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Differential Morphologic and Metabolic Alterations in Permissive versus Nonpermissive Murine Macrophages Infected with *Legionella pneumophila*

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Legionella pneumophila **infection of macrophages from permissive guinea pigs and from A/J mice compared with infection of cells from nonpermissive BDF_1 mice was studied by electron microscopy. The cells from the BDF_1 mice were nonpermissive for legionella growth in vitro and showed few if any bacteria in phagosomes by electron microscopic examination. Similar electron micrographic examination of macrophages from A/J mice permissive for legionella growth showed numerous intact intracellular bacteria within 24 to 48 h of culture and the transition of intracellular bacteria from localization in a few large vacuoles early in the course of infection to later localization in areas surrounded and studded by ribosomes. These electron microscopic observations were similar to those seen in the case of guinea pig macrophages infected with legionellae. Biochemical studies of macrophages from permissive versus nonpermissive animals showed little or no differences in respiratory burst and lysosomal enzyme activity for macrophages from all animals tested. However, when zymosan was used as a stimulant, macrophages from the nonpermissive mouse strain produced a larger amount of H_2O_2 and O_2^- than did cells from permissive guinea pigs or A/J mice. However, legionella vaccine itself induced no detectable or very little H_2O_2 and O_2^- in macrophages tested from any source. These results suggest that permissiveness of A/J mouse macrophages to legionella growth may involve mechanisms similar to those occurring in guinea pig macrophages in terms of morphologic and possibly even biochemical events. The relatively higher production of reactive oxygens by BDF_1 mouse macrophages in response to zymosan correlated with nonpermissiveness for legionella growth, although further analysis is necessary to link these observations.**

Legionella pneumophila is a facultative intracellular bacterium considered the etiologic agent of opportunistic infection, especially pneumonia. Humans appear to be generally resistant to infection by the bacteria. However, active infection has been shown to correlate with the status of a host's cellular immune competence. Nevertheless, these bacteria can replicate readily in all human monocytes as well as in guinea pig macrophages, which are highly susceptible to legionella infection (4, 7–9, 21, 24). Studies from a number of laboratories, including ours, indicated that macrophages from the mouse strains tested did not appear permissive for legionella growth, but recent studies in this laboratory showed that at least one mouse strain, the A/J strain, is permissive for infection with these bacteria and that legionellae readily grow in macrophages from this mouse strain (22).

The importance of macrophages in resistance to legionella infection has been indicated by a number of investigations. We reported that peritoneal macrophages from permissive guinea pigs replicate the organisms readily, and the ability of the bacteria to grow in macrophages from these animals seems related to their immune status (19). In addition, we have shown that peritoneal macrophages from A/J mice are permissive for legionella growth in vitro, but macrophages from other mouse strains are not (22). The mechanisms for such differences are not clear. In this study, we examined morphologic differences between macrophages from a permissive mouse strain and guinea pigs versus a nonpermissive mouse strain, using electron micrographic analysis. Attempts were also made to correlate such permissiveness with metabolic activities of the macrophages.

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MATERIALS AND METHODS

Animals. Inbred A/J and BDF_1 female mice, 6 to 8 weeks old at the initiation of an experiment, were obtained from Jackson Laboratory, Bar Harbor, Maine. Female strain 2 guinea pigs were also used and were obtained from the National Cancer Institute, Frederick, Md. The guinea pigs were approximately 300 to 400 g each at the initiation of an experiment. All animals were housed and cared for according to National Institutes of Health guidelines.

Bacteria. A virulent strain of *L. pneumophila*, serogroup 1, was obtained at autopsy from a case of fatal legionellosis at Tampa General Hospital, Tampa, Fla., and cultured on buffered charcoal yeast extract medium (GIBCO Laboratories, Madison, Wis.) exactly as described previously (6, 19, 21). A formalin-killed legionella vaccine was prepared by using 0.5% formalin, also as described previously (6).

Macrophages. Thioglycolate-elicited peritoneal macrophages were obtained from each animal 4 days after intraperitoneal injection of sterile thioglycolate medium (Difco Laboratories, Detroit, Mich.). The elicited macrophages were suspended in RPMI 1640 medium (GIBCO) supplemented with 15% heat-inactivated fetal calf serum and allowed to adhere to 6- or 24-well tissue culture plates (Costar, Cambridge, Mass.) with or without glass coverslips for 2 h in 5% CO_2 at 37°C. The resulting cell monolayers were washed with Hanks' balanced salt solution (HBSS) and used for the experiments.

