APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1997, p. 1219–1224 Vol. 63, No. 4
0099-2240/97/$04.00+0

Multiplication of Different *Legionella* Species in Mono Mac 6 Cells and in *Acanthamoeba castellanii*

B. NEUMEISTER,[1,2]* S. SCHÖNIGER,[1] M. FAIGLE,[2] M. EICHNER,[3] AND K. DIETZ[3]

Abteilung Medizinische Mikrobiologie und Hygiene, Universität Ulm, 89081 Ulm,[1] Abteilung Transfusionsmedizin, Universität Tübingen, 72076 Tübingen,[2] and Institut für Medizinische Biometrie, Universität Tübingen, 72070 Tübingen,[3] Germany

Received 15 October 1996/Accepted 14 January 1997

Survival and distribution of legionellae in the environment are assumed to be associated with their multiplication in amoebae, whereas the ability to multiply in macrophages is usually regarded to correspond to pathogenicity. Since most investigations focused on *Legionella pneumophila* serogroup 1, we examined the intracellular multiplication of different *Legionella* species in Mono Mac 6 cells, which express phenotypic and functional features of mature monocytes, and in *Acanthamoeba castellanii*, an environmental host of *Legionella* spp. According to the bacterial doubling time in Mono Mac 6 cells and in *A. castellanii*, seven clusters of legionellae could be defined which could be split further with regard to finer differences. *L. longbeachae* serogroup 1, *L. jordanis*, and *L. anisa* were not able to multiply in either *A. castellanii* or Mono Mac 6 cells and are members of the first cluster. *L. dumoffi* did not multiply in Mono Mac 6 cells but showed a delayed multiplication in *A. castellanii* 72 h after infection and is the only member of the second cluster. *L. steigerwaltii*, *L. gormanii*, *L. pneumophila* serogroup 6 ATCC 33215, *L. bozemanii*, and *L. micdadei* showed a stable bacterial count in Mono Mac 6 cells after infection but a decreasing count in amoebae. They can be regarded as members of the third cluster. As the only member of the fourth cluster, *L. oakridgensis* was able to multiply slightly in Mono Mac 6 cells but was killed within amoebae. A strain of *L. pneumophila* serogroup 1 Philadelphia obtained after 30 passages on SMH agar and a strain of *L. pneumophila* serogroup 1 Philadelphia obtained after intraperitoneal growth in guinea pigs are members of the fifth cluster, which showed multiplication in Mono Mac 6 cells but a decrease of bacterial counts in *A. castellanii*. The sixth cluster is characterized by intracellular multiplication in both host cell systems and consists of several strains of *L. pneumophila* serogroup 1 Philadelphia, a strain of *L. pneumophila* serogroup 2, and a fresh clinical isolate of *L. pneumophila* serogroup 6. Members of the seventh cluster are a strain of agar-adapted *L. pneumophila* serogroup 1 Bellingham and a strain of *L. pneumophila* serogroup 1 Bellingham which was passaged fewer than three times on BCYEα agar after inoculation and intraperitoneal growth in guinea pigs. In comparison to members of the sixth cluster, both strains showed a slightly enhanced multiplication in Mono Mac 6 cells but a reduced multiplication in amoebae. From our investigations, we could demonstrate a correlation between prevalence of a given *Legionella* species and their intracellular multiplication in Mono Mac 6 cells. Multiplication of members of the genus *Legionella* in *A. castellanii* seems to be dependent on mechanisms different from those in monocytes.

Members of the genus *Legionella* are facultative intracellular bacteria which are able to multiply within human mononuclear phagocytes (22). The ability to multiply is usually regarded to correspond to pathogenicity (8, 14, 23, 29, 38, 40). *Legionella pneumophila* (predominantly serogroup 1) is detected in most cases of legionellosis, while other species can mainly be found in the aquatic environment and only occasionally cause infections (4, 13, 16, 27). The differences in prevalence may result from environmental factors, bacterial virulence, and host factors. In addition, the clinical significance of species other than *L. pneumophila* may be underestimated if diagnosis is based only on serology, since the majority of serological tests are directed against *L. pneumophila.*

