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Effect of Bacteria on Survival and Growth of *Acanthamoeba castellanii*

Xiaohui Wang, Donald G. Ahearn

Georgia State University, Biology Department, P.O. Box 4010, Atlanta, GA 30303-4010, USA

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Abstract. The growth and survival of *Acanthamoeba castellanii* in the presence of Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Serratia marcescens*, and *Stenotrophomonas maltophilia* varied with the densities and species of bacteria. All species of bacteria suspended in a buffered saline at densities of 10^5 to 10^6/ml supported the growth and survival of 10^6/ml trophozoites of *Acanthamoeba castellanii* in a buffered saline solution. At densities of bacteria to amoebae of 100:1 or greater, growth and survival of *A. castellanii* were suppressed, particularly by *P. aeruginosa*. In an enrichment medium, the rapid growth of most co-inoculated bacteria inhibited the growth and survival of the amoeba.

Acanthamoeba spp. are predators of a variety of microorganisms, particularly Gram-negative bacteria such as *Pseudomonas fluorescens* and *Klebsiella aerogenes* [19]. Viable *Escherichia coli* and *K. aerogenes* have been reported to be excellent food for *Acanthamoeba* spp., which had been grown axenically [19]. *E. coli* is a traditionally used, growth-supporting species and is also the standard co-cultivation species used in the isolation of amoebae from clinical specimens [18].

Acanthamoeba spp. have shown differential capabilities for the binding and internalization of different species of bacteria [14]. Directly after initial binding, bacteria were clustered in patches on the surface of the amoebae (focal clustering phenomenon) prior to internalization. This was noted most frequently with polar flagellate bacteria, such as *Pseudomonas* spp. and *Stenotrophomonas* (*Xanthomonas*) spp., less so with *E. coli*, and not at all with *Staphylococcus epidermidis* and *S. aureus*. *P. aeruginosa* was found to be inhibitory to *Acanthamoeba castellanii* upon their co-cultivation; 92.5% of the amoebae died in mixed culture in 48 h, whereas only 28% of the amoebae died in saline without the pseumonad [5].

The toxic effects of pigmented bacteria, such as *S. marcescens* and *P. aeruginosa*, for amoebae have been frequently noted [9]. Nevertheless, *A. castellanii* has been reported to show positive chemotactic responses to *S. marcescens* and *P. aeruginosa* [6].

Acanthamoeba keratitis, most often attributed to *A. castellanii*, is a rare eye infection in the USA, associated mainly with contact lens wearers who used home-prepared saline or tap water while rinsing their lenses [1, 13, 17]. The incidence of *Acanthamoeba* keratitis in the USA has decreased from that indicated in the 1980's, apparently because of the withdrawal from the market of the tablets for home-prepared saline [2]. In England and elsewhere, *Acanthamoeba* keratitis remains constant and seems to be associated with domestic reservoir, tap-water systems [10, 11].

Qureshi et al. [15] theorized that *P. aeruginosa* produced amoebicial exoenzymes, possibly phosphalipase C, various proteases, and other materials such as pyocyanin that might inhibit the growth and survival of *Acanthamoeba* spp. They speculated that an apparent exclusivity in the occurrences of *P. areuginosa* and *Acanthamoeba* spp. in cases of clinical keratitis was related to the inhibitory effects of the bacterium for the amoebae. Donzis et al. [7], however, isolated both *P. aeruginosa* and amoebae simultaneously from lens cases.

The present investigation compares growth and survival of *A. castellanii* in the presence of bacteria that commonly contaminate contact lens cases and also with growth and survival in the presence of bacteria reported to be excellent food sources.

Materials and Methods

Culture conditions

Amoebae. Acanthamoeba castellanii (ATCC 30868), initially isolated from a human cornea, was obtained from the American Type Culture

