Ingested *Listeria monocytogenes* survive and multiply in protozoa

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Summary. *Listeria monocytogenes* cells are ingested by protozoa such as *Acanthamoeba* sp. or *Tetrahymena pyriformis*. However, they are not killed, but survive within the protozoa and may multiply intracellularly. The protozoa are lysed within about 8 days, releasing viable *L. monocytogenes*. No co-existence was observed between *L. monocytogenes* and *Tetrahymena*. A co-culture of *L. monocytogenes* and *Acanthamoeba* sp. showed a decay of locomotive forms and release of listeria from vegetative protozoan cells whereas the bacteria were destroyed in cysts. These phenomena provide an insight into the pathogenesis of listeria infection in man and warm-blooded animals because intracellular processes occurring in protozoa after ingestion of *L. monocytogenes* may be similar to those observed in mammalian cells.

Introduction

Listeria monocytogenes is an ubiquitous bacteria occurring in all regions of the world. However, its ecology is unclear.¹ On the one hand, the organisms have been isolated frequently from all sorts of soil samples, superficial sewage, and wastewater.² Furthermore, they grow at temperatures as low as 4°C. This evidence seems to point to the environment as the natural habitat of *Listeria* spp. On the other hand, *L. monocytogenes* is a pathogen, causing disease in many species of warm-blooded animals.³ Not only raw vegetables such as cabbage⁴ or lettuce⁵ but also foods of animal origin such as meat, milk and cheese are sources of human infection.² However, in contrast to most other saprophytes that are occasional pathogens, but rather like some obligatory parasitic organisms, *L. monocytogenes* is able to multiply inside many host cells, e.g., in macrophages, enterocytes and hepatocytes. This is hard to understand. The known facts about the behaviour and properties as well as the ecological niche of *L. monocytogenes* cannot explain how this saprophytic, environmental organism has acquired the ability to survive within mammalian cells.

The similarity of free-living, bacteria-scavenging protozoa to macrophages suggests that protists are the missing link in the ecology and pathology of listeria. Therefore, this study was initiated to investigate the complex food chain of the two groups of organisms.

Materials and methods

Protozoa

Acanthamoeba sp. were isolated by filtering fresh water through 3 µm nitrocellulose membrane filters (SM 11302, Sartorius, Göttingen, FRG). The filters were placed on non-nutrient agar plates spread with a 0.1-ml suspension of heat-killed *Escherichia coli* (10⁸ cfu/ml). Plates were incubated at 20°C for 1 week in a moist chamber. Growth of amoebae occurred after 6 days. The amoebae were cloned and a pure isolate of *Acanthamoeba* sp. was identified as described by Lee⁶ and Page.⁷ They were maintained on non-nutrient agar as described above.

A *Tetrahymena pyriformis* culture was from a stock strain grown in Diamond's medium⁶ at 20°C. When needed, *T. pyriformis* organisms were collected on a 3-µm nitrocellulose membrane filter, washed, and suspended in sterile tap water.

Listeria monocytogenes

L. monocytogenes was isolated from wastewater as described elsewhere.^{8,9} Biochemical characterisation was by standard methods.^{10–12} The organisms were maintained on blood agar at 20°C. After removal from agar, pure suspensions were obtained by washing in sterile tap water.

Counting

T. pyriformis cells were counted by adding 0.1 ml of the culture to 0.05 ml of Türk's solution (acetic acid-gentian violet solution) and 0.05 ml distilled water and then counting the number of cells in a Neubauer chamber (0.1 mm × 0.0025 mm²) under bright-field illumination at

a magnification of 10. *Acanthamoeba* sp. were counted by adding 0.1 ml of the protozoan suspension to 0.1 ml of aqueous methylene blue 0.005% and counting as above. All preparations of protozoa were tested for the viability of ingested listeria by a fluorescent staining procedure with fluorescein diacetate.^{13,14} Viable *L. monocytogenes* cfus were counted on acriflavine-ceftazidime (AC) agar.¹⁵

Microscopical examination

T. pyriformis were examined by light microscopy *in situ* in solution in petri dishes with a Zeiss stereomicroscope DR. *Acanthamoeba* sp. were suspended in one drop of sterile tap water on a cover slip and examined with a Zeiss Axioscope.

Co-cultivation experiments

The interaction of *L. monocytogenes* with *Acanthamoeba* sp. and *T. pyriformis* was studied by mixing appropriate concentrations of the organisms in sterile tap water (25 ml) and incubating at 36°C for up to 5 weeks. As control, a pure suspension of *L. monocytogenes* was incubated in the same conditions; similar controls with *Acanthamoeba* sp. and *T. pyriformis* were set up, although the initial concentrations of the protozoa were higher than in the co-cultivation experiments because of difficulties in counting lower numbers.

Determination of phagocytosis of *L. monocytogenes* by protozoa

Acanthamoeba sp. and *T. pyriformis* fed upon *L. monocytogenes* by co-cultivation during 6–8 days; they

were then separated from extracellular *L. monocytogenes* by filtration as described above and washed with a solution of gentamicin 0.5 µg/ml for 1 h. After these procedures, no extracellular viable *L. monocytogenes* were detected. The washed protozoa suspended in sterile distilled water were disrupted by ultrasonication for 10 s. Complete disintegration of protozoa was confirmed by microscopy. However, no loss of viability of *L. monocytogenes* occurred as shown by examination of controls of bacteria without protozoa. Samples of the lysed suspensions were plated on AC agar and cfus of *L. monocytogenes* were counted.