Since growth of legionellae in the absence of protozoa has not been documented, it is generally accepted that the number of legionellae in an environmental source results from the number of protozoa and the capability of a given *Legionella* species to multiply within (1, 17–19, 21, 31). On the other hand, differences in pathogenicity should also contribute to the prevalence of a given *Legionella* species. To answer the question whether the reported differences in prevalence can be partially explained by these environmental and bacterial factors, we tested several *Legionella* species with regard to multiplication within protozoa and to pathogenicity. As a representative of an environmental host, *Acanthamoeba castellanii* was used. The measure of pathogenicity was multiplication within a macrophage-like cell line. We used the Mono Mac 6 cell line instead of the histiocytic lymphoma cell line U937 or the promyelocytic leukemia cell line HL-60 (24, 29, 33) since Mono Mac 6 cells represent a more mature macrophage-like cell line which expresses phenotypic and functional properties of mature monocytes and which does not need to be stimulated by phorbol esters or 1,25-dihydroxyvitamin D_3 (24, 29, 33, 41).

MATERIALS AND METHODS

***Legionella* bacteria.** *L. pneumophila* serogroup 1 Philadelphia ATCC 33152 (isolated from human lung), *L. pneumophila* serogroup 1 Bellingham ATCC 43111 (isolated from human lung), *L. pneumophila* serogroup 2 Togus ATCC 33154 (isolated from human lung), *L. pneumophila* serogroup 6 Chicago ATCC 33215 (isolated from human lung biopsy), *L. gormanii* ATCC 32979 (isolated from soil of a creek bank), *L. bozemanii* ATCC 33217 (isolated from lung tissue), *L. longbeachae* serogroup 1 ATCC 33462 (isolated from human lung), *L. dumoffii* ATCC 33279 (isolated from a cooling tower), *L. micdadei* ATCC 33218 (isolated from human blood via yolk sac), *L. anisa* ATCC 35292 (isolated from tap water), *L. steigerwaltii* ATCC 35302 (isolated from tap water), *L. jordanis* ATCC 33623

* Corresponding author. Mailing address: Abteilung Transfusionsmedizin, Hoppe-Seyler-Strasse 3, Universität Tübingen, 72076 Tübingen, Germany. Phone: 0049 7071 296658. Fax: 0049 7071 296593.

(isolated from Jordan River), and *L. oakridgensis* ATCC 33761 (isolated from industrial cooling tower water) were obtained from the American Type Culture Collection (ATCC).

All ATCC strains were obtained after an unknown number of passages. They were grown on BCYEα agar (Oxoid, Wesel, Germany) at 35°C in 3% CO_2 for 3 to 5 days.

The following strains were used as controls: a strain of *L. pneumophila* serogroup 1 and a strain of *L. pneumophila* serogroup 6 which were isolated from patients with acute pneumonia and passaged less than three times on BCYEα agar; a strain of *L. pneumophila* serogroup 1 Philadelphia and a strain of *L. pneumophila* serogroup 1 Bellingham (ATCC 43111) which were passaged fewer than three times on BCYEα agar after inoculation and intraperitoneal growth in guinea pigs as described by Elliott and Johnson (15) (kindly supplied by P. C. Lück and J. H. Helbig, University of Dresden, Dresden, Germany); a strain of *L. pneumophila* serogroup 1 Philadelphia (ATCC 33152) which was passaged six times in MRC-5 cells as described by Wong et al. (36, 37); a strain of *L. pneumophila* serogroup 1 Philadelphia (ATCC 33152) obtained after 30 passages on Mueller-Hinton agar (Oxoid) supplemented with 0.025% ferric citrate and 0.025% cysteine (SMH agar) as described by Catrenich and Johnson (6); a Mip-negative mutant of *L. pneumophila* serogroup 1 Philadelphia (ATCC 33152; kindly donated by J. Hacker, University of Würzburg, Würzburg, Germany) (35).