Electron microscopy. Macrophage monolayers on glass

coverslips (approximately 10^6 cells) were infected with 1×10^6 to 10×10^6 legionella organisms for 30 min at 37°C and then washed with HBSS to remove nonphagocytized bacteria. The cells were then incubated for various time periods at 37°C in RPMI 1640 medium with 15% fetal calf serum. At appropriate times after incubation, the macrophages on the coverslips were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 h at 4°C. Postfixation was with 1% OsO_4 in 0.1 M phosphate buffer (pH 7.4) for 45 min at 4°C. The cells were then embedded in Epon 812. The coverslips were removed by quick immersion in liquid nitrogen, cut, stained with uranyl acetate and lead nitrate, and examined with an electron microscope (Philips 301).

H_2O_2 assay. Production of hydrogen peroxide (H_2O_2)-mediated and horseradish peroxidase-dependent oxidation by the macrophages was measured by the method of Pick and Keisari (16), which is based on H_2O_2 conversion of phenol red to a product, the absorbance of which was read at 610 nm. Mezerein (Sigma Chemical Co., St. Louis, Mo.) was used as a nonspecific stimulator. Zymosan (Sigma) and legionella vaccine were also used as stimulators.

O_2^- assay. Generation of superoxide anion (O_2^-) was measured by reduction of ferricytochrome *c* (1, 2). In brief, macrophage monolayers in individual wells (approximately 2×10^6 cells per well) were covered with 1.0 ml of an 80 μM solution of ferricytochrome *c* (type III; Sigma) in phenol red-free Earle's balanced salt solution (GIBCO) with or without a stimulant (mezerein, zymosan, or legionella vaccine) and incubated for 2 h at 37°C. After incubation, the culture supernatants were measured in a spectrophotometer at 550 nm against blanks consisting of cytochrome solution from wells without cells. The specificity of cytochrome reduction was controlled by inclusion in some samples of 300 U of superoxide dismutase (type I; Sigma) per ml. H_2O_2 and O_2^- production was expressed as nanomoles per milligram of cell protein.

β-Glucuronidase assay. β-Glucuronidase activity was measured by the method of Fishman et al. (5), with some modifications. As the substrate for the enzyme, 0.03 M phenolphthalein glucuronic acid (Sigma) was used. Cell monolayers (approximately 2×10^6 cells) were lysed with 1.0 ml of distilled water. Lysates were mixed with the substrate in 0.2 M acetate buffer (pH 4.5) and incubated for 60 min at 37°C. The amount of phenolphthalein release was determined with a spectrophotometer at 550 nm after addition of 0.1 M 2-amino-2-methyl-1-propanol buffer (Sigma), pH 11.0. Activity was expressed as the concentration of phenolphthalein per milligram of protein. Protein contents were determined by using the method of Lowry et al. (15), with bovine serum albumin as the standard.

Statistical analysis. Statistical analysis was performed by use of the two-tailed Student *t* test.

