Differential binding capacity and internalisation of bacterial substrates as factors in growth rate of *Acanthamoeba* spp.

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Summary. *Acanthamoeba* spp. are free-living predators that selectively feed on bacteria. Adherence of the bacterial food source to the trophozoite membrane is followed by internalisation and digestion. Through co-cultivation of *A. castellanii* and *A. polyphaga*, individually, with *Xanthomonas maltophilia*, *Escherichia coli*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* (despite the amoebicidal properties of the latter organism), specificity with regard to the preferred bacterial substrate was judged. *X. maltophilia* and *P. aeruginosa* adhered almost immediately forming a multilayered mantle of bacilli around trophozoites of both species of amoebae. *E. coli* adhered to fewer trophozoites and in smaller numbers. *X. maltophilia* was readily internalised after co-cultivation for 8 h, whereas *P. aeruginosa*, *E. coli* and *S. epidermidis* were not internalised even after co-cultivation for 24 h. These data suggest that the suitability of a bacterial food source for the *Acanthamoeba* spp. studied is associated not only with the proclivity with which the bacterial species binds to the trophozoite surface, but also with the rate of its internalisation.

Introduction

Acanthamoeba spp. are free-living predators that selectively feed on diverse bacteria encountered in the environment. Germane to their feeding pattern is adherence of the bacterial food source to the trophozoite membrane with subsequent internalisation within phagosomes and digestion. Therefore, adherence and internalisation kinetics determine the suitability of the bacterial substrate which is in turn reflected in the growth rate of the acanthamoebae.

Our previous report¹ showed that several water-borne environmental bacteria such as *Xanthomonas maltophilia*, *Flavobacterium breve* and *Pseudomonas paucimobilis*, which co-habitate with *Acanthamoeba* spp., supported growth of acanthamoebae better than the non-environmentally derived, but recommended, co-cultivating bacterial species *Escherichia coli*.² In that study, counts of viable amoebae were made over a 96-h period, but little attention was paid to the definitive amoeba-bacterium interactions underscoring growth rate and selectivity of the bacterial substrate. In this study, through co-cultivation, light microscopy and electronmicroscopy, we examined the rate of adherence and internalisation of several gram-negative and gram-positive bacteria to *A. castellanii* and *A. polyphaga*.

Materials and methods

Adherence and internalisation of bacteria

A. castellanii (ATCC 30461) and *A. polyphaga* (ATCC 30010) axenically grown in proteose peptone-yeast extract-glucose medium² were co-cultivated individually in sterile saline at 35°C with clinical isolates of *X. maltophilia*, *E. coli*, *Staphylococcus epidermidis* and, even though lethal to *Acanthamoeba* spp., *P. aeruginosa*.^{3,4} To individual saline suspensions containing 2×10^5 acanthamoebae (70% trophozoites, 30% cysts)/ml, 1×10^5 cells of each bacterial species from bacterial growth harvested from sheep blood agar cultures incubated overnight at 35°C were added and gently mixed.

Immediately, and at 2-h intervals for 8 h, and after 24 h, a drop of the mixture was removed for examination by phase contrast microscopy and Gram's stain. Ten contiguous trophozoites were evaluated at each interval. The remaining suspension was processed for transmission electronmicroscopy (TEM). The cells for TEM were fixed in glutaraldehyde 3%, buffered to pH 7.4 with sodium cacodylate, post-treated with osmium tetroxide 1%, dehydrated in increasing concentrations of ethanol and embedded in EMbed 812.

Fig. 1. Gram-stained smear showing linear adherence of *X. maltophilia* to *A. polyphaga* trophozoites after co-cultivation for 2 min in sterile saline ($\times 1000$). (Similar results were noted for *A. castellanii*.)