Mono Mac 6 cells. Mono Mac 6 cells were kindly donated by H. W. L. Ziegler-Heitbrock (University of Munich). They were cultured as replicative nonadherent monocytes under lipopolysaccharide-free conditions in 250-ml flasks (Nunc, Roskilde, Denmark) in 50 ml of RPMI 1640 medium (Gibco, Eggenstein, Germany) supplemented with 10% selected fetal calf serum (Myoclone plus; Gibco), 2 mM L-glutamine (Gibco), 1 mM pyruvic acid (Fluka, Buchs, Switzerland), 1% nonessential amino acids (Gibco), 9 μg of insulin (Sigma, Munich, Germany) per ml, and 1 mM oxalacetate (Sigma; Mono Mac 6 medium) at 35°C in 5% CO_2 as described by Ziegler-Heitbrock et al. (42) and diluted 1:3 twice a week in fresh medium.

A. castellanii (ATCC 30234) was grown in 250-ml flasks (Nunc) in 50 ml of PYE broth [2% proteose peptone no. 3 (Difco, Detroit, Mich.), 0.1% yeast extract (Difco), 0.1 M glucose, 4 mM $MgSO_4$, 0.4 M $CaCl_2$, 0.1% sodium citrate dihydrate, 0.05 mM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 2.5 mM NaH_2PO_3, 2.5 mM K_2HPO_3 (Sigma; pH 6.5)] at 35°C in 5% CO_2 as described by Moffat and Tompkins (26) and diluted 1:2 twice a week.

Intracellular multiplication. Nonadherent Mono Mac 6 cells were harvested by centrifugation at 400 × *g* for 10 min. The pellet was washed twice in Mono Mac 6 medium without fetal calf serum (Mono Mac coincubation medium [MCM]). Legionellae were harvested from BCYEα agar, suspended in MCM, and adjusted to an optical density at 578 nm of 0.2 (spectrophotometer 1101 M [Eppendorf, Hamburg, Germany) corresponding to a concentration of approximately 3×10^8 legionellae per ml. For concentration, bacteria were centrifuged at 3,000 × *g* for 10 min. Mono Mac 6 cells (2×10^7) were pelleted and resuspended with 2×10^9 legionellae in a volume of 1.5 ml in a well of a six-well tissue culture plate (Nunc) to provide a bacterium-to-cell ratio of 100:1. Cocultures were then incubated at 35°C in 5% CO_2 for 2 h. After this period, nonphagocytized bacteria were killed by the addition of 4.5 ml of MCM containing 100 μg of gentamicin per ml for 1 h at 35°C in 5% CO_2. After three washes by centrifugation at 400 × *g* for 10 min, the cells were resuspended in 10 ml of MCM and distributed in 1-ml aliquots into the wells of a 24-well tissue culture plate (Nunc), giving a concentration of 2×10^6 infected Mono Mac 6 cells per well. This time point was defined as 0. The cells were then incubated for an additional 72 h at 35°C in 5% CO_2. Every 24 h, the contents of two wells were aspirated and pelleted by centrifugation at 400 × *g* for 10 min. Supernatant was transferred to a sterile tube. One milliliter of sterile distilled water was added to the pellet, and final disruption of the cells was performed by aspirating the suspension through a 27-gauge needle. Supernatant and lysis fluid were pooled, and serial 10-fold dilutions were made. One hundred microliters of each dilution was inoculated onto BCYEα agar to determine the number of viable legionellae after multiplication in Mono Mac 6 cells. Colonies on the agar were counted on day 5 after incubation at 35°C in 5% CO_2.

As a control, 1 ml of the original legionella suspension in MCM but without cells was incubated in a well of the tissue culture plate and serial 10-fold dilutions were made at the same time points as those indicated above.

The viability of Mono Mac 6 cells was determined by trypan blue exclusion at the same time points as those indicated above.

Coculture with *A. castellanii* was performed in an identical manner except that legionellae were suspended in amoebae buffer (PYE broth without glucose) and the culture was incubated for 96 h.

To compare the intracellular growth kinetics of *L. pneumophila* in Mono Mac 6 cells with the intracellular bacterial growth in more traditional cells, adherent human blood mononuclear cells of healthy blood donors were infected with *L. pneumophila* serogroup 1 Philadelphia (ATCC 33152) as described by Horwitz (22) with an infectivity ratio of 100 legionellae per monocyte. The number of intracellular bacteria was determined every 24 h as described above. All experiments were done at least in triplicate.