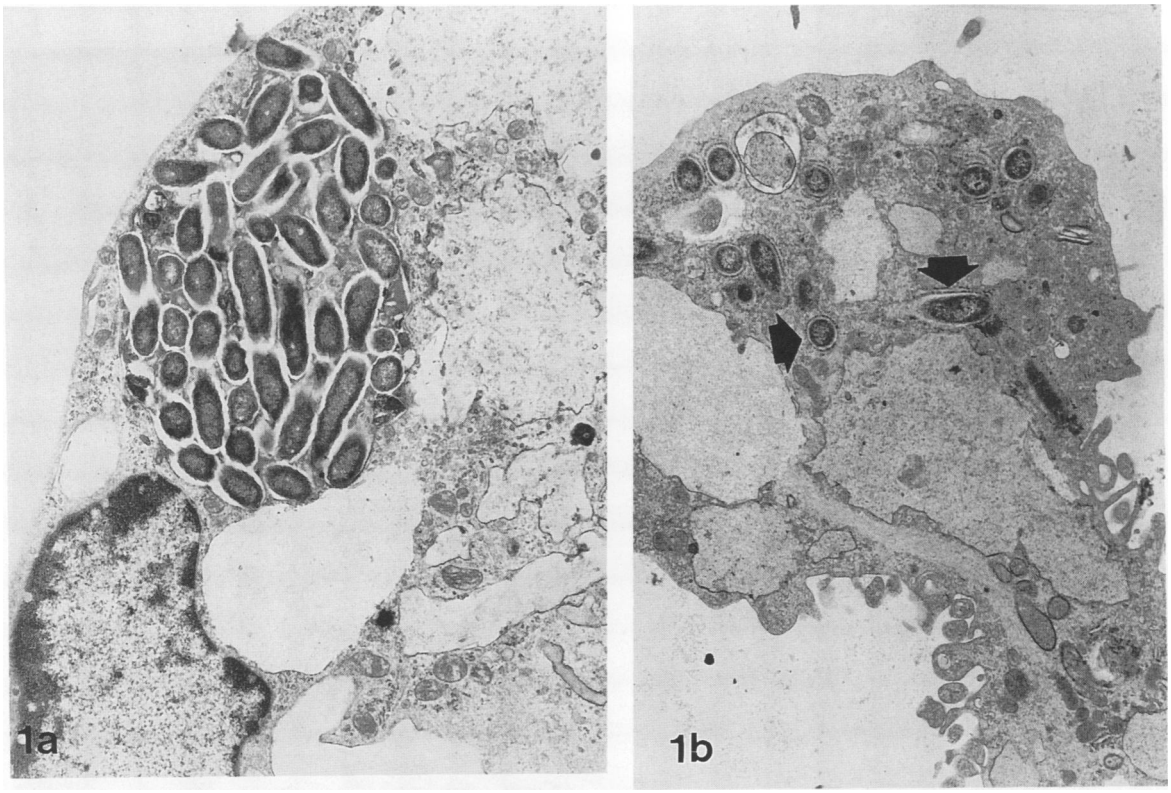
RESULTS

The interaction between legionellae and macrophages from guinea pigs (permissive for legionella growth) versus those from A/J (permissive) or BDF_1 (nonpermissive) mice was examined by electron microscopy. The bacteria grew rapidly in cultures of macrophages from A/J mice (22). As is apparent in Fig. 1, cells from these permissive A/J mice showed numerous bacteria in their cytoplasm. Furthermore, the uptake of legionellae by these macrophages appeared to be due to phagosome formation. After 24 h of incubation, the A/J mouse macrophages contained many endosomal clusters of legionellae contained within a membrane-bound cytoplasmic vesicle (Fig. 1a and b). By 48 h after infection, there was an overwhelming number of bacteria in the cells (Fig. 1c). Most of the bacteria appeared diffused into the cytoplasm within an individual vacuole. At higher-power magnification, legionella organisms were found completely within phagosomes studded by ribosomes.

Similar phagocytosis was noted in the electron micrographs of peritoneal cells from the permissive guinea pigs (Fig. 2). Examination of cell cultures from these guinea pigs showed a rapid increase in the number of intracellular bacteria in the macrophages after infection. In contrast, peritoneal macrophages from the BDF_1 mice showed no evidence of multiplication of bacteria. Macrophages from these nonpermissive mice did not support legionella growth, and only an occasional bacterium was seen within a cell by electron microscopy (Fig. 3). At 48 h after incubation, some phagolysosomes which contained what looked like digested bacteria were observed in the BDF_1 macrophages.

The possible difference in production of H_2O_2 or O_2^- by macrophages from the permissive versus nonpermissive animals stimulated with either nonspecific stimulators or the legionella vaccine was examined. Macrophages from the A/J mice produced relatively low concentrations of H_2O_2 when stimulated with mezerein, but the guinea pig and BDF_1 mouse macrophages produced relatively large amounts of H_2O_2 at nearly similar levels (Table 1). On the other hand, when zymosan was used as the stimulator, the BDF_1 cells produced more H_2O_2 than did the cells from the A/J mice or the guinea pigs. These differences were statistically significant (Table 1). The legionella vaccine did not induce detectable amounts of H_2O_2 in any macrophage culture tested. These findings suggest that H_2O_2 production measured after stimulation with the nonspecific membrane activator mezerein did not correlate with legionella growth in the permissive versus nonpermissive macrophages tested. However, the response of macrophages to zymosan correlated much better with permissiveness of the cells; i.e., relatively higher production of H_2O_2 by the nonpermissive macrophages stimulated with the zymosan was evident. There was no difference in the uptake or phagocytosis of bacteria by the macrophages from the A/J and BDF_1 mice (data not shown).