Results

The *X. maltophilia*, *P. aeruginosa* and *E. coli* isolates adhered to trophozoites of both species of *Acanthamoeba* almost immediately; smears prepared immediately on mixing amoebae and bacteria showed bacilli in close proximity to the trophozoite membrane. However, counts of bacteria adhering to 10 trophozoites showed that the *X. maltophilia* and *P. aeruginosa* isolates were more adherent than *E. coli*. After co-cultivation for 2 min with *X. maltophilia* and *P. aeruginosa*, a mantle of bacilli encircled the trophozoite membrane and cyst forms (fig. 1). Initially, as noted with *X. maltophilia*, bacteria approached the trophozoite head-on and adhered in such a way that the distal end pointed into the medium. In fact, it appeared that the bacteria became entrapped in a surface matrix for an otherwise motile bacterium could be seen struggling to free itself.

By electronmicroscopy (fig. 2), the nature of the head-on articulation of *X. maltophilia* with the amoeba could be discerned better. At the attachment sites of the bacilli, the trophozoite plasmalemma was thicker and the trophozoite glycocalyx appeared to be lost. By way of contrast, the glycocalyx on the amoebic surface remained intact in the absence of full articulation by

Fig. 2. Electronmicrograph showing two *X. maltophilia* cells articulating head-on with an *A. polyphaga* trophozoite at sites of plasmalemma thickening. A third *X. maltophilia* cell is seen approaching the trophozoite glycocalyx which is still intact ($\times 37000$).

an approaching bacterial cell. In other fields, *X. maltophilia* was seen to attach to two distinct receptor sites on the same trophozoite and between three different trophozoites (fig. 3).

Subsequent to head-on articulation with the amoebic surface, these gram-negative bacteria assumed a linear position encircling the trophozoite membrane. In some instances, the mantle of adherent gram-negative bacteria delineated a clear zone distal to the cytoplasmic membrane of the trophozoite. This area may represent a potential glycocalyx which serves as the initial binding site leading to the gradual transposing of the attached bacteria to the amoebic membrane.

The adherence of the *S. epidermidis* isolate was similar to that observed for the gram-negative bacteria and included trophozoite plasmalemma thickening (fig. 4). Adherence seemed to be mediated through fibrils extending from the staphylococcal cell wall.

Fig. 3. Electronmicrograph showing linear arrangement of *X. maltophilia* on *A. polyphaga* trophozoite surface subsequent to head-on attachment. Note trophozoite plasmalemma thickening at two sites of bacterial attachment (upper right), and attachment of one bacterial cell to membranes of three trophozoites (lower left) ($\times 22000$).

Fig. 4. Electronmicrograph showing adherence of a staphylococcal cell through fibrillar matrix to amoebic trophozoite membrane. Note trophozoite plasmalemma thickening at attachment sites ($\times 37000$).

An interesting adherence phenomenon observed after co-cultivation for 24 h with *X. maltophilia* and, especially, with *P. aeruginosa*, was the focal clustering of multiple layers of bacteria to the trophozoite surface, and what appeared to be bacterial cell-to-cell adhesion forming a crown of attached organisms (fig. 5). Thus there was clear evidence for two definitive focal attachment sites on the amoebic surface. One comprised a two-three-cell layer and the other a mantle of adherent cells encompassing 75% of the

trophozoite surface as if the bacteria were enveloped in a matrix secreted by the amoeba. However, by electronmicroscopy, there was an abundance of *P. aeruginosa* cells surrounding the trophozoite, but the absence of a clearly defined matrix to account for the focal adherence and the apparent cell-to-cell adherence.

Phagocytosis of adherent bacteria was most evident with *X. maltophilia*, occurring as early as within 8 h of co-cultivation. Electronmicroscopy revealed that

Fig. 5. Phase contrast photomicrograph of *A. polyphaga* trophozoite showing "capped" aggregation of *P. aeruginosa* cells after co-cultivation for 24 h. Smaller cluster of adherent cells noted on lower aspect of trophozoite membrane ($\times 900$). (Similar results were observed for *A. castellanii*).

