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Differential Growth of *Legionella pneumophila* in Guinea Pig versus Mouse Macrophage Cultures

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***Legionella pneumophila* is a facultative intracellular bacterium which replicated well in inbred guinea pig strain 2 peritoneal macrophages at a low infectivity ratio. In contrast, the growth of this organism was markedly restricted in mouse (BDF_1) peritoneal macrophages, even at a relatively high infectivity ratio. The initial uptake of *L. pneumophila* organisms by macrophages was similar in both animal species, and both groups of macrophages supported the growth of *Listeria monocytogenes*. Treatment of *L. pneumophila* with immune guinea pig serum did not result in restriction of bacterial growth in macrophages, but guinea pig macrophages were readily induced to suppress the growth of *L. pneumophila* by preincubation with supernatants obtained from mitogen-activated normal guinea pig splenocyte cultures. Thus, lymphokines generated from mitogen-stimulated guinea pig lymphocytes induced a restriction of growth of these organisms similar to that observed naturally with macrophages from mice, which are considered highly resistant to these bacteria. Although guinea pigs are considered highly susceptible to *L. pneumophila* infections and mice are considered relatively resistant, the mechanism of this difference is not clear. The results of the present study suggest that the restriction of *L. pneumophila* growth by macrophages relates to host susceptibility to infection and that cell populations permissive for *L. pneumophila* can be transformed to nonpermissive by products from stimulated lymphocytes but not by opsonization with immune serum.**

Legionella pneumophila is a facultative intracellular bacterium, and infections caused by this organism appear to correlate with the cellular immune system of the host (2, 18). In this regard, the importance of macrophages in legionellosis has been suggested by a number of investigators (9, 13, 15, 16). This disease was initially discovered in humans, but guinea pigs have been widely used as an animal model for legionellosis (1, 3, 4, 14). These animals are highly susceptible to infection with various strains of *L. pneumophila*. Although mice have been found to be highly useful for studying infection and immunity to a wide variety of bacteria, this animal species is relatively resistant to *L. pneumophila* infections (14). Furthermore, normal mouse lymphocytes show a high degree of reactivity to *L. pneumophila* antigens, including killed organisms (5, 6). In contrast, lymphocytes from noninfected normal guinea pigs are generally unresponsive to these organisms and their antigens. Such differences between guinea pigs and mice with regard to susceptibility and responsiveness to *L. pneumophila* appear to be valuable in elucidating the mechanisms of infection by these organisms. In the present study, variations in the growth of *L. pneumophila* in macrophage cultures from guinea pigs versus mice are reported, indicating that the different fate of *L. pneumophila* in macrophages from these animal species may correlate with susceptibility to infection by these organisms.

MATERIALS AND METHODS

Animals. Female strain 2 guinea pigs, were obtained from the National Cancer Institute, Frederick, Md., and weighed about 250 to 350 g at the start of each experiment. Inbred strain BDF_1 mice were obtained from Jackson Laboratory, Bar Harbor, Maine, and were 10 to 12 weeks old at the initiation of each experiment. All of the animals were housed and cared for in accordance with National Institutes of Health guidelines.

Bacteria. A virulent strain of *L. pneumophila* serogroup 1 was obtained at autopsy from a patient at Tampa General Hospital and cultured on buffered charcoal-yeast extract agar (GIBCO Diagnostics, Madison, Wis.) exactly as described previously (7). Virulence was maintained by periodic passage through guinea pigs. For in vitro studies, the bacteria were grown on buffered charcoal-yeast extract agar for 48 h at 37°C and harvested into pyrogen-free saline. *Listeria monocytogenes* EGD was used in control experiments. *L. monocytogenes* was maintained in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) at −70°C. Before each experiment, a sample was thawed, inoculated into broth, and incubated for 18 h at 37°C. Both bacteria were adjusted to working concentrations with a spectrophotometer (620 nm).

