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Intraphagocytic Growth Induces an Antibiotic-Resistant Phenotype of *Legionella pneumophila*

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The antimicrobial susceptibilities of *Legionella pneumophila* isolates grown either in U937 human monocytic cells or in *Acanthamoeba polyphaga* were studied after release from the host cells without further subculture. Time-survival studies showed that exposure of *L. pneumophila* cells, grown exclusively in vitro, to 5 μg of rifampin per ml resulted in at least 99.9% killing after 6 h and no detectable survivors at 24 h. Similar rates of killing were observed for in vitro-grown cells tested by exposure to ciprofloxacin. Conversely, time-survival studies revealed that macrophage-grown and amoeba-grown cells were ca. 1,000-fold more resistant to the activities of both drugs. Macrophage-grown cells treated with 5 μg of rifampin per ml showed 70 and 62% survival after 6 and 24 h, respectively. Intracellularly grown legionellae were also highly resistant to erythromycin (8 μg/ml). After 24 h of exposure to the drug, there was 70 and 60% survival for amoeba-grown and macrophage-grown legionellae, respectively, whereas in vitro-grown cells showed a 2-$\log_{10}$ reduction in viable count. When intracellularly grown *L. pneumophila* cells were subcultured in broth for 48 h, they reverted to the phenotype characteristic of in vitro growth. Morphologically, the cells were larger than their intracellularly grown counterparts and resistance characteristics were lost. The susceptibilities of the subcultured cells to all three drugs were similar to those of *Legionella* cells grown exclusively in vitro. In view of these findings, the successful treatment of Legionnaires' disease may be related as much to the resistance phenotype induced by intramacrophage growth as to the ability of the antibiotic to enter phagocytic cells.

Legionella pneumophila, the causative organism of Legionnaires' disease, can infect and kill specific species of amoebae in aquatic environments and can multiply as an intracellular parasite in human phagocytic cells (3). Infection results from the inhalation of bacteria, some of which may have previously grown inside a protozoal host. The ability of *L. pneumophila* to multiply within and kill alveolar macrophages is a major virulence determinant (27). This intracellular niche affords protection from the host's humoral response and against some antibiotics which are effective in vitro but not in vivo. Clinical experience has shown that the most effective treatment for Legionnaires' disease is with antibiotics capable of accumulating in phagocytic cells, such as erythromycin and rifampin (10). However, antibiotic treatment is not always successful when these drugs are used (29), and in vitro studies with *L. pneumophila*-infected macrophages have shown that although erythromycin and rifampin inhibited the growth of *L. pneumophila*, when the drugs were removed, the intracellular bacteria resumed multiplication (28). Further studies with *L. pneumophila*-infected macrophages have confirmed that although erythromycin (20, 43) and also ciprofloxacin (20) inhibited intracellular growth, after removal of the extracellular antimicrobial agents, regrowth occurred.

It is not clear what nutrient limitations are imposed on legionellae as they grow intracellularly, but it has been suggested that iron availability may regulate intracellular growth in human monocytes stimulated with gamma interferon (15, 16) and in murine macrophages (24). It is also suggested that intracellular growth of legionellae may be regulated by the availability of the amino acid tryptophan (25). Whatever nutrient deprivations the organism encounters as a result of intracellular growth, these deprivations likely induce physiological changes in the cell. It is well known that bacteria grown under nutrient-restricted conditions have altered phenotypic properties, which affect the organisms response to host defenses and susceptibility to antimicrobial compounds (11, 13, 14). We have previously shown that intra-amoebic growth has a profound effect on the physiological properties of *L. pneumophila*, affecting its response to biocides (5) and its surface properties (8). The purpose of the investigation described here was to examine the antibiotic susceptibilities of *L. pneumophila* isolates grown either in *Acanthamoeba polyphaga* or in undifferentiated U937 human monocytes immediately upon release from the host cells without prior subculture in vitro. The U937 cell line has been shown by other investigators to support the growth of virulent strains of *L. pneumophila* (30, 35, 36) and to have properties similar to those of human alveolar macrophages (41).

MATERIALS AND METHODS

Organisms and cultivation. A virulent strain of *L. pneumophila* serogroup 1 (subgroup Knoxville), the causative organism in an outbreak of Legionnaires' disease in Stafford, England (2), was used throughout the study (available from Legionella Reference Laboratory, Central Public Health Laboratory, Colindale, London, United Kingdom). The strain was stored at −70°C as described previously (9), and recovery was achieved by growth on buffered charcoal-yeast extract (BCYE) agar supplemented with L-cysteine, α-ketoglutarate, and ferric pyrophosphate (19). To prepare inocula for antimicrobial studies, *L. pneumophila* was grown in yeast extract (YE) broth (7) by shaking cultures at 35°C. Growth was monitored spectrophotometrically at A_{660} until the stationary phase (ca. 72 h), when the cells were harvested by centrifuging (2,080 × *g* for 30 min) and were resuspended in fresh YE broth to give ca. 10^6 cells per ml.