Differences in O_2^- production by permissive versus nonpermissive macrophages were also examined in cells from guinea pigs and BDF_1 mice (Table 2). There was only a slight to moderate difference in O_2^- production after stimulation with mezerein, but this difference appeared to be marginal and was not statistically significant. Stimulation of the macrophages with zymosan also induced the nonpermissive BDF_1 mouse macrophages to produce moderately larger amounts of O_2^- compared with production by permissive guinea pig cells, but this difference was again marginal. When legionella vaccine was used as a stimulator, the BDF_1 cells produced only a slight amount of O_2^- and the guinea pig cells did not produce a detectable amount (Table 2). β-Glucuronidase activity in the macrophages obtained from guinea pigs versus mice, i.e., either the A/J or BDF_1 strain, was also examined. No significant difference in activity of this enzyme (expressed as mean micrograms of phenolphthalein per milligram of macrophage protein ± standard deviation) was found in cells from the permissive versus the nonpermissive mice (125.0 ± 16.1 for A/J and 178.1 ± 37.3 for BDF_1) or the permissive guinea pigs (125.0 ± 16.1).



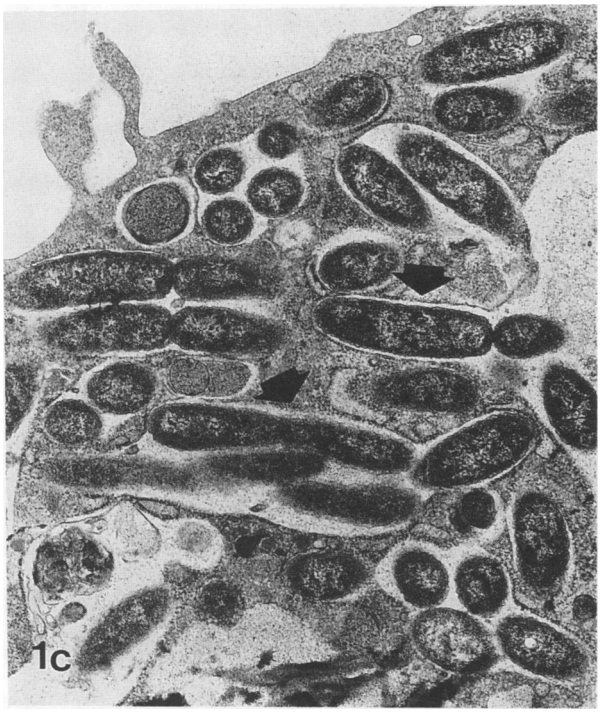


FIG. 1. Electron micrographs of macrophages from A/J mice infected with *L. pneumophila*. (a) 24 h postinfection, showing numerous intracellular bacteria (magnification, ×10,920); (b) 24 h postinfection, showing bacteria (arrows) within vacuoles in ribosome-studded endoplasmic reticulum (magnification, ×8,734); (c) 48 h postinfection, showing bacteria (arrows) within vacuoles surrounded by ribosome-studded endoplasmic reticulum (magnification, ×18,000).

DISCUSSION

We previously reported that elicited peritoneal macrophages from either guinea pigs or A/J mice are permissive for legionella growth but that macrophages from other mouse strains, including BDF_1, DBA/2, C_3H/HeN, C57BL/6, and BALB/c, are nonpermissive, as measured by the CFU assay for viable bacteria replicating in the cells (22). The observation that legionellae do not replicate well in macrophages of most mouse strains has been reported a number of times previously by others and by us. Thus, it is now well accepted that most mouse strains except the A/J strain are generally resistant to infection with these bacteria. In contrast, guinea pigs are considered susceptible to infection with virulent as well as less virulent strains of legionellae. The differences in permissiveness of guinea pigs versus mice in replicating these bacteria in macrophages appears to correlate well with susceptibility versus resistance of the intact animal to infection by these organisms. Thus, permissive and nonpermissive macrophages are now available for comparative legionella studies. Especially, innate permissive and nonpermissive macrophages from A/J and BDF_1 mice are useful for analysis of the interaction between legionellae and macrophages. Recently our own and other studies have demonstrated differences in genetic background of permissive versus nonpermissive mice, which we used to examine mouse macrophage growth of legionellae (20, 23). However, there was little or no information available on possible mechanisms concerning permissiveness of these mouse macrophages for legionellae, except for this study on morphologic and biochemical differences in legionella responses by permissive versus nonpermissive macrophages.