Collection and cultivation of peritoneal macrophages. Peritoneal exudate cells (PEC) were obtained from guinea pigs or mice 3 to 4 days after intraperitoneal (i.p.) injection with 30 ml (guinea pigs) or 3 ml (mice) of sterile thioglycolate broth (Difco Laboratories, Detroit, Mich.). The PEC were suspended in Dulbecco modified Eagle medium (GIBCO) supplemented with 10% heat-inactivated fetal calf serum (FCS; Hyclone Laboratories, Logan, Utah) or RPMI 1640 medium (GIBCO) supplemented with 15% heat-inactivated FCS. Macrophages were identified by microscopic examination with Camco Quik stain (American Scientific Products, McGaw Park, Ill.). The viability of the cells was determined by their ability to exclude trypan blue stain (GIBCO). The PEC from guinea pigs were found to contain at least 70 to 80% macrophages on the basis of morphology, and more than 95% were viable. The PEC from mice contained 80 to

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TABLE 1. Intracellular fate of *L. pneumophila* and *L. monocytogenes* in macrophages cultivated from guinea pigs and mice[a]

Animals	No. of viable intracellular bacteria/culture[b] at indicated incubation time after phagocytosis			
	L. pneumophila		*L. monocytogenes*	
	Zero time	24 h	Zero time	24 h
Guinea pigs	$5.3 \times 10^3 \pm 0.4$	$2.4 \times 10^4 \pm 0.6$	$1.8 \times 10^3 \pm 0.1$	$1.8 \times 10^4 \pm 0.6$
Mice	$3.7 \times 10^3 \pm 0.5$	$1.9 \times 10^3 \pm 0.5$	$1.6 \times 10^4 \pm 0.4$	$9.2 \times 10^4 \pm 1.5$[c]

[a] PEC from guinea pigs and mice were prepared, washed, and adjusted to 10^6 cells per ml. A total of 10^6 cells were allowed to adhere to tissue culture plates (24 wells) for 2 h in a 5% CO_2 atmosphere at 37°C. The resulting cell monolayers were washed with HBSS and supplied with 500 μl of Dulbecco modified Eagle medium containing 10% FCS. The *L. pneumophila* or *L. monocytogenes* suspension (50 μl) was added to macrophages, resulting in an infectivity ratio of 10^5 bacteria per 10^6 PEC. Phagocytosis was allowed to proceed for 30 min. Cells were then washed with HBSS, supplied with Dulbecco modified Eagle medium containing 10% FCS, and incubated. In the case of *L. monocytogenes*, the culture medium was changed several times for the duration of the experiment.

[b] Data in each experiment represent the average ± standard deviation of determinations for three separate macrophage cultures.

[c] This value was obtained 4 h after phagocytosis.

85% macrophages, and more than 95% were viable. The PEC suspensions were diluted to contain 10^6 morphologically distinct macrophages per ml and allowed to adhere to tissue culture plates (24 wells, 1.6 cm in diameter; Costar, Cambridge, Mass.) for 2 h in 5% CO_2 at 37°C. The resulting cell monolayers were washed with Hanks balanced salt solution (HBSS; GIBCO) and then incubated with 0.5 ml of medium.

Infection and enumeration of cultures. Briefly, macrophage monolayers (approximately 10^6 macrophages per well) were pulsed (50 μl) with different numbers of *L. pneumophila*, yielding infectivity ratios ranging from 65:1 to 0.002:1 (bacteria/macrophages). After 30 min of phagocytosis in a 5% CO_2 atmosphere at 37°C, the monolayers were washed three times with HBSS to remove free bacteria, supplied with 0.5 ml of medium, and incubated for 4 to 72 h at 37°C. Because of the possibility that *L. monocytogenes* would grow in tissue culture medium, the culture medium was replaced several times during the course of incubation. This technique of frequent washing was previously reported (17) to diminish the contribution of extracellular CFU to cell-associated CFU. Following culturing, the monolayers were washed, and the number of viable intracellular bacteria was determined by plate counts of macrophages lysed with sterile distilled water. Cell lysates and culture supernatants (extracellular bacteria) were appropriately diluted with HBSS and plated on buffered charcoal-yeast extract agar plates or Trypticase soy agar plates. The plates were incubated at 37°C for 24 h in the case of *L. monocytogenes* or 72 h in the case of *L. pneumophila* and then counted for CFU.