Correspondence to: D.G. Ahearn

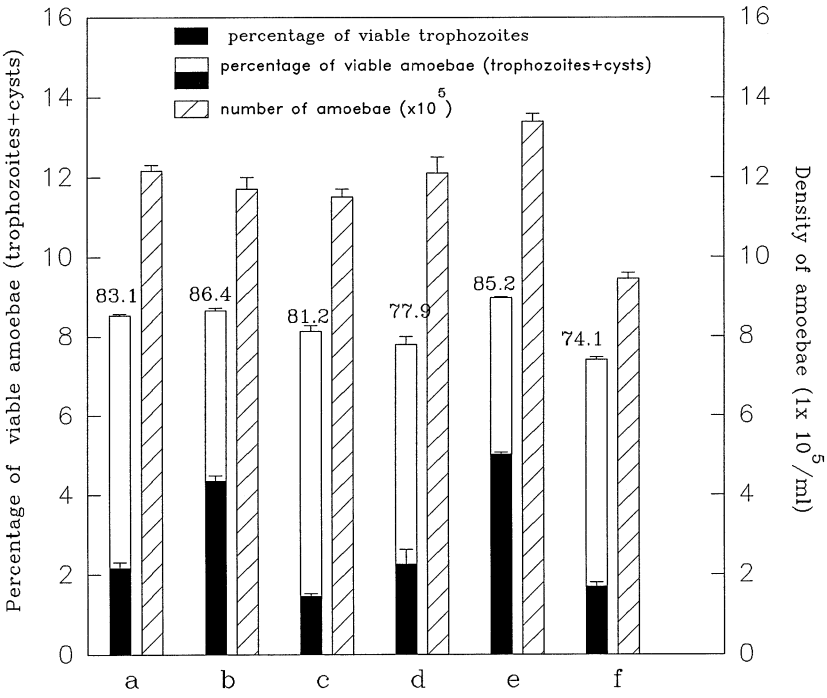

Fig. 1. Effects of various bacteria—(a) *Serratia marcescens*, (b) *E. coli*, (c) *Staphylococcus epidermidis*, (d) *P. aeruginosa*, (e) *Stenotrophomonas maltophilia*, (f) amoebae alone—on the densities of viable trophozoites (VT) of *A. castellanii* after 48 h in PBS. Vertical bars represent standard error of the mean (N = 6). Initial inocula were 10^6/ml amoebae and 10^6/ml bacteria.

Collection (ATCC, Rockville, MD, USA). *Acanthamoeba castellanii* was grown in peptone (2.0%)–yeast extract (1.0%)–glucose (1.8%) (PYG) medium fortified with penicillin G sulfate (400 U) and streptomycin sulfate (400 U) [16]. The amoebae (0.1 ml) were transferred to antibiotic-free PYG medium in 75-cm^2 tissue culture flasks (Corning Inc., Corning, NY, USA). Flasks were examined daily with an inverted microscope. Trophozoites were harvested after 48 h of incubation at room temperature (19°–24°C) when the amoebae were in the logarithmic growth phase. Cysts were harvested after 45 days or incubation. The amoebae were centrifuged at 94 *g* for 5 min. Pellets (≈0.5 ml) were washed three times with 10 ml phosphate-buffered saline (PBS: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2PO_4, 0.24 g KH_2PO_4 in 1000 ml distilled water, pH 7.4).

Bacteria. Pseudomonas aeruginosa (from human keratitis, GSU#3), *Staphylococcus epidermidis* (ATCC 155), *Escherichia coli* (ATCC 13902), and *Serratia marcescens* (ATCC 8100) were cultured at 37°C, and *Stenotrophomonas maltophilia* (ATCC 420207) was cultured at 25°C in trypticase soy (TS) broth (Difco Laboratories, Detroit, MI, USA) in shaken culture for 18 h. The bacteria were harvested by centrifugation, and the cells were washed three times with 10 ml PBS.

Concentrations of amoebae and bacteria. The bacteria were suspended in 5 ml PBS and adjusted to optical densities equivalent to final concentrations of from 10^5 to 10^{10} colony forming units (CFU) per ml. The bacteria (*E. coli* and *P. aeruginosa*) in certain experiments were killed by incubation in a 80°C water bath for 15 min. Amoebae used for inocula were suspended in PBS, and the density was adjusted to 10^6/ml from hemocytometer counts. Inocula contained more than 92% viable trophozoites. Co-inoculated PBS or PYG medium were mixed vigorously and incubated in static culture for 48 h at 24°C.

Determination of the viability of amoebae. PBS (0.1 ml) containing amoebae alone or mixtures of amoebae and bacteria was transferred into 0.2 ml 0.3% basic methylene blue in 1.5-ml Eppendorf tubes [4]. The tubes were immediately and vigorously vortexed. The number of unstained and stained cysts and trophozoites were counted in a 0.1-ml counting chamber (Hausser Scientific, Inc., Horsham, PA, USA) within 10 min after staining. At least 100 amoebae were counted in each of three separate samples, and the percentages of viable trophozoites and cysts (methylene blue excluding) were calculated. All experiments were repeated at least in triplicate.