Statistical analysis of data. Based on the maximum-likelihood method, assuming a Poisson distribution, the resulting number of bacteria in each experiment was estimated by dividing the sum of bacterial counts for different dilutions by the sum of dilution factors. The estimates obtained had a log normal distribution. The whole set of data was then subjected to an analysis of covariance with the continuous factor of time and the nominal factors of species, host, and number of experiment together with the appropriate interaction terms. The model provides estimates for the growth rates which are specific for bacterial species and host cells. A dendrogram summarizing the results of a cluster analysis was calculated by the average linkage method of Sokal and Michener (32). The linear model provides estimates for the standard errors of the growth rates. These were used to exhibit 95% confidence ellipses for the growth rates. Statistical analysis was performed with the statistics package JMP, version 3.1.5.

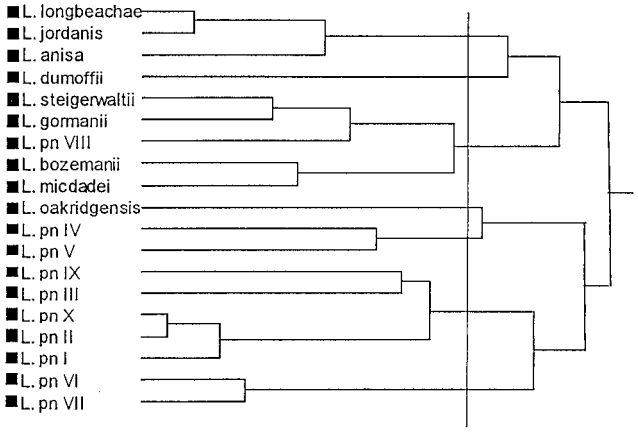

FIG. 1. Dendrogram calculated from the growth rates of different *Legionella* species in Mono Mac 6 cells and in *A. castellanii*. The vertical line determines the subgrouping into seven clusters. Abbreviations: L. pn I, *L. pneumophila* serogroup 1 Philadelphia (ATCC 33152); L. pn II, *L. pneumophila* serogroup 2 Togus (ATCC 33154), fresh clinical isolate of *L. pneumophila* serogroup 6; L. pn III, *L. pneumophila* serogroup 1 isolated from a patient with acute pneumonia, passaged fewer than three times on BCYEα agar; L. pn IV, *L. pneumophila* serogroup 1 Philadelphia (ATCC 33152) obtained after 30 passages on Mueller-Hinton agar supplemented with 0.025% ferric citrate and 0.025% cysteine (SMH agar); L. pn V, *L. pneumophila* serogroup 1 Philadelphia passaged fewer than three times on BCYEα agar after inoculation and intraperitoneal growth in guinea pigs; L. pn VI, *L. pneumophila* serogroup 1 Bellingham (ATCC 43111) passaged fewer than three times on BCYEα agar after inoculation and intraperitoneal growth in guinea pigs; L. pn VII, *L. pneumophila* serogroup 1 Bellingham (ATCC 43111); L. pn VIII, *L. pneumophila* serogroup 6 Chicago (ATCC 33215); L. pn IX, Mip-negative mutant of *L. pneumophila* serogroup 1 Philadelphia (ATCC 33152); L. pn X, *L. pneumophila* serogroup 1 Philadelphia (ATCC 33152) passaged six times in MRC-5 cells.

RESULTS

According to the bacterial doubling time in Mono Mac 6 cells and in *A. castellanii*, seven clusters of legionellae which could be split further with regard to finer differences could be defined (Fig. 1 and 2). *L. longbeachae* serogroup 1, *L. jordanis*, and *L. anisa*, which were not able to multiply in either *A. castellanii* or Mono Mac 6 cells, are members of the first cluster. *L. dumoffii* did not multiply in Mono Mac 6 cells but showed a delayed multiplication in *A. castellanii* 72 h after infection and is the only member of the second cluster. *L. steigerwaltii*, *L. gormanii*, *L. pneumophila* serogroup 6 ATCC 33215, *L. bozemanii*, and *L. micdadei* showed more or less constant bacterial counts in Mono Mac 6 cells after infection but decreasing counts in amoebae, and thus they form the third cluster. As the only member of the fourth cluster, *L. oakridgensis* was able to multiply slightly in Mono Mac 6 cells but was killed within amoebae. A strain of *L. pneumophila* serogroup 1 Philadelphia (ATCC 33152) obtained after 30 passages on SMH agar and a strain of *L. pneumophila* serogroup 1 Philadelphia obtained after intraperitoneal growth in guinea pigs are members of the fifth cluster, which showed multiplication in Mono Mac 6 cells but a decrease of bacterial counts in *A.*

