INFECTION AND IMMUNITY, Nov. 1987, p. 2631–2635
0019-9567/87/112631-05$02.00/0

Vol. 55, No. 11

Pulmonary Clearance of *Mycoplasma pulmonis* in C57BL/6N and C3H/HeN Mice

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Received 20 May 1987/Accepted 5 August 1987

In C57BL/6N and C3H/HeN mice known to be free of all murine pathogens and matched for age, sex, and environmental factors, pulmonary clearance was measured over a 72-h time period after exposure to infectious aerosols of ^{35}S-labeled *Mycoplasma pulmonis*. Reduced clearance of *M. pulmonis* in C3H/HeN mice relative to C57BL/6N mice was primarily due to impaired mycoplasmacidal activity in the lungs of the C3H/HeN mice. The C3H/HeN mice also had a slightly slower rate of mechanical transport of radiolabel from the lungs in the first 4 h after infection relative to the C57BL/6N mice but not at any later times. By 72 h after infection (relative to 0 h, C3H/HeN mice had an over 4,000% (1.75×10^7 versus 4.30×10^5) increase in neutrophils and an over 18,000% (more than 2 orders of magnitude) increase in numbers of *M. pulmonis* recovered from mechanically disaggregated lungs. In contrast, C57BL/6N mice reduced the number of *M. pulmonis* present by over 83% (nearly 2 orders of magnitude) before any increase in inflammatory cells, which was only a slight increase in lymphocytes and macrophages at 24 h after infection. These results directly link decreased mycoplasmal pulmonary clearance in C3H/HeN mice with the increased susceptibility to, and severity of, murine respiratory mycoplasmosis observed in this strain. The resistance of C57BL/6N mice appears to be related to nonspecific host defense mechanisms responsible for limiting the extent of infection.

Murine respiratory mycoplasmosis (MRM), a naturally occurring disease of laboratory mice and rats due to *Mycoplasma pulmonis*, provides excellent animal models for the study of infectious respiratory disease (1, 22). We have previously shown that C3H/HeN mice are more susceptible than C57BL/6N mice to MRM experimentally induced via intranasal inoculation (10) or aerosol exposure (11). C3H/HeN mice are not more susceptible than the C57BL/6N mice to infection, but they are more susceptible to disease as quantitated by gross and microscopic analyses of tissues, 50% gross pneumonia dose, 50% microscopic lesion dose, and 50% lethal dose. Also, more organisms can be recovered from the lungs of C3H/HeN mice than from those of C57BL/6N mice.

We wanted to determine whether C3H/HeN mice were less efficient than C57BL/6N mice at clearing *M. pulmonis* from the lungs by nonspecific defense mechanisms. To directly link the findings of these pulmonary clearance studies with the strain differences reported in the aerosol studies, we have performed experiments by identical exposure protocols (11). The present studies used radiolabeled *M. pulmonis* organisms in C57BL/6N and C3H/HeN mice known to be free of all other murine pathogens to determine the relative contributions of physical clearance and intrapulmonary killing of mycoplasmas in clearance of these organisms from the lungs of both strains of mice over a 72-h period after infection. The numbers of total inflammatory cells, lymphocytes, macrophages, and neutrophils recoverable from the lungs in both mouse strains were enumerated to establish the relationship of the kinetics of these cell populations with clearance of *M. pulmonis* from the lung. Increased neutrophil numbers could not be correlated with clearance of *M. pulmonis*, since C57BL/6N mice reduced organism numbers without any increase in recovery of these cells from the lung, and C3H/HeN mice did not control proliferation of the organisms, even though the mice showed large increases in the number of neutrophils recovered from the lungs after infection.

