INTERACTIONS OF *LISTERIA MONOCYTOGENES*, *LISTERIA SEELIGERI*, AND *LISTERIA INNOCUA* WITH PROTOZOANS

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Cocultures of three *Listeria* species, *L. monocytogenes*, *L. seeligeri*, and *L. innocua* with *Acanthamoeba castellanii*, and *Tetrahymena pyriformis*, respectively, were investigated. The *Listeria* were ingested intracellularly by the protozoans. However, they were not killed, on the contrary, they survived. The two hemolytic *Listeria* species, i.e. *L. monocytogenes* and *L. seeligeri* ruptured their host cells completely after a few days and became free. Subsequently, they died.

On the other hand, the apathogenic *L. innocua* partially lysed the protozoans and coexisted intracellularly as well as extracellularly for some weeks. Additionally, cocultures of *L. monocytogenes*, *Escherichia coli*, *Salmonella typhimurium*, and *A. castellanii*, *T. pyriformis*, respectively, were studied. The results showed that both protozoans possess similar distinct preferences for the *Salmonella* and *Listeria*, but *E. coli* was not desirable 'prey.' Ingested *S. typhimurium* were killed but *L. monocytogenes* survived.

The observed phenomena provide a special insight regarding *L. monocytogenes* infection in man and animals in respect to the different behaviour patterns of the three *Listeria* species investigated.

Many observations suggest that species of the genus *Listeria* are considered soil microorganisms (19, 20). Notwithstanding, *L. monocytogenes* is the causative agent of listeriosis that affects humans as well as many species of mammals and birds (11, 12). Of course, some other *Listeria* species which have recently been described, show similar biochemical and ecological properties but they are nonpathogenic.

The virulence of *L. monocytogenes* seems to be related to its intracellular multiplication in host cells. However, it is puzzling how saprophytic soil bacteria

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have or acquire the ability to survive and multiply within cells of warm-blooded hosts. Lower animals are not considered because *Listeria* species normally do not exist in such hosts, as a previous publication has shown (12). Therefore, we examined the hypothesis that amebas or other free-living protozoans can serve as natural hosts to *Listeria* because they have properties similar to the macrophages of warm-blooded hosts in which *L. monocytogenes* multiplies.

MATERIALS AND METHODS

Bacteria. *L. monocytogenes*, strain L119, *L. seeligeri*, strain L106, and *L. innocua*, strain L118, were isolated from waste-water and characterized biochemically and serologically as described in previous papers (5, 6).

Escherichia coli and *Salmonella typhimurium* were freshly isolated from human feces. Their biochemical and serological characteristics were tested according to standard procedures (3, 4, 9).

Protozoa. *Acanthamoeba castellanii* was isolated from fresh water by filtration through cellulosic nitrate membranes with a pore size of 3 μ m (SM 11302, Sartorius, Göttingen, FRG). The filters were placed on non-nutrient agar plates spread with a 0.1-ml suspension of heat-killed *E. coli* (10^8 CFU/ml). The plates were incubated at about 20°C for 1 week in a moist chamber. Differentiation was performed according to appropriate keys (8, 13, 22). They were maintained on non-nutrient agar as described above.

Tetrahymena pyriformis was from a stock strain grown in Diamond's medium (8) at 20°C. The cells were collected by filtering through 3 μ m filters of cellulosic nitrate membranes. They were washed and suspended in sterile tap water.

Counting. Amoebae were enumerated by adding 0.1 ml of the suspension of protozoans to 0.1 ml of a 0.005% solution of methylene blue and counting the number of cells in a Neubauer chamber (0.1×0.0025 mm²) using bright-field illumination at 10 $\times$ magnification. Each counting was performed twice and the average counts were recorded.

T. pyriformis were enumerated by adding 0.1 ml of the protozoans to 0.05 ml of Türk's solution (acetic acid gentian violet solution) and 0.05 ml distilled water. The counting was performed in a Neubauer chamber as described above.

Listeria were enumerated by plating and counting CFUs on an acriflavine-ceftazidine agar plate incubated at 30°C (1, 2). *E. coli* and *S. typhimurium* were plated and counted on MacConkey agar (3, 9). All plates were prepared in duplicate with different dilutions. Only plates with 30 to 300 colonies were counted. The bacterial counts per ml were computed by multiplying the average number of colonies per plate by dilution used.