Results

Growth of amoebae in the presence of bacteria. All bacteria supported the growth of *A. castellanii* after 48 h. As reported by Bottone et al. [5], *Stenotrophomonas maltophilia* was the best food source (Fig. 1). At densities of bacteria of 10^5/ml to 10^6/ml of amoebae, *P. aeruginosa*, *E. coli*, and *Serratia marcescens* (Fig. 2) and *Stenotrophomonas maltophilia* (data not shown), both viable and heat-killed, all supported growth and survival of *A. castellanii* in PBS. Although the total numbers of amoebae produced were similar with live and dead cells of the different bacterial species, the amoeba grown on *P. aeruginosa* more rapidly converted to cysts. Higher initial densities of bacteria, particularly of *P. aeruginosa*, depressed the growth and survival of *A. castellanii*; even *E. coli*, which was an excellent food source for the amoebae, tended to be inhibitory at higher densities (Fig. 3). Viable *E. coli* yielded a higher number of viable trophozoites than dead *E. coli* when the densities of bacteria were equal to the densities of amoebae. A high density of viable *P. aeruginosa* (10^8/ml) depressed the growth and survival of *A. castellanii* (10^6/ml) more than 10^8/ml of nonviable bacterium cells.

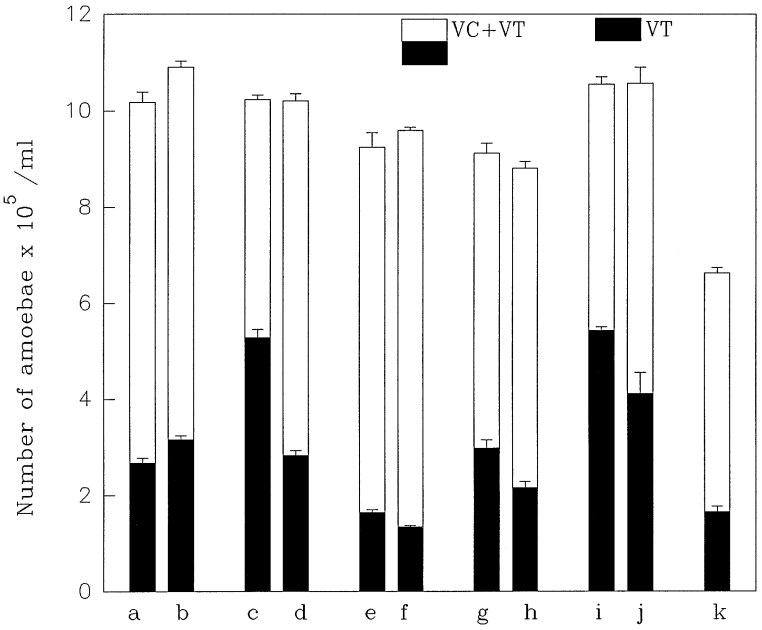

Fig. 2. Densities of viable trophozoites (VT) and viable cysts (VC) of *A. castellanii* after 48 h in PBS with *Serratia marcescens*, viable (a), dead (b); *Escherichia coli*, viable (c), dead (d); *Staphylococcus epidermidis*, viable (e), dead (f); *Pseudomonas aeruginosa*, viable (g), dead (h); *Stenotrophomonas maltophilia*, viable (i), dead (j); no bacteria (k). Vertical bars represent the standard error of the mean (N = 6). Initial inocula were 10^6/ml bacteria and 10^6/ml amoebae.

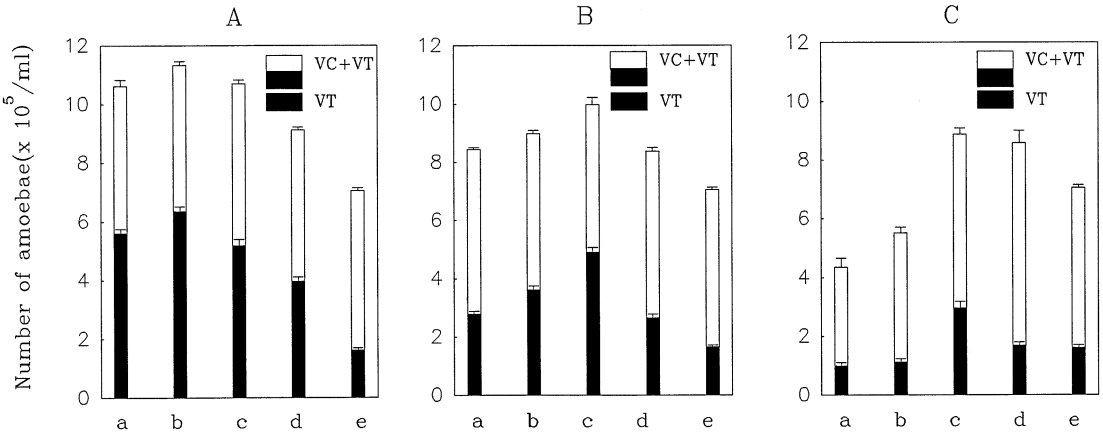

Fig. 3. Effect of initial densities of viable bacteria (A) *S. maltophilia*, (B) *E. coli*, (C) *P. aeruginosa*: (a) 10^{10}, (b) 10^8, (c) 10^6, (d) 10^5, (e) 0 (amoebae alone), on the densities of viable trophozoites (VT) and viable cysts (VC) of *A. castellanii* after 48 h in PBS. Vertical bars represent the standard error of mean (N = 6). Initial inocula of amoebae were 10^6/ml.