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MATERIALS AND METHODS

Animals. All of the mice used in these studies were 6- to 10-week-old C57BL/6N and C3H/HeN mice from breeding colonies maintained at the University of Alabama at Birmingham. The colonies were monitored monthly for the presence of mycoplasmas by enzyme-linked immunosorbent assay (15) for immunoglobulin G and M antibodies to *M. pulmonis* and *Mycoplasma arthritidis* (2, 3, 8) and quarterly for other murine pathogens by fecal cultures, necropsy, and histological examination of retired breeders and serologic tests for rodent viruses on sera from retired breeders and mice used in experiments. Mice were tested by either hemagglutination inhibition, complement fixation, or enzyme-linked immunosorbent assay by Microbiological Associates (Bethesda, Md.) for pneumonia virus of mice, reovirus-3, Theiler's GDVII virus, polyomavirus, Sendai virus, minute virus of mice, ectromelia, mouse adenovirus, mouse hepatitis virus, and lymphocytic choriomeningitis virus. No murine pathogens were detected within the colony for the past 3 years. Animals used for experiments were matched for age and sex. Experimental mice were maintained in Trexler-type plastic film isolators (Germ-Free Supply Division, Standard Safety Equipment Co., Palatine, Ill.) in shoebox cages before exposure and in sterile shoebox cages equipped with filter tops after exposure. All cages were provided with sterile hardwood chip bedding (PJ Murphy Forest Products, Rochelle Park, N.J.), sterile food (Agway, Inc., Syracuse, N.Y.), and sterile water ad libitum. The level of intracage ammonia was measured during experiments and was consistently less than 0.025 ml/liter.

Mycoplasmas. All experiments were performed with the CT strain of *M. pulmonis* (10). Animals were exposed to mycoplasmal aerosols as previously described (11). Briefly, animals were exposed to aerosols generated with log 9.99 ± 8.88 CFU of *M. pulmonis* in the nebulizer. Exposure lasted for 30 min with replacement of the culture in the nebulizer every 5 min, with a diluting air flow of 20 liters/min and a nebulizer air flow of 5 liters/min. The aerodynamic diameter for each exposure was determined with mass data. The mass median aerodynamic diameter averaged 1.93 μm (standard error of the mean [SEM], 0.10; range, 1.83 to 2.03), and the sigma G, a measure of particle size variability, averaged 2.57 (SEM, 0.10; range, 2.47 to 2.67). Previous experiments have shown no significant variation in the aerosol dose delivered to the different animal exposure ports in the nose-only chamber (11).

Radiolabeled *M. pulmonis*. Radiolabeled *M. pulmonis* was prepared by pelleting 1,800 ml of early-log-phase cultures grown in dialyzed media (33) and suspending the pellet in 90 ml of labeling medium containing Hanks balanced salt solution (GIBCO Laboratories, Grand Island, N.Y.), 5% horse serum (GIBCO) dialyzed against phosphate-buffered saline (PBS), 0.05% thallium acetate (Sergent-Welch, Skokie, Ill.), 0.5% glucose, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and 8 μCi of [^{35}S]methionine (Amersham Corp., Arlington, Ill.) per ml (25). After incubation for 4 h at 37°C, an equal volume of complete mycoplasma broth was added and incubation was continued for 2 more h. The culture was then pelleted, washed with PBS, and suspended in RPMI 1640 for a total volume of 30 ml. In all experiments, the disintegrations per min and the number of CFU were determined; the ratio of dpm/CFU ranged from 1/100 to 1/1,000.

Quantitation of radioactivity and *M. pulmonis* in tissues. Quantitative mycoplasma cultures of tissues from experimental animals were done as described previously (10). Lungs were collected after death by overdose of pentobarbital, homogenized in 1 ml of PBS, and sonicated at a rate known to release, but not kill, cell-bound organisms (10). Separate samples of the lung homogenates were cultured or examined for radioactivity. Cultures were incubated for 2 weeks at 37°C before they were considered negative. For purposes of demonstration, the culture data are expressed as log change in CFU in the lung (statistical analysis actually performed on the log CFU in the lung). For determination of radioactivity, lung homogenates were solubilized (Protosol; New England Nuclear Corp., Boston, Mass.), bleached with H_2O_2, and counted with Aquasol (New England Nuclear Corp.) in a Betatrac 6895 (TM Analytic, Elk Grove Village, Ill.).