Fig. 6. Electronmicrograph taken after co-cultivation for 8 h of *X. maltophilia* with *A. polyphaga* showing adherent bacterial cells and several internalised within endocytic vacuoles ($\times 37000$).

while there were still numerous *X. maltophilia* cells in linear adherence to the amoebic surface, many clearly discernible, internalised bacteria were observed within phagocytic vacuoles (fig. 6). After co-cultivation for 24 h, some vacuoles contained up to 16 phagocytosed bacilli (fig. 7). By contrast, when *E. coli* was co-cultivated with *A. polyphaga* and *A. castellanii* for 24 h, despite the presence of numerous *E. coli* surrounding trophozoites, no internalisation of the bacteria could be seen. *P. aeruginosa*, which is lethal for both *Acanthamoeba* species,³ was found to adhere in large numbers to the amoebic surface, but only rarely was a bacterial cell internalised after co-cultivation for

24 h. This finding supports our earlier report³ that the pseudomonas amoebicidal activity ensues rapidly.

Interestingly, in gram-stained smears prepared after co-cultivation for 24 h with *S. epidermidis* (or *S. aureus*), cells were found layered around the entire trophozoite surface delineating its contour (fig. 8). However, internalisation was sparse, with only an occasional coccus found within a phagocytic vacuole. These observations were confirmed by electron-microscopy.

Fig. 7. Electronmicrograph showing 16 *X. maltophilia* cells within an endocytic vacuole in an *A. polyphaga* trophozoite ($\times 15000$).

Fig. 8. Gram-stained smear of *S. epidermidis* co-cultivated with *A. polyphaga* for 24 h showing rim of multilayered staphylococcal cells surrounding trophozoite but sparse internalised cocci.

Discussion

Our studies have confirmed and advanced the earlier observations of Singh⁵ who showed a differential feeding tropism of free-living amoebae toward bacteria in their environment. Furthermore, we have shown that the suitability of a bacterial food source for *Acanthamoeba* spp. is intimately associated with the proclivity with which the bacteria bind to the trophozoite (and cyst) surface and the time course over which the bacterial substrate is internalised.

However, binding and internalisation are not equivalent steps in the growth cycle of *Acanthamoeba* spp. but vary with the nature of the bacteria. Thus, while many gram-negative and gram-positive bacteria bind to the amoebic surface, phagocytosis of bound bacteria, for an as yet unknown reason, is not

equivalent for all species. Thus, whereas *X. maltophilia* was bound readily, and internalised within 8 h of co-cultivation, *E. coli*, which also attached readily to the trophozoite surface, was not internalised even after co-cultivation for 24 h. These data augment our previous observations¹ of increased and earlier growth of *A. castellanii* and *A. polyphaga* in the presence of *X. maltophilia* over that with *E. coli*.

P. aeruginosa also bound readily to both the *Acanthamoeba* spp., forming a dense multilayered rim of adherent bacilli. From this repository, however, *P. aeruginosa* exerted an amoebicidal activity.³ In the present study, despite unbridled adherence, very few *P. aeruginosa* cells could be seen to be internalised after co-cultivation for 24 h. As endocytosis by *Acanthamoeba* spp. is energy-dependent,⁶ failure to internalise what appears, by an adherence phenom-

enon, to be a desirable food source, may actually be attributed to a pseudomonas-induced membrane lesion leading to the death of the amoebae.³

Acanthamoebae have also been shown to use certain gram-positive bacteria such as *S. epidermidis* as growth substrates.⁷ Our data showing marked adherence of *S. epidermidis* to trophozoites but delayed and sparse internalisation, as also noted with *E. coli*, confirm our previous observations on the equivalence of these two species as poor growth substrates for *Acanthamoeba* spp. Furthermore, our data provide a plausible explanation for the observations of Larkin and Easty,⁷ who showed that incubation for 6 days was required to determine the efficacy of *S. epidermidis* and *E. coli* as food sources for *Acanthamoeba* spp. inoculated on to lawns of these bacteria on agar.

