are known to be susceptible to *Mycobacterium bovis* BCG (Pasteur) or *Mycobacterium tuberculosis*. The bacteria (10^7 viable units) were injected intravenously into groups of six mice. Two mice of each group were killed on day 1, and the rest were killed on day 21. The numbers of bacteria in the spleens were determined as CFU on T-Ogawa medium. We used T-Ogawa medium for the CFU counts because most of the *M. intracellulare* strains showed better growth on T-Ogawa medium than on Ogawa medium. Ten strains of *M. intracellulare* showed various in vivo growth rates (2.18 to 48.0), whereas the other six strains showed no growth in vivo (Table 1). Strain K4 was the most virulent strain, and strain Mino was the second most virulent. Since strain K4 grew poorly on the medium, we chose the Mino strain for further experiments.

Growth of *M. intracellulare* Mino in different mouse strains. Figure 1 shows growth curves obtained after intravenous injection of 5×10^6 organisms of the Mino strain into various mouse strains. There were striking differences in the rates of growth among the strains. However, there was no difference in the viable count on day 1 after challenge. The viable counts increased thereafter in B6, B10, BALB/c, and B10 congenic mice through day 21 of infection. In contrast, the viable counts of bacilli in CBA, DBA/2, A/J, and C3H mice did not increase. From these results, we divided the mouse strains into two types: the susceptible type, which permitted bacterial growth; and the resistant type, which did not. Strain B6 was of the susceptible type, and strain C3H was of the resistant type. It should be noted that multiplication means the net growth of bacteria in vivo.

TABLE 1. In vivo growth of 16 strains of *M. intracellulare* in CF1 mice

Bacterial strain	No. of bacteria per spleen (log_{10}) on day:		Bacterial growth rate[a]
	1	21	
ATCC 15984	5.78[b]	3.35 ± 0.30[c]	0.004
K2	6.10	6.44 ± 0.08	2.18
K3	6.06	6.86 ± 0.19	6.30
K4	6.07	7.75 ± 0.14	48.0
K6	5.33	5.16 ± 0.17	0.68
K8	4.49	5.74 ± 0.26	17.80
K9	5.70	6.86 ± 0.26	14.45
K10	5.70	4.90 ± 0.30	0.16
K14	6.03	5.05 ± 0.09	0.10
K16	6.21	6.11 ± 0.10	0.80
Ninomiya	4.53	5.66 ± 0.37	13.48
Tanabe	5.27	4.65 ± 0.12	0.24
Mino	5.25	6.77 ± 0.36	33.09
Teshiogi	5.97	7.37 ± 0.09	25.08
YP-7	6.17	7.58 ± 0.18	25.68
YP-10	6.69	8.09 ± 0.30	25.10

[a] Bacterial growth rate = (mean number of bacteria on day 21)/(mean number of bacteria on day 1).
[b] Mean of two mice.
[c] Mean ± standard deviation of four mice.

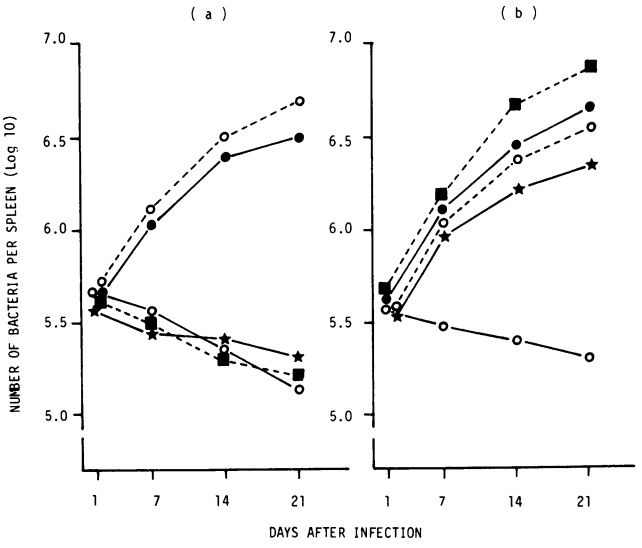

FIG. 1. Time course of growth of *M. intracellulare* Mino in the spleens of various inbred strains of mice. Viable bacteria (10^7) were injected intravenously into mice of each strain. The mice were killed at various times after infection, and the number of bacteria in each spleen was determined by counting the CFU on T-Ogawa medium. (a) Symbols: O- - -O, B10.BR; ●, B10.A; ■, A/J; O——O, C3H; ★, CBA. (b) Symbols: ■, B10.D2; ●, B6; ★, BALB/c; O——O, DBA/2; O- - -O, B10.