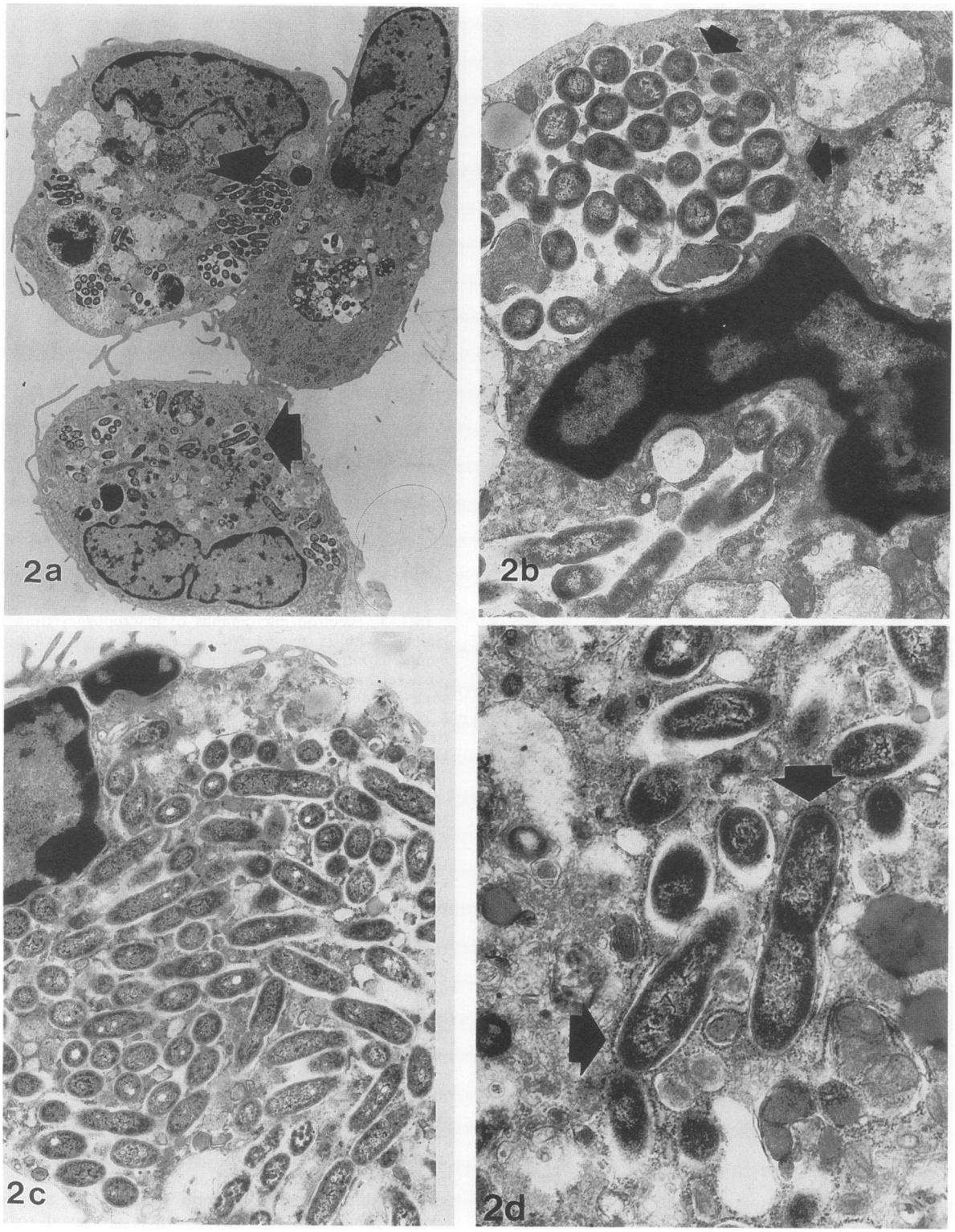


FIG. 2. Electron micrographs of guinea pig infected macrophages showing ingested and replicating *L. pneumophila*. (a) 24 h postinfection, showing clusters of bacteria (arrows) in endosomes (magnification, ×3,000); (b) 24 h postinfection, showing clusters of bacteria (arrows) in endosomes (magnification, ×18,000); (c) 48 h postinfection, showing a diffuse pattern of bacteria within the cell (magnification, ×13,200); (d) 48 h postinfection, showing bacteria enclosed in vacuoles in the rough endoplasmic reticulum (arrows) (magnification, ×22,800).