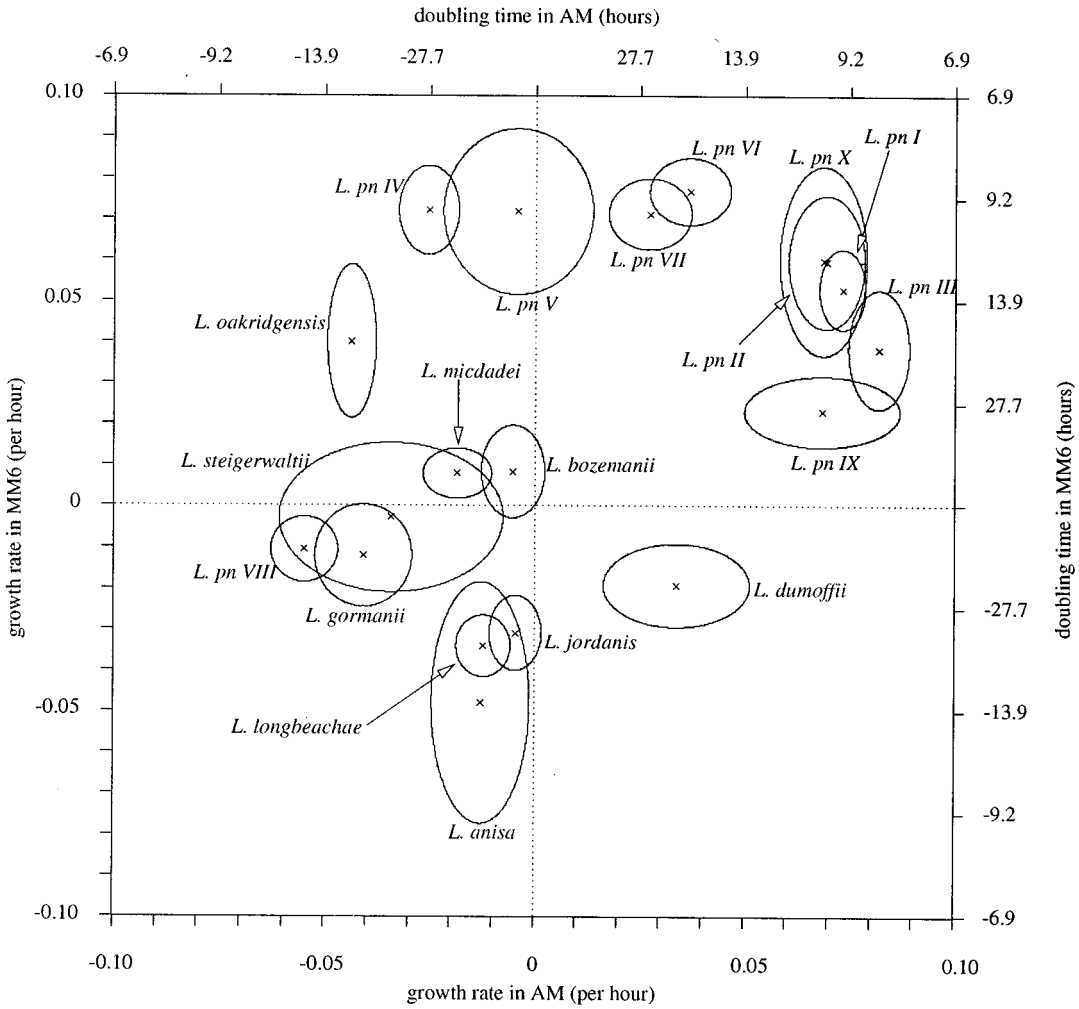

FIG. 2. Ninety-five percent confidence ellipses of growth rates of different *Legionella* species in Mono Mac 6 cells (MM6) and in *A. castellanii* (AM). Results are expressed as doubling times of bacterial counts (in hours) and as exponential growth rates (per hour). For the definition of L. pn I to X, see the legend of Fig. 1.