Phenotypic expression of susceptibility to *M. intracellulare* in B6 and C3H mice. To determine whether the dose of *M. intracellulare* could affect the phenotypic expression of susceptibility in mice, we challenged B6 and C3H mice with 10^4, 5×10^6, or 10^7 cells of *M. intracellulare* Mino and examined them for numbers of bacteria in the spleens at various times (Fig. 2). Strain C3H did not permit bacterial growth in the spleen at any dose of bacteria, whereas strain B6 did. This means that the phenotype of susceptibility of *M. intracellulare* was not altered by the dose of the bacteria.

DTH to *M. intracellulare* in B6 and C3H mice. The activity of PPD-I was tested by the footpad reaction in B6 and C3H mice immunized with 1 mg of heat-killed *M. intracellulare* 2 weeks before. Various doses of PPD-I were injected separately into hind footpads of either B6 or C3H mice. Normal mice of both strains were given various doses of PPD-I to check for nonspecific reactivity. A 10-μg amount of PPD-I elicited marked footpad swelling in both B6 and C3H mice immunized with *M. intracellulare*, although the values for C3H mice were much higher than those for B6 mice (Fig. 3a). It was found that 10μg of PPD-I did not elicit footpad swelling in normal mice, but 25 μg of PPD-I did; therefore, 10 μg of PPD-I was used for the evaluation of DTH in the subsequent experiments.

The kinetics of the footpad reaction to 10 μg of PPD-I are shown in Fig. 3b. C3H mice showed a stronger response than did B6 mice. The highest response in both strains was observed 24 h after the eliciting antigen was injected.

DTH to PPD-I in the mice immunized with living *M. intracellulare* was tested by either the footpad reaction or the peritoneal macrophage disappearance test. The mice were immunized by intravenous injection of living *M. intracellulare* at a dose of 10^4 or 10^7. The DTH reactions were checked on days 10 and 20. The agglutinin antibody titer was also measured on these days. A significant DTH reaction was observed only in B6 mice that had received 10^7 living bacteria 20 days before (Table 2). When the mice received 10^4 bacteria, no DTH was induced after infection. Even

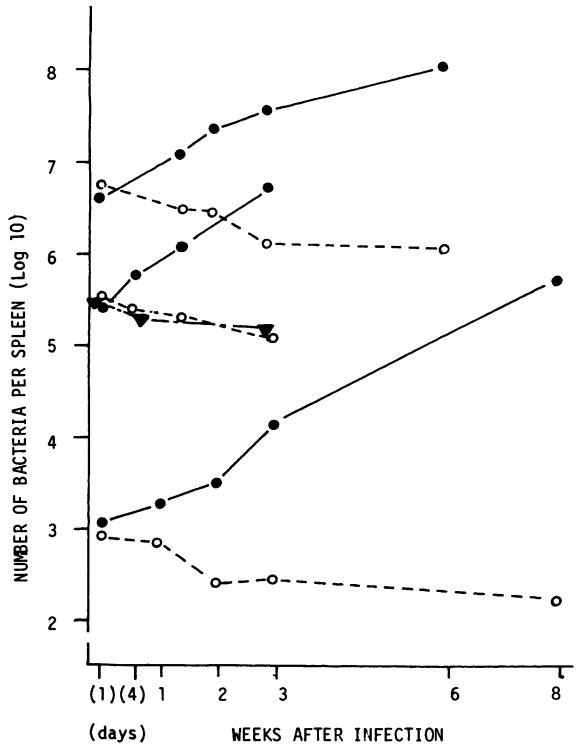

FIG. 2. Time course of growth of *M. intracellulare* Mino in the spleens of B6 (●), C3H (○), and B6C3F1 (▼) mice. A group of mice of each strain received a high dose (10^7), a medium dose (5×10^6), or a low dose (10^4) of bacteria intravenously. The mice were killed at various times after the infection, and the number of the bacteria in each spleen was determined.