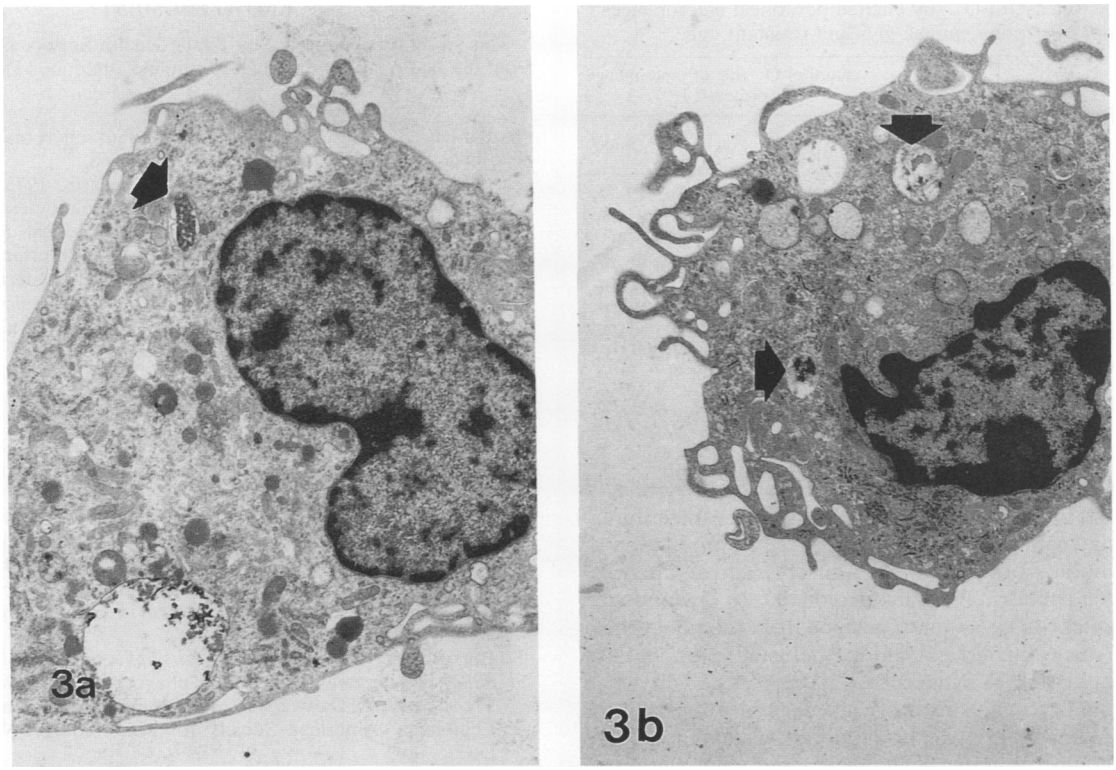


FIG. 3. Electron micrographs of macrophages from BDF_1 mouse infected with *L. pneumophila*. (a) 24 h postinfection, showing bacteria enclosed in vacuoles (arrow) (magnification, ×9,260); (b) 48 h postinfection, showing phagolysosomes (arrows) with digested bacteria (magnification, ×7,200).

In this study, we used electron microscopy to examine morphologic differences in permissive (guinea pig and A/J mouse) and nonpermissive (BDF_1 mouse) macrophages with regard to legionella growth. Results of this study support our previous conclusions based on assay for viable bacteria by colony count analysis. Electron micrographs of macrophages from the BDF_1 mice after legionella infection showed only a few organisms, most of which seemed to be digested by the cells. In contrast, macrophages from permissive guinea pigs exhibited many bacteria within the cells, and by 48 h, these cells contained numerous bacteria. Macrophages from A/J mice also showed marked permissiveness for growth of the bacteria, and within 48 h of infection, many of the cells contained numerous bacteria. Furthermore, the electron microscopic results of this study indicated a transient and early localization of legionellae in a few large vacuoles early in the course of infection of macrophages from permissive guinea pigs and A/J mice. Within a short time thereafter, the bacteria appeared to be completely surrounded by a membrane studded with ribosomes. Similar observations were first reported by Horwitz and Silverstein, using peripheral blood monocytes from humans infected with legionellae (9). Thus, these morphologic studies of permissive mouse macrophages, in comparison with study of permissive guinea pig macrophages, indicated that the permissive macrophages from either animal source show generally similar morphologic events during the course of infection, regardless of species.