During this study, we observed several phenomena relating to bacteria-amoebae adherence worthy of comment. By phase contrast microscopy of wet preparations, *X. maltophilia* cells were seen to be entrapped on the trophozoite surface and writhing *in situ*. This observation is similar to that reported by Preston and King⁸ who showed that *P. fluorescens*, a polar-flagellate bacterium analogous to *X. maltophilia*, attached to *A. castellanii* by its polar flagellum and, while anchored in this fashion, "produced a jostling monolayer around each amoeba". Our electronmicrographs clearly demonstrate *X. maltophilia* directly attached to and linking trophozoites. However, in several preparations, there was a clear space separating *X. maltophilia* cells from the amoebal surface which may have represented articulation through the flagellum which was either sheared off during preparation for electronmicroscopy or in a different section plane. Subsequent to attachment by flagella to the amoebic surface, the bacteria became apposed linearly on the trophozoite surface and, if determined suitable, endocytosed.

The second distinct phenomenon, occurring subsequent to the attachment of motile bacteria to trophozoites was the transposition of large numbers of the entrapped bacteria to a focal point(s) on the amoebic surface. It appeared that the amoebae were secreting a substance that ensnared and fixed multiple bacterial cells to their surface. In this fashion, phagocytosis of the entrapped food supply could proceed either gradually over time or as an entire unit. Compelling evidence for the secretion of an entrapping substance by the amoebae can be derived from a report by Weisman and Korn.⁶ Working with chemically inert latex beads, these investigators showed that, prior to phagocytosis, acanthamoebae first accumulated beads in closely packed aggregates on

their membrane, then enveloped the beads with the plasma membrane and internalised them in a phagocytic vacuole.

Ray⁹ also described a similar phenomenon for *Hartmanella* (*Acanthamoeba*) spp. in the presence of bacteria possessing a single polar flagellum. She showed that when *P. aeruginosa* and *P. (Aeromonas) hydrophila* impinged against the trophozoite surface, they stuck to and covered the trophozoite membrane. Subsequent bacterial impaction resulted in their accumulation on the amoebic surface and to each other until they were multilayered. The accumulated bacteria then began to focally coalesce or were "capped" on the amoebic surface. Ray further noted that pseudopodia arose occasionally from the amoebic surface on either side of the capped bacterial mass, converged toward each other, and endocytosed into a food vacuole a section of the bacterial mass abutting the cytoplasmic membrane. If undisturbed, the remaining bacterial mass remained fixed to the amoebic surface for continued digestion and new bacterial cells were added to the outermost layer. In our study, as well as that of Ray⁹ and a 1902 report,¹⁰ capping was noted most frequently with polar flagellate bacteria such as *Pseudomonas* and *Xanthomonas* spp., less so with *E. coli* and not at all with *S. aureus* and *S. epidermidis*.

Our observations, through electronmicroscopy and light microscopy, of the feeding patterns of *A. castellanii* and *A. polyphaga* show that the suitability of a bacterial food source correlated directly with its ability to adhere to the trophozoite surface. In the case of polar flagellate environmental bacteria, such as *Pseudomonas* spp. (including *P. aeruginosa*) and *X. maltophilia*, attachment to available trophozoites in the co-cultivation system was uniform and immediate. Adhering bacteria could be focalised ("capped") and multilayered, suggesting the secretion by the amoebae of a "sticky" substance entrapping edible bacterial cells on their surface for internalisation, either sequentially or *en masse*.

Capping was not observed with *E. coli*, which is usually peritrichously flagellate, or the non-motile *S. epidermidis*, suggesting that this particular mode of feeding was absent for these bacteria. Thus, although these bacterial food sources bound readily to the amoebic surface, the delay in their internalisation in food vacuoles may be a function of the failure to be "capped" as a pre-requisite to phagocytosis. Interestingly, Ray⁹ showed that peritrichously flagellate bacterial cells formed haphazard clumps on the amoebic surface which may impede or delay phagocytosis.

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