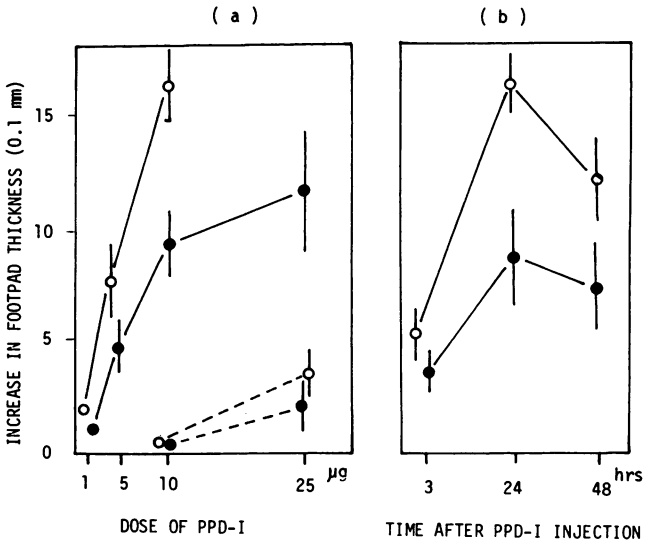

FIG. 3. Footpad reaction to PPD-I in immunized B6 and C3H mice. The mice were injected with 0.1 mg of heat-killed *M. intracellulare* and tested for the footpad reaction with 10 μg of PPD-I 2 weeks later. (a) Various doses of PPD-I were used for eliciting the footpad swelling in immunized B6 (●——●) and C3H (○——○) or normal B6 (●- - -●) and C3H (○- - -○) mice. (b) Time course of the footpad reaction in immunized B6 (●——●) and C3H (○- - -○) mice.

TABLE 2. Macrophage disappearance and footpad reaction to PPD-I in *M. intracellulare*-infected B6 and C3H mice

Mouse strain	Days after infection[a]	PPD-I	No. of macrophage[b] ($\log_{10}$ ± SD)	FPR[c] (0.1 mm ± SD)
B6	0	−	6.56 ± 0.04	NT[d]
		+	6.60 ± 0.10	
	10	−	6.64 ± 0.06	0.6 ± 0.4
		+	6.68 ± 0.04	
	20	−	6.66 ± 0.06	3.7 ± 0.5[e]
		+	5.60 ± 0.12[e]	
C3H	0	−	6.30 ± 0.08	NT
		+	6.32 ± 0.06	
	10	−	6.34 ± 0.08	0.9 ± 0.1
		+	6.32 ± 0.04	
	20	−	6.30 ± 0.10	1.3 ± 0.5
		+	6.00 ± 0.12	

[a] Intravenous injection of 10^7 bacteria.
[b] Total number of macrophage from the peritoneal cavity per mouse.
[c] FPR, Footpad reaction (swelling at 24 h after PPD-I injection).
[d] NT, Not tested.
[e] Significantly different ($P < 0.05$).

when the mice received 10^7 bacteria, no DTH was observed on day 10. In C3H mice, no DTH reaction occurred under any conditions in this experiment. Agglutinin antibody was not detected in either strain of mice on day 10, but a low level of antibodies was observed on day 20 in both strains when the mice received 10^7 bacteria (data not shown).

Histopathology. The livers of *M. intracellulare*-infected mice were examined histopathologically. Granulomas were observed in both B6 and C3H mice 2 weeks after injection of 10^7 bacteria. More granulomas were observed in B6 than in C3H mice at this time. The number of the granulomas increased in B6 mice thereafter, but not in C3H mice. When 10^4 bacteria were injected, a few granulomas were observed in B6 mice only at 8 weeks after infection, but no granulomas were observed in C3H mice at any time.

Protective immunity against secondary challenge with *M. intracellulare* in susceptible (B6) and resistant (C3H) mice. B6 and C3H mice were infected with 5×10^4 *M. intracellulare*. After 2 weeks, the mice were challenged with 10^7 bacilli. Normal B6 and C3H mice of the same age were infected with 10^7 bacteria as controls. Two mice in each strain were killed 7 days later, and the others were killed 14 days later to determine the numbers of bacteria in the spleens (Table 3).

In the susceptible strain, B6, the increase in bacteria was smaller in the immunized group than in the control group, but not very significantly. In the resistant strain, C3H, on the other hand, the number of bacteria decreased in the same manner in the preinfected and the control groups (Table 3).

