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Acanthamoeba: Could it be an environmental host of *Shigella*?

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Abstract

Shigellosis is a serious public health problem in Korea, because large outbreaks of *Shigella sonnei* infections were recorded in many parts of the country during the period 1998–2000. However, the epidemiological features of shigellosis are not well known. In this study, we devised conditions suitable for the growth and replication of *Shigella* in an amoebic intracellular environment, and investigate whether medium conditions affect the survival and replication of *Shigella* within *Acanthamoeba*. We evaluated the uptake rates of invasive and non invasive *S. sonnei* strains by three *Acanthamoeba* species, namely, *A. castellanii* Neff, *A. astronyxis* Ray & Hayes, and *A. healyi* OC-3A. When *A. castellanii* Neff was infected with *S. sonnei* 99OBS1 or 80DH248, shigellae was maintained for a longer time in cytoplasms than in other *Acanthamoeba* species. *S. sonnei* 99OBS1 strain (a virulent strain) was recovered in higher numbers than the non-virulent *S. sonnei* 80DH248 strain in all experiments. Moreover, *S. sonnei* was more easily engulfed by *Acanthamoeba* at 18 °C. The shigellae uptake rates of Neff strain, which was cultured in free-media (less nutrition), were higher (>10-fold) than those observed in original amoeba culture media (PYG medium) in all time points. *S. sonnei* 99OBS1 was localized, with an intact membrane, to the vacuoles of *Acanthamoeba*. We conclude that free-living amoebae more likely act as environmental hosts for shigellae, and thus, may have contributed to outbreaks of shigellosis in Korea.

Index Descriptors and Abbreviations: *Acanthamoeba castellanii*; Environmental host; Uptake rate; *Shigella sonnei*

1. Introduction

Free-living amoebae and bacteria interact in complex ways. It is known that amoebae act as environmental hosts for several intracellular pathogens, such as, *Legionella*, *Chlamydia*, *Mycobacterium*, and *Listeria* spp. (Amann et al., 1997; Ly and Muller, 1990; Neumeister et al., 1997; Steinert et al., 1998). Interestingly, some of the gene functions required by *Legionella* spp. for infecting protozoans are also required for infecting mammalian cells (Hales and Shuman, 1999; Segal and Shuman, 1999). It was even suggested that growth in an amoebic intracellular environment might assist bacteria adaptation to the mammalian phagocytic cell environment (Harb and Kwaik, 2000; Harb et al., 2000). Moreover, the incorporation of bacteria into amoebic cysts has been shown to allow bacteria to survive under adverse environmental conditions, for example, exposure to biocidal agents (Barker and Brown, 1994).

Bacillary dysentery caused by *Shigella* species is an important cause of acute diarrheal disease in both developing and industrialized countries (Vila et al., 1994). Shigellosis is viewed as a serious public health problem in Korea, because large outbreaks of *Shigella sonnei* infections in several parts of the country occurred during the period 1998–2000. The annual incidence of shigellosis was estimated at about 10 cases before 1997, but increased explosively to

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about 1000–2500 cases during the period 1998–2000 (National Institute of Health in Korea, 2002). Nevertheless, comparatively little is known about the epidemiological features of shigellosis, or of vectors capable of transmitting *Shigella*, which can only infect primates.

Free-living amoebae, such as *Acanthamoeba* spp., are commonly found in natural aquatic systems and in soil (Martinez and Visvesvara, 1997), and even within the intestines of humans (Zaman et al., 1999) and reptiles (Sesma and Ramos, 1989), and hence, they are expected to encounter and ingest *Shigella*. In fact, King et al. (1988) reported on the survival of *Salmonella enterica serovar Typhimurium* within *Acanthamoeba castellanii* during chlorination, and suggested that the amoeba afforded a protective intracellular habitat for the bacteria. In addition, *Escherichia coli* O157 can survive in a soil protozoan (Barker et al., 1999). However, the role played by protozoa in the environmental survival of *Shigella* has not been studied, and there is growing concern about the survival of these pathogens in sewage sludge disposed on land now that green laws limit sea dumping. Soils contaminated with organic matter and sewage waste contain vast numbers of protozoans like Acanthamoebae (Rodriguez-Zaragoza, 1994). Thus, it is probable that *Shigella* in soil and slurry will be preyed on by free-living amoebae, which could be potential vectors for the spread of this pathogen. Here, using laboratory microcosms we studied the ability of the ubiquitous free-living protozoan, *Acanthamoeba*, to support the growth of *Shigella*.