All experiments were repeated several times. The figures, displayed graphically, are typical and were reproducible. However, the data obtained from different assays were not expressed numerically for statistical presentation.

Results

L. monocytogenes and *Acanthamoeba* sp.

Sequential counts of *L. monocytogenes* and *Acanthamoeba* sp. are shown in fig. 1. Pure suspensions of *L. monocytogenes* and *Acanthamoeba* sp. served as controls and their numbers decreased slowly and continuously. However, in co-culture, *L. monocytogenes* showed first a major fall to a minimum of about 1 cfu/ml around day 8 and thereafter numbers rose again. In contrast, the *Acanthamoeba* sp. showed inverse kinetics. First, the number of amoebae increased to reach a maximum around day 8. Fluorescein diacetate staining showed that

Fig. 1. Counts of *L. monocytogenes* (cfu/ml) and *Acanthamoeba* sp. (cells/ml) in co-culture and in control suspension incubated at 36°C for 34 days. *L. monocytogenes* in co-culture with *Acanthamoeba* sp. △; *Acanthamoeba* sp. in co-culture with *L. monocytogenes* ▲; *L. monocytogenes* control, ○; *Acanthamoeba* sp. control ●.

amoebae contained hundreds of living listeria intracellularly. Viable counts of listeria from gentamicin-washed and then lysed cells of *Acanthamoeba* sp. confirmed the microscopic findings. Beginning on day 8, the *Acanthamoeba* sp. encysted, although some of the cells ruptured and released viable *L. monocytogenes*. On day 10, about 90% of the amoebae were locomotive forms and only 10% were cysts. However, on day 34, almost all were cysts. Ingested and so far viable *L. monocytogenes* died during formation of the cysts.

L. monocytogenes and *T. pyriformis*

Growth kinetics of *L. monocytogenes* and *T. pyriformis* are given in fig. 2. During the first 5–7 days, *T. pyriformis* ingested all cells of *L. monocytogenes*. During this period *T. pyriformis* multiplied greatly and large numbers of aggregated cells appeared. However, in contrast to amoebae, *Tetrahymena* does not develop cysts. Microscopic examination of cells stained by fluorescein diacetate as well as lysis experiments showed thousands of living listeria intracellularly within one *Tetrahymena* cell. After 8–15 days, *T. pyriformis* cells lysed and released viable *L. monocytogenes*. The viable counts of *L. monocytogenes* now reached 10^8 cfu/ml and were higher than the initial inoculum. Therefore, intracellular multiplication of listerias in *T. pyriformis* occurred because they did not multiply extra-

cellularly. On the other hand, the population of *T. pyriformis* broke down completely within 3–5 weeks and no cells were alive after that; the *L. monocytogenes* also died.

Discussion

Our results show that *Acanthamoeba* sp. as well as *T. pyriformis* ingest *L. monocytogenes*. The bacteria survive in the protozoa, multiply at least in *T. pyriformis* and may also stimulate cell division. After some days, the infected protozoa are destroyed and release *L. monocytogenes*. Whereas cells of *T. pyriformis* die completely, *Acanthamoeba* sp. develop cysts in which *L. monocytogenes* dies. The phenomenon of intracellular multiplication of *L. monocytogenes* in protozoa is a further example of an obvious general principle: saprophytic bacteria living in environmental niches and yet pathogenic for man or animals by intracellular parasitism may be virulent by virtue of the ability to survive in the same way as they survive in and destroy protozoa.

Rowbotham observed that the pathogenicity of *Legionella pneumophila* is due to its ability to multiply within freshwater and soil amoebae;^{16,17} *L. monocytogenes* shows a similar behaviour. Normally, *Listeria* and *Legionella* spp., as well as many other bacteria, serve as food for secondary feeders, i.e., protozoa, as part of the microbial food chain. However, *Listeria* and *Legionella* spp. have suc-

Fig. 2. Counts of *L. monocytogenes* (cfu/ml) and *T. pyriformis* (cells/ml) in co-culture and in control suspensions incubated at 36°C for 34 days. *L. monocytogenes* in co-culture with *T. pyriformis* △; *T. pyriformis* in co-culture with *L. monocytogenes* ▲; *L. monocytogenes* control ○; *T. pyriformis* control ●.

ceeded in turning the tables on their predators and growing within the protozoa. Some other environmental bacteria are also intracellular pathogens of man or animals and may possess similar properties, e.g., ubiquitous non-tuberculosis mycobacteria (MOTT bacilli), possibly *M. leprae*, and *Yersinia* spp.^{18,19} Resistance to digestion by protozoa and destruction of the feeding cells give us a special insight into the pathogenesis of infections by such pathogens. Intracellular processes occurring in protozoa after ingestion of listeria may be similar to those observed in macrophages or other mammalian cells from hosts with listeriosis.

The fact that higher organisms possess abundant iron in the enzymes and functional proteins of many cells, but that such reserves are not available to a similar extent in most lower animals (e.g.,

insects) may explain the occurrence and pathogenicity of *L. monocytogenes* in warm-blooded hosts.³ Of course, the organisms produce listeriolysin O which is an important virulence factor^{20,21} and mobilises from host cells iron which is a growth limiting factor. On the other hand, lower animals contain more copper and less iron. Therefore, they are not natural hosts of *L. monocytogenes*.¹ Interestingly, *Legionella* shows a similar requirement for iron and also does not possess siderophores because they are not primary parasites of warm-blooded hosts. There is no need for siderophores to mobilise iron in their natural environment.

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