Genetic analysis of susceptibility and resistance markers with F1 and backcross mice. F1 hybrids of B6 and C3H mice, B6C3F1, were resistant to *M. intracellulare* infection (Fig. 4). This suggests that resistance to *M. intracellulare* is dominant over the susceptibility character. To analyze this character genetically, B6C3F1 × B6 or B6C3F1 × C3H mice were examined for susceptibility to *M. intracellulare*. The mice were challenged with 5×10^6 *M. intracellulare*, and the numbers of bacteria recovered from the spleens on days 1 and 20 were determined. The average number of CFU in the spleens of either strain on day 1 was $10^{5.8}$ (Fig. 4). We determined that the mice were susceptible or resistant by comparing the viable count on day 1 with that on day 20. Approximately 50% of the B6C3F1 × B6 backcross mice were susceptible. The offspring of the converse backcross onto the resistant parent, B6C3F1 × C3H, were 100% resistant. In the F2 generation, the ratio of resistant mice to susceptible mice was ca. 3:1 (Fig. 4). Analysis of B6C3F1 × B6 mice revealed that there was no correlation between susceptibility to *M. intracellulare* and coat colar (data not shown).

DISCUSSION

Of 16 strains of *M. intracellulare*, the Mino strain, which had been isolated from an atypical mycobacteriosis patient, was selected as the standard strain in this study because it was highly virulent for CF1 mice and its pathogenicity remained unchanged when maintained on T-Ogawa medium.

TABLE 3. Growth of *M. intracellulare* in B6 and C3H mice with or without primary infection

Mouse strain	Days after secondary infection	No. of bacteria per spleen ($\log_{10}$)	
		Normal[a]	Immunized[b]
B6	7	6.35 ± 0.19	6.31 ± 0.12
	14	7.14 ± 0.26	6.85 ± 0.21
C3H	7	6.24 ± 0.20	6.23 ± 0.19
	14	5.39 ± 0.18	5.35 ± 0.18

[a] Mice without primary infection.
[b] Mice with primary infection (5×10^4 bacteria).

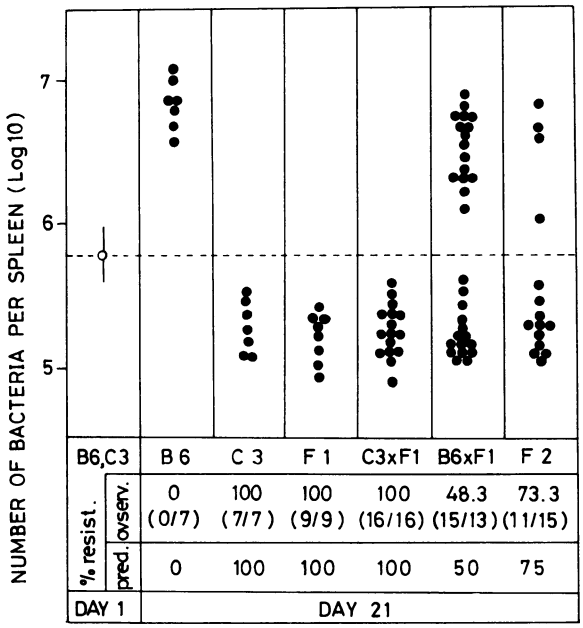

FIG. 4. Cells (5×10^6) of *M. intracellulare* Mino were injected intravenously into B6, C3H, B6C3F1, C3 × F1, B6 × F1, and F2 mice. On day 21, the mice were killed, and a spleen homogenate from each mouse was cultured on T-Ogawa medium. CFU were counted 3 weeks later. On day 1 of infection, the number of bacteria per spleen was $10^{5.8}$ in either C3H or B6 mice. Symbols: ●, number of bacteria per spleen in one mouse; ○, mean number of bacteria per spleen on day 1 of infection. The vertical bar shows the standard deviation. % resist., Percentage of mice resistant to *M. intracellulare*; pred., predicted; observ., observed. Numbers in parentheses show actual number of mice.