Preparation of ConA supernatants. Cultures of 5×10^6 normal guinea pig spleen cells in 1.0 ml of RPMI 1640 medium supplemented with 10% FCS and 5×10^{-5} M mercaptoethanol (Sigma Chemical Co., St. Louis, Mo.) were stimulated with 1 to 16 μg of concanavalin A (ConA; Sigma) per ml for 24 h in a 5% CO_2 atmosphere at 37°C. Control spleen cells were incubated in the same way but without ConA. After incubation, the cultures were centrifuged at 200 × *g* for 10 min, and the resultant supernatants were sterilized by filtration with membrane filters (0.2-μm-pore Millex GV; Millipore Corp., Bedford, Mass.). The macrophage monolayers were preincubated with 30% (vol/vol) ConA supernatants for 24 h at 37°C and then infected with *L. pneumophila* (10^5 bacteria per 10^6 macrophages) for 30 min. Control supernatants were spiked with ConA and then added to macrophage cultures. After infection, the monolayers were washed with HBSS and supplied with RPMI 1640 medium supplemented with 15% FCS and 30% supernatants. The fate of *L. pneumophila* in the cultures was determined as described above after 24 to 48 h of incubation in a 5% CO_2 atmosphere at 37°C. All values represent the mean ± standard deviation for at least three separate macrophage cultures per experiment.

Preparation of antiserum. Antiserum to *L. pneumophila* was prepared in guinea pigs. Guinea pigs were immunized by three i.p. injections with sublethal doses (1×10^6, 5×10^6, and 5×10^6 cells) of viable *L. pneumophila* at 4- to 6-week intervals. Blood was obtained from the animals by cardiac puncture at 10 days after the last injection, and the serum was collected and stored at −70°C until used. The serum titer was determined by microagglutination exactly as described previously (19) and was demonstrated to be 640. For opsonization studies, a 50-μl suspension of *L. pneumophila* containing 2×10^5 bacteria was added to 1 ml of antiserum or normal guinea pig serum and incubated for 30 min at 37°C. Macrophage monolayers were then pulsed with the treated bacteria for 30 min. After phagocytosis, the monolayers were washed, supplied with RPMI 1640 medium containing 15% FCS, and incubated for 24 to 48 h in a 5% CO_2 atmosphere at 37°C. As a control, culture medium instead of serum was used for pretreatment of the bacteria.

RESULTS

Growth of *L. pneumophila* and *L. monocytogenes* in cultured macrophages. The fate of *L. pneumophila* in cultures of macrophage monolayers induced by i.p. injection of thioglycolate from guinea pigs or mice was compared with the fate of *L. monocytogenes*. PEC obtained from both animal species were allowed to adhere to 24-well plates and were then infected with either bacterium. The infectivity ratio was 0.1 bacterium per macrophage. *L. monocytogenes* is well known as a typical facultative intracellular bacterium which replicates well in macrophages from both animal species. In experiments in this laboratory, *L. monocytogenes* was similarly found to grow in macrophages from both species (Table 1). However, *L. pneumophila* showed different growth patterns with regard to macrophages from these animals. In guinea pig macrophages, *L. pneumophila* grew at least fourfold in terms of CFU during 24 h of incubation. In mouse macrophages, in contrast, growth was markedly restricted.