A. polyphaga amoebae were obtained from T. Rowbotham, Leeds Public Health Laboratory, Leeds, United Kingdom. This strain was associated with an outbreak of Legionnaires' disease in the United Kingdom caused by *L. pneumophila* serogroup 1. The amoebae were maintained axenically at 35°C in PYG broth (39) as monolayers in 75-cm^2 tissue culture flasks (5).

Intra-amoebic culture of *L. pneumophila*. Intra-amoebic cultivation and har-

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vesting of *L. pneumophila* cells were achieved as described previously (5). Essentially, *L. pneumophila* cells were grown in YE broth, and water-washed cells (10^7 CFU/ml) were inoculated into amoebic saline (39) suspensions of *A. polyphaga* (10^5 trophozoites per ml). Cultures were monitored by phase-contrast microscopy, which indicated the presence of infective, highly motile, intra-amoebic legionellae after 3 days of incubation at 35°C. The coculture was vortexed for 1 min to release the legionellae from the amoebic. Legionellae were harvested from the suspension by centrifugation initially at 400 × *g* for 6 min at room temperature to deposit the amoebic cells and the cell debris. The bacterial cells in the supernatant were deposited by further centrifugation (2,080 × *g* for 15 min at room temperature) and were then washed in amoebic saline. The resulting bacterial suspension, containing ca. 10^8 bacteria per ml, was found by phase-contrast microscopy to be free of amoebic cells or fragments. *L. pneumophila* cells were routinely passaged in amoebic saline cocultures without subculture in vitro to produce bacteria characteristic of intra-amoebic growth.

Growth of *L. pneumophila* in U937 cells. Undifferentiated U937 monocytic cells were maintained in replicative, nonadherent cultures in HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffered RPMI medium (Sigma) supplemented with heat-inactivated fetal calf serum (10%; vol/vol) and glutamine (4 mM). The cell lines were incubated in a humid atmosphere with 5% CO_2. The origin of the U937 cell line has been described previously (38). For coculture, U937 cells were centrifuged (400 × *g* for 5 min) and were resuspended in fresh RPMI medium to give a suspension containing about 10^5 cells per ml, as determined with a hemocytometer. YE broth cultures of *L. pneumophila* were centrifuged, washed, and resuspended in RPMI broth to give ca. 10^9 cells per ml. The U937 cell lines were inoculated to give a ratio of approximately 100 legionellae per monocyte. Preliminary experiments had shown that this level of inoculum resulted in the presence of small, highly motile legionellae within the cytoplasm of the host cells after 4 days of incubation at 35°C in 5% CO_2. The intracellularly grown bacteria were passaged through the U937 cell lines on five separate occasions before they were used for the antibiotic assays. Harvesting of the intracellular bacteria was carried out as described for the amoeba-grown legionellae. Previous studies have revealed that in cocultures with either *A. polyhaga* (5) or in U937 cell lines (35), *L. pneumophila* grows intracellularly and not in the suspending menstruum.

Antimicrobial susceptibility. The MICs of erythromycin, ciprofloxacin, and rifampin were determined by serial (one-half) dilution of the compounds in YE broth and inoculation with *L. pneumophila* (10^5 cells per ml). The tubes were assessed for visible signs of growth after incubation for 48 h at 35°C, and the MIC was determined as the lowest concentration of antibiotic that prevented growth. MICs were also determined with *L. pneumophila* cells (10^5 cells per ml) that had been grown either in amoebic or in U937 cells without prior subculture in vitro.

Time-survival data were determined for variously grown *L. pneumophila* suspensions in YE broth by exposure of 10^6 cells per ml to erythromycin (8 μg/ml), rifampin (5 μg/ml), or ciprofloxacin (1 μg/ml). The bacterial inoculum was obtained by diluting standardized cell suspensions (ca. 10^8 cells per ml) prepared by using McFarland opacity tubes. Aliquots (0.1 ml) of suspensions were taken at the commencement of the exposure and at 6 and 24 h. Serial dilutions were made in amoebic saline, and survival was assessed by spreading the dilutions onto the surface of predried BCYE agar plates. BCYE agar has previously been shown to be a suitable medium for survival studies with antibiotics because it inhibits the activities of compounds such as rifampin and ciprofloxacin (6). Colony counts were determined after 9 days of incubation at 35°C, and the results were expressed as percent survival. The minimum number of detectable bacteria was 10 CFU/ml.