castellanii. The sixth cluster is characterized by intracellular multiplication in both host cell systems and consists of *L. pneumophila* serogroup 1 Philadelphia (ATCC 33152), *L. pneumophila* serogroup 2 Togus (ATCC 33154), a strain of *L. pneumophila* serogroup 1 and a strain of *L. pneumophila* serogroup 6 isolated from patients with acute pneumonia and passaged fewer than three times on BCYEα agar, a strain of *L. pneumophila* serogroup 1 Philadelphia (ATCC 33152) which was passaged six times in MRC-5 cells, and a Mip-negative mutant of *L. pneumophila* serogroup 1 Philadelphia (ATCC 33152). From 72 to 96 h, colony counts of *L. pneumophila* increased 100- to 200-fold in Mono Mac 6 cells and nearly 1,000-fold in *A. castellanii*. In Mono Mac 6 cells, *L. pneumophila* serogroup 1 Philadelphia (ATCC 33152) showed a growth rate of 0.053 per h with a 95% confidence interval between 0.044 and 0.062. In adherent human blood mononuclear cells of healthy blood donors, the same strain showed a growth rate of 0.051 per h with a 95% confidence interval between 0.037 and 0.064. Peak bacterial concentrations were observed after 24 to 48 h in Mono Mac 6 cells and after 72 h in amoebae. *L. pneumophila* serogroup 2 and a fresh clinical isolate of *L. pneumophila* serogroup 6 showed identical growth characteristics which were equivalent to those of *L. pneumophila* serogroup 1. In Mono Mac 6 cells, a strain of *L. pneumophila* serogroup 1 isolated from a patient with acute pneumonia which was passaged fewer than three times on BCYEα agar multiplied less than the *L. pneumophila* serogroup 1 Philadelphia (ATCC 33152) strain which was passaged several times on BCYEα agar. In *A. castellanii*, both strains showed nearly identical multiplication rates. Passage of *L. pneumophila* serogroup 1 Philadelphia (ATCC 33152) in MRC-5 cells did not sufficiently increase the ability of this strain to multiply within Mono Mac 6 cells or *A. castellanii*. In contrast, the Mip-negative mutant showed a delayed multiplication within Mono Mac 6 cells but not within *A. castellanii*. Members of the seventh cluster are a strain of *L. pneumophila* serogroup 1 Bellingham (ATCC 43111) which was passaged several times on BCYEα agar and a strain of *L. pneumophila* serogroup 1 Bellingham (ATCC 43111) which was passaged fewer than three times on BCYEα agar after inoculation and intraperitoneal growth in guinea pigs. In comparison to *L. pneumophila* serogroup 1 Philadelphia (ATCC 33152), *L. pneumophila* serogroup 2 Togus (ATCC 33154), and the fresh clinical isolate of *L. pneumophila* serogroup 6, both strains in cluster 7 dem-

onstrated a slightly enhanced multiplication in Mono Mac 6 cells but a reduced multiplication in amoebae.

DISCUSSION

In this study, different *Legionella* species were investigated with respect to their intracellular multiplication within Mono Mac 6 cells and within *A. castellanii*. *L. pneumophila* serogroups 1, 2, and 6, the most common causes of Legionnaires' disease, were the only strains which multiplied in both cell types. This corresponds well to prevalence and clinical importance (16). Since the use of Mono Mac 6 cells to study intracellular growth kinetics of legionellae was not reported in any previous publication, the intracellular growth of *L. pneumophila* serogroup 1 Philadelphia (ATCC 33152) in Mono Mac 6 cells was compared with the bacterial growth of this strain in adherent human blood mononuclear cells of healthy blood donors by using the traditional method described by Horwitz (22). Mono Mac 6 cells supported the growth of *L. pneumophila* serogroup 1 in the same manner as adherent blood mononuclear cells did. Since Mono Mac 6 cells are expressing phenotypic and functional properties of mature monocytes, do not need to be stimulated by phorbol esters or 1,25-dihydroxyvitamin D_3 to become capable of phagocytosis (41, 42), and, in comparison to blood monocytes, show an identical regulation of intracellular growth of *L. pneumophila*, this cell line can be used as a constant host cell model for the study of intracellular multiplication or growth restriction of *Legionella* species.