Intracellular and extracellular fate of *L. pneumophila* in macrophages from guinea pigs versus mice. The intracellular and extracellular fate of *L. pneumophila* in cultivated guinea pig peritoneal macrophages after phagocytosis was compared with that in mouse peritoneal macrophages. The bacteria were added to the monolayers at infectivity ratios of 0.1 and 0.5 bacterium per macrophage and examined for growth, stasis, or killing at zero time, 1 day, and 2 days after

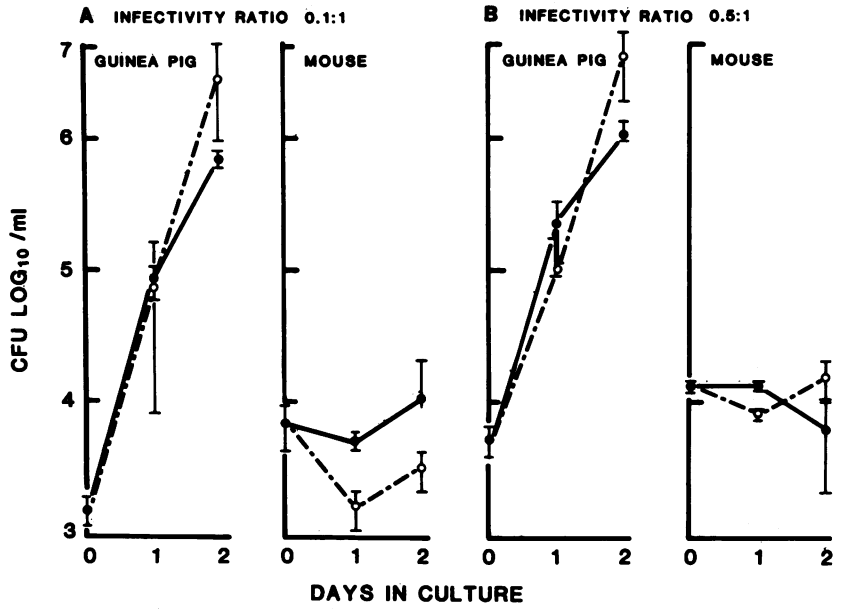

FIG. 1. Intracellular and extracellular fate of *L. pneumophila* in guinea pig and mouse macrophages. The numbers of viable extracellular (○) and intracellular (●) bacteria were determined by plate counts of supernatants of macrophage cultures and macrophage lysates, respectively. Each point represents the mean ± standard deviation for three separate macrophage cultures. Infectivity ratios were 1×10^5 bacteria per 10^6 PEC (A) and 5×10^5 bacteria per 10^6 PEC (B).

phagocytosis was initiated. In a typical experiment (Fig. 1), guinea pig macrophages permitted growth from approximately 10^3 to 10^6 CFU/ml in both the extracellular and intracellular sites at the different infectivity ratios used. In contrast, in mouse macrophages, growth was restricted at both infectivity ratios.

Growth of *L. pneumophila* in guinea pig and mouse macrophages at different infectivity ratios. Figures 2 and 3 show data concerning the fate of *L. pneumophila* in cultured macrophage monolayers from guinea pigs versus mice at different infectivity ratios. Different concentrations of bacteria were added to the macrophage monolayers and, after phagocytosis, washed and incubated for 1 to 5 days at 37°C. The fate of bacteria in cultivated guinea pig peritoneal macrophages is shown in Fig. 2. The bacteria were added to the monolayers at infectivity ratios of 0.002, 0.02, 0.2, and 2 bacteria (i.e., CFU) per macrophage and examined for growth at 1, 2, and 3 days after phagocytosis. At all infectivity ratios, guinea pig macrophages readily supported the growth of *L. pneumophila*. The fate of *L. pneumophila* in cultivated mouse peritoneal macrophages is shown in Fig. 3. The bacteria were added to the macrophages at infectivity ratios of 0.006, 0.06, 0.6, 6.5, and 65 bacteria (CFU) per macrophage. Mouse macrophages restricted growth at an infectivity ratio as high as 65 bacteria per macrophage. At all infectivity ratios, *L. pneumophila* was completely restricted in growth or killed within the macrophages.