Quantitation of inflammatory cells. After death by overdose of pentobarbital, cells were collected from the lungs by a previously described modification (9) of the technique described by Hunninghake and Fauci (17). The lungs were removed, placed in PBS, and mechanically disaggregated for 1.5 min with a Stomacher 80 (Dynatech Laboratories, Inc., Alexandria, Va.). The resulting suspension was passed through a 250-μm-mesh nylon screen. After lysis of erythrocytes by hypotonic shock (26), total cells in the suspension were counted with a hemacytometer and differential counts of Wright stained smears (Fisher Scientific Co., Fairlawn, N.J.) were done according to standard methods (24). The percentage of cells other than leukocytes made up 2 to 5% of the total sample and did not vary between strains or with time. The total leukocyte and differential cell counts were then calculated from the total cell count. Lymphocytes, macrophages, and neutrophils collectively accounted for ≥98% of the total leukocyte count. Basophils, mast cells, and eosinophils collectively made up less than 2% of the total leukocyte count and did not vary significantly in numbers between the strains or with time.

Calculation of physical clearance. Physical clearance of radioactive label at 0, 1, 4, 8, 24, and 72 h was expressed as a percentage of the mean of the disintegrations per minute at 0 h (immediately after infection) for each strain of mice (13) (statistical analysis actually performed on the disintegrations per minute of the samples).

Calculation of intrapulmonary mycoplasmacidal activity. The mycoplasmacidal activity was defined as the change in the proportion of viable to total mycoplasmas in the lung and was calculated in each mouse by the radioactive ratio method of Green and Goldstein (13). The ratio of CFU to radioactive counts (disintegrations per minute [dpm]) was determined for the organisms in the aerosol and in the lungs. The mycoplasmacidal activity was calculated from the change that occurred between the two ratios and is expressed as follows: mycoplasmacidal activity = (CFU/dpm ratio of lung homogenates)/(CFU/dpm ratio of aerosol). Thus, an activity equal to the activity at 0 h indicates absence of killing of organisms in the lung. An activity less than the activity at 0 h indicates killing of organisms within the lung. An activity at any time greater than the activity at 0 h indicates proliferation of organisms within the lung.

Statistical analysis. All data were analyzed with the analysis of variance technique (37) and, when appropriate, with Duncan multiple mean comparison test (20). Antibody level, CFU, and mycoplasmacidal activity data were logarithmically transformed before data analysis. A probability of 0.05 or less was accepted as significant.

RESULTS

Experimental design. In all experiments, groups of C57BL/6N and C3H/HeN mice were simultaneously exposed to aerosols of *M. pulmonis*. Data were collected at 0 (immediately after exposure), 1, 4, 8, 24, and 72 h after exposure. Clearance data and inflammatory-cell data are derived from separate groups of mice exposed simultaneously. All data are derived from two repetitions of each experiment and are presented as group means ± the standard errors of the means (SEMs).

Mycoplasmal clearance. Animals killed immediately after exposure (0 h) had (7.00 ± 1.03) × 10^4 CFU of *M. pulmonis* in the lungs. The two mouse strains did not differ in the number of organisms recovered at this time. C57BL/6N mice were more efficient than the C3H/HeN mice at clearing *M. pulmonis* from the lungs (Fig. 1). The C57BL/6N mice reduced mycoplasma numbers by 83% (nearly 2 orders of magnitude) by 4 h after exposure ($P < 0.05$). C3H/HeN mice never significantly reduced the numbers of organisms in the lungs, and in fact, organism numbers had increased ($P < 0.05$) over 2 orders of magnitude (over 18,000%) by 72 h after infection in this strain.

Mycoplasmacidal activity and physical clearance. C57BL/6N mice killed more mycoplasmas than the C3H/HeN mice did (Fig. 2). Theoretically, the mycoplasmacidal activity ratio should be 10^0 at 0 h. The slightly lower ratio seen in both strains at this time is most likely due to the inevitable loss of viability of organisms that occurs during nebulization (11). Killing by C57BL/6N mice was apparent by 4 h after infection ($P \leq 0.05$), but C3H/HeN mice never killed significant numbers of mycoplasmas in the lungs. C57BL/6N