Correlative biochemical studies indicated that macrophages from the nonpermissive mouse strain (i.e., BDF_1 mice) produced more H_2O_2 in response to zymosan than did macrophages from legionella-permissive animals (i.e.,

TABLE 1. Release of H_2O_2 by elicited peritoneal macrophages from guinea pigs and mice

Stimulating agent	Concn	nmol of H_2O_2/mg of macrophage protein/2 h[a]		
		Guinea pig strain 2	Mouse strain	
			A/J	BDF_1
None		≤15	34.4 ± 3.4	43.5 ± 9.3
Mezerein	50 ng/ml	615.3 ± 73.3	167.6 ± 19.1[b]	402.3 ± 117.5
Zymosan	0.5 mg/ml	90.5 ± 40.2	90.5 ± 7.9	393.3 ± 131.7[c]
Legionella vaccine	10^8 cells/ml	≤15	≤15	≤15

[a] Macrophage monolayers (2×10^6 cells) incubated with or without stimulating agent for 2 h at 37°C. Data represent means ± standard deviations of three to five experiments.
[b] $P < 0.02$ compared with guinea pigs.
[c] $P < 0.02$ compared with A/J mice.

TABLE 2. Release of O_2^- by elicited peritoneal macrophages from susceptible guinea pigs and resistant mice

Stimulating agent	Concn	nmol of O_2^-/mg of macrophage protein/2 h[a]	
		Guinea pig strain 2	BDF_1 mice
None		≤4.0	≤4.0
Mezerein	50 ng/ml	92.5 ± 61.0	168.1 ± 45.9
Zymosan	0.5 mg/ml	64.3 ± 4.2	117.2 ± 25.4
Legionella vaccine	10^8 cells/ml	≤4.0	8.0 ± 4.6

[a] Macrophage monolayers (2×10^6 cells) incubated with or without stimulating agent for 2 h at 37°C in HBSS containing 80 μM ferricytochrome *c*. Data represent means ± standard deviations of three experiments; there was no significant difference between guinea pigs and BDF_1 mice.

guinea pigs and A/J mice). However, when mezerein, a diterpene ester considered a nonspecific membrane stimulator (17), was used, there was no difference in production of these metabolites between the permissive and nonpermissive animals. The formalin-killed legionella vaccine induced a slight amount of O_2^- in macrophages from the nonpermissive BDF_1 mice but did not induce detectable amounts of H_2O_2 in cells from A/J mice or guinea pigs. Even when viable legionella organisms were used as a stimulator for H_2O_2 production, there was no induction of this metabolite (data not shown). Whether legionellae can induce the respiratory burst in macrophages is still unclear because the phenol red oxidation method for H_2O_2 detection and the cytochrome *c* reduction method used for O_2^- detection are not considered highly sensitive as detection methods for intracellular release of H_2O_2 and O_2^-. These differences between macrophages from legionella-susceptible versus -nonsusceptible mice did not appear to be due to differences in phagocytic capability of the cells, since phagocytosis of legionellae was generally similar.

Oxygen-dependent and -independent components, including the key oxygen metabolites, are considered important in basic antimicrobicidal activity and macrophage activation (16, 19, 21). For example, Jacobs et al. reported that the legionella-killing activity of primate alveolar cells was related to oxygen metabolites (10). Locksley et al. also reported the susceptibility of legionellae to oxygen-dependent microbicidal systems (14). These observations suggest the possibility that oxygen-dependent microbicidal mechanisms serve as a restriction mechanism for legionella growth in nonpermissive cells. However, reactive oxygen-independent mechanisms may still be involved. Recent studies by us and by others showed that gamma interferon-induced resistance to legionella growth in permissive macrophages such as human monocytes or A/J macrophages does not seem to be due to a reactive oxygen-dependent antimicrobial system (3, 13). However, it is not clear whether gamma interferon-induced nonpermissiveness of A/J macrophages or human monocytes is similar to the nonpermissive nature of BDF_1 mouse macrophages. However, further studies to examine metabolic events associated with susceptibility versus resistance of macrophages to legionella infection are now possible with the availability of permissive and nonpermissive strains of mice, and correlation of metabolic events with susceptibility of macrophages to infection is now readily feasible.

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