Effect of ConA supernatants. The effect of culture supernatants from guinea pig spleen cells activated by ConA on the bactericidal capacity of the macrophage monolayers is shown in Fig. 4. Guinea pig macrophage monolayers were preincubated with 30% ConA supernatants at 37°C for 24 h and then infected with *L. pneumophila*. After 1 day, ConA supernatants inhibited the growth of *L. pneumophila* in the macrophage cultures in a dose-dependent manner. After 2 days, culture supernatants from splenocytes stimulated with 4 or 16 μg of ConA per ml showed marked growth restriction. Growth of the bacteria in the cultures was approxi-

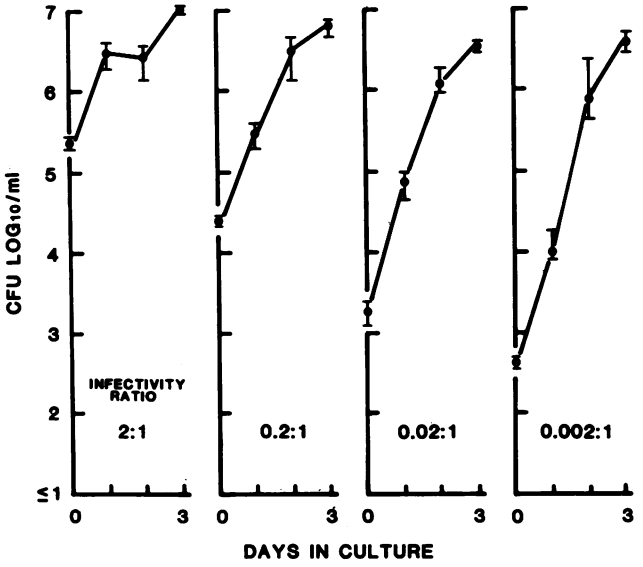

FIG. 2. Fate of *L. pneumophila* in guinea pig macrophages at different infectivity ratios. A total of 10^6 guinea pig macrophages were allowed to adhere to tissue culture plates (24 wells). The resulting cell monolayers were washed with HBSS and supplied with 500 μl of RPMI 1640 medium containing 15% FCS. *L. pneumophila* suspensions were serially diluted from 2×10^6 to 2×10^3 bacteria per 50 μl of saline and added to macrophage monolayers, and phagocytosis was allowed to proceed for 30 min. Cells were then washed with HBSS, supplied with RPMI 1640 medium containing 15% FCS, and incubated. The numbers of viable bacteria in macrophage cultures were determined by plate counts of combined supernatants of macrophage cultures and lysates. Each point represents the mean ± standard deviation for three separate macrophage cultures. Infectivity ratios were 2×10^6 bacteria per 10^6 macrophages (2:1) to 2×10^3 bacteria per 10^6 macrophages (0.002:1).

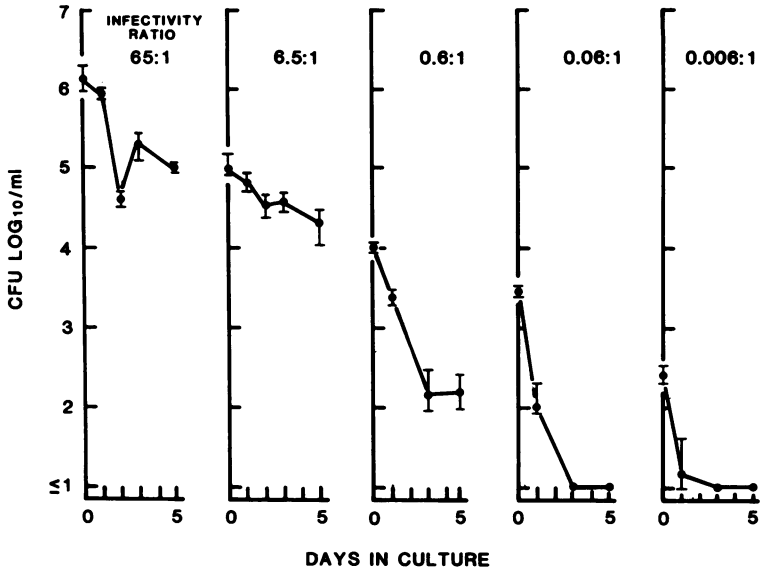

FIG. 3. Fate of *L. pneumophila* in mouse macrophages at different infectivity ratios. See the legend to Fig. 2 for details. Infectivity ratios were 6.5×10^7 bacteria per 10^6 macrophages (65:1) to 6.5×10^3 bacteria per 10^6 macrophages (0.006:1).