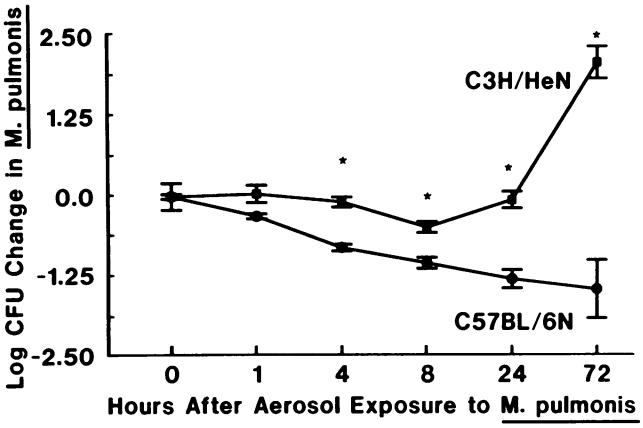

FIG. 1. Mycoplasmal clearance. Average log change ± SEM (n = 6) in the CFU of *M. pulmonis* in the lungs of C57BL/6N and C3H/HeN mice exposed to infectious aerosols generated with log 9.99 ± 8.88 CFU of ^{35}S-labeled *M. pulmonis* in the nebulizer.

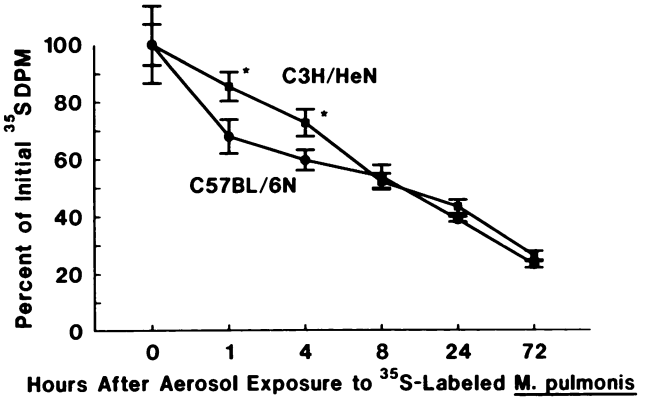

FIG. 3. Physical clearance. Average percent ± SEM (n = 6) of the ^{35}S label remaining in the lungs of C57BL/6N and C3H/HeN mice exposed to infectious aerosols generated with log 9.99 ± 8.88 CFU of ^{35}S-labeled *M. pulmonis* in the nebulizer.

removed slightly more radiolabel from the lungs immediately after aerosol exposure than the C3H/HeN mice did (Fig. 3). However, by 8 h after exposure, there were no differences between the two strains in the rate of physical transport of radiolabel from the lungs.

Cell quantitation. C3H/HeN mice had more total leukocytes recovered from the lungs at 72 h after infection than C57BL/6N mice (Table 1). This difference is almost totally due to the large increase in the number of neutrophils recovered in the C3H/HeN mice. At 72 h, the numbers of neutrophils recovered had increased over 40-fold ($P < 0.05$), whereas the numbers of lymphocytes and macrophages had only increased approximately threefold over the initial numbers recovered ($P < 0.05$). In contrast, recovery of total leukocytes or neutrophils did not increase in the C57BL/6N mice at any time after infection, although the numbers of lymphocytes and macrophages recovered at 24 h were slightly increased ($P < 0.05$).

DISCUSSION

Nonspecific host defenses play an important role in pulmonary clearance of inhaled bacteria (12). Our finding of more efficient pulmonary clearance of mycoplasmas in the C57BL/6N mouse strain, which is more resistant than the C3H/HeN mouse strain to MRM, indicates that clearance of mycoplasmas from the lung by nonspecific defense mechanisms is likely an important determinant in the outcome of *M. pulmonis* infection in mice. C3H/HeN mice did not exhibit significant mycoplasmacidal activity or significant clearance of the organisms from the lungs, and in fact, the mycoplasmas proliferated in the lungs of this mouse strain. In contrast, the C57BL/6N mice had significant mycoplasmacidal activity and clearance of the organisms from the lungs by 4 h after infection. The C57BL/6N mice had slightly higher removal of radiolabel from the lungs than the C3H/HeN mice, but the two strains did not differ in the rate of physical clearance at later times when the differences in mycoplasmal clearance between the two strains were the largest. Thus, physical clearance of the organisms does not have a major role in the strain differences in susceptibility to MRM.