Microscopy. *A. castellanii* were examined by a suspension in one drop of sterile tap water on a cover slip using Zeiss Axioscope. *T. pyriformis* were examined microscopically in situ in Petri dishes with a Zeiss stereomicroscope DR.

All preparations of protozoans having ingested *Listeria* were tested for viability

of the intracellular bacteria by a fluorescent staining procedure using fluorescein diacetate (7, 15).

Determination of phagocytosis of Listeria by protozoans. *A. castellanii*, and *T. pyriformis*, respectively, fed in cocultures with *Listeria* were separated at different times from extracellular *Listeria* by filtration and washed for 1 h with a solution of gentamicin (0.5 µg/ml) and then three times with 100 ml of sterile tap water. Thereafter, the washed protozoans were suspended and no extracellular viable *Listeria* was detected. The disruption of the protozoans was performed by ultrasonication for 10 s. The complete disintegration of protozoans was confirmed by a microscopic examination and no loss of viability of *Listeria* occurred under these conditions. Portions of lysed suspensions were plated on acriflavine-ceftazidine agar plates and *Listeria* CFUs were counted. Survival curves were drawn by plotting the log 10 number of survivors against time.

Cocultivation experiments. Each experiment consisted of three tests:

- (a) a pure suspension of *A. castellanii* or *T. pyriformis* in sterile tap water as controls,
- (b) a pure suspension of *Listeria* spec., *E. coli*, *S. typhimurium*, respectively, in sterile tap water as controls, and
- (c) the coculture. equal volumes (25 ml) of appropriate concentrations of organisms suspended in sterile tap water were mixed, incubated at 36°C up to 5 weeks, and investigated as described above.

All experiments were performed repeatedly. The figures are typical and reproducible regarding the course of graphs. However, the data obtained from different assays were not suitable for a summarizing statistical presentation.

RESULTS

Sequential counts of coculture suspensions of *A. castellanii* infected with *L. monocytogenes*, *L. seeligeri* or *L. innocua* are given in Fig. 1. As the controls show in Fig. 3, the *Listeria* died within 18 to 34 days and the number of amoebas decreased. Of course, later they encysted and the counts remained constant. The behaviour of amoebas in the three cocultures was more or less the same. The numbers rose to a maximum after 1 week, decreased slightly and remained constant as cysts. The three *Listeria* species showed a similar behaviour only in the first week. Their extracellular level decreased by 7 to 8 orders of magnitude. But after that time, *L. monocytogenes*, and *L. seeligeri* disappeared completely from the extracellular compartment. Thereafter, the counts rose by 3 to 5 orders of magnitude and then decreased again. In contrast, after an initial fall the number of *L. innocua* remained constant over 4 weeks. The number and fate of ingested and intracellular *Listeria* were similar in the three cocultures with *A. castellanii*. After the first few days, the amoebas contained hundreds of living listerias intracellularly as shown by fluorescein diacetate. In about the second week, some of the amoebas ruptured and set free listerias, recognizable because of the increasing counts of extracellular

Fig. 1. Cocultures of *Acanthamoeba castellanii* (A.c.) with A) *Listeria innocua* (L.i.), B) *Listeria seeligeri* (L.s.), and C) *Listeria monocytogenes* (L.m.). The dotted lines give the concentrations (CFU/ml) of intra- and extracellular listerias in toto. The stretched lines represent only the extracellular organisms.

L. monocytogenes, or *L. seeligeri*. But *L. innocua* were not released and remained constant at low counts extracellularly. Most of the amoebas developed cysts, and, synchronously, the *Listeria* ingested were destroyed (Fig. 1).

The cocultures with *T. pyriformis* are given in Fig. 2. In contrast to amoebas, *T. pyriformis* do not develop cysts. Of course, they multiply abundantly in the presence of *Listeria* sp. by increasing the cell number by about 3 orders of magnitude due to fission into small cells. In a manner similar to coculture with *A. castellanii*, the concentration of listerias decreased in coculture with *T. pyriformis*, and reached zero after about 6 days in the cases of *L. monocytogenes* and *L. seeligeri*, whereas low concentrations of *L. innocua* remained detectable extracellularly. *T. pyriformis* cells

Fig. 2. Cocultures of *Tetrahymena pyriformis* (T.p.) with A) *Listeria innocua* (L.i.), B) *Listeria seeligeri* (L.s.), and C) *Listeria monocytogenes* (L.m.). The dotted lines give the concentrations (CFU/ml) of intra- and extracellular listerias in toto. The stretched lines represent only the extracellular organisms.