RESULTS

The MICs of the antibiotics for in vitro-, amoeba-, and macrophage-grown *L. pneumophila* cells determined in YE broth after 48 h of incubation were identical. The MICs of erythromycin, ciprofloxacin, and rifampin were 0.25, 0.06, and 0.008 μg/ml, respectively. These results are within the MIC ranges of these drugs for *L. pneumophila* reported previously (6, 23, 43).

The relative susceptibilities of YE broth-, macrophage-, and amoeba-grown *L. pneumophila* cells to rifampin, determined by time-kill studies, are shown in Fig. 1. The activity of rifampin at 5 μg/ml, a therapeutic concentration of the drug (42), was markedly bactericidal against cells grown exclusively in vitro, resulting in >99.9% killing after 6 h and no detectable survivors at 24 h. Conversely, intracellularly grown *L. pneumophila* cells were ca. 1,000-fold more resistant to the activity of rifampin; 62% of macrophage-grown cells and 71% of amoeba-grown cells survived after 24 h of exposure. Broadly similar results were obtained with ciprofloxacin (1 μg/ml). In vitro-grown cells were rapidly killed after 6 h of contact, and there

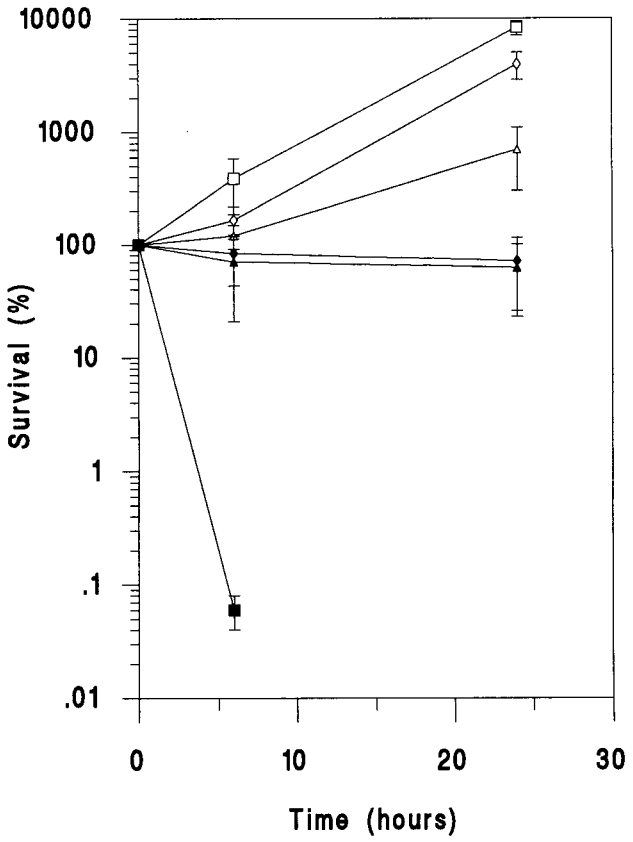

FIG. 1. Survival of *L. pneumophila* cells after growth in YE broth (■), passage through U937 cells (▲), or passage through amoebic (◆) after exposure to rifampin (5 μg/ml) at 35°C in YE broth. Results for controls to which antibiotic was not added are also shown (□, △, and ◇, respectively). Bars represent the standard errors of the means for three replicate experiments.

were few detectable survivors at 24 h, whereas 10% of macrophage-grown cells and 6% of amoeba-grown cells survived at 24 h (Fig. 2). Intracellularly grown legionellae were also profoundly resistant to erythromycin (8 μg/ml). After 24 h of exposure to the drug, there was 70 and 60% survival for amoeba-grown and macrophage-grown legionellae, respectively, whereas in vitro-grown cells showed a 2-$\log_{10}$ reduction in viable count (Fig. 3).

Time-survival studies against rifampin were repeated on macrophage-grown *L. pneumophila* cells after subsequent incubation in YE broth at 35°C. When the cells were tested after 24 h of growth in broth, there was a notable decrease in the resistance of the organism, with 99% killing, after exposure to the drug for 24 h. For the same batch of cells tested immediately upon release from the macrophages only 30% killing was shown. However, when macrophage-grown legionellae were grown in YE broth for 48 h and then tested against rifampin, there were no detectable survivors after 24 h of exposure to the drug. Thus, the cells had the level of susceptibility associated with cells grown in vitro. Similarly, the resistance of intracellularly grown legionellae to erythromycin and ciprofloxacin was lost when the cells were subcultured in broth for 48 h.

Microscopic observations revealed that when macrophage-grown legionellae, measuring ca. 1.0 by 0.5 μm, were subcultured in YE broth for 24 h at 35°C, there were profound changes in their morphologies. It was noted that none of the cells demonstrated the rapid darting motility characteristic of

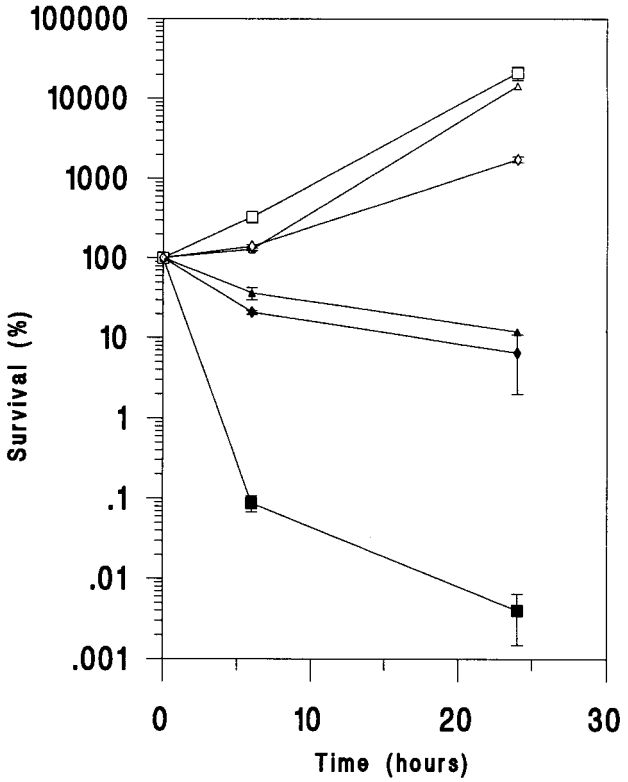

FIG. 2. Survival of *L. pneumophila* cells after growth in YE broth (■), passage through U937 cells (▲), or passage through amoebic (◆) after exposure to ciprofloxacin (1 μg/ml) at 35°C in YE broth. Results for controls to which antibiotic was not added are also shown (□, △, and ◇, respectively). Bars represent the standard errors of the means for three replicate experiments.

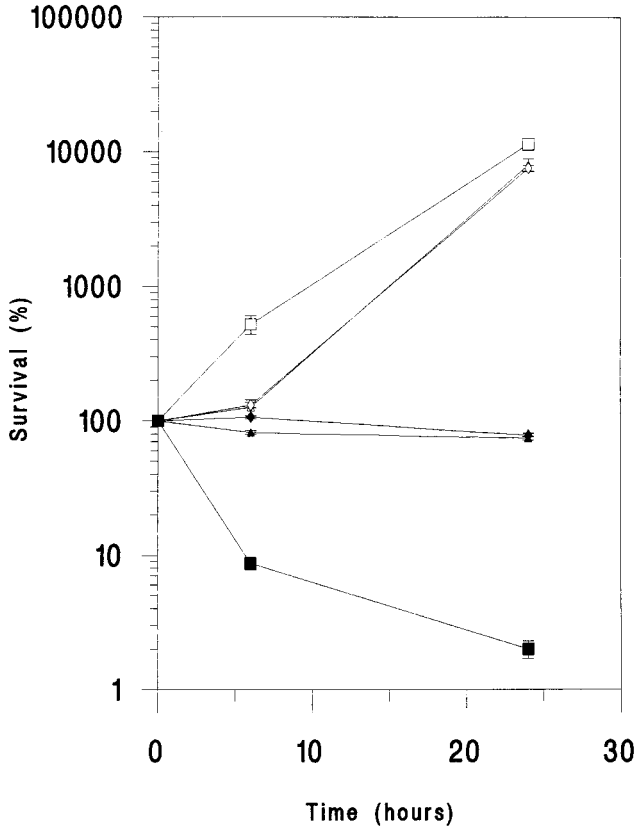

FIG. 3. Survival of *L. pneumophila* cells after growth in YE broth (■), passage through U937 cells (▲), or passage through amoebic (◆) after exposure to erythromycin (8 μg/ml) at 35°C in YE broth. Results for controls to which antibiotic was not added are also shown (□, △, and ◇, respectively). Bars represent the standard errors of the means for three replicate experiments.

intracellular growth. The bacteria were larger than their in vivo-grown counterparts, with individual cells being ca. 2.0 by 0.5 μm, i.e., the morphological phenotype typically associated with broth-grown cells. By continuing the incubation for 48 h, longer cells and filaments of up to 20 μm were observed.

DISCUSSION

There have been various reports of the effectiveness of erythromycin in killing *L. pneumophila* within phagocytes grown in tissue culture systems (20, 23, 28, 43). In general, conclusions that an antibiotic is bactericidal against intracellular *L. pneumophila* have been based on the absence of regrowth or very slow regrowth of the bacterium after drug removal from tissue culture wells of infected phagocytes and continued incubation (20, 21, 28, 43). Although such studies demonstrate the ability of the antibiotic to penetrate and concentrate within various types of phagocytic cells, i.e., macrophage-like cells (e.g., U937 cells) or professional phagocytic cells, they do not give any indication of the level of resistance of the intracellular bacterium. By separating the small, highly motile legionellae from macrophages after completing intracellular growth, it is possible to determine the physiological properties of the cells as they are influenced by intracellular environmental conditions (5). It is clear from the present study that growth within both macrophages and amoebic induces a phenotype in which the cells have similar morphological characteristics, i.e., small and highly motile, with increased levels of resistance to antimicrobial compounds compared with the level of resistance of cells grown in vitro. Previous morphological studies of *L. pneumophila* grown in *Acanthamoeba* spp. (5, 18, 39, 40) have revealed the presence of small flagellated cells, in contrast to in vitro growth, which results in cells which are predominantly nonmotile and often filamentous (7, 37). Similarities in the morphologies and ultrastructures of *L. pneumophila* grown in amoebic and in human monocytes have also been noted (18, 32).

In a previous study (20) antibiotics were found to be less effective at killing intracellular bacteria when the antibiotics were added to infected macrophages 1 day after inoculating the macrophages with in vitro-grown *L. pneumophila* than when the antibiotics were added to the infected macrophages 2 h after inoculating the macrophages with the organism. This difference in response to antibiotics may be related to the change in phenotype as intracellular growth progresses. We have previously shown that the response of *L. pneumophila* cells to inactivation by various biocides is also greatly affected by the phenotype induced by intra-amoebic growth and results in a ca. 1,000-fold increase in the level of resistance compared with that of cells grown in vitro (5). The reason for this increase in resistance is not clear, but the growth of intracellular bacteria may be less optimal than that of in vitro-grown bacteria and may be controlled by limited access to essential nutrients such as iron (15, 16). There are a number of clinical situations in which the bacteria are in a nearly dormant state (17), and such cells with slow growth or zero growth rates have been shown to be less susceptible to antimicrobial agents (11, 22, 26). Because the intracellular growth of *L. pneumophila*

appears to result in increased levels of resistance to antimicrobial agents in general, we have speculated that the intracellular environment induces the formation of cells in a hypometabolic state (4). The intra-amoebic growth of *L. pneumophila* increases the production of poly-β-hydroxybutyric acid (PHB) (40), and the formation of reserves such as PHB is a noted characteristic of some bacterial species in a dormant state, as is the formation of small cells (31, 33). We were the first to report that the growth of *L. pneumophila* in amoebic also affects the surface properties of the organism by altering proteins, lipopolysaccharides, and fatty acid content (8). The expression of new proteins in amoeba-grown *L. pneumophila* cells has also been noted by Cirillo et al. (18). Macrophage-grown *L. pneumophila* cells also show changes in protein composition compared with that of cells grown in vitro (34). It has been suggested that changes in the surface composition of pathogenic bacteria may cause alterations in the ability of antibacterial molecules to cross the cell envelope (1, 12). Whatever the reasons for the decrease in susceptibility of intracellularly grown *L. pneumophila*, after subculture of the organisms in broth for 48 h the cells reverted to the susceptible phenotype associated with in vitro growth. Because the MICs for in vitro-grown and intracellularly grown *L. pneumophila* cells were the same, we believe that this reflects the phenotypic changes taking place as the organism responds to growth in YE broth. Thus, intracellular growth induces resistance in *L. pneumophila* which is lost when the cells are incubated in nutrient-rich medium. It is therefore important that intracellularly grown bacteria be used to test the efficacies of antimicrobial agents against *L. pneumophila*.

Because some antibiotics may only inhibit, rather than kill, intracellular legionellae, the ability of the host's immune system to identify and destroy infected phagocytic cells probably has a central role in determining the outcome of legionella infections (44). It is probably for this reason that Legionnaires' disease patients with impaired cell-mediated immunity are often more prone to protracted illness and show higher mortality rates (29) even when they are treated with antibiotics. However, in view of our findings, successful treatment of Legionnaires' disease may be related as much to the resistance phenotype induced by intramacrophage growth as to the ability of the antibiotic to enter phagocytic cells.

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