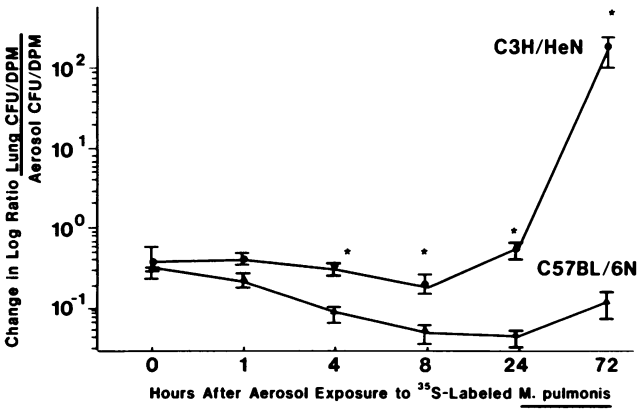

FIG. 2. Mycoplasmal killing. Average log ± SEM (n = 6) of the mycoplasmacidal activity in the lungs of C57BL/6N and C3H/HeN mice exposed to infectious aerosols generated with log 9.99 ± 8.88 CFU of ^{35}S-labeled *M. pulmonis* in the nebulizer.

Even the resistant C57BL/6N mice were not able to entirely eliminate the mycoplasmas from the lungs by 3 days after infection. This suggests that nonspecific mechanisms may not entirely account for the elimination of mycoplasmas from the lung and raises the possibility that specific host immune responses may also play a role in elimination of the organisms from the lungs. In preliminary studies, the C3H/HeN strain appeared to have higher levels of specific anti-*M. pulmonis* immunoglobulin M antibodies in the serum, relative to C57BL/6N mice, by 3 days after infection (data not shown). This strain difference in serum antibody response also occurs at 7 and 14 days after infection (10, 11). Collectively, these findings suggest that the increase in antibody levels is in response to increased numbers of *M. pulmonis* in the lungs of the C3H/HeN mice and that systemic specific anti-*M. pulmonis* antibodies are not likely of primary importance in pulmonary clearance of mycoplasmas. However, this does not eliminate the possibility that local antibody production could be involved.

Even though the C3H/HeN mice had a strong cellular response in the lungs by 72 h, particularly by neutrophils, *M. pulmonis* not only persisted in the lung but actually proliferated. The rapid increase in numbers of organisms after 8 h in this strain was associated with a dramatic increase in the numbers of neutrophils recovered from the lungs. In contrast, the C57BL/6N mice significantly decreased mycoplasmal numbers in the lungs before any significant changes in

TABLE 1. Inflammatory cells recovered from the lungs of C57BL/6N and C3H/HeN mice[a]

Time (h) postinfection	Strain	No. of cells (10^6) (mean ± SEM)			
		Total leukocytes	Lymphocytes (%)[b]	Macrophages (%)	Neutrophils (%)
0	C57BL/6N	3.08 ± 0.51	0.82 ± 0.30 (22)	1.87 ± 0.29 (60)	0.39 ± 0.12 (16)
	C3H/HeN	3.43 ± 0.49	0.63 ± 0.16 (17)	2.37 ± 0.31 (68)	0.43 ± 0.07 (13)
1	C57BL/6N	2.45 ± 0.25	0.57 ± 0.10 (23)	1.64 ± 0.24 (64)	0.25 ± 0.09 (11)
	C3H/HeN	3.35 ± 0.26	0.49 ± 0.14 (16)	2.40 ± 0.34 (69)	0.46 ± 0.11 (13)
4	C57BL/6N	3.33 ± 0.40	0.72 ± 0.11 (21)	2.41 ± 0.33 (71)	0.21 ± 0.03 (7)
	C3H/HeN	3.18 ± 0.31	0.44 ± 0.81 (14)	2.14 ± 0.15 (67)	0.60 ± 0.18 (18)
8	C57BL/6N	4.59 ± 1.50	0.36 ± 0.07 (8)	3.78 ± 0.35 (83)	0.36 ± 0.10 (8)
	C3H/HeN	5.06 ± 0.78	0.42 ± 0.04 (6)	4.30 ± 0.26 (63)	2.20 ± 0.54 (31)
24	C57BL/6N	5.89 ± 0.96	1.52 ± 0.28 (25)	3.57 ± 0.83 (58)	0.80 ± 0.17 (16)
	C3H/HeN	7.11 ± 1.15	1.17 ± 0.26[c] (16)	2.97 ± 0.49 (44)	2.97 ± 0.87 (39)
72	C57BL/6N	4.11 ± 0.65	0.64 ± 0.20 (15)	2.82 ± 0.32 (70)	0.65 ± 0.33 (15)
	C3H/HeN	26.80 ± 2.00[c]	2.60 ± 0.58[c] (9)	6.72 ± 1.31 (25)	17.50 ± 1.50[c] (66)