Table 1. Effect of *P. aeruginosa* and *E. coli* on the viability of *A. castellanii* in PBS and diluted PYG medium

Medium	Axenic culture	*E. coli*	*P. aeruginosa*
PBS	72.4 ± 3.1[a]	81.8 ± 2.3	73.1 ± 1.5
PYG	86.4 ± 4.6	76.3 ± 2.4	41.3 ± 4.3

[a] Percentage of viable cells of amoebae after 48 h; inocula of both amoebae (>90% trophozoites) and bacteria ~10^6 cell/ml (n = 3).

In diluted PYG medium after 48 h of incubation, the growth of *P. aeruginosa* (cell densities increased from 10^6/ml to 10^9/ml) suppressed the growth of the amoebae (Table 1). In co-culture with *P. aeruginosa*, more than 50% of the amoebae were nonviable after 48 h.

Discussion

The survival and growth of *A. castellanii* in the presence of bacteria were markedly influenced by the species and densities of bacteria, especially Gram-negative bacteria. At low densities, all Gram-negative bacteria in PBS supported the growth of *A. castellanii*. However, when the density of amoebae to the density of bacteria exceeded 1:10^4, Gram-negative bacteria (even *E. coli*) had an inhibitory effect on the amoebae. In PYG enrichment medium the growth of Gram-negative bacteria, particularly *P. aeruginosa*, overwhelmed the amoebae. The optimal ratio between bacteria and *A. castellanii* ranged from 10:1 to 1:1. Avery et al. [3] reported that ingestion of inert particles (diameter = 1 μm) by *A. castellanii* in-

creased linearly as the density of beads increased until phagocytosis became saturated. When the particle-to-cell ratio reached 10:1, further increase in bead concentrations caused no further increase in the number of internalized beads. After phagocytosis became saturated, the number of particles binding on the surface of *A. castellanii* continued to increase as the density of the beads increased. The suppressive effect of an excessive density of food-source bacteria (such as *E. coli*) might result, in part, from the excessive number of bacteria on the surface of amoebae and the extensive change of pH rather than from an actual toxic intermediate. The amoebae can survive and grow from pH 4 to pH 8.5 in proteose peptone-glucose medium and autoclaved yeast media [12]. The decrease of pH by large numbers of bacteria might be inhibitory to amoebae and also might exacerbate encystment and death of trophyzoite inocula. The production by the bacteria of inhibitory by-products such as phospholipase may also contribute to the decline of the amoebae [15].

John et al. [8] reported that *Acanthamoeba* spp. used *E. coli* as a food source; viable *E. coli* as a substrate provided a 51% higher yield of trophozoites than did nonviable *E. coli.* We had similar results with all Gram-negative bacteria tested but not with Gram-positive bacteria.

Escherichia coli and *Stenotrophomonas maltophilia* served more readily as a food source for *A. castellanii* than *Staphylococcus epidermidis*, *Serratia marcescens*, and *P. aeruginosa*. *P. aeruginosa*, in enrichment culture or at high densities in saline, showed the greatest inhibition of *A. castellanii*. Bottone et al. [4] reported *S. maltophilia* easily adhered to amoeba cysts and trophozoites; and many bacteria were observed to be phagocytized by amoebae within 2 h. They also stated that the numbers of amoebae increased much faster in the presence of *S. maltophilia* than in the presence of *E. coli*. These phenomena were observed also in our experiments.

Most cases of *Acanthamoeba* keratitis have been related to improper cleaning or disinfection of contact lenses, resulting in contamination with both bacteria and amoebae [17]. In saline or diluted contact lens holding and rewetting solutions, bacteria may grow slowly (saline) or the bacterial growth may be inhibited (preserved holding solutions), but bacterial cell-lysis may not necessarily occur. *A. castellanii* may survive and multiply under these conditions and may utilize otherwise inhibitory *P. aeruginosa* as a food source. Encystment of the amoebae may occur, and evidence of initial contamination by bacteria or co-contamination of the solutions may not be evident. Conversely, dependent upon the densities and species of bacteria present, amoebae may not be recoverable. The dynamics of mixed populations of amoebae and bacteria, including strain variations, in contact lens systems is complex and requires further study.

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