In this study, we describe the establishment of conditions required to support the uptake of *Shigella* in an amoebic intracellular environment.

2. Materials and methods

2.1. Bacterial strains

Two *S. sonnei* strains were used in this study, 80DH248 and 99OBS1, both were isolated from human infections in Korea. 80DH248 strain was isolated at 1980 and had become avirulent after being maintained in artificial medium and being allowed to reproduce many times. The other strain, 99OBS1, was isolated during a significant outbreak shigellosis and had retained its virulence.

2.2. Cell lines and culture conditions

Three amoebae isolates were obtained from the American Type Culture Collection (ATCC), namely, *A. castellanii* Neff strain (ATCC #30010), *Acanthamoeba astronyxis* Ray & Hayes strain (ATCC #30137), and *Acanthamoeba healyi* OC-3A strain (ATCC #30866). Table 1 shows characteristics of these isolates. All three isolates were maintained in an axenic culture medium, i.e., peptone-yeast extract/glucose (PYG) medium (Chung et al., 1996), in 25-cm^3 tissue culture flasks and incubated at 24 °C, until near confluent. Amoebic suspensions were examined under bright-field microscopy before use, and cell numbers were determined by counting in a Burker chamber. HeLa cells (a phagocytic cell-line) from the ATCC were maintained in RPMI 1640 medium supplemented with L-glutamine (final concentration, 2 mM), Hepes (final concentration, 10 mM), and fetal bovine serum (final concentration, 10% [vol/vol]).

2.3. Plaque assay of bacterial strains

Shigellae grown to the late logarithmic growth phase in LB were added to a culture flask of HeLa cells grown to the late logarithmic growth phase (No. of bacteria:No. of HeLa cells = 10:1), and after incubation at 37 °C for 2 h, cells were washed three times with phosphate-buffered saline (PBS). The fresh cell culture medium contained gentamicin sulfate (final concentration, 50 μg/ml) added to the culture flask and after incubation for various times at 37 °C. Invasion ability (virulence) of Shigellae was determined by formed cell plaques.

2.4. Intracellular bacterial growth in Acanthamoeba

Amoebae were grown in 5 ml of PYG medium. Tissue culture flasks were gently shaken, and PYG containing non-adherent amoebae were removed. New PYG was then added, and amoebae were taken off by incubation of culture flasks on ice for 30 min. Suspensions obtained were centrifuged for 5 min at 200*g*, and pellets were washed with PBS and resuspended in PYG. This suspension was then added to each well of a six-well plate (10^6 amoebae/well), and amoebae were incubated for 24 h at 30 °C to allow them to adhere. Numbers of amoebae/well were determined before infection. To infect amoebae, invasive or non invasive bacterial inocula were diluted in PYG or free medium (proteose peptone, yeast extract, glucose exclude in PYG medium; nutrient free medium) to a final concentration of 10 bacteria per amoeba. Plates were gently centrifuged (for 5 min at 500*g*) to promote contact between bacteria and amoebae, and were then incubated for various times (30 min–24 h) and temperatures (18, 24, and 37 °C). After these incubations, medium was replaced

Table 1
The list of *Acanthameoba* species examined

Strain	# ATCC	Environmental source	Species designation	Cyst morphology[a]
Ray & Hayes	30137	Soil	*A. astronyxis*	Group I
Neff	30010	Soil	*A. castellanii*	Group II
OC-3A	30866	GAE[b]	*A. healyi*	Group III

[a] Grouping was performed according to method of Pussard and Pons (1970).
[b] Granulomatous amoebic encephalitis.