[a] Mice (n = 6) were infected with aerosols generated with log 9.99 ± 8.88 CFU of *M. pulmonis* per ml in the nebulizer.
[b] Percentage of total leukocyte count.
[c] Significantly different from C57BL/6N.

leukocyte numbers in the lungs. Unlike prior findings indicating the importance of neutrophils in clearance of gram-negative bacteria (27, 32) and higher doses of gram-positive bacteria (23, 31, 34) from the lung, our findings suggest that neutrophils have little impact on clearance of *M. pulmonis* from the lung. Other investigators have also found a similar lack of antimycoplasmal activity by mouse neutrophils in experiments with *M. pulmonis* in vivo (16).

Previous studies in C3H/HeN and C57BL/6N mice used 7- and 14-day periods to demonstrate the strain differences in susceptibility to MRM (10, 11). At both of these times after exposure, one of the striking differences in microscopic lesion severity between the mouse strains is in the amount of leukocytic exudates (composed of neutrophils and macrophages) in the lungs. Also, for most doses of *M. pulmonis*, C3H/HeN mice had significantly higher numbers of organisms in the lungs at both 7 and 14 days after infection. The present studies indicate that the intensity of leukocytic responses of the two mouse strains differs within hours of infection and that reduction of mycoplasmal numbers by nonspecific mechanisms as early as 4 h after infection may be required to prevent the increase in neutrophils in the lungs. Collectively, the findings of increased numbers of mycoplasmas, neutrophils, and lesion severity in the C3H/HeN mice and exactly the opposite findings in C57BL/6N mice strongly suggest that the host response to *M. pulmonis* may be a major determinant in the severity of MRM.

The host mechanisms responsible for clearance of *M. pulmonis* from the lungs of mice soon after infection are unknown at this time. Our studies indicate that it is unlikely that recruited leukocytes or humoral immune responses are responsible for early clearance of *M. pulmonis* from the lungs of mice. Although the absence of mycoplasmal killing in C3H/HeN mice agrees with findings of previous in vitro experiments that demonstrated a lack of activity against *M. pulmonis* by mouse macrophages in the absence of specific serum antibodies (5, 9, 16, 18), killing of *M. pulmonis* by C57BL/6N mice presents a paradox. Pulmonary clearance of bacteria was initially ascribed to the activity of alveolar macrophages (14), but experiments with *Staphylococcus aureus* (21, 29, 30) and *Klebsiella pneumoniae* (32) have recently shown that bactericidal activity in the lungs is not solely dependent on alveolar macrophages alone. Thus, although alveolar macrophages may play a role in clearance of *M. pulmonis* from the lungs, other factors are likely also involved. Other lung defenses that may contribute to this early clearance of *M. pulmonis* from the lungs include alveolar lining material (19, 21, 28), opsonic activity of complement (35), fibronectin (30), serum components (4), natural killer cells (36), and extracellular bactericidal substances (6, 7).

ACKNOWLEDGMENTS

This work was supported by the Public Health Service grant HL 19741 to G.H.C. from the National Institutes of Health. R.F.P. and J.W.S. are supported by Public Health Service training grant 5T32 HL07553 from the National Heart, Lung, and Blood Institute (to G.H.C.).

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