having phagocytized *L. monocytogenes* and *L. seeligeri*, began to lyse and after a further 1 to 2 weeks, they disappeared and were seen no more. To the same extent listerias became free and were detectable extracellularly. As rupture experiments showed, each *T. pyriformis* cell contained about $6-9 \times 10^3$ listerias. But notwithstanding that *T. pyriformis* contained so many *L. innocua*, only a few of the host cells ruptured and released *L. innocua*. Up to day 33, concentrations of *T. pyriformis* cells, of *L. innocua* extracellular as well as intracellular within

Fig. 3. A) The behaviour patterns of pure suspensions of bacteria as controls without protozoans: *Escherichia coli* (E.c.), *Salmonella typhimurium* (S.t.), *Listeria innocua* (L.i.), *Listeria seeligeri* (L.s.), and *Listeria monocytogenes* (L.m.). B) Cocultures of *Tetrahymena pyriformis* with three bacterial species: *Escherichia coli* (E.c.), *Listeria monocytogenes* (L.m.), and *Salmonella typhimurium* (S.t.).

T. pyriformis remained constant, as Fig. 2 shows.

In a further set of experiments, cocultures with protozoans, *L. monocytogenes* and two additional bacterial species, i.e. *E. coli* and *S. typhimurium*, were investigated (Fig. 3). Both, *L. monocytogenes* and *S. typhimurium* were preferred 'prey' for both protozoans and their counts dropped during the first week. While *S. typhimurium* disappeared completely between days 17 and 19, *L. monocytogenes* showed increasing cell counts after the first week due to the rupture of protozoans. However, *E. coli* did not show this phenomenon. Its concentrations remained nearly constant.

DISCUSSION

Regarding the three *Listeria* species, there are significant differences in the behaviour patterns of the cocultures analogous to their pathogenicity. The most pathogenic *L. monocytogenes* as well as the 'apathogenic' but hemolytic *L. seeligeri* lysed their host cells. Even a multiplication occurred in *T. pyriformis*. In this context, it is worthwhile to note that *L. seeligeri* possess pathogenic potential to some extent, as patient with meningitis due to *L. seeligeri* showed (16). Obviously, hemolysin is an important virulence factor (10, 14) which may also be due to the ability to rupture the protozoans. In contrast, *L. innocua* is apathogenic and non-hemolytic.

The microorganisms were ingested and survived within protozoans. They were killed by *A. castellanii* only while encystment occurred. However, a transient coexistence of *T. pyriformis* and *L. innocua* lasted for 5 weeks. As the experiments of cocultures of protozoans with three different bacterial species showed, *A. castellanii* and *T. pyriformis* have the same distinct preferences for *Listeria* and *Salmonella*, two species which also have the ability to survive intracellularly in warm-blooded hosts. *E. coli*, however, was phagocytized to a lesser degree.

The ecological niche of listerias was seen up to now to be in soil and mud (19, 20). However, they seem to be not only saprophytic organisms, but are also intracellular parasites of protozoans. *L. innocua* seem to be adapted best to their phagocytizing hosts, because they are able to coexist with and in protozoans for a longer period of time. But *L. monocytogenes* and *L. seeligeri* are more pathogenic for protozoans as well as mammals and human beings. Especially, *L. monocytogenes* are able to survive and multiply inside numerous host cells, i.e. macrophages and, furthermore, in enterocytes or hepatocytes (11, 19, 20). This property seems to be related to the lysis of protozoans. Of course, it is unclear up to now whether the hemolysis is the responsible factor for both effects. However, the phenomenon of survival within and rupture of protozoans provides a special insight into an obviously general principle: facultative pathogenic water and soil bacteria, with ecological niches in the environment and the ability to multiply intracellularly in host cells, have probably acquired this property in protozoans. Some years ago, Rowbotham reported that legionellas ingested by amebas multiply intracellularly and then rupture them (17, 18). Now listerias are a further example. Supposedly, other pathogens living in the environment and proliferating intracellularly have acquired the virulence for humans in a similar manner by means of their ability to survive and multiply within protozoans, i.e. *Mycobacterium leprae*, ubiquitous atypical nontuberculous mycobacteria, or *Yersinia* species.

More knowledge is needed to understand the ecological niches for such microorganisms and the correlations with virulence and pathogenicity for warm-blooded hosts.

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