When the Mino strain was used as the pathogen, various mouse strains were found to be divided into two types according to their responses. One was the susceptible type that permitted bacterial growth in vivo; these infected mice died 40 to 50 weeks after infection with 10^7 bacteria. The number of bacteria in the spleen reached 10^9 at this time. The other was the resistant type in which the bacteria did not grow, and, consequently, the inoculated mice did not die from the infection. B6, B10, B10.BR, B10.A, B10.D2, and BALB/c mice were susceptible, and C3H/He, CBA, A/J, and DBA/2 mice were resistant. The difference in these types was very clear. The histological data were parallel to the bacterial growth data in vivo. Severe granulomatous lesions were found only in the susceptible mice, wherein the bacteria propagated well, but almost no lesions were observed in the resistant mice.

Genetic analysis of F1 hybrids of a susceptible strain (B6) and resistant strain (C3H) and analysis of F2 and BC mice showed that the resistance to *M. intracellulare* infection is possibly controlled by a single dominant gene. Interestingly, the segregation pattern of resistance to *M. intracellulare* among the various mouse strains matched very well the pattern of their natural resistance to *M. bovis* BCG, *Salmonella*, and *Leishmania* infections (2, 8, 12, 22). Therefore, it seems very likely that the gene that controls the resistance to *M. intracellulare* is the same as or is linked to the *Bcg*, *Ity*, or *Lsh* gene on chromosome 1 (22).

On the other hand, there are strain differences among mice

in their resistance to other intracellular pathogens such as *Listeria* spp. (4, 23), rickettsiae (15), or fungi (13). Resistance to these parasites is also reported to be genetically controlled. The gene regulating *Listeria* infection has been mapped on chromosome 2 (7), whereas the gene regulating rickettsial infection has been mapped on chromosome 5 (10). It is interesting to know the mechanisms of resistance of the host against various intracellular pathogens. Gros et al. (9) showed that the *Bcg* gene is expressed on a mature cell of the mononuclear phagocyte lineage in function. It has been suggested that the mechanisms of resistance against *M. bovis* BCG, *Salmonella* spp., *Leishmania* spp., and *M. intracellulare* would be regulated basically in the same manner, which may differ from that of resistance against *Listeria* spp. or rickettsiae. Genetic studies with macrophages are currently under way.

Recently, Orme and Collins (18) reported that *M. bovis* BCG Pasteur grew well in Bcg^r mice and also that the growth pattern of *M. bovis* BCG in Bcg^r mice was not the same among organs such as the spleen, liver, and lungs. For *M. intracellulare* infection, however, the growth patterns of the bacteria was the same in the spleen, liver, and lungs (Y. (Goto, unpublished data). It seems possible that the virulence of the bacteria used would modulate the effect of the natural resistance of the host cells in various organs. Lung macrophages are known to behave differently from the macrophages of other organs in cytotoxic activity (T. Tokunaga and K. S. Akagawa, *Proceedings of the 4th Leiden Conference on Mononuclear Phagocytes*, in press). Therefore, it would be possible that the expression of natural resistance in macrophages may differ depending on the organs infected.

It is not clear whether natural resistance and acquired resistance have any relationship to each other. A strain naturally resistant to *M. bovis* BCG has been shown to develop neither DTH nor protective immunity within first 2 to 3 weeks of infection (8, 19), whereas a Bcg^s strain has. However, the same dose of *M. bovis* BCG has induced clear protective immunity in both Bcg^r and Bcg^s strains 6 weeks after infection in a different experiment (18). For *M. intracellulare*, a low dose of the primary challenge seemed to have induced a slight inhibition of growth of the secondary challenging bacteria in a Bcg^s strain. However, the difference between the controls was not very significant. This may be a result of the small antigenic burden of the primary challenge. Further studies with various doses and time intervals are necessary because the discrepancy between previous experiments (18, 19) seems to be the result of the difference in doses and time courses selected.

DTH was induced in B6 mice injected with a high dose of *M. intracellulare*, but not in C3H mice. This result is consistent with the fact that DTH can be induced by injection with *M. bovis* BCG in Bcg^s but not in Bcg^r mice (8), except in the present study the dose of *M. bovis* BCG was much lower than that of *M. intracellulare*. It is interesting to know whether or not suppressor T cells are induced in C3H mice with *M. intracellulare* infection because suppressor T cells are induced by a large dose of injected *M. bovis* BCG or *Mycobacterium avium* (17, 25).

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