BCYEα agar-adapted strains of *L. pneumophila* serogroup 1 (Philadelphia and Bellingham) showed intracellular multiplication rates in Mono Mac 6 cells and in *A. castellanii* comparable to corresponding strains which were passaged fewer than three times on BCYEα agar after inoculation and intraperitoneal growth in guinea pigs (15), after passage in MRC-5 cells (36, 37), or after isolation from a case of legionellosis—measures that are commonly used to obtain virulent strains of *L. pneumophila*. This result supports the observation of Yamamoto et al. that virulence of *L. pneumophila* is maintained after multiple passages on BCYEα agar (39). As opposed to this, a strain of *L. pneumophila* serogroup 1 Philadelphia which was obtained after 30 passages on SMH agar (6) was able to multiply in Mono Mac 6 cells but failed to grow in *A. castellanii*. In contrast to BCYEα agar, this suboptimal medium seems to exclusively affect the capability of *L. pneumophila* to grow within *A. castellanii*. This result is only partly in concordance with the observations of Moffat and Tompkins (26) and Fields et al. (17, 19), who found an impaired multiplication of *L. pneumophila* in macrophages as well as in the amoebae *Tetrahymena pyriformis*, *Hartmanella vermiformis*, and *A. castellanii* after multiple transfers on Mueller-Hinton agar. The cause of this phenomenon is not clear, but it seems not to be induced by an impaired function of the Mip protein, since in our study a Mip-negative mutant failed to replicate inside Mono Mac 6 cells but was able to grow in *A. castellanii*. This is supported by the results of Ciancotto et al., who deleted the 24-kDa Mip protein by side-specific mutagenesis and found that, compared with the isogenic parent, the mutant was significantly impaired in its ability to infect transformed U937 cells as well as human alveolar macrophages (8) and was less virulent in guinea pigs via the intratracheal route of infection (9). Unlike our results with *A. castellanii*, this mutant also showed a delay of intracellular multiplication in *H. vermiformis* and *T. pyriformis* (10), amoebae that are known to support the intracellular growth of legionellae. Since the ability of members of the genus *Legionella* to invade host cells, multiply intracellularly, and cause cell damage is regarded to correlate directly with the virulence of a given strain (8, 23, 29, 38, 40), Mip-negative *L. pneumophila* is not avirulent with respect to the intracellular growth in *A. castellanii* and only partially attenuated with respect to the multiplication in Mono Mac 6 cells, since in the latter cells they show only a delay in intracellular multiplication.

L. oakridgensis was able to multiply slightly in Mono Mac 6 cells but was killed within amoebae, whereas *L. dumoffii* showed a delayed multiplication in *A. castellanii* 72 h after infection but was killed in Mono Mac 6 cells. *L. micdadei*, *L. bozemanii*, *L. pneumophila* serogroup 6 ATCC 33215, *L. gormanii*, *L. steigerwaltii*, *L. longbeachae* serogroup 1, *L. jordanis*, and *L. anisa* were not able to multiply in either *A. castellanii* or Mono Mac 6 cells. Most of these species are regarded to be less pathogenic to humans; however, *L. micdadei* and *L. bozemanii* can cause legionellosis in patients undergoing immunosuppressive therapy, and *L. pneumophila* serogroup 6 is occasionally detected in patients with legionellosis (16, 27). Whereas *L. micdadei* and *L. bozemanii* showed a limited tendency to multiply within Mono Mac 6 cells, which can explain their clinical importance, *L. pneumophila* serogroup 6 ATCC 33215 was not able to multiply. The latter strain seems to be attenuated through a cause as yet unknown since a fresh clinical isolate of *L. pneumophila* serogroup 6 showed an intracellular multiplication in both host cell systems identical to that of *L. pneumophila* serogroups 1 and 2. The ability of *L. oakridgensis* to multiply within Mono Mac 6 cells leads to the assumption that this species may be of greater clinical importance than previously known, whereas the exclusive multiplication of *L. dumoffii* in *A. castellanii* may be a sign of low human virulence and specialized adaptation to an amoebal host.