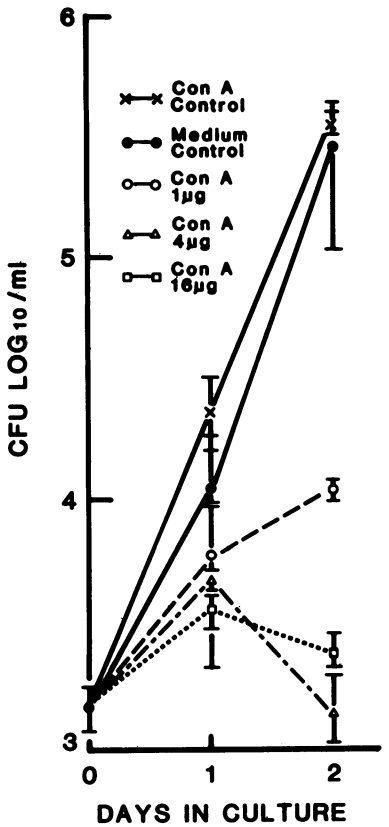

FIG. 4. Effect of culture supernatants from splenocytes stimulated with ConA on the growth of *L. pneumophila* in guinea pig macrophages. Guinea pig macrophages were preincubated with 30% (vol/vol) culture supernatants from guinea pig splenocytes at 37°C for 24 h and then infected with *L. pneumophila* (infectivity ratio, 10^5 bacteria per 10^6 macrophages). Culture supernatants were obtained from 5×10^6 normal guinea pig splenocytes per ml stimulated with ConA (final concentrations, 16, 4, or 1 μg/ml) for 24 h. Control macrophages were incubated with either supernatants from splenocyte cultures from which ConA was omitted (medium control) or supernatants from splenocyte cultures spiked with ConA prior to the addition to macrophages (ConA control). Each point represents the mean ± standard deviation for three separate macrophage cultures.

mately 2 orders of CFU less than that in macrophages treated with control supernatants spiked with ConA.

Opsonization with immune serum. The effect of immune guinea pig serum on the growth of *L. pneumophila* in guinea pig macrophages is shown in Fig. 5. When *L. pneumophila* was pretreated with antiserum, the uptake of bacteria by macrophages was somewhat higher than that in controls treated with normal guinea pig serum. However, antiserum treatment did not induce the restriction of *L. pneumophila* growth in guinea pig macrophages.

DISCUSSION

Guinea pigs have been widely used for experimental infections with *L. pneumophila*, probably because these animals readily develop pulmonary disease symptomatically and histologically similar to that observed in humans and thus appear to be highly susceptible to *L. pneumophila* infections. Mice, on the other hand, are considered to be relatively resistant to disease or death or both following i.p. infection with these bacteria (14). In our experiments, the 50% lethal doses for i.p. infected BDF_1 mice and strain 2 guinea pigs were 5×10^7 and 1×10^6 bacteria per animal, respectively. The mechanism of this difference in susceptibility to *L. pneumophila* infections is not clear. *L. pneumophila* is a facultative intracellular pathogen, and a close relationship between the bacteria and macrophages is evident in vivo in humans as well as in guinea pigs (9). Thus, it is widely assumed that macrophages play a major role in *L. pneumophila* infections and pathogenesis.

L. pneumophila has been reported to multiply in vitro in the presence of human peripheral blood monocytes, human alveolar macrophages, monkey alveolar macrophages, guinea pig macrophages, and other cell lines (10, 13, 15, 16, 20; T. W. Nash, D. M. Libby, and M. A. Horwitz, Program

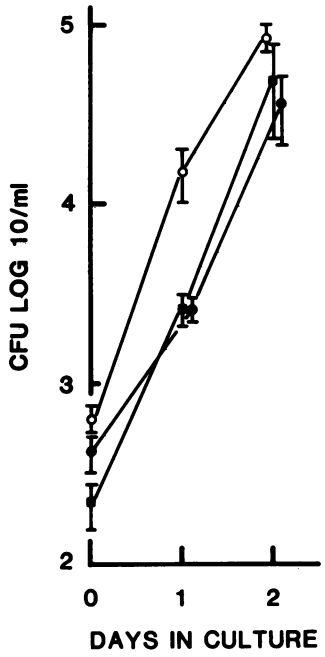

FIG. 5. Effect of specific guinea pig antiserum on the growth of *L. pneumophila* in guinea pig macrophages. One milliliter of fresh antiserum or normal guinea pig serum containing 2×10^5 bacteria was incubated for 30 min at 37°C, and then 50 μl of the preparation was added to macrophage monolayers. The final infectivity ratio was 0.01:1 (bacterium/macrophage). Symbols: ●, nontreated control; ○, *L. pneumophila* treated with immune serum; ■, *L. pneumophila* treated with normal guinea pig serum.

Abstr. 22nd Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 93, 1982). However, to better understand the mechanism involved in susceptibility, it seems important to examine the interaction of these bacteria with macrophages from susceptible and resistant species such as guinea pigs and mice. Recently, Yoshida and Mizuguchi (21) demonstrated in a study comparing various animal species that in the case of guinea pigs versus mice, the permissive nature of the peritoneal macrophages correlated with susceptibility to infection. However, no such association occurred in hamsters. Our results confirm and extend these observations and showed that in inbred guinea pig peritoneal macrophages, *L. pneumophila* grew even when an infectivity ratio as low as 0.002 bacterium per macrophage was used. In contrast, BDF_1 mouse peritoneal macrophages markedly restricted the growth of bacteria at ratios as high as 65:1. Furthermore, the initial uptake of *L. pneumophila* by macrophages from both guinea pigs and mice appeared to be almost the same on the basis of CFU per macrophage protein (data not shown). The failure of *L. pneumophila* to grow in mouse macrophages appears to be somewhat unique in that another facultative intracellular bacterium, *L. monocytogenes* (8), was shown in the present study to grow in association with both guinea pig and mouse macrophage cultures. Also, our data showed that both extracellular growth and intracellular growth of *L. pneumophila* were parallel in both guinea pig and mouse macrophages. Since the culture medium was the same in both cultures, these results suggest that the growth of *L. pneumophila* observed in guinea pig cultures was dependent upon the macrophages rather than the medium. Taken together, these results suggest a fundamental difference between exudate macrophages from guinea pigs and mice in the way they deal with *L. pneumophila* once the microorganism is internalized.

The innate deficiency of guinea pig macrophages was corrected by preincubation with supernatants obtained from ConA-activated guinea pig splenocyte cultures. However, prior treatment of *L. pneumophila* with immune serum containing a specific antibody was not effective in this regard. Horwitz and Silverstein previously obtained similar results with human monocytes in vitro (10–12). The biochemical, morphological, and functional similarities and differences between lymphokine-treated and nontreated guinea pig macrophages and nontreated mouse macrophages are currently under investigation. Horwitz reported that *L. pneumophila* inhibits the fusion of phagosomes with lysosomes in monocytes and suggested that the inhibition of such fusion may be an important mechanism for the survival of *L. pneumophila* in phagocytes (9). Further comparative analysis of permissive and nonpermissive macrophage populations such as that described here should better define other molecular mechanisms responsible for the restriction of *L. pneumophila* growth.

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