Only a few publications describe the investigation of species other than *L. pneumophila* in cell culture or amoebae. In an early report, Weinbaum et al. (34) demonstrated the multiplication of *L. micdadei* in human peripheral monocytes by using a bacterium-to-cell ratio of 1:1. When the input multiplicity was greater, no intracellular growth occurred. Fields et al. (18) investigated an *L. anisa* strain that was isolated from an indoor fountain implicated in an outbreak of Pontiac fever. This strain was able to multiply in the simultaneously isolated amoeba *H. vermiformis*, which seemed to be its natural host in the fountain, but it failed to infect guinea pigs and did not multiply in other amoebae or in human mononuclear cells, suggesting that different *Legionella* species use certain amoeba species as hosts to multiply within. Moffat and Tompkins (26) found that *L. pneumophila* serogroup 1 as well as *L. dumoffii* was able to replicate in *A. castellanii* and in U937 cells at 37°C, whereas, in comparison to these pathogens, *L. feeleii* demonstrated a markedly reduced multiplication rate. Fields et al. (17) examined 17 strains of *Legionella* including 11 species and found that *L. pneumophila*, *L. micdadei*, *L. oakridgensis*, and *L. hackelia* multiplied in *T. pyriformis* and killed guinea pigs. *L. dumoffii*, *L. gormanii*, and *L. bozemanii* were able to multiply in the amoeba and to infect guinea pigs but could not kill them. *L. feeleii* could multiply in amoebae and was lethal to guinea pigs only at high concentrations. *L. longbeachae* serogroup 1 and *L. anisa* could neither multiply in amoebae nor infect or kill guinea pigs.

With the exception of the *L. pneumophila* serogroup 6 ATCC 33215 strain, in this study, we were able to demonstrate a correlation between pathogenicity of a given *Legionella* species (4, 13, 16, 27) and its intracellular multiplication in Mono Mac 6 cells. Our results indicate that different mechanisms are involved in invasion and intracellular multiplication of legionellae in amoebae and macrophages. This point of view is

supported by experiments of Abu-Kwaik et al. (1), who found that inhibitors of eukaryotic protein synthesis, such as cycloheximide and emetine, had no effect on the uptake of *L. pneumophila* by macrophages but completely abolished ingestion by the amoeba *H. vermiformis*. They concluded that host cell protein synthesis was required for this bacterium to infect the amoebae but not to infect human macrophages. In addition, different amoeba species may support the intracellular growth of different *Legionella* species.

Many *Legionella* species and serogroups of *L. pneumophila* investigated in this study express factors which are regarded to be associated with virulence such as macrophage infectivity potentiator (Mip) (7, 20), major outer membrane protein (5), flagella (3), zinc metalloprotease (30), and the serogroup- and species-specific lipopolysaccharide (11, 12, 28). The presence of a *dot* or *icm* gene locus in *Legionella* species other than *L. pneumophila* remains to be demonstrated (2, 25). Thus, the differences in intracellular multiplication of legionellae in Mono Mac 6 cells and/or *A. castellanii* demonstrated in this study cannot be explained by possible differences in expression of these virulence factors. More than one virulence factor may be necessary to induce pathogenicity for human beings.

In this study, we could establish the suitability of a new macrophage-like cell line (Mono Mac 6) for investigation of intracellular multiplication of different *Legionella* species. Correlation between the degree of intracellular replication in Mono Mac 6 cells and the reported clinical prevalence of a given *Legionella* species could be demonstrated. The results were compared with the ability of these *Legionella* strains to multiply within *A. castellanii*. Multiplication of members of the genus *Legionella* in *A. castellanii* seems to be partly dependent on mechanisms different from those in monocytes.

Mono Mac 6 cells represent a more mature macrophage-like cell line which expresses phenotypic and functional properties of mature monocytes and which does not need to be stimulated by unphysiological stimuli like phorbol esters or 1,25-dihydroxyvitamin D_3. They support the intracellular replication of *L. pneumophila* and are able to discriminate between species of high and low human virulence by showing different rates of intracellular multiplication. Therefore, this cell line may be useful for further investigations to characterize virulence factors and pathogenesis of *